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Chloroplast PNPase exists as a homo-multimer enzyme complex that is distinct from the *Escherichia coli* degradosome

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ABSTRACT

In *Escherichia coli*, the exoribonuclease polynucleotide phosphorylase (PNPase), the endoribonuclease RNase E, a DEAD-RNA helicase and the glycolytic enzyme enolase are associated with a high molecular weight complex, the degradosome. This complex has an important role in processing and degradation of RNA. Chloroplasts contain an exoribonuclease homologous to *E. coli* PNPase. Size exclusion chromatography revealed that chloroplast PNPase elutes as a 580–600 kDa complex, suggesting that it can form an enzyme complex similar to the *E. coli* degradosome. Biochemical and mass-spectrometric analysis showed, however, that PNPase is the only protein associated with the 580–600 kDa complex. Similarly, a purified recombinant chloroplast PNPase also eluted as a 580–600 kDa complex after gel filtration chromatography. These results suggest that chloroplast PNPase exists as a homo-multimer complex. No other chloroplast proteins were found to associate with chloroplast PNPase during affinity chromatography. Database analysis of proteins homologous to *E. coli* RNase E revealed that chloroplast and cyanobacterial proteins lack the C-terminal domain of the *E. coli* protein that is involved in assembly of the degradosome. Together, our results suggest that PNPase does not form a degradosome-like complex in the chloroplast. Thus, RNA processing and degradation in this organelle differ in several respects from those in *E. coli*.

Keywords: chloroplast gene expression; degradosome complex composition; exoribonuclease; higher plant; mRNA stability

INTRODUCTION

Structural proteins and enzymes in the chloroplast are encoded in both nuclear and chloroplast genomes. The expression of plastid-encoded genes during chloroplast development is tightly controlled at different levels, including processing and accumulation of their mRNAs. Plastid RNA metabolism is regulated by mechanisms that depend on RNA secondary structures, nucleases, and regulatory RNA-binding proteins (reviewed in Barkan & Stern, 1998; Hayes et al., 1999; Schuster et al., 1999). Similar to bacterial mRNAs, most chloroplast mRNAs contain an inverted repeat sequence in their 3′ untranslated region (UTR) that can fold into a stable stem-loop structure (Barkan & Stern, 1998). These stem-loop structures do not function as efficient transcription terminators, resulting in the transcription of an extended precursor RNA. This precursor RNA is processed to a mature mRNA, which terminates in the 3′ stem-loop structure (Barkan & Stern, 1998). RNA processing can be reproduced in vitro using synthetic RNA molecules and a chloroplast soluble extract, which facilitated the isolation and biochemical characterization of a 100-kDa RNA-binding protein that is homologous to the bacterial exoribonuclease polynucleotide phosphorylase or PNPase (Hayes et al., 1996).

The molecular mechanisms of RNA processing and degradation in the chloroplast have been studied in detail during the last few years and in certain aspects

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resemble RNA degradation mechanisms in *Escherichia coli* (Hayes et al., 1999; Schuster et al., 1999). In both systems, RNA degradation is thought to be initiated mainly by endoribonucleolytic cleavage of the RNA molecule followed by the addition of a poly(A) tail in *E. coli* and poly(A)-rich tail in the chloroplast (Kudla et al., 1996; Lisitsky et al., 1996, 1997). The polyadenylated cleavage products are then removed by rapid exonucleolytic degradation involving PNPase and RNase II in *E. coli*, and PNPase (possibly in concert with other exoribonucleases) in the chloroplast (Hayes et al., 1999; Lisitsky et al., 1996, 1997). Thus, RNA polyadenylation is part of the RNA degradation pathway in bacteria, chloroplasts, and also plant mitochondria (Carpousis et al., 1999; Coburn & Mackie, 1999; Gagliardi & Leaver, 1999; Hayes et al., 1999; Lupold et al., 1999; Schuster et al., 1999; Regnier & Arraiano, 2000; Steege, 2000). In *E. coli*, about 10–20% of the PNPase population is associated with other proteins in a high molecular weight complex, called the "degradosome" (Carpousis et al., 1999; Coburn & Mackie, 1999; Liou et al., 2001; Regnier & Arraiano, 2000). The proteins identified in the complex include RNase E, the DEAD-RNA helicase Rhl B, and the glycolytic enzyme enolase. A possible association of other proteins in substoichiometric amounts, including the chaperons GroEL and DnaK as well as the enzyme polyphosphate kinase (PPK), was also reported (Blum et al., 1997). Together, these proteins appear to work together during RNA processing and degradation. RNase E most likely initiates the degradation (and perhaps processing) process, whereas the RNA helicase is probably required for unwinding secondary structures (Py et al., 1996).

