Analysis of the human polynucleotide phosphorylase (PNPase) reveals differences in RNA binding and response to phosphate compared to its bacterial and chloroplast counterparts

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ABSTRACT
PNPase is a major exoribonuclease that plays an important role in the degradation, processing, and polyadenylation of RNA in prokaryotes and organelles. This phosphorolytic processive enzyme uses inorganic phosphate and nucleotide diphosphate for degradation and polymerization activities, respectively. Its structure and activities are similar to the archaeal exosome complex. The human PNPase was recently localized to the intermembrane space (IMS) of the mitochondria, and is, therefore, most likely not directly involved in RNA metabolism, unlike in bacteria and other organelles. In this work, the degradation, polymerization, and RNA-binding properties of the human PNPase were analyzed and compared to its bacterial and organellar counterparts. Phosphorolytic activity was displayed at lower optimum concentrations of inorganic phosphate. Also, the RNA-binding properties to ribohomopolymers varied significantly from those of its bacterial and organellar enzymes. The purified enzyme did not preferentially bind RNA harboring a poly(A) tail at the 3' end, compared to a molecule lacking this tail. Several site-directed mutations at conserved amino acid positions either eliminated or modified degradation/polymerization activity in different manners than observed for the Escherichia coli PNPase and the archaeal and human exosomes. In light of these results, a possible function of the human PNPase in the mitochondrial IMS is discussed.

Keywords: polyadenylation; RNA degradation; PNPase; human; phosphorolysis

INTRODUCTION
Polynucleotide phosphorylase (PNPase) (EC 2.7.7.8) was the first enzyme to be identified that catalyzes the formation of polynucleotides from ribonucleotides. However, unlike RNA polymerases, PNPase neither requires a template nor can transcribe one. When a mixture of ribonucleotide diphosphates (NDPs) serves as a substrate for the polymerization reaction, the ensuing polymerization reaction forms a random copolymer.

PNPase catalyzes both processive 3' → 5' phosphorolysis and 5' → 3' polymerization of RNA (Littauer and Soreq 1982; Grunberg-Manago 1999; Littauer and Grunberg-Manago 1999). In Escherichia coli, PNPase is mostly active in 3' → 5' phosphorolysis during RNA degradation and 3' end processing, but recently a substantial degree of activity in the polymerization of heteropolymeric tails has also been reported (Mohanty and Kushner 2000; Mohanty et al. 2004). Moreover, PNPase is suggested to be the major polyadenylating enzyme in spinach chloroplasts, cyanobacteria, and Gram-positive bacteria (Yehudai-Resheff et al. 2001; Rott et al. 2003; Slomovic et al. 2006). PNPase was also reported to be a global regulator of virulence and persistence in Salmonella enterica (Clements et al. 2002). The crystallographic structure analysis of PNPase from the bacteria, Streptomyces antibioticus, revealed a homotrimeric, circular-shaped complex (Symmons et al. 2000, 2002). Part of the PNPase population in E. coli is associated with the endoribonuclease, RNase E, an RNA helicase, enolase, and possibly other proteins in a high-molecular weight complex termed the degradosome (Marcaida et al. 2006). However, PNPase in the chloroplast was revealed to be a homotrimeric complex without the association of other proteins (Baginsky et al. 2001). It is also possible that two homotrimeric complexes are associated together in...
spinach chloroplasts, as PNpase elutes from a size-exclusion column at about 600 kDa (Baginsky et al. 2001).

The amino acid sequences of PNPsases from bacteria, as well as from the nuclear genomes of plants and mammals, display a high level of identity and feature similar structures composed of five motifs (Yehudai-Resheff et al. 2003; Leszczyniecka et al. 2004). There are two core domains that display different degrees of homology with the *E. coli* phosphorylase RNase PH, an α-helical domain between the two core domains, and two adjacent RNA-binding domains called KH and S1. These RNA-binding domains were characterized in other RNA-binding proteins. The domain structure of the bacterial and organellar PNPsases is very well conserved in the exosomes of archaea and eukaryotic cells (Buttner et al. 2006; Houseley et al. 2006; Lin-Chao et al. 2007). Indeed, the archaeal exosome is a phosphorylolytic complex that is very similar to PNpase and, in systems in which it exists, is responsible for the degradation and polymerization of RNA, resembling the PNpase found in cyanobacteria (Portnoy et al. 2005; Portnoy and Schuster 2006). The core structure of the eukaryotic exosome, which is responsible for the 3′ → 5′ exonucleolytic degradation of RNA in the nucleus and cytoplasm is also very similar to the PNpase and the archaeal exosome. While the human complex contains a phosphorylolytic activity site, it could not be detected in the yeast exosome (Liu et al. 2006; Dziembowski et al. 2007). The *E. coli* and chloroplast PNPsases display high binding affinity to poly(A); the responsible site is located in the S1 domain (Yehudai-Resheff et al. 2003). This phenomenon is important for its function in the polyadenylation-stimulated degradation pathway, in which the addition of poly(A) or poly(A)-rich sequences induces rapid exonucleolytic degradation (Lisitsky et al. 1997; Lisitsky and Schuster 1999). The enzymatic activity is significantly enhanced by inorganic phosphate at concentrations of 10–20 mM, and is sensitive to secondary structures in the RNA substrate (Yehudai-Resheff et al. 2001, 2003).

