Homogeneous pyrimidine nucleotidase from human erythrocytes: enzymic and molecular properties

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A pyrimidine nucleotidase with unique specificity has been obtained for the first time as an homogeneous protein from the cytosolic fraction of human erythrocytes. Both conventional chromatography and f.p.l.c. techniques have been used in the purification procedure. The final enzyme preparation gave a single protein band of $M_r = 23\,500$ on SDS/PAGE under both reducing and non-reducing conditions. The native enzyme was eluted at $M_r = 45\,000$ in gel filtration chromatography on Superose 12, suggesting a dimeric structure. Amino acid analysis was consistent with an acidic isoelectric point and revealed the presence of six half-cystine and two methionine residues per

subunit. The enzyme was active on a variety of pyrimidine nucleoside monophosphates, being most active on the 3'-monophosphates. K_m values for 3'dUMP, 3'UMP, 5'dUMP, 5'UMP, 5-fluoro-2'dUMP, ranged from $192\ \mu\text{M}$ to $1.15\ \text{mM}$. The enzyme activity was inhibited by both reaction products, orthophosphate, and the nucleoside formed. Product inhibition studies suggested an Ordered Uni-Bi mechanism for the reaction. The enzyme required Mg^{2+} for its activity, while heavy metals cations were strongly inhibitory. The enzyme activity was inhibited by metal chelating agents and it was sensitive to thioreactive reagents. The isoelectric point was 5.4 and the optimum activity pH was 6.5.

INTRODUCTION

5'-Nucleotidases (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) are widely distributed in nature and a voluminous literature has appeared on the enzyme from vertebrate tissues, snake venom, yeast, and bacteria. At least three mammalian 5'-nucleotidases have been identified in liver, one lysosomal, one cytoplasmic and one membrane-bound. The lysosomal enzyme is unique in that it hydrolyses 2', 3'- and 5'-mononucleotides equally well (Arsenis and Touster 1968). 5'-Nucleotidases have been characterized also from intestinal mucosa, pituitary gland, brain, cardiac tissue and skeletal muscle. Furthermore evidence has accumulated on the existence of isoenzymic forms of this enzyme from several tissues (Drummond and Yamamoto 1971). Valentine et al. (1974) first reported the presence of a pyrimidine-specific 5'-nucleotidase in the soluble fraction of human erythrocytes. Unlike nucleotidases from other sources, the human erythrocyte enzyme was inactive towards purine nucleotides. This unique substrate restriction appeared to match with the needs of red cell metabolism, which critically depends upon a limited reservoir of ATP, whose base cannot be synthesized *de novo* in the erythrocyte. Since the components of the adenine nucleotide pool, in erythrocytes, are in rapid equilibrium through the mediation of adenylate kinase, the presence of a nucleotidase acting on AMP and producing diffusible adenosine should impose their inexorable drain (Paglia et al., 1984). It is well established that hereditary deficiency of pyrimidine 5'-nucleotidase results in non-spherocytic haemolytic anaemia. Such a condition results in a marked accumulation of pyrimidine nucleotides in the erythrocytes, which are detectable either spectrophotometrically (Valentine et al., 1974) or by h.p.l.c. analysis of deproteinized samples (Simmonds et al., 1988), and is accompanied by a distinctive basophilic stippling of erythrocytes upon Wright staining of blood smears. The enzyme has been isolated and partially purified by Torrance et al. (1977). Subsequently it has been shown (Swallow et al., 1983; Paglia et al., 1983, 1984) that

affected patients, although defective in erythrocyte pyrimidine 5'-nucleotidase activity towards UMP, CMP and dCMP, conserved normal activity towards dUMP and dTMP. These findings pointed to the presence of two distinct pyrimidine 5'-nucleotidase (P5N) activities, which were partially separated by Hirono et al. (1985, 1987) and indicated as P5N type I (P5N-I) and P5N type II (P5N-II). The two P5N activities exhibited both different thermostability and enzymic and molecular properties, consistent with the existence of two different enzymes, coded by two distinct genes (Hirono et al., 1985). Recently, additional interest in the study of erythrocyte nucleotide metabolism has derived from the use of autologous erythrocytes as carriers and bioreactors for the conversion of encapsulated pyrimidine derivative pro-drugs into their pharmacologically active form. De Flora et al. (1988) reported on the involvement of the erythrocyte dephosphorylating machinery in the conversion of 5-fluoro-2'dUMP (5'FdUMP) to diffusible 5-fluoro-2'deoxyuridine (FdUrd), a potent anti-neoplastic agent. Thus the study of the major molecular and kinetic properties of erythrocyte 5'-nucleotidases might provide a remarkable contribution towards achieving an appropriate modulation of the drug delivery rate. The present report deals with the purification to homogeneity and the characterization of a soluble 5'-nucleotidase from human erythrocytes whose features are consistent with those described for P5N-II.