The association of a 3′-5′exonuclease with other proteins has been recently described for yeast mitochondria as well. In this organelle, a 3′-5′ exonuclease possibly related to RNase II is associated with a putative NTP-dependent RNA helicase (Suv3p) and a 75-kDa protein of unknown function. This complex is involved in the degradation of excised group I introns (Min & Zassenhaus, 1993; Margossian et al., 1996).

Work in chloroplasts initially identified an exoribonuclease homologous to the bacterial PNPase as a 100-kDa protein that eluted as a 580–600-kDa complex in size-exclusion chromatography, compared to the 500-kDa elution profile of the *E. coli* degradosome (Py et al., 1994; Hayes et al., 1996). In addition, a 67-kDa protein that cross-reacted with different preparations of antibodies against the *E. coli* RNase E was also detected in this molecular weight range of the column (Hayes et al., 1996; Kudla et al., 1996). Thus, it was possible that similar to the situation in *E. coli*, a degradosome-like protein complex directs RNA processing and degradation in chloroplasts and other plastid types as well (Carpousis et al., 1999; Hayes et al., 1999; Schuster et al., 1999). In the work reported here, we analyzed the chloroplast high molecular weight PNPase complex in more detail to determine its protein components. Unlike in *E. coli*, we found that chloroplast PNPase is not associated with other degradosome-related proteins to form a regulatory RNA processing complex. Several plant and algal amino acid sequences with homology to the bacterial RNase E also revealed the absence of a C-terminal domain that provides the platform for binding the degradosome components in *E. coli*. These results, together with the recent observation that spinach chloroplasts lack a homolog of the poly(A)-polymerase, the major polyadenylation enzyme in *E. coli* (Yehudai-Resheff et al., 2001), demonstrate that although the basic mechanism of RNA polyadenylation and degradation is similar, there are major differences between *E. coli* and chloroplast.

RESULTS

Purification of the HMW complex from spinach chloroplasts

When chloroplast soluble proteins were fractionated by size-exclusion chromatography on a Superdex 200 column and the protein profile was analyzed by SDS-PAGE, several high molecular weight complexes were observed. This included the ribosomal small subunit at about 700 kDa and the CO_2-fixing enzyme complex of RuBP-carboxylase-oxigenase at 550 kDa (Shteiman-Kotler & Schuster, 2000). As previously described, when the molecular weight of the chloroplast PNPase was determined following fractionation on this column and using specific antibodies, it was found exclusively at about 580–600 kDa (Hayes et al., 1996; Fig. 1A). No other fractions of the column were found to contain PNPase, indicating that all of the PNPase species in the chloroplast are associated in the complex of 580–600 kDa. It is therefore conceivable that PNPase performs its function as a 3′-5′ exoribonuclease in association with this protein complex in vivo. Because PNPase in *E. coli* is partially associated with the 500-kDa degradosome complex (Carpousis et al., 1999; Regnier & Arraiano, 2000), we investigated the possibility that a similar situation exists in the chloroplast.

The PNPase complex was further purified using ion exchange chromatography followed by chromatography on a single-strand DNA affinity column (Materials and Methods). Finally, the fraction that eluted from the single-strand DNA column was subjected to size-exclusion chromatography. PNPase was monitored during the fractionation procedure using specific antibodies and UV crosslinking to RNA (Hayes et al., 1996). The enzyme was again found to elute at 580–600 kDa, indicating that the PNPase-containing complex most likely remains intact during the purification procedure (Fig. 1B). As an important functional control for the activity of PNPase, we performed in vitro RNA degradation assays with the fractionated PNPase complex.

