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Research Article

Evidence for essential catalytic determinants for human erythrocyte pyrimidine 5′-nucleotidase

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Abstract. Human erythrocyte pyrimidine 5′-nucleotidase, PN-I, catalyzes the dephosphorylation of pyrimidine nucleoside monophosphates. The enzyme also possesses phosphotransferase activity, transferring phosphate groups between pyrimidine nucleoside monophosphates and various pyrimidine nucleosides. Deficiency of the enzyme activity is associated with a hemolytic anemia. PN-I cDNA has been expressed in *Escherichia coli*, yielding a fully active recombinant enzyme, which was purified to homogeneity and extensively characterized. Multiple sequence alignment of PN-I and homologues proteins revealed the existence of conserved regions, whose importance in catalysis was examined by performing experiments designed to intercept covalent intermediates as strongly suggested by our previous kinetic studies. Furthermore, a functional analysis of the enzyme was carried out through site-directed mutagenesis designed on the basis of the sequence of the identified conserved regions as well as mutations observed in PN-I-deficient patients.

Key words. Pyrimidine nucleotide; enzyme; site-directed mutagenesis; kinetic analysis.

During human erythrocyte maturation, the nucleus is extruded and most of the ribosomal RNA is degraded [1]. Several enzymes are involved in the metabolism of nucleotides derived from the nucleic acid degradation, the last step of which is catalyzed by nucleotidases, causing the formation of membrane-diffusible nucleosides [2–4]. In erythrocytes, pyrimidine nucleoside 5′-monophosphates are dephosphorylated by a specific nucleotidase, PN-I (EC 3.1.3.5) [5] and the homeostasis of pyrimidine nucleotides is controlled by the balance between kinase and nucleotidase activities. Indeed, in patients lacking PN-I activity, the accumulation of pyrimidine nucleotides was observed both by a spectrophotometric method [6] and by HPLC determination [7, 8]. The deficiency of PN-I has been associated with a hemolytic anemia characterized by marked basophilic stippling on blood smearing [9–11], very likely caused by the accumulation of ribonucleic acid material, suggesting an association of PN-I deficiency with incomplete RNA degradation [12]. Interestingly, in red blood cells of human fetuses, PN-I activity was remarkably higher than in mature erythrocytes [13].

We have previously characterized PN-I both as the wild-type and recombinant enzyme [14, 15]. We demonstrated that PN-I possesses phosphotransferase activity specific for pyrimidine nucleotides, suggesting an additional role of this enzyme in nucleotide metabolism. Of particular importance in this context is the ability of PN-I to utilize, in addition to its natural substrates uridine and cytidine, important pyrimidine nucleoside analogs, like 3′-azido-3′-deoxy-thymidine (AZT) and cytosine-R-D-arabinofuranoside (AraC), which are widely used in chemotherapy. During our investigation on the structural properties of PN-I we have also been able to report on the identity of PN-I with p36, a protein associated with lupus inclusion formation in response to α-interferon [14].

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The PN-I cDNA sequence [14] was used, by our collaborators, to study three families with PN-I deficiency and, by GenBank search, to identify the gene for PN-I. It was found on chromosome 7, showing a total length of 26.8 kb and consisting of ten exons with alternative splicing of exon 2, producing 286 and 297-amino-acid-residue proteins [16]. In that study, three mutations were identified in patients of the examined families: codon 98, GAT→GTT, Asp→Val; codon 177, CAA→TAA, Gln→termination; IVS9-1, G→T, loss of exon 9 [16].

The present report deals with a comprehensive characterization of the catalytic and functional properties of PN-I. The catalytic study was carried out by performing experiments to identy amino acid residues involved in the formation of covalent intermediates, as strongly suggested by our previous kinetic investigations [15, 17]. The functional analysis was carried out by conducting site-directed mutagenesis experiments on the basis of the mutations observed in PN-I-deficient patients, as well as the presence of two conserved regions containing the consensus sequence DXDX(T/V), as demonstrated by multiple sequence alignment analysis [18, 19].

Materials and methods

Materials

All general laboratory reagents were of analytical grade if not otherwise stated. Oligonucleotide primers were obtained from Sigma-Genosys.