The human PNpase was identified in an overlapping pathway screen to discover genes displaying coordinated expression as a consequence of terminal differentiation and senescence of melanoma cells (Leszczyniecka et al. 2002). Unlike chloroplast and plant mitochondria, in which PNpase is located in the stroma and matrix, the human PNpase is mostly or exclusively located in the mitochondrial intermembrane space (IMS) (Chen et al. 2006; Rainey et al. 2006). In addition, PNpase was purified as an interacting protein with TCLI, a nonenzymatic oncoprotein promoting B- and T-cell malignancies and apoptotic stimuli caused PNpase mobilization following cytochrome c release to the cytoplasm (Chen et al. 2006; French et al. 2007). Considering its IMS location, it most likely does not play a major or direct role in mitochondrial RNA metabolism, contrary to its role in bacteria, chloroplast, and plant mitochondria (Slomovic et al. 2006). There is no obvious RNA substrate for the human PNpase in the IMS of the mitochondria, and its function and possible RNA target are still unknown. As the first step to reveal the biological function of this enzyme in human cells, we considered biochemical characterization in comparison to the homologous bacterial and chloroplast enzymes. To this end, the RNA phosphorolysis, binding, and complex formation of the human PNpase were analyzed. We found that when expressed in *E. coli* and purified the human PNpase is active in both polymerization and degradation and, like the bacterial enzyme, forms a trimeric homopolymer. However, the human PNpase differs from the bacterial and chloroplast enzymes in its optimal inorganic phosphate concentration for degradation activity as well as its RNA-binding properties. Several site-directed mutations that eliminated the phosphorolytic activity of the *E. coli* enzyme or the archaeal and human exosomes have different effects on the human PNpase.

RESULTS

Human PNpase has a similar structure to that of the bacterial enzyme

The human PNpase belongs to an evolutionarily conserved enzyme family whose members were characterized in bacteria and organelles (Yehudai-Resheff et al. 2003; Leszczyniecka et al. 2004). This enzyme contains an N-terminal transit peptide of 37 amino acids that guides the protein into the mitochondrial IMS and is, thereafter, removed by cleavage (Chen et al. 2006; Rainey et al. 2006). Similar to its bacterial and organellar counterparts, the protein is composed of two core domains homologous to the bacterial phosphorylase/exoribonuclease RNase PH (Fig. 1). The phosphorylase activity site was located at the second core domain in the bacteria, *Streptomyces antibioticus* (Symmons et al. 2000) as well as in the homologous archaeal exosome protein Rrp41 (Lorentzen et al. 2005). In spinach chloroplasts, in addition to the activity of the second core domain, the first core domain is active in the degradation of polyadenylated RNA while the situation in *E. coli* is still unknown (Yehudai-Resheff et al. 2003). The first core domain is followed by an α-helical domain and the second core domain, by KH and S1 domains, which were shown in the bacterial and chloroplast enzymes to be involved in RNA binding (Fig. 1; Jarrige et al. 2002; Symmons et al. 2002; Yehudai-Resheff et al. 2003; Stickney et al. 2005). The S1 and KH domain motifs characterize many other RNA-binding proteins (Worrall and Luisi 2007).

In order to observe the putative structure of the human PNpase, we used the coordinates of the homologous enzyme from the bacteria, *S. antibioticus*, that was deciphered by crystallization and X-ray diffraction analysis (Symmons et al. 2000). Figure 1B presents the predicted trimeric structure compared to the bacterial enzyme.
The human PNPase exhibits a very similar structure to the bacterial enzyme, having a doughnut-shaped trimer with a central channel that is of the correct dimensions to accommodate a single-stranded RNA molecule (Symmons et al. 2000). The circular structure is composed of the first and second core domains, while the KH and S1 RNA-binding domains are located at the top, adjacent to the gate entrance into the circle (Symmons et al. 2000; Lorentzen and Conti 2005; Lorentzen et al. 2005; Buttner et al. 2006). In Figure 1B, in which the trimer structure is presented as viewed from above, the KH domain (in yellow) is shown, located above the circular structure.