EXPERIMENTAL

Materials

Nucleosides, nucleotides, thioglycolic acid, *p*-chloromercuribenzoic acid (*p*-CMB), imidazole, Tris, Bistris and Tricine were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate, dithiothreitol (DTT), EDTA and h.p.l.c. grade methanol were from Merck (Darmstadt, Germany). Other reagents were of analytical grade.

Abbreviations used: P5N-I, pyrimidine 5'-nucleotidase type I; P5N-II, pyrimidine 5'-nucleotidase type II; 5'FdUMP, 5-fluoro-2'dUMP; FdUrd, 5-fluoro-2'-deoxyuridine; *p*-CMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; ACD, acid/citrate/dextrose.

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Enzyme assay

Nucleotidase activity was measured by an h.p.l.c.-based assay. The standard reaction mixture contained: 50 mM Tris/HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM DTT, 1 mM substrate, and the appropriate amount of enzyme sample, in a final volume of 500 μ l. Incubations were carried out at 37 °C for 30 min. The reaction was stopped by adding 100 μ l of assay mixture to 50 μ l of ice-cold 1.2 M $HClO_4$ in an Eppendorf tube. After 10 min at 0 °C the protein pellet was removed by centrifugation. Supernatant (130 μ l) was neutralized by the addition of 35 μ l of 1 M K_2CO_3 and the $KClO_4$ was removed by centrifugation. A 20 μ l aliquot of neutralized supernatant was injected into the h.p.l.c. apparatus and analysed as described by Amici et al. (1994). Alternatively the enzyme activity was assayed by measuring the amount of phosphate released, according to the method described by Martensen (1984).

One unit of enzyme activity is defined as the amount of enzyme hydrolysing 1 μ mol of nucleoside monophosphate/min under the standard assay conditions.

Purification procedure

All steps in the purification procedure were carried out at 4 °C; all buffer solutions contained 1 mM DTT, 1 mM $MgCl_2$ and 0.5 mM EDTA unless otherwise specified.

Washed erythrocytes

ACD (acid/citrate/dextrose) outdated bank blood was centrifuged and the plasma and white cells layer was removed by aspiration. The packed cells were washed in 4 vol. of physiological saline (0.9 % NaCl) followed by 10 min centrifugation at 1200 g and the supernatant and residual white cells layer were aspirated to yield leucocyte-free red blood cells after three washes.

Step 1: Haemolysate

Erythrocytes were haemolysed by the addition of 4 vol. of deionized water and pH was adjusted to 6.5 with HCl. Stroma was removed by centrifugation at 13000 g and the clear supernatant taken as the haemolysate.

Step 2: DEAE-cellulose chromatography

Haemolysate was chromatographed on an 80 mm $\times$ 255 mm DEAE-cellulose column equilibrated with 10 mM imidazole/HCl buffer at pH 6.5 at a flow rate of 50 ml/min. The column was eluted stepwise by adding 100 mM NaCl to the equilibration buffer. Active fractions were pooled.

Step 3: Ammonium sulphate fractionation

Solid ammonium sulphate was added to the active fractions at up to 90 % saturation and pH was readjusted to 6.5. After 2 h at 0 °C the precipitate was collected by centrifugation at 13000 g, and was dissolved in a minimal volume of 10 mM imidazole, pH 6.5. The residual ammonium sulphate was removed by dialysis against the same buffer with several changes.

Step 4: Phenyl-Sepharose hydrophobic interaction chromatography

The dialysed sample was adjusted to 2 M KCl and loaded on to a 60 mm $\times$ 190 mm phenyl-Sepharose CL-4B column equilibrated with 50 mM imidazole/HCl, pH 6.5, containing 2 M KCl at a flow rate of 20 ml/min. The column was washed with the same buffer and eluted by using a linear gradient of 2–0 M KCl. Activity was eluted as a single peak at 1 M KCl. The pooled

active fractions were concentrated through a YM 10 (Amicon) membrane and dialysed against 10 mM potassium phosphate buffer, pH 6.5.