Cloning of human PN-I cDNA

The synthetic oligonucleotide primers 5′-GTACATATG ATGATGCCAGAATTCCAGAAA-3′ and 5’-GCCGGG ATCCTTATAGAATCTTCTG-3′ were used in a polymerase chain reaction (PCR) to amplify the PN-I gene (GenBank accession number BC066915) and to insert the *Nde*I and *Bam*HI restriction sites (underlined bases) at its 5′ and 3′ end, respectively. PCR was performed using 1 µl of human placenta library (Clontech Laboratories) as the template and 25 pmol of each primer in a final volume of 50 µl. After a 7-min initial denaturation at 95 °C, each cycle was set for 30 s of denaturation at 95 °C, 30 s of annealing at 55 °C, and 1 min of elongation at 72 °C, and 30 reaction cycles were carried out in a DNA thermal cycler (GeneAmp PCR System 2400; Perkin Elmer Life Science). The 880-bp product was purified from an agarose gel, digested with *Nde*I and *Bam*HI, and ligated into a similarly digested pET11a plasmid vector (Novagen). The ligation mixture was used to transform *Escherichia coli* TOP10 F′ (Invitrogen) cells and growth was selected on Luria-Bertani (LB) medium containing ampicillin (100 µg/ml). A representative recombinant plasmid (referred to as pET11a-pn1) was sequenced to confirm the absence of PCR errors.

Site directed mutagenesis

Site-directed mutagenesis to change the selected residues was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutagenic primers are summarized in table 1. Plasmid pET11a-pn1 was used as

Table 1. Primers for site-directed mutagenesis.

Primer	Sequence
D49N f	CTTCAGATAATAACG**A**ACTTTGATATGACACTCAG
D49N r	CTGAGTGTCATATCAAAGT**T**CGTTATTATCTGAAG
F50A f	CAGATAATAACGGAC**GC**TGATATGACACTCAG
F50A r	CTGAGTGTCATATCA**GC**GTCCGTTATTATCTG
D51N f	CCAAACTTCAGATAATAACGGACTTT**A**ATATGACACTCAGTAG
D51N r	CTACTGAGTGTCATAT**T**AAAGTCCGTTATTATCTGAAGTTTGG
E96D f	CTACGCTATTGA**T**GTTGATCCTGTTCTTACTGTAGAAGAG
E96D r	CTCTTCTACAGTAAGAACAGGATCAAC**A**TCAATAGCGTAG
D98V f	CTACGCTATTGAAGTTG**T**TCCTGTTCTTACTGTAGAAGAG
D98V r	CTCTTCTACAGTAAGAACAGGA**A**CAACTTCAATAGCGTAG
L142P f	GGCAGAATCTGACGTTATGC**C**CAAAGAAGGATATG
L142P r	CATATCCTTCTTTG**G**GCATAACGTCAGATTCTGCC
N190S f	GTCAAAGTTGTGTCCA**G**TTTTATGGATTTTGATGAAACTGGGG
N190S r	CCCCAGTTTCATCAAAATCCATAAAA**C**TGGACACAACTTTGAC
D193N f	GTGTCCAATTTTATG**A**ATTTTGATGAAACTGGGGTGC
D193N r	GCACCCCAGTTTCATCAAAAT**T**CATAAAATTGGACAC
F194A f	CCAATTTTATGGAT**GC**TGATGAAACTGGGGTGCTC
F194A r	GAGCACCCCAGTTTCATCA**GC**ATCCATAAAATTGG
D195N f	GTCCAATTTTATGGATTTT**A**ATGAAACTGGGGTGC
D195N r	GCACCCCAGTTTCAT**T**AAAATCCATAAAATTGGAC
G241R f	GGGAGACTCCCAA**A**GAGACTTAAGAATGGCAGATGG
G241R r	CCATCTGCCATTCTTAAGTCTC**T**TTGGGAGTCTCCC

Mutations are underlined.

the template for the PCR mutagenesis reaction. Briefly, 25 ng of template DNA was incubated with the appropriate mutagenic primers, dNTPs, and Pfu DNA polymerase using the cycling parameters recommended in the manual of the supplier. After the temperature cycling step, the parental template was digested by the restriction enzyme *Dpn*I, which cuts only dam-methylated DNA. The mutagenized plasmids were then transformed into *E. coli* TOP10 F′ cells. The mutant plasmids were extracted, purified and sequenced to verify incorporation of the desired modification and to ensure the absence of random mutations.

Expression of human wild-type PN-I and mutants

For protein expression, pET11a-pn1 and mutagenized plasmids were transformed into *E. coli* BL21 (DE3) (Novagen) cells. Single colonies were inoculated into 400 ml of LB medium containing 100 μg/ml ampicillin and grown at 37 °C. Expression was induced with 1 mM isopropylthiogalactoside (IPTG) at an A_{600} of 0.4, and cells were collected after 2 h induction.