To achieve a detailed analysis of the degradation, polymerization, and RNA-binding activities of the human PNPase, the protein was expressed in bacteria and purified to apparent homogeneity (Fig. 2A). In addition, several amino acids that are highly conserved when aligning the sequences of known PNPases (Fig. 1C), as well as the corresponding subunits of the archaeal, human, and yeast exosomes, which were predicted to be involved in the formation of the phosphorolysis site or the trimeric complex, based on the structural analysis, were modified by site-directed mutagenesis (Fig. 1B; Symmons et al. 2000; Lorentzen and Conti 2005; Lorentzen et al. 2005). The corresponding proteins were expressed in bacteria and purified also (Fig. 2A). Several additional conserved residues were modified as well; however, the corresponding proteins were not obtained in a soluble form when expressed in bacteria.

Phosphorolytic activity of the human PNPase

As a phosphorylase, PNPase incorporates Pi and ADP in degradation and polymerization processes, respectively (Littauer and Grunberg-Manago 1999). When a 24-nucleotide (nt) RNA molecule was incubated with the enzyme in the presence of Pi and without the addition of ADP, only the degradation of the RNA was observed (Fig. 2B, left).
However, when the same substrate was incubated with the enzyme in the presence of ADP but without Pi, both degradation and polymerization activities were detected (Fig. 2B, middle panel). When both ADP and Pi were present at the reaction mixture, the direction of activity, either polyadenylation or degradation, was dependent on their relative concentrations (Fig. 2B, left panel). As expected of a phosphorylase, the byproduct of the degradation of [32P]UTP-labeled RNA was identified as UDP and the reaction was significantly stimulated by Pi (Fig. 2C). Optimal degradation activity was obtained at the relatively low concentration of 0.1 mM (Fig. 2D). This optimal concentration differs significantly from that of bacterial and chloroplast PNPases, in which the degradation reaction was stimulated at much higher Pi concentrations of 10–20 mM (Littauer and Grunberg-Manago 1999; Yehudai-Resheff et al. 2001; Jarrige et al. 2002). Indeed, at a Pi concentration of 10 mM, the degradation activity of the human PNPase was severely inhibited (Fig. 2C; data not shown). The low activity observed here without the addition of Pi was eliminated when CaCl₂ was included in the reaction mixture in order to quench traces of Pi that were present in the reaction (cf Fig. 2B,C, in which 50 μM CaCl₂ was added). This result suggested that the human PNPase can respond to much lower concentrations of Pi, changing its mode of activity between degradation and polymerization, compared to bacterial and chloroplast enzymes. The specificity of the enzyme for the polymerization reaction is, like that of the E. coli PNPase, high for ADP, with much less activity when incubated with other NDPs. No activity was observed with ATP nor the other NTPs, as well as mono phosphate nucleotides (Fig. 3).

Human PNPase degrades polyadenylated and nonpolyadenylated RNA at similar rates

The PNPases of bacteria and chloroplasts preferentially degrade and polyadenylate RNA molecules containing a poly(A) tail at the 3′ end. This preferential activity on polyadenylated transcripts has been attributed to the high binding affinity to poly(A) tracks located at the S1 domain in the spinach PNPase (Lisitsky et al. 1997; Lisitsky and Schuster 1999; Yehudai-Resheff et al. 2003). To analyze whether the human PNPase shares this preferential activity, RNA related to a region of the mitochondrial COX1 gene, either with or without the addition of a 49 nt poly(A) tail, was incubated with the human PNPase in either optimal polymerization or degradation conditions. The results revealed no differences in either the polymerization or the degradation activities (Fig. 4). Similar results were obtained with polyadenylated or nonpolyadenylated RNA substrates of varying lengths, derived from additional genes (Fig. 4E; data not shown). Moreover, when polyadenylated and nonpolyadenylated transcripts related to the ubiquitin mRNA were mixed together and assayed for degradation, the same rate was observed for both substrates, indicating no competition of the polyadenylated RNA (Fig. 4E). In contrast, competition between polyadenylated and nonpolyadenylated transcripts, resulting with the stabilization of the nonpolyadenylated one, has been described previously for the E. coli and spinach chloroplast PNPases (Lisitsky et al. 1997; Lisitsky and Schuster 1999; Yehudai-Resheff et al. 2003). Therefore, we concluded that, unlike bacteria and chloroplast PNPases, the human enzyme does not degrade polyadenylated RNA preferentially. This conclusion was strengthened by the observation described in the continuation of the manuscript that human PNPase does not display high binding affinity to poly(A). Our results differ from a previous analysis of this protein in which preferential degradation of polyadenylated RNA was detected (French et al. 2007).

In order to determine the degradation efficiency that the human PNPase displays when incubated with RNA molecules containing extensive structural features, we analyzed the RNAI of E. coli. This RNA, which oversees the plasmid replication in the bacterial cell, is characterized...
by a tRNA-like structure, shown to hamper degradation by bacterial PNPases, to a certain degree (Kaberdin et al. 1996). When the human or E. coli PNPases were incubated at the same concentration with the RNAI transcript, it was successfully degraded, resulting in a very similar pattern of degradation products (Fig. 5). We concluded that once titrated to conditions for optimal degradation activity in terms of the concentrations of Pi and ADP, both the human and E. coli PNPases similarly degrade structured RNA molecules.