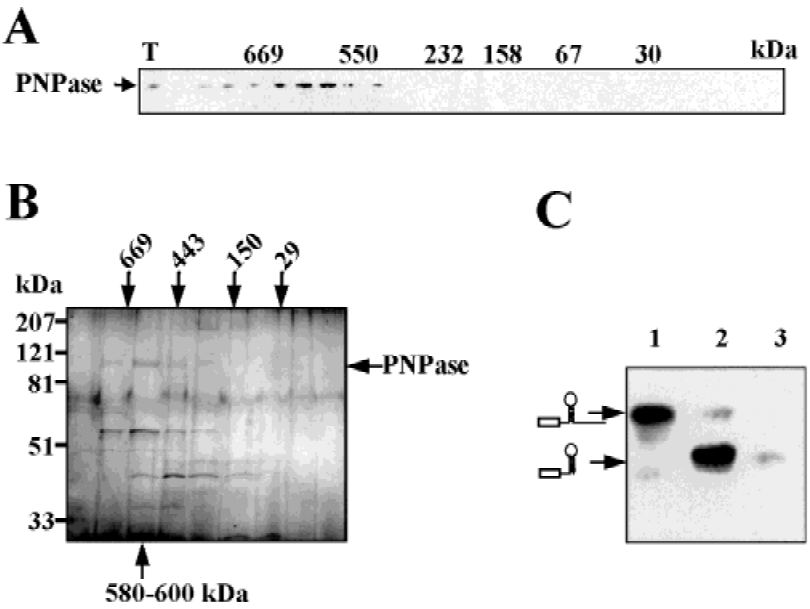

FIGURE 1. Purification of the PNPase complex. **A:** Chloroplast soluble proteins were fractionated by Superdex-200 size exclusion chromatography and the fractions analyzed by immunoblot using specific antibodies to PNPase. Conditions were as described in Materials and Methods; Buffer E (pH 7.9) containing 60 mM KCl was used throughout the size fractionation procedure. **B:** The PNPase was purified by a combination of ion exchange (EconoQ) and single-strand DNA chromatography as described in Materials and Methods. PNPase fractions eluted from the ssDNA column were dialyzed in Buffer E containing 60 mM KCl and then applied to Superose 6 size exclusion chromatography under the same buffer conditions used for the dialysis. Proteins in individual fractions were analyzed by silver staining. The migration of the size markers for the size exclusion column are shown on top; the SDS-PAGE markers are shown on the left. **C:** RNA-degradation activity of the purified PNPase complex. [^{32}P]-RNA corresponding to the 3′ untranslated region of the chloroplast gene *petD* was incubated without proteins (lane 1), with chloroplast soluble proteins (lane 2), or with the purified PNPase fraction (marked 580–600 kDa) shown in **B** (lane 3). Following incubation for 45 min in buffer E with the addition of 2 mM P_i, the RNA was purified and analyzed by denaturing TBE gel electrophoresis and autoradiography.

The 580–600-kDa fraction was incubated with an in vitro-synthesized RNA substrate representing the 3′ untranslated region of the chloroplast gene *petD*. The RNA was degraded, indicating that the chloroplast PNPase has maintained its structural and functional integrity during the purification procedures (Fig. 1C). As previously described (Hayes et al., 1996), PNPase pauses at the stem loop structure, but can degrade this structure in the presence of moderate levels of P_i, resulting in the complete degradation of the precursor RNA (Fig. 1C).

Figure 1B shows the protein profile of the final size-exclusion column step. Analysis of the protein bands revealed that most of the proteins present in the 580–600-kDa fractions have elution profiles that differ from PNPase, and therefore are likely not associated with the complex (Fig. 1B). Nevertheless, we next decided to identify the proteins that were included in the 580–600 kDa fractions in order to verify that these are not associated with PNPase to form a degradosome-like complex.

Identification of proteins coeluting with PNPase

The proteins in the 580–600-kDa fractions obtained from the final size-exclusion chromatography were identified by mass spectrometric analysis using n-HPLC μ-ESI MS (see Materials and Methods). The proteins that coelute with chloroplast PNPase were found to be the large subunit of ribulose-1,5-bisphosphate carboxylase, aldolase, and glutamine synthetase (see also Fig. 2A). According to their apparent molecular weight determined by SDS-PAGE, the bands corresponding to these proteins are labeled in Figure 2A with: a: PNPase, b: the large subunit of Rubisco, c: glutamine synthetase, and d: aldolase. All of the proteins we identified are known to form high molecular weight complexes in the chloroplast and, except for PNPase, they are unrelated to proteins reported to form the *E. coli* degradosome.