Step 5: Hydroxyapatite chromatography

The dialysed pool from the phenyl-Sepharose column was loaded onto a 25 mm $\times$ 200 mm hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer, pH 6.5. The enzyme was eluted by using a linear gradient of 0–0.5 M KCl. The pool of active fractions was concentrated through a YM 10 (Amicon) membrane and dialysed against 10 mM imidazole buffer, pH 6.5.

Step 6: F.p.l.c. anion exchange chromatography

The f.p.l.c. system (LKB) consisted of a single pump (model 2150), a gradient controller (model 2152) a fixed wavelength detector (Uvicord SD, model 2158) with a 279 nm filter, a two-channel recorder (model 2210), and an automatic fraction collector (Helirac, model 2212). Samples were injected through a Valco valve equipped with a 1.0 ml loop. The active pool from the hydroxyapatite step was injected into a TSK DEAE-5PW f.p.l.c. (8 mm $\times$ 100 mm) column equilibrated with 10 mM Bistris buffer, pH 6.5, at a flow rate of 1.0 ml/min. After a 10 min wash with equilibration buffer, the column was eluted with a 20 min linear gradient of 0–50 mM NaCl, followed by a 30 min linear gradient of 50–200 mM NaCl. The column was then washed with a 5 min linear gradient of 200–500 mM NaCl before re-equilibration with the starting buffer. Active fractions were pooled and concentrated on the Amicon YM 10.

Step 7: F.p.l.c. hydrophobic interaction chromatography

The column was equilibrated with 50 mM BisTris/1 M $(NH_4)_2SO_4$, pH 6.5. Sample was adjusted to 1 M $(NH_4)_2SO_4$ before injection. After 5 min washing with the equilibration buffer, the elution was performed with a 150-min linear decreasing gradient of $(NH_4)_2SO_4$ from 1 to 0 M. Active fractions were pooled, and concentrated on the Amicon YM 10.

Analytical procedures

SDS/PAGE

PAGE under denaturing condition was conducted essentially as described by Schagger and Von Jagow (1987) with Tris/Tricine buffer, 10 % acrylamide/bisacrylamide ($T = 3\%$) running gel and a 4 % stacking gel. After electrophoresis, gels were silver stained according to the procedure of Oakley et al. (1980). Molecular mass standards were: phosphorylase b, 93000; BSA 66000; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500 and lysozyme, 14400.

Isoelectric point determination

Isoelectric point determination was carried out by isoelectrofocusing on a 110 ml LKB 8100 ampholine column using a linear gradient of glycerol (0–65 % w/v) and 1 % (w/v), pH 3.5–10, ampholine carrier ampholytes. The sample was added to the dense solution before gradient delivery to the column. Isoelectrofocusing was conducted at 15 W constant power and 1600 V upper limit for 16 h. pH and activity of the eluate were determined.

Molecular mass determination

Native molecular mass determination according to Andrews (1965) was performed on a Superose 12 HR 10/30 column

Figure 1 Phenyl-Sepharose CL-4B chromatography

DEAE-cellulose enzyme fractions were applied to a column of phenyl-Sepharose CL-4B equilibrated with 50 mM imidazole/HCl, pH 6.5/1 mM DTT/1 mM MgCl_2 /0.5 mM EDTA/2 M KCl, and eluted by a decreasing linear gradient of KCl (---) as described in the text. P5N-II (●) was quantitatively separated from acid phosphatase (○).

connected to the above-mentioned f.p.l.c. system. The column was equilibrated and eluted by using 50 mM Bistris, pH 6.5, and 0.5 M NaCl at a flow rate of 0.5 ml/min. Samples of pure P5N-II and molecular mass markers were injected together in the same run and 0.5 ml fractions were collected.

Amino acid analysis

Pure protein was extensively dialysed against water; duplicate samples (2 μg) were hydrolysed for 30 and 45 min at 155 °C in 6 M HCl/0.1 % phenol, in sealed, evacuated tubes. The analysis was performed on a Chromakon (Kontron Instruments) amino acid analyser, as described by Hare (1977). Amino acids were detected fluorimetrically after post-column derivatization with *o*-phthalaldehyde. Cysteine and cystine were determined as cysteic acid after performic acid oxidation (Moore, 1963).