Purification of wild-type recombinant PN-I and mutants

All the buffers contained 1 mM dithiothreitol (DTT) and 1 mM $MgCl_2$ and all steps were performed at 4 °C if not otherwise stated. Induced cells were harvested by centrifugation at 5000 g for 10 min and resuspended in 20 ml of lysis buffer 20 mM Tris/HCl, pH 7.5. The suspension was sonicated for 45 s, with 0.5-s intervals, at 50 W and centrifuged at 39,000 g for 20 min. Streptomycin sulfate was added to the supernatant (crude extract) at 1% final concentration and after centrifugation at 39,000 g for 20 min, the supernatant was loaded onto a hydroxyapatite Bio-scale CHT5-I column (BioRad) equilibrated with 10 mM potassium phosphate, pH 6.5. After washing with the same buffer, proteins were eluted by a linear gradient of potassium phosphate from 10 to 250 mM. Active fractions were pooled and concentrated by ultrafiltration on a YM10 membrane (Amicon-Millipore). After dialysis against 10 mM Tris/HCl, pH 7.5, the pool was applied in 1-ml aliquots to a TSK-DEAE 5PW column (Tosho), equilibrated with the dialysis buffer. After washing with the same buffer, elution was performed with a gradient of increasing NaCl concentrations (0–0.1 M) in 10 mM Tris/HCl, pH 7.5. Active fractions were pooled and concentrated by ultrafiltration as described above. Samples of crude extract, partially purified and final protein preparations were subjected to SDS-PAGE to monitor expression and purification progress. Final preparations were stored at 4 °C.

N-terminal sequencing

All purified proteins were subjected to amino-terminal sequencing by Edman degradation performed on a Procise 491 Protein Sequencer (Applied Biosystems), to ensure their identity.

Activity assay and substrate specificity

Nucleotidase and phosphotransferase activities were measured by an HPLC-based assay as reported in Amici et al. [15]. V_{max} and K_m values were obtained from the double reciprocal plots of substrate versus initial velocity. One unit of enzyme activity (U) was defined as the amount of enzyme producing 1 μmol product (nucleoside or nucleoside monophosphate) per minute under the standard assay conditions.

Phosphoenzyme covalent intermediate trapping and analysis of tryptic peptides

Pure PN-I (190 μg) was incubated in 50 mM Tris/HCl buffer, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, in the absence (control) or presence of 0.5 mM CMP at 0 °C, in a final volume of 200 μl [18]. After 15 s, the reactions were stopped by addition of 5 vol cold 10% TCA and centrifuged for 10 min at 4300 g, at 4 °C. Chemical entrapment of the acyl phosphate intermediate was carried out in the presence of $[^3H]NaBH_4$ (16 Ci/mmol), as reported in Allegrini et al. [18]. Samples were resuspended in 200 μl 0.4% NH_4HCO_3, pH 8.5, containing 6 μg trypsin and digested at 37 °C for 18 h. Tryptic digests were lyophilized and dissolved in 5% acetronitrile containing 0.1% TFA. Peptide mixtures were fractionated by reverse-phase HPLC on a C18 column (0.5 mm × 150 mm, 300 Å; Applied Biosystem) connected to a capillary HPLC (ABI 173A Microblotter capillary HPLC System; Applied Biosystems). Peptides were eluted by a linear gradient from 5 to 60% acetonitrile containing 0.1% TFA, over 160 min, at a flow rate of 5 μl/min. Peptides were spotted on a PVDF membrane and counted for radioactivity.

Woodward reagent K inactivation

PN-I (1 μM, 36 μg/ml) was incubated at 25 °C in 0.2 M MES buffer, pH 6.0, with concentrations of Woodward reagent K (WRK) ranging from 0.01 to 2 mM. At 1, 2, 3 and 4 min of incubation, samples were withdrawn and assayed for PN-I activity, as described above. The protection effect of substrates (10 mM), products (5 mM), and $MgCl_2$ (5 mM) were also evaluated during WRK inactivation. The WRK-modified PN-I was subjected to size exclusion chromatography on a Superose 12 column. Absorbance was monitored at 280 nm and 340 nm for total protein and WRK-bound protein determinations, respectively. The number of modified carboxyl groups was estimated by the increase in absorbance at 340 nm ($\varepsilon = 7000\ M^{-1}\ cm^{-1}$) [18, 20, 21].