p67 is related to GroEL

We previously found that PNPase copurified with p67, a protein with a molecular mass of 67 kDa that cross-reacts with an antibody against the *E. coli* RNase E (Hayes et al., 1996). Mass spectrometric analysis of the PNPase-containing protein fractions from the purification scheme described above did not reveal a protein with sequence similarity to *E. coli* RNase E. We therefore decided to purify p67 using a strategy in which PNPase and p67 copurify. The soluble chloroplast protein extract was first fractionated by size-exclusion chromatography on Superdex 200. The 580–600-kDa fraction obtained from this chromatography showed PNPase activity and a p67 protein that cross-reacts to the RNase E antibody (data not shown). Proteins in this fraction were further separated by chromatography on an anion exchange MonoQ column. The fractions that contained PNPase and p67 were analyzed for their protein profile by gel electrophoresis and silver stain-

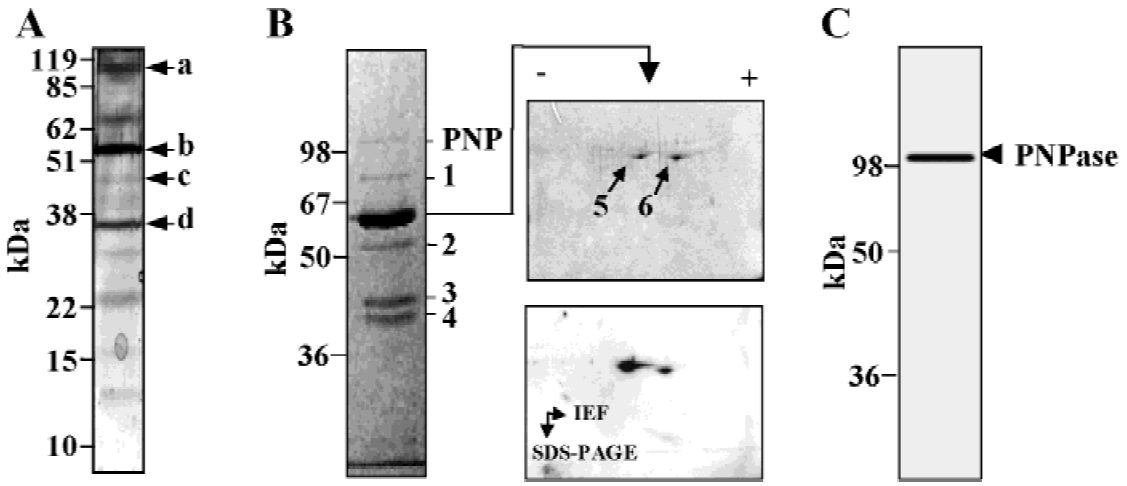

FIGURE 2. Identification of proteins coeluting with PNPase during the purification procedures. **A:** The proteins that were copurified with the PNPase as described in the Materials and Methods section were analyzed by n-HPLC-μESI-MS and are visualized by SDS PAGE and subsequent silver staining. The proteins were identified as: a: PNPase, b: the large subunit of RuBP carboxylase/oxygenase, c: glutamine synthetase, and d: plastidic form of aldolase. **B:** The PNPase was isolated together with the 67-kDa protein that cross-reacts to RNase E antibodies as described in Materials and Methods. Shown here is the silver-stained protein profile of the purified PNPase fraction. The proteins of this fraction were identified using LC-mass spectroscopy. PNP: PNPase, 1: Dna K, 2: the large subunit of Rubisco, 3: Glyceraldehyde 3-phosphate dehydrogenase, and 4: the precursor of Phosphoribulose kinase. The two bands at 67 kDa were further fractionated on two-dimensional PAGE that was silver stained (upper panel). Another two-dimensional PAGE was analyzed by immunoblotting and decoration with antibodies to RNase E of *Synechocystis* (lower panel). These proteins were identified as the α (5) and β (6) subunits of the chaperon GroEL. **C:** The PNPase was purified to homogeneity as described in Materials and Methods. Purified PNPase was analyzed by SDS PAGE and subsequent silver staining.