Other analyses

Haemoglobin was determined spectrophotometrically as cyanomethaemoglobin. Protein concentration was determined as described by Bradford (1976). Optimum pH studies were conducted in 50 mM Bistris and 50 mM Tris/HCl buffer in the range 5.5–9.0. The actual pH of the incubation mixture was determined both before and after the 30 min incubation period by using a Radiometer model PHM 82 digital pH meter. The effects of metal ions on P5N-II activity were studied by using a protein preparation dialysed against 100 mM Bistris/HCl, pH 6.5, buffer omitting EDTA and DTT from the standard assay mixture. Protein phosphate content was determined according to Martensen (1984).

RESULTS

Purification

We had previously observed that in human erythrocytes P5N-II activity is present only in the soluble fraction of lysate. Removal of stroma by high-speed centrifugation was not accompanied by any decrease of P5N-II activity in the supernatant lysate, nor was there perceptible activity in reconstituted stroma (results not

Figure 2 Hydroxyapatite column chromatography

Phenyl-Sepharose enzyme fractions were applied to a column of hydroxyapatite equilibrated with 10 mM potassium phosphate, pH 6.5/1 mM DTT/1 mM MgCl_2 /0.5 mM EDTA. Two pyrimidine nucleotidases were separated by KCl gradients (---): P5N-I (○) and P5N-II (●).

shown). P5N-II activity was stable in stored erythrocytes with little loss (20 %) during 30 days at 4 °C. Therefore outdated bank blood was judged suitable as the enzyme source for purification purposes. Outdated bank blood (20 units; 6 l) was used for the purification procedure. Haemoglobin was removed with the unbound material during the DEAE-cellulose chromatography step. Acid phosphatase activity was removed during the washing step of the phenyl-Sepharose chromatography column since it did not bind under the conditions used (Figure 1). Figure 2 shows the elution profile from the hydroxyapatite which was the crucial step for the separation of P5N-I from P5N-II. The last step of the purification procedure, corresponding to a TSK-phenyl 5PW chromatography, is depicted in Figure 3 where a symmetrical activity peak closely matched the major protein peak. The constant value of the specific activity throughout the

Figure 3 TSK-phenyl 5PW chromatography

The 90 % pure enzymic activity obtained from TSK-DEAE 5PW chromatography was injected on to a TSK-phenyl 5PW column equilibrated in 50 mM Bistris buffer, pH 6.5/1 mM DTT/1 mM MgCl_2 /0.5 mM EDTA/1 M $(\text{NH}_4)_2\text{SO}_4$, and eluted with a descending gradient of $(\text{NH}_4)_2\text{SO}_4$ as described in the text. Enzyme activity (●) was recovered corresponding to a single absorbance peak (---).

Figure 4 SDS/PAGE of pure P5N-II

Enzyme protein (1 μ g), obtained from the last purification step, was analysed on a 10% polyacrylamide gel as described by Schagger and Von Jagow (1987). M_r standards were: phosphorylase b, 93 000; BSA, 66 000; ovalbumin, 45 000; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 000; lysozyme, 14 000. The gel was stained with silver nitrate according to the procedure of Oakley et al. (1980).

Table 1 Purification of human erythrocyte P5N-II

Step	Activity* (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Puri- fication (-fold)
Lysate	259	698 000	0.000371	100	1
DEAE-cellulose	237	4200	0.0564	91.5	152
Ammonium sulphate	245	3000	0.0817	94.6	220
Phenyl-Sepharose CL4B	147	455	0.323	56.8	871
Hydroxyapatite	102	11.0	9.27	39.4	25 000
TSK-DEAE 5PW	14.5	0.512	28.3	5.6	76 200
TSK-Phenyl 5PW	13.6	0.185	73.9	5.3	199 000

* Activity was assayed by using 3'UMP as the substrate.

peak was strongly suggestive of a pure enzyme preparation. SDS/PAGE analysis of the individual active fractions gave a single protein band upon silver staining, Figure 4. A typical purification procedure is summarized in Table 1. The presence of DTT during the purification procedure was required for stabilizing the enzyme activity and for ensuring reproducibility of the method. The specific activity of the final preparation was about 74 units/mg.

Molecular mass determination

A native M_r of 45 000, Figure 5, was estimated by gel filtration according to Andrews (1965) by using a superose 12 HR 10/30 column, as described in the Analytical procedures section. The M_r of 23 000 determined by SDS/PAGE (Figure 4) as described above was consistent with a dimeric structure for the enzyme. An identical pattern was obtained in the presence and in the absence of 2-mercaptoethanol with or without heat treatment at 100 °C for 5 min.