Size exclusion chromatography

A Superose 12 column (Pharmacia, Sweden) was equilibrated and eluted with 50 mM Tris/HCl, pH 7.5, 1 mM

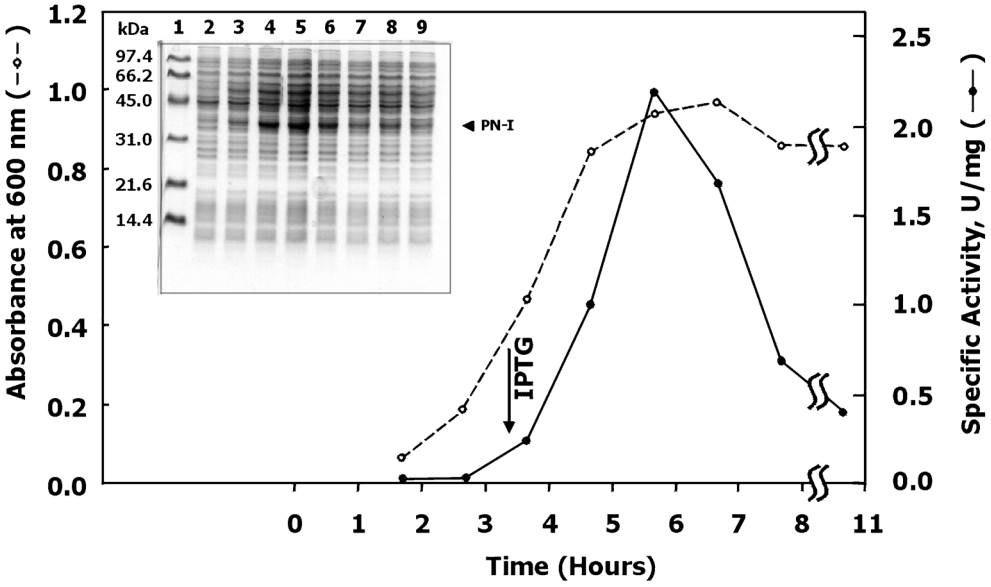

Figure 1. Overexpression of recombinant PN-I in *E. coli* BL21 (DE3). *E. coli* cells, transformed with pET11a-pn1 plasmid, were grown as described in Materials and methods. The vertical arrow indicates IPTG induction time. At different incubation times, both the absorbance at 600 nm of the culture and PN-I specific activity in the soluble cell extracts were determined. Inset: SDS-PAGE (15%) of soluble extracts before (lane 2) and after 0.5, 1, 2, 3, 4, 6 and 7 h induction (lanes 3–9). Lane 1 contains molecular-weight markers.

$MgCl_2$, 1 mM EDTA, 1 mM DTT, and 0.5 M NaCl. BSA (66,000 MW), ovalbumin (45,000 MW) and carbonic anhydrase (29,000 MW) were used as the molecular-weight standards [22].

Other methods

SDS-PAGE was carried out according to Laemmli [23]. Protein concentration was determined by the method of Bradford [24].

Results and discussion

Cloning, expression, purification and characterization of recombinant PN-I

PN-I was cloned, expressed, and purified as described in Materials and methods. Figure 1 shows the expression profile of the recombinant protein, indicating that the maximum activity is achieved after 2 h of IPTG induction. As can be seen from the inset of the same figure, the decline in activity parallels a decrease in the recombinant protein band intensity owing to the formation of inclusion bodies (not shown). The recombinant enzyme was subsequently purified to homogeneity, as depicted in figure 2 and table 2. To confirm the identity of recombinant PN-I, the pure protein was submitted to N-terminal sequencing, as described in Materials and methods. The sequence of the first 11 residues corresponded exactly to that found for the native enzyme sequence [14]. The homogeneous preparation was subjected to a detailed molecular and kinetic characterization in order to compare its features with those possessed by the wild-type enzyme [14, 15]. The molecular weight calculated from SDS-PAGE was estimated to be 36,000, as expected [15] (fig. 2). Gel filtration experiments indicated a native molecular weight of 35,500, consistent with a monomeric structure, as it has already been shown for the in-vivo-derived protein [15]. Kinetic analysis of recombinant PN-I, determined using CMP as the substrate, indicating K_m, V_{max}, and k_{cat} values of 66 μM, 31 U/mg, and 19 s^{-1}, respectively, suggested a catalytic behavior very similar to that of the in-vivo-derived enzyme [14]. Substrate specificity examined