Human PNPase displays different RNA-binding properties compared to bacterial and organellar PNPases

Bacterial and spinach PNPase proteins were previously analyzed and displayed high binding affinity to poly(A) and poly(U), in correlation with this enzyme’s necessity to compete for polyadenylated RNA (Lisitsky et al. 1997; Lisitsky and Schuster 1999). In order to determine the binding curve, RNA-binding of the purified protein was analyzed in a UV-cross-linking assay using increasing amounts of PNPase. As shown in Figure 6A, while the E. coli enzyme displayed a saturation binding curve with a Kd of 11 nM, in agreement with previous reports (Bermudez-Cruz et al. 2002; Schubert et al. 2004; Stickney et al. 2005; Amblar et al. 2007), an apparent sigmoid curve, typical of cooperative binding, was observed for the human enzyme. The Kd observed for the human PNPase was ~16 nM.

Next, we examined whether the human PNPase, similar to other PNPases analyzed to date, binds poly(A) with higher affinity than poly(G) and poly(C). To this end, competition UV-cross-linking assays, in which increasing amounts of the corresponding ribohomopolymer compete with [32P]-(GU)12 RNA for binding of the protein, were carried out. The efficiency by which the ribohomopolymer competes for protein binding reflects its affinity. Surprisingly, while, as previously reported, the E. coli enzyme displayed high binding affinities to poly(U) and poly(A) (Lisitsky and Schuster 1999), the human PNPase bound poly(U) and poly(G) with high affinity and displayed very low binding affinity to poly(A) (Fig. 6B,C). The low affinity to poly(A) sequences is in line with the observation that, unlike the case of the E. coli and chloroplast PNPases, the human enzyme displayed no preferential activity for polyadenylated RNA (Fig. 4).

Unlike the spinach chloroplast PNPase, the human PNPase does not bind the E. coli enzyme to form a heterotrimeric complex when expressed in E. coli

When expressing the recombinant spinach PNPase protein in E. coli, we previously found that a heterotrimeric functional complex, consisting of both spinach and E. coli subunits, was formed (Yehudai-Resheff et al. 2003). In this case, the presence of bacterial subunits in the trimer interfered with the determination of the activities and RNA-binding properties of the recombinant spinach PNPase. We then, therefore, expressed the spinach protein in an E. coli strain in which the endogenous PNPase gene was interrupted by the genomic insertion of DNA and therefore, the bacterial protein was not expressed (Lopez et al. 1999). In the present work, since we were concerned that a similar phenomenon could hamper characterization of the human PNPase, we initially expressed the protein in the E. coli strain in which PNPase is not expressed. In order to examine whether, similar to the spinach PNPase, the human enzyme can form a heterotrimeric complex composed of E. coli and human PNPase subunits, the human PNPase was produced in nonmutated E. coli strain expressing the intrinsic protein. The recombinant his6 tagged human PNPase was purified by affinity chromatography and analyzed on SDS-PAGE in order to see if an associated functional complex, consisting of both spinach and human PNPase subunits, the human PNPase subunits, the human PNPase copurified with the his6 tagged human PNPase, indicating that a heterotrimeric PNPase complex was not obtained (Fig. 7A, lane 3). However, when spinach chloroplast PNPase was expressed in this strain, the E. coli polypeptide copurified with the recombinant spinach protein (Fig. 7A, lane 4). In order to
verifying that even a minute amount of *E. coli* PNPase did not bind the human polypeptide, we analyzed the purified recombinant proteins with specific antibodies. No signal was obtained with antibodies specific to the *E. coli* PNPase when probing the purified human PNPase (Fig. 7B, left panel, lane 2). We concluded that, unlike the spinach chloroplast PNPase, human polypeptides cannot form a heterotrimer with the *E. coli* protein.

**Recombinant human PNPase forms a homotrimeric complex; formation is impeded by a D135G mutation located at the first core**

Next, we analyzed whether human PNPase, like the *E. coli* enzyme, forms a homotrimer of ~240 kDa (Amblar et al. 2007). Alternatively, the spinach chloroplast enzyme is characterized as a 580-kDa complex that is possibly comprised of two homotrimers (Symmons et al. 2000; Baginsky et al. 2001). Analysis of the complex formation of the various enzymes by fractionation on a size-exclusion column revealed that the human enzyme migrated at a molecular weight of 230 kDa, similar to *E. coli* and unlike the spinach chloroplast homolog (Fig. 7C). The same size was measured previously, upon fractionation on a blue-native gel (Chen et al. 2006). A site-directed mutant, in which one amino acid located in the first core domain was changed (D135G), was characterized by the inability to form the homotrimer complex, and the protein was eluted from the column in a monomeric form of ~90 kDa (Fig. 7C).