Absorption spectrum

The absorption spectrum of the pure enzyme showed a maximum at 280 nm and a minimum at 250 nm. The A_{280}/A_{260} ratio was 1.84 and the percentage absorption coefficient $E_{280}^{1\%}$ was 9.7.

Figure 5 Native P5N-II molecular mass determination

The enzyme obtained from the last purification step was concentrated to 0.5 ml and subjected to Superose 12 gel-filtration chromatography as described in the text. The column was calibrated with BSA (M_r = 66 000), ovalbumin (OVA, M_r = 45 000), and carbonic anhydrase (CA, M_r = 30 000). V_t column total volume, V_0 column void volume. The semi-logarithmic inset shows the interpolation curve calculated by a least squares analysis of molecular masses and K_d values of proteins.

Substrate specificity and inhibitors

The kinetic behaviour of P5N-II was examined in the pure protein with several substrates. Table 2 shows that the homogeneous enzyme catalyses the dephosphorylation of several pyridine nucleoside monophosphates according to the order: 3'dTMP > 3'dUMP > 3'UMP > 5'FdUMP > 2'UMP > 3'GMP > 5'dIMP > 5'dUMP > 5'dTMP > 3'dGMP > 5'IMP > 5'UMP > 2'GMP. P5N-II does not possess phosphohydrolase activity towards any of the cyclic nucleotides, nucleoside diphosphates and nucleoside triphosphates. In addition no activity has been observed towards nucleoside polyphosphates, like AppA, ApppA, AppppA, at 1 mM concentration, and against bis-*p*-nitrophenylphosphate, whereas *p*-nitrophenylphosphate was a very poor substrate with a K_m value higher than 10 mM. The K_m values for several substrates, as calculated from a Lineweaver-Burk plot, were: 0.192 mM (3'-dUMP), 0.250 mM (3'-UMP), 0.3 mM (3'-dTMP), 0.412 mM (5'-dUMP), 0.66 mM (5'-UMP), 1.15 mM (5'-FdUMP). Among a broad variety of compounds tested as possible effectors of the enzyme activity, only the reaction products, nucleosides and phosphate, exerted an inhibitory action.

Product inhibition studies

Kinetic analysis of the inhibition exerted by the reaction products was carried out by using substrate (3'-UMP) concentrations ranging from 0.1 to 1 mM, at different fixed concentrations of both phosphate (1–2 mM) and uridine (10–20 mM). The inhibition exerted by phosphate appeared to be of the competitive type, with a K_i value of 3.5 mM, whereas uridine exerted non-competitive inhibition, with a K_i value of 21 mM. Such an inhibition pattern suggested an Ordered Uni-Bi mechanism for the reaction, according to Cleland's nomenclature.

Metals ions effect

P5N-II activity was dependent on Mg^{2+} and it was fully active at 1 mM $MgCl_2$. Cd^{2+} , Cu^{2+} , Hg^{2+} , Ni^{2+} , Pb^{2+} were strongly in-

Table 2 Substrate specificity of purified P5N-II (relative activity*)

Phosphate position	Nucleoside base type											
	A	dA	C	dC	G	dG	I	di	U	dU	dT	5FdU
Activity (%)												
5'-MP	0	0	0	0	0	0	2.8	21.7	2.6	16.6	7.9	50.7
3'-MP	0	0	0	0	29.3	4.2	—	—	72.5	94.2	100	—
2'-MP	0	—†	0	—	1.8	—	—	—	36.4	—	—	—
c3',5'-MP	0	—	0	—	0	—	—	—	0	—	—	—
c2',3'-MP	0	—	0	—	0	—	—	—	0	—	—	—

* Activity is expressed as percentage relative to substrate 3'-dTMP

† —, not determined.

Table 3 Effect of the addition of metal ions on P5N-II activity*

Addition	Activity (%)	Addition	Activity (%)
None	100	Hg ²⁺	7
Ca ²⁺	17	Li ⁺	44
Cd ²⁺	9	Mn ²⁺	20
Co ²⁺	16	Ni ²⁺	7
Cr ³⁺	120	Pb ²⁺	4
Cu ²⁺	8	V ⁵⁺	15
Fe ³⁺	29	Zn ²⁺	11

* The enzyme activity has been assayed throughout by using 3'UMP as the substrate, in the presence of 1 mM MgCl₂, as described under 'Analytical procedures', with and without the addition of the indicated 1 mM metal chloride salts.

hibitory (Table 3). Like other Mg²⁺-dependent enzymes, P5N-II activity was fully inhibited by EDTA (results not shown).

pH optimum

The pH optimum of the enzyme was 6.5 as determined by assaying the activity in the pH range 5.5–9.0, as described in the Analytical procedures section.