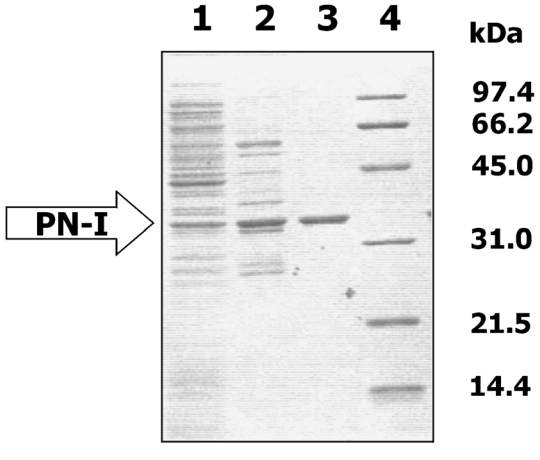

Figure 2. Recombinant PN-I-purification. SDS-PAGE (15%) of PN-I-containing samples at different stages of purification. Lanes: 1, crude extract; 2, hydroxylapatite column; 3, TSK-DEAE 5PW column; 4, molecular-weigth markers.

Table 2. Purification of recombinant PN-I.

Step	Activity (U)	Specific activity	Recovery (%)
Crude extract	1899.6	8.87	100
Hydroxyapatite	1945.8	14.5	102
TSK_DEAE	580.7	31.1	31

Activity units were obtained starting from 1 l of *E. coli* culture; activity was assayed as described in Materials and methods. Specific activity is expressed as U/mg.

using a variety of metabolites, including CMP, UMP, dCMP, dUMP, and dTMP, also corresponded to that exibited by the in-vivo-derived protein, indicating CMP as the best substrate. Furthermore, the enzyme was also active toward AZT-MP and Ara-CMP, known as important antiviral and antineoplastic agents [15]. In our laboratory we showed that PN-I possessed phosphotransferase activity specific for pyrimidine derivatives [15]. Like the in-vivo-derived PN-I, the recombinant enzyme is also endowed with the same activity. In particular, it has the ability to catalyze the transfer of a phosphoryl group from a pyrimidine nucleotide donor to a nucleoside acceptor. Kinetic analysis of the phosphotransferase activity revealed K_m values for CMP, as the donor, and uridine, as the acceptor, of 0.018 mM and 0.56 mM, respectively. In addition, the efficiency for the phosphotransferase activity, i.e., the percentage of phosphate transferred with respect to the total phosphate hydrolyzed, was evaluated to be 29%. These results indicate that the phosphotranferase capacity of the recombinant enzyme is almost identical to that of the in-vivo-derived enzyme [15].

Phosphoenzyme covalent intermediate trapping

In previous investigations on the catalytic mechanism exhibited by PN-I, as far as the nucleotidase and the phosphotransferase reactions are concerned, we observed a kinetic behavior providing convincing evidence for the formation of a covalent enzyme-phosphate intermediate [15, 17]. Moreover, inspection of the PN-I amino acid sequence revealed a signature motif, DXDX(T/V), which is common to a number of phosphomutases, phosphatases, nucleotidases, and phosphotransferases [18, 19]. The first aspartate of the signature has been suggested to be involved in the formation of a phosphoryl-intermediate, although the covalent modified residue has been isolated in only a few cases [19, 25]. This failure has been interpreted as the consequence of the extreme lability of the putative covalent phosphoryl-intermediate [18, 19]. Indeed, many enzymes forming covalent phosphoryl-intermediates during catalysis often go unnoticed because of the cryptic nature of the acylated enzyme-phosphate intermediate, which can be formed on aspartate and glutamate side chains. Since phosphoaspartyl and phosphoglutamyl residues are too labile to resist the procedures used for isolation of phosphorylated amino acids, we reduced the phosphate adduct with $[^3H]NaBH_4$ to obtain a stable radiolabeled hydroxymethyl-containing derivative [18, 26]. Samples were subsequently digested by trypsin treatment and the resulting peptides were separated and counted for radioactivity. We also included control samples in which the phosphate donor (CMP) was omitted. The radiolabeling distribution among tryptic peptides was essentially identical both in the presence of CMP and in the control (not shown). No specific radioactive peaks were identified, suggesting an extremely labile phosphorylated intermediate, which remains to be determined, if it exists at all.