ing. Figure 2B shows that the MonoQ fraction has five proteins that can be detected at this sensitivity in addition to p67. The protein with an apparent molecular mass of 105 kDa was identified as PNPase using specific antibodies. To identify the proteins shown in Figure 2B, we extracted them from the gel and analyzed them by HPLC μ-ESI MS. The amino acid sequences of their peptides revealed that the proteins correspond to PNPase (105 kDa) and DnaK (75 kDa), the large subunit of RuBP carboxylase-oxygenase (55 kDa), glyceraldehyde 3-phosphate dehydrogenase (41 kDa), and the 40-kDa precursor of phosphoribulose kinase (labeled 1–4 in Fig. 2B, respectively). Because two bands were visible in the range of proteins with the molecular mass of p67, the proteins were further separated by two-dimensional gel electrophoresis (Fig. 2B). After immunoblotting, both spots on the two-dimensional gel that correspond to the p67 bands cross-reacted with antibodies against the *Synechocystis* RNase E (Fig. 2B, lower panel). Identification of the proteins by mass spectroscopy showed, however, that they are the α and β subunits of the chloroplast chaperon GroEL (labeled 5 and 6, respectively, in Fig. 2B; Dickson et al., 2000). Cross-reactivity of GroEL to many different IgG molecules is a well-known phenomenon of this protein (Hajeer & Bernstein, 1993; Sohlberg et al., 1993; Alconada et al., 1994), and may therefore explain the cross-reaction of p67 to the RNase E antibody as well.

Because the PNPase was not purified to homogeneity with the purification protocols described so far, we applied additional purification steps using fractionation on heparin and MonoQ columns (Materials and Methods; Lisitsky et al., 1997; Yehudai-Resheff et al., 2001). Using this protocol, the PNPase was purified to homogeneity and appeared as a single silver-stained band (Fig. 2C). This result reinforces the conclusion that PNPase is not associated with other proteins to form a complex similar to the *E. coli* degradosome. The fraction containing the PNPase purified to homogeneity was fully active in P_i-dependent RNA degradation in vitro (Yehudai-Resheff et al., 2001) indicating that PNPase can catalyze the phosphorolytic RNA degradation without associating with additional protein factors.

Native PAGE analysis of the chloroplast protein complexes

To further substantiate that chloroplast PNPase does not associate with the proteins identified in the 580-kDa fraction (Fig. 1), we analyzed chloroplast protein complexes using blue-native PAGE (Schaegger & von Jagow, 1991). When chloroplast soluble proteins were fractionated on a single-strand DNA column and the bound fraction was analyzed using blue-native PAGE, three protein complexes were visible and clearly separated in the gel (labeled I, II, and III in Fig. 3A). Of these, only complexes I and II were obtained when the 580–600-kDa fraction of the size-exclusion chromatography was analyzed (data not shown). Complex III could be separated from the PNPase complex by size-

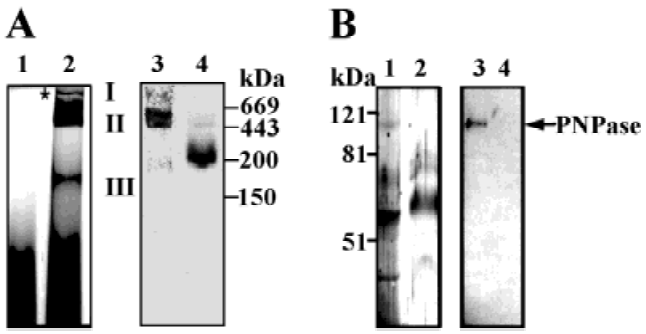

FIGURE 3. Blue-native PAGE analysis of protein complex composition. **A:** The soluble chloroplast protein extract was fractionated by ion exchange and affinity chromatography on ssDNA (see Materials and Methods). The PNPase containing fractions were pooled and analyzed by blue-native PAGE. Lane 1 represents a "no protein" control. Three protein complexes were identified in the ssDNA bound fraction (labeled I, II, and III, lane 2), of which only complexes I and II were observed when this fraction was further purified by size exclusion chromatography (not shown). The presence of PNPase was checked by immunoblotting with PNPase-specific antibodies after running the sample under native (lane 3) or dissociating (1.6 M urea, 10 mM DTT, lane 4) conditions in the blue-native gel system. The native PNPase complex migrates with Rubisco in complex II. **B:** The PNPase-containing fractions after ssDNA chromatography were fractionated on a size exclusion Superose 6 column and the fractions of 580–600 kDa containing the PNPase were analyzed by SDS-PAGE and silver staining (lane 1) and immunoblotting with specific PNPase antibodies (lane 3). The complex I of the blue-native PAGE described in **A** was cut out, eluted from the gel, and subsequently subjected to SDS-PAGE, silver staining (lane 2), and immunoblotting with specific PNPase antibodies (lane 4). Although complex I included the proteins that comigrated with PNPase at 580–600 kDa, it does not contain PNPase, which was found in complex II together with the highly abundant Rubisco complex (**A**).

exclusion chromatography and was therefore not analyzed further (not shown).