Thiol reagents sensitivity

The enzyme was sensitive to 1 mM thioreactive reagents like p-CMB and 5,5'-dithiobis(2'-nitrobenzoic acid). P5N-II activity was totally inactivated when 2 mol of p-CMB/mol of protein were present in the assay mixture deprived of DTT.

Amino acid composition

The amino acid composition of the homogeneous enzyme, shown in Table 4, may be consistent with the acidic isoelectric point of the protein (see below), as suggested by the presence of a relative excess of acidic residues. Of particular relevance appears to be the presence of six half-cystine and two methionine residues per subunit.

Isoelectric point

The activity and pH profiles of an eluate from an electrofocusing column (see the Analytical procedures section), showed one catalitically active component with an isoelectric pH value of 5.4.

Table 4 Amino acid composition of human erythrocyte P5N-II

Amino acid	Residues per subunit*
Half-cysteine	6
Aspartic acid and asparagine	25
Threonine	11
Serine	10
Glutamic acid and glutamine	30
Proline	n.d.
Glycine	19
Alanine	16
Valine	19
Methionine	2
Isoleucine	9
Leucine	31
Tyrosine	6
Phenylalanine	12
Lysine	10
Histidine	8
Tryptophan	n.d.
Arginine	30

* All calculations are based on a *M_r* of 23 000.

Phosphate content

Total phosphate analysis was conducted on 50 µg of P5N-II by using the ammonium molybdate–Malachite Green procedure as described in the Analytical procedures section. No phosphate was detected.

DISCUSSION

This work presents the first purification to homogeneity of a human pyrimidine nucleotidase enzyme from red blood cells, which, on the basis both of pH optimum and substrate specificity, can be identified with the P5N-II form previously described by others (Swallow et al., 1983; Paglia et al., 1984; Hirano et al., 1985). In particular, the pH optimum of our enzyme activity is 6.5, which is close to the value found for the deoxyribonucleotidase identified by Paglia et al. (1984). Furthermore, the relative enzyme activity towards various substrates indicates a

higher dephosphorylation rate of 3'- versus 5'-nucleotides, in agreement with the results reported for P5N-II (Hirono et al., 1987). However, in contrast with these data, our findings show the absolute inability of pure P5N-II to dephosphorylate 5'CMP and 5'dCMP and negligible activity towards p-nitrophenyl-phosphate. These discrepancies can be ascribed to the presence of interfering activities in the preparations used by the above-mentioned authors. In addition our enzyme is active on 2'GMP and 2'UMP. It is ineffective towards adenine and cytidine nucleotides, nucleoside di- and tri-phosphates and dinucleosides di- tri- and tetra-phosphates. Of particular interest appears to be the remarkable hydrolytic activity towards 5'FdUMP, which is not a substrate for P5N-I (A. Amici et al. unpublished work). This strict specificity possibly identifies our enzyme with the nucleotidase activity responsible for the hydrolysis of 5'FdUMP in bioconversion experiments of this encapsulated prodrug, as reported by De Flora et al. (1988). In this regard it assumes particular relevance for the study of possible inhibitors of P5N-II in that they might act as appropriate modulators of the conversion of 5'FdUMP to the diffusible product FdUrd, which in turn is released from the erythrocyte thus effecting its pharmacological action. Among a broad variety of compounds tested, including nucleoside tri-, di-, and mono-phosphates, nucleosides, bases and base analogues, only orthophosphate and uridine results in inhibitory action. However, because of their diffusibility these compounds do not seem to be suitable as efficient modulators of the intraerythrocytic deoxyribonucleotide-dephosphorylating machinery. The results obtained upon investigation of the molecular properties of the enzyme reveal significant differences with respect to those observed for P5N-I. Our enzymic protein appears to be a dimer with a subunit M_r of 23000, whereas P5N-I is a monomer of M_r 34000. This value agrees with the M_r of 36000 reported for native P5N-I (Amici et al., 1991). These findings, together with the above-described remarkable differences in the pH optimum and substrate specificity existing between the two nucleotidases, led us to the

conclusion that our homogeneous protein, rather than being a pyrimidine 5'-nucleotidase isoform, is indeed a different enzyme endowed with a catalytic activity preferentially dephosphorylating pyrimidine 3'-nucleotides.

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