WRK inactivation

To ascertain if carboxyl groups were involved in the PN-I active site, the carboxyl-group-modifying WRK was employed. Incubation of PN-I with WRK markedly inactivated the enzyme depending on incubation time and reagent concentration (not shown). The same behavior has been described for other nucleotidases/phosphotransferases which form a labile phospho-acyl-enzyme intermediate during catalysis [18]. Figure 3 shows the stoichiometry of residual nucleotidase activity as a function of WRK incorporation into homogeneous PN-I. Inactivation of PN-I by WRK appeared to accompany the reaction of a single carboxyl group up to approximately 80% inhibition (fig. 3). At higher levels of inactivation (where higher concentrations of WRK were used), additional carboxyl groups also reacted. Figure 3 indicates that the essential carboxyl is the most reactive of a class of at least three reactive groups. Although this sensitive response of activity inhibition by WRK strongly supports the hypothesis of the involvement of a carboxyl group in the catalytic mechanism, the possibility remains that this group only indirectly affects the activity and is not directly involved as originally postulated. To discriminate between these two possibilities, experiments aiming at the effects of the

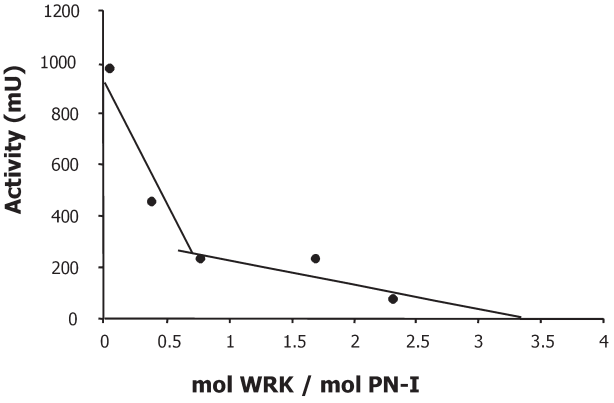

Figure 3. PN-I inactivation by WRK at different extents of WRK incorporation. The incubation conditions and the activity assay are described in Materials and methods.

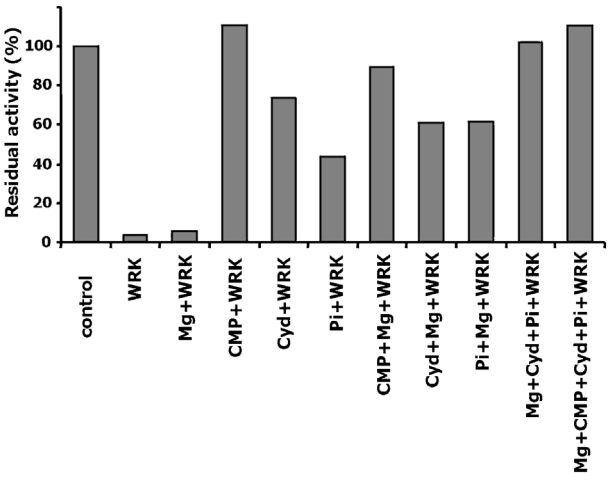

Figure 4. Protective action of PN-I effectors on WRK inactivation. PN-I was incubated for 5 min at 37 °C in the presence of 600 μM WRK and various effectors at the concentration indicated in Materials and methods.

substrates on the inhibition exerted by WRK were carried out. As depicted in figure 4, the presence of CMP, cytidine and phosphate, substrates and product of the nucleotidase/phosphotransferase reactions, brought about a marked protection of the enzyme activity, in the order: CMP > cytidine > phosphate. Furthermore the presence of Mg^{2+} by itself or together with the other effectors did not influence the action exerted by WRK. Together, these results speak in favor of a direct involvement in catalysis of specific carboxyl groups.

Site-directed mutagenesis of PN-I

Analysis of the PN-I amino acid sequence demonstrated the presence of two regions containing the motif DXDX (T/V) which has been shown to be a signature characteristic of a class of nucleotidases/phosphotransferases (fig. 5) [18, 19]. Data have been reported on the identification of the first aspartate of the signature as the phosphoryl acceptor residue during catalysis [18, 19]. Multiple alignment of PN-I from several organisms, ranging from nematodes to plants to animals, showed that while the first region containing the DXDX(T/V) motif is conserved, the second region was present only in humans and rodents. To ascertain if both motifs were involved in catalysis, selected residues were chosen for mutation. D49, D51, D193, and D195 were replaced individually with asparagine, and F50 and F194 were changed to alanine using site-directed mutagenesis, as described in Materials and methods.