**Changing conserved amino acids resulted in the altering of phosphorolytic and RNA-binding activities**

In order to determine which amino acids are important for the phosphorolytic and RNA-binding activities of PNPase, several residues that are evolutionarily conserved between bacterial and organelar PNPases, as well as the archaeal, yeast, and human exosomes, were modified via site-directed mutagenesis (Fig. 1). The corresponding modified proteins were then analyzed for activity and RNA-binding (Fig. 8). Although other conserved amino acids, such as G509D and G513R, were modified as well, the corresponding proteins could not be obtained when expressed in bacteria in a soluble form and could therefore not be analyzed.

Changing the aspartic acid residue at position 544, which is involved in the formation of the catalytic site, to glycine (D544G), significantly reduced degradation activity but surprisingly enhanced polymerization. In contrast, in the *E. coli* PNPase, the equivalent change, (D493G), resulted in the loss of phosphorolytic activity but also abolished polyadenylation activities by 90% (Table 1; Jarrige et al. 2002). Similar results of enhancing polymerization and significantly inhibiting degradation activities were obtained when arginines, 445 and 446, involved in RNA binding at the catalytic site, were converted to glutamic acid (R445,446E). This observation is interesting, since modifying the corresponding conserved arginines in the *E. coli* PNPase, (R398,399D), the archaeal exosome, (R94V,K95A of Rrp41), and human exosome, (R398V,K95A of Rrp41), resulted in the complete inhibition of phosphorolytic activity for *E. coli* PNPase and the human exosome, as well as the degradation activity for the archaeal complex (Table 1; Jarrige et al. 2002; Lorentzen et al. 2005; Liu et al. 2006). Changing serine 484 and aspartic acid 538 to alanine (S484G and D538A) practically eliminated both the polyadenylation and degradation activities of the human PNPase (Fig. 8). When the corresponding residues in the human and archaeal exosomes, involved in the formation of the P" binding and the catalytic active sites, were modified, the result was elimination of activity, as well (Table 1).

Interestingly, changing aspartic acid 135, which is located in the first core domain, to glycine, (D135G), a mutation shown above to cause the inability to form the homotrimeric complex (Fig. 7), resulted in the almost complete inhibition of degradation and polyadenylation activities. The corresponding mutation in the *E. coli* PNPase, (D96G), resulted with almost complete loss of the degradation activity but only slightly reduced the polymerization activity (Table 1; Jarrige et al. 2002).

The RNA-binding property of the modified proteins, as disclosed by the UV-cross-linking assay, was drastically reduced compared to the wild-type and the *E. coli* proteins even in the D544G and R445,446E mutants, in which the polymerization activity was significantly enhanced (Fig. 8C). Together, the mutational analyses revealed both similarities and differences between the activities of the mutated PNPase, compared with parallel mutations in the *E. coli* enzyme and the archaeal and human exosomes (Table 1).
Domain swapping of the S1 domain between the E. coli and human PNPases, conferred high poly(A) binding affinity to the human enzyme

Previously, we have found that the S1 domain of the spinach chloroplast PNPase is responsible for high binding affinity to poly(A) (Yehudai-Resheff et al. 2003). This phenomenon is important for the activity of this enzyme in bacteria, chloroplast, and plant mitochondria in the polyadenylation stimulated RNA degradation pathway (Lisitsky et al. 1997; Lisitsky and Schuster 1999; Yehudai-Resheff et al. 2003). The human PNPase is located in the mitochondria IMS and does not seem to be directly involved in this pathway, and accordingly, displayed low binding affinity to poly(A) (Fig. 6). Alignment of the amino acids sequence of the S1 domain of the E. coli, spinach chloroplast, and human PNPase revealed significant amounts of conserved amino acids but not drastic differences that could possibly account for the diverse binding properties (Fig. 9A). Nevertheless, when the S1 domain of the human enzyme was replaced with that of the E. coli PNPase, high binding affinity to poly(A) was observed, indicating that the S1 domain is responsible for this phenomenon (Fig. 9B). Interestingly, the high binding affinity to poly(G), a characteristic of human but not E. coli PNPase, was still observed in the human PNPase harboring the E. coli S1 domain (Fig. 9B). This result suggest that the high affinity to poly(G) characteristic of the human PNPase is not located at the S1 domain.

DISCUSSION

In this study, the phosphorolytic activity and RNA-binding properties of the human PNPase were studied and compared to bacterial and organellar PNPases. In addition, conserved amino acids in PNPases and the archaea and human exosomes, which were shown to be located at the activity sites, were mutated, and the modified proteins analyzed for activity and RNA-binding properties. The human PNPase was found to be active in the phosphorolysis of RNA, resembling its bacterial and organellar

FIGURE 4. Similar activities of human PNPase on nonpolyadenylated and polyadenylated substrates. [32P] RNA corresponding to part of the mitochondrial gene COX1, either without (A,C) or with the addition of 49 adenosins at the 3' end (B and D) was incubated with the protein and 0.1 mM Pi (C,D) or 2.5 mM ADP (A,B). In panel E, degradation assay was performed to non-polyadenylated ubiquitin transcript (I), the same transcript with the addition of 64 adenosines (II), and the two transcripts mixed together (III). Samples were withdrawn at 0, 15, 30, and 120 min, and the RNA analyzed by denaturing PAGE and autoradiography.
homologs, as previously described (Hayakawa and Sekiguchi 2006; Sarkar and Fisher 2006; French et al. 2007). Differences in the $P_i$ concentrations for optimal activity and RNA-binding properties, between the human PNPase and other PNPases, were detected as well.

The characterization of the human PNPase is of great interest because of its distinct location in the human cell in comparison to plant organellar PNPases. While in plant organelles PNPase is located in the mitochondrial matrix and the chloroplast stroma, the human PNPase is located in the IMS of the mitochondrion, where no RNA has yet been observed. This unique location is probably reflected by diverse functions acquired in the evolution of plant and animal PNPases. In bacteria, PNPase is directly involved in the degradation, processing and nontemplate polymerization of RNA (Kushner 2004; Deutscher 2006). Indeed, in cyanobacteria, PNPase is exclusively responsible for the polyadenylation activity of the RNA degradation pathway, and deletion of the PNPase gene is lethal (Rott et al. 2003). Similarly, in hyperthermophilic and methanogenic archaea, the archaeal exosome, which is very similar to PNPase, is responsible for both polyadenylation and degradation of RNA (Portnoy et al. 2005; Portnoy and Schuster 2006). PNPase also plays a direct role in the degradation and processing of organellar transcripts in the mitochondria and chloroplast of plants (Walter et al. 2002; Perrin et al. 2004a,b). However, no PNPase exists in yeast; possibly a general case for all fungi (Dziembowski et al. 2003). Interestingly, it was recently observed that the yeast exosome exhibits no phosphorolytic activity, implying that with neither PNPase nor a phosphorolytic exosome, no RNA phosphorolytic activity occurs in yeast (Liu et al. 2006; Dziembowski et al. 2007).

As described above, the human PNPase was recently found to be located in the IMS of mitochondria and is, therefore, excluded from the matrix where the mitochondrial RNA, contrary to the case of plants. On the other hand, silencing the expression of PNPase in human cells was found to significantly affect, perhaps indirectly, the amount and length of stable poly(A) tails which characterized the 3' ends of mitochondrial transcripts (Slomovic and Schuster 2008). Therefore, and since there is not an obvious known RNA substrate in the IMS, it was of great interest to analyze the phosphorolytic and RNA-binding properties of the human PNPase in comparison to the bacterial and plant PNPases that are directly involved in RNA metabolism.

**Differences between human and previously characterized PNPases**

The RNA degradation activity of human PNPase has been previously shown for purified recombinant or in vitro translated protein (Leszczyniecka et al. 2002; Sarkar et al.
The results of the present work disclosed that the human PNPase exhibits phosphorolytic activity that could efficiently polymerize or degrade all of the RNA molecules that were examined. There are two major differences between the human and the thus far characterized bacterial and organellar PNPases. The first difference is that while the bacterial and plant organellar enzymes disclosed a high binding affinity to poly(A), the human PNPase binds poly(A) with very low affinity (Fig. 6). The high affinity poly(A)-binding of bacterial and plant organellar PNPases is required for activity because of their direct involvement in the polyadenylation-stimulated RNA degradation pathway. Due to its binding preference to polyadenylated RNA, following the polyadenylation of RNA by poly(A)-polymerase or PNPase, PNPase binds and degrades the polyadenylated molecule. Thus, polyadenylated RNA molecules compete with nonpolyadenylated molecules for PNPase binding (or additional exoribonucleases) in bacteria and plant organelles (Schuster et al. 1999; Regnier and Arraiano 2000; Dreyfus and Regnier 2002; Slomovic et al. 2006). Since, in human mitochondria PNPase is mostly or exclusively not located in the matrix, it is most likely not directly involved in a polyadenylation-stimulated RNA degradation pathway, and therefore high binding affinity to poly(A) is not required. All characterized PNPases bind the ribohomopolymer, poly(U), with high affinity. However, while bacterial and chloroplast PNPases exhibited low binding affinity to poly(G) (Lisitsky and Schuster 1999; Yehudai-Resheff et al. 2003), the human enzyme binds this ribohomopolymer with high affinity.