SDS-PAGE of the cell extracts showed that all of the mutants were expressed as soluble proteins, migrating at the same position (36 kDa) as the wild type (fig. 6). All six mutants were purified to homogeneity as described for the wild-type enzyme; all of them showed the same elution profile when purified on hydroxylapatite and TSK-DEAE columns (not shown). The kinetic properties of the pure mutants were determined and compared with those of the wild-type enzyme (table 3). The nucleotidase activity was assayed using CMP as the substrate; the phosphotransferase activity was measured in the presence of CMP as the donor and uridine was used as the acceptor. Table 3 indicates that substitution with asparagine of the aspartate residues present in the first region completely abolished the enzyme activities. On the other hand, mutation of the aspartate residues present in the second region did not significantly affect the kinetic parameters. Replacement of phenylalanine residues (present in both regions) with alanine almost completely abolished nucleotidase and phosphotransferase activities. The V_{max} values of F50A and F194A decreased by 99% and 96%, respectively. Furthermore, an increase in the K_m values for CMP, of about 45- and 21-fold, respectively, was observed. Therefore, substitution of F50 and F194 residues caused a decrease in the catalytic efficiency of about 6400- and 600-fold, respectively. These results, together with the observation that the two phenylalanine residues are conserved among all organisms (fig. 5), strongly suggest their involvement in catalysis. Furthermore, mutations of F50 and F194 to alanine had a clear effect on the phosphotransferase activity; in fact, V_{max} values decreased by about 99 and 94%, respectively. However, F50A exhibited a K_m value for uridine similar to that calculated for the wild-type protein, and F194A showed only a twofold increase in the K_m value for the acceptor. As a result, the catalytic efficiency was 84 and 38 times lower, respectively, than that exhibited by the wild-type enzyme. Therefore, the mutations, while affecting the V_{max}, did not alter the binding affinity for uridine. In particular, phenylalanine residue substitution preferentially altered the hydrolytic ability possessed by PN-I with respect to its

	FIRST REGION											SECOND REGION										
Human	46	I	I	T	**D**	F	**D**	M	**T**	L	54	191	F	M	**D**	F	**D**	E	**T**	G	198
Mus musc.	46	I	I	T	**D**	F	**D**	M	**T**	L	54	191	F	M	**D**	F	**D**	E	**T**	G	198
Gallus gal.	45	I	I	T	**D**	F	**D**	M	**T**	L	53	190	F	M	**D**	F	**D**	E	N	G	197
Danio rer.	69	I	I	T	**D**	F	**D**	M	**T**	L	77	214	F	M	**D**	F	**D**	D	N	G	221
Dros. mel.	52	I	V	S	**D**	F	**D**	Y	**T**	I	60	199	F	L	Q	F	R	D	–	G	205
Cae. eleg.	116	V	I	S	**D**	F	**D**	Y	**T**	L	124	265	M	I	L	F	**D**	E	D	D	272

Fig. 5. Multiple sequence alignment of PN-I and homologous proteins. The two regions containing the DXDX(T/V) motif are shown.

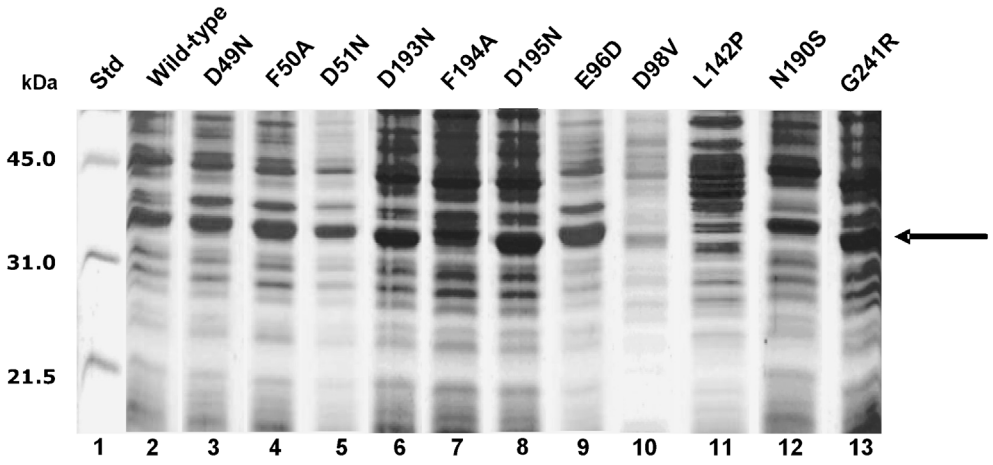

Fig. 6. SDS-PAGE (15%) of soluble fractions of crude extracts from *E. coli* cells expressing the wild-type PN-I (lane 2) and the mutated proteins (lanes 3–13). Lane 1 contains molecular-mass markers.