**FIGURE 7.** Unlike the spinach chloroplast PNPase, the human protein does not form a complex with its *E. coli* counterpart. (A) Human (lanes 1 and 3), *E. coli* (lane 2), or spinach chloroplast (lane 4) PNPases were expressed in *E. coli* strain in which the expression of the endogenous PNPase was knocked-out (lane 1), or in *E. coli* strain containing the endogenous PNPase (lanes 2, 3, and 4). The expressed proteins were purified as described in Materials and Methods and analyzed on SDS-PAGE followed by silver staining. The identities of the different PNPases is indicated on the right. The *E. coli* endogenous PNPase was copurified with the spinach protein (lane 4) but not with the human one (lane 3). (B) Recombinant human PNPase was expressed and purified in *E. coli* strains either lacking (lane 1) or containing (lane 2) endogenous PNPase. In lane 3, a purified recombinant *E. coli* PNPase was loaded. The proteins were analyzed by SDS-PAGE and immunoblotted to a nitrocellulose membrane that was incubated with antibodies against the *E. coli* PNPase or the human PNPase, as indicated in the figure. (C) Recombinant human PNPase forms a high molecular weight complex of ~300 kDa. Recombinant proteins (~5 µg) were fractionated on a Superdex 200 size-exclusion column. The elution profile of molecular-weight markers: Thyroglobulin 665 kDa, Ferritin 440 kDa, Catalase 232 kDa, Aldolase 158 kDa, and Albumin 67 kDa, fractionated on the same column, is indicated at the top.

**FIGURE 8.** Polymerization, degradation, and RNA-binding activities of the different mutants. (A,B) [*32P*]-labeled RNA oligonucleotide (GU)12 was incubated with wild-type (wt) PNPase or site-directed mutants as indicated. Reactions contained 2.5 mM of ADP and incubated for 10 min in (A) and 0.1 mM Pi incubating for 15 min in (B). RNA was purified and analyzed by denaturing PAGE and autoradiography. The input RNA, as well as the polymerized and degradation products, are indicated on the left. A control reaction in which no protein was added is shown in lane NP. (C) Wild-type (wt) human PNPase and its site-directed mutants (10 ng each) were analyzed for RNA-binding by the UV light cross-linking assay using [*32P*] RNA corresponding to the (GU)12. Following UV cross-linking and ribonuclease digestion, the label transfer from RNA to protein was analyzed by SDS-PAGE and autoradiography. In the lane labeled NP, no protein was added to the assay as a control. Ec, purified *E. coli* PNPase.
Another possibility is that, similar to endonuclease G, PNPase does not have a designated function within the IMS; rather, it is stored in this compartment, released during apoptosis, and functions in the phosphorylolyis of a substrate found in the cytoplasm (Chen et al. 2006). Further studies concerning the biological and molecular properties of the human PNPase will explore these hypotheses and reveal the biological function of this enzyme.

**MATERIALS AND METHODS**

**Production of recombinant PNPase and its mutated versions**

The corresponding DNA sequence of the mature protein (without the transit peptide) was amplified by PCR using the primers GGTACCGAGCTGTTGGCGCTGACCTTA and GTTCGACTCAGGAATTTAGATAGTA TGACTGTTGA and the PNPase cDNA, kindly obtained from P.B. Fisher (Columbia University). The Acc65I and Sall restriction sites that were added are bold and underlined. The different point mutations in the full-length (wild-type [wt]) protein were introduced using the site-directed mutagenesis kit (Stratagene). To create the chimera protein in which the S1 domain of human PNPase was replaced by that from the *E. coli* enzyme, a BamHI site was introduced between KH and S1 domains using the site-directed mutagenesis kit (Stratagene). The *E. coli* S1 domain, to which the same restriction site was introduced, was cloned using this site and the Xhol restriction site located in the pet20b vector.

For expression in *E. coli*, the PCR products were inserted into the Pet20b vector with the addition of a His6 tag at the C terminus. The proteins were expressed in the BL-21 DE3 pLYS5 or the strain ENS134-3 lacking the endogenous PNPase and containing the T7 RNA polymerase [BL21(DE3)(lacZ::Tn10 mulPp534:PT7lacZ-Arg5)(pnp::Tn5)] (Lopez et al. 1999), kindly obtained from Marc Dreyfus (Ecole Normale Superieure). The proteins were purified according to the manufacturer's protocol using a NTA-agarose affinity column with additional purification steps using MonoQ and Heparin columns (Pharmacia). The wt and mutant D91G proteins were purified additionally by fractionation on the size-exclusion column Superdex 200. All of the proteins were purified to the stage of one Coomassie-stained band on SDS-PAGE (Fig. 1), and no contaminating ribonuclease activity was detected. In order to examine possible RNA content of the PNPase preparation, RNA was extracted from a purified fraction and analyzed by gel fractionation and silver staining. Less than 1 ng of RNA was detected (data not shown). In addition, incubation of the enzyme with P1 for 30 min (phosphorylolyis) or extensive RNase A treatment did not reveal any differences. Therefore, the purified PNPase contains only minor traces, if any, of RNA. Specific antibodies for the *E. coli* and