phosphotransferase activity. Particularly interesting to observe was the effect caused by the mutation indicated in table 3 as E96D. Inspection of the primary structure in the region encompassing residues 96–100 showed the presence of an -EVDPV- sequence, closely resembling the DXDX(T/V) motif, in that the first aspartate of the latter is replaced by its homologous glutamate. To investigate the possible involvement of such a region in PN-I catalysis, the glutamate residue E96 was changed to D. The mutated protein was expressed in *E. coli* (fig. 6) and purified to homogeneity, exhibiting a chromatographic behavior identical to the wild-type enzyme (not shown). The kinetic characterization, as described in table 3, showed a marked reduction of the K_m value for CMP and, more importantly, an almost complete suppression of the phosphotransferase activity, the V_{max} value of the nucleotidase activity remaining essentially identical to that exhibited by the wild-type enzyme. This finding will be instrumental for designing experiments aimed at a catalytic analysis of a PN-I variant devoid of the phosphotransferase activity.

PN-I deficiency is associated with mild to moderate anemia and in 10% of reported patients is linked to variable degrees of delayed development and learning difficulties [10, 11]. Furthermore, the enzyme activity reduction has also been associated with the conversion of hemoglobin E disease into an unstable hemoglobinopathy-like disease [27]. Previous genetic studies carried out by our collaborators [16] as well as by others have identified several different mutations in the PN-I gene of patients with hemolytic anemia [28–30]. Four of the mutations have been shown to be missense and consequently the mutated proteins would be expected to be endowed with a profoundly altered functionality. Therefore, site-directed mutagenesis experiments were designed to establish the relationship between genotype and phenotype. To this end, D98V, L142P, N190S and G241R recombinant proteins were produced and their catalytic properties were compared to those exibited by the wild-type enzyme.

Selected mutants were overexpressed in *E. coli* cells as described in Materials and methods of cell crude extracts after IPTG induction is shown in figure 6. Only two of the mutants, N190S and G241R, were expressed as soluble proteins migrating at the same position (36 kDa) as the wild type, whereas D98V and L142P were expressed in the form of inclusion bodies (not shown). Soluble mutated proteins were catalytically inactive. To determine whether the loss of activity might be attributed to a disturbed oligomerization process, N190S and G241R mutated proteins were purified to homogeneity following the same procedure described for the wild-type enzyme and pure proteins were subjected to gel filtration

Table 3. Kinetic properties of wild-type PN-I and mutants.

			First region			Second region			
		wild-type	D49N	F50A	D51N	D193N	F194A	D195N	E96D
CMP	K_m (μM)	10±5	nd	450±100	nd	32±10	215±50	107±40	450±100
	V_{max} (U/mg)	28±2	0	0.2±0.05	0	33±2	1.0±0.2	31±3	25±2
Urd	K_m (μM)	640±70	nd	765±70	nd	668±60	1400±100	670±60	nd
	V_{max} (U/mg)	7±2	0	0.1±0.02	0	10±2	0.4±0.1	9.6±3	0.1±0.02

Values are given as the mean ± SD of triplicate measurements. Nd, values not determined.

analysis as described in Materials and methods. The elution profile of both mutated enzymes corresponded to that exhibited by the wild-type enzyme (not shown), strongly suggesting that, for all other mutants described above, the loss of activity was due to a specific involvement of mutated residues in catalysis.

This report represents the first experimental evidence identifying specific amino acids as the catalytic determinants of human PN-I enzyme. Solving the three-dimensional structure of the protein, currently in progress with our collaborators, will be instrumental for verifying our observations.

Addendum: During the revision of the manuscript a report on a partial functional analysis of the enzyme has been published by Chiarelli L. R. et al. (Chiarelli L. R., Bianchi P., Fermo E., Galizzi A., Iadarola P., Mattevi A., Zanella A. and Valentini G. (2005) Functional analysis of pyrimidine 5′-nucleotidase mutants causing nonsferocytic hemolytic anemia. Blood **105:** 3340–3345)

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