<table>
<thead>
<tr>
<th>Organism</th>
<th>Function</th>
<th>Trimer formation</th>
<th>Active site (RNA binding)</th>
<th>Active site (catalytic site)</th>
<th>Active site (Pi binding)</th>
<th>Active site (catalytic site)</th>
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<tr>
<td>Human PNPase</td>
<td>wt</td>
<td>DI35G</td>
<td>R34E, R34E</td>
<td>S346G</td>
<td>S186A</td>
<td>D32A</td>
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<tr>
<td><em>E. coli</em> PNPase</td>
<td>wt</td>
<td>D96G</td>
<td>R34E, R34E</td>
<td>D346G</td>
<td>S186A</td>
<td>D32A</td>
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<td>Archaea</td>
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<td>Human Exosome</td>
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<tr>
<td>Yeast Exosome</td>
<td>wt</td>
<td>E1098</td>
<td>R95, R95 (RPrp1)</td>
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<td>G334 (RPrp1)</td>
<td>E179G (RPrp1)</td>
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The corresponding residues of the yeast exosome that is phosphorolytically inactive are shown as well (Liu et al. 2006; Dziembowski et al. 2007). A minus sign (–) indicates no activity detected. The numbers of plus signs indicate the relative strength of the activity. ND, activity not determined; Pol., polymerization activity; Deg., RNA degradation activity.
human PNPases were kindly obtained from Agamemnon J. Carpousis (Centre National de la Recherche Scientifique) and Paul B. Fisher (Columbia University), respectively.

**Synthetic RNAs and antibodies**

cDNAs encoding the 100 nt of the 3' end of the human mitochondrial transcript COX1 with or without the addition of 49 adenosines were described (Slomovic et al. 2005). Part of the cDNA encoding 267 nt of the ubb (ubiquitin), with or without the addition of 62 adenosines was used to generate the corresponding transcript. These cDNAs were cloned to the pGEM vector, and the RNAs were transcribed using T7 RNA polymerase and radioactively labeled with [32P]UTP (Portnoy and Schuster 2006). The full-length transcription products were purified by 5% denaturing polyacrylamide gels. The plasmid for the transcription of full-length transcription products were purified from 5% denaturing polyacrylamide gels. The plasmid for the transcription of (GU)12 oligonucleotide was radioactively labeled at the 5' end using [32P] γ-ATP and polynucleotide kinase.

**Polyadenylation and degradation assays**

Polyadenylation and degradation activities of the recombinant proteins were assayed as previously described (Yehudai-Resheff et al. 2003). [32P]-RNA was incubated with the corresponding proteins in buffer E (20 mM HEPES, pH 7.9, 60 mM KCl, 12.5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, and 17% glycerol) at 37°C with the addition of P3. When polyadenylation was assayed, ADP or the corresponding nucleotide was added and the Pi omitted. Following incubation, the RNA was isolated and analyzed by denaturing PAGE and autoradiography. For a thin-layer chromatography analysis of the degradation products, an aliquot of each sample was spotted on a polyethyleneimine thin layer chromatography plate, which was then developed with 0.9 M GnCl, dried, and exposed to autoradiography (Yehudai-Resheff et al. 2003). Nucleosides di-, and triphosphates (5 μg of each) were separated on the same plate and visualized by fluorescence quenching.

**UV light cross-linking assay**

UV light cross-linking of proteins to radio-labeled RNA was performed as previously described (Lisitsky et al. 1997). The proteins were mixed with [32P]-RNA in a buffer containing 10 mM HEPES-NaOH, pH 7.9, 30 mM KCl, 6 mM MgCl2, 0.05 mM EDTA, 2 mM DTT, and 8% glycerol and cross-linked immediately at UV light cross-linker that was set at 1.8 J (Hoefer). This was followed by digestion of the RNA with 10 μg of RNase A and 30 units of RNase T1 at 37°C for 1 h. The proteins were then fractionated by SDS-PAGE and analyzed by autoradiography. For the competition assay, the protein was first mixed with the ribohomopolymers and then the radiolabeled RNA was added.

**Structure prediction and complex model building**

Homology-based modeling of the three-dimensional structure of human PNPase was performed using the Phyre Server at http://www.sbg.bio.ic.ac.uk/~3dpssm/. The visualization of the human PNPase trimer complex was performed by inserting the monomeric model described above into a pseudorhombohedral (H32) space group with dimensions similar to those of the Streptomyces antibioticus crystal structure (PDB code 1E3H) using PyMOL (Delano).

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