To verify the presence of PNPase, we electroblotted the native gel to a nitrocellulose membrane and probed this membrane with a PNPase specific antibody. This analysis revealed that PNPase migrates in complex II together with the highly abundant Rubisco complex (Fig. 3, lane 3). To exclude the possibility that the antibody reaction resulted from the large amount of blue dye bound by the Rubisco complex that was still visible on the nitrocellulose membrane, we dissociated both complexes by adding 1,6 M urea and 10 mM DTT to the protein fraction prior to BN-PAGE. In control experiments, we ascertained that the addition of urea and DTT did not change the migration characteristics of monomeric marker proteins in this gel system (not shown). This treatment resulted in the dissociation of Rubisco and the PNPase complex and led to a clear separation of the subunits in this gel system with the large subunit of Rubisco migrating at 55 kDa and the PNPase migrating at a position close to the 200-kDa marker protein (Fig. 3A, lane 4). Although most of the dye was bound to the large subunit of Rubisco (not shown), the PNPase antibody reacts exclusively with PNPase (Fig. 3A, lane 4). All attempts to elute the proteins present in complex II failed because of protein precipitation during the elution process (not shown). The observed precipitation probably resulted from the high concentration of Rubisco, therefore causing inefficient recovery of PNPase from the gel.

We next attempted to identify the proteins in the 580-kDa fraction obtained after gel filtration. Complex I (Fig. 3A) was excised from the gel and the proteins analyzed by SDS-PAGE and immunodetection with the PNPase antibody. As a control, we included the proteins in the 580–600-kDa PNPase fraction obtained from size-exclusion chromatography. The analysis of the silver-stained gel (Fig. 3B, lanes 1 and 2) and the immunoblot (Fig. 3B, lanes 3 and 4) showed that the blue-native gel complex I contained the proteins that are also detectable in the 580–600-kDa complex. PNPase could not be detected in this complex (Fig. 3B), but was found exclusively in the blue-native gel complex II that also contained the comigrating RuBP carboxylase-oxygenase complex (Fig. 3A). This result confirms that the mixture of proteins present in the 580–600-kDa PNPase complex and identified by mass spectrometry do not form a complex with PNPase. They most likely participate in other protein complexes that elute during size-exclusion chromatography in a molecular mass range similar to that of the 580–600-kDa PNPase complex.

Chloroplast PNPase can form a homo-multimer complex

If the chloroplast PNPase is not associated with other proteins, then it is possible that the enzyme forms a homo-multimer complex composed of several monomers. This view is consistent with the results from the gel filtration (Fig. 1B) and the blue-native PAGE analyses (Fig. 3). To investigate the possibility of a PNPase homo-multimer complex, we expressed chloroplast PNPase in *E. coli*, purified the enzyme to homogeneity (Fig. 4A; Lisitsky & Schuster, 1999) and analyzed its elution profile using size-exclusion chromatography. The recombinant purified protein bound RNA in a UV-crosslinking assay (Fig. 4A,B) and degraded RNA exonucleolytically (Fig. 4C). The degradation activity was stimulated in the presence of P_i ions, a characteristic of phosphorolytic RNA degradation (Fig. 4D). We found maximal RNA-degradation activity in the presence of 20 mM P_i, a value which is in good correlation with the determined in vivo concentration of P_i in the chloroplast (Yehudai-Resheff et al., 2001). When the catalytically active (Fig. 4B–D) recombinant PNPase was fractionated by size-exclusion chromatography, it eluted as a 580–600-kDa complex (Fig. 4E). Similar results were obtained when the purified protein from spinach chloroplasts (Fig. 2C) was analyzed for its elution characteristics in gel filtration (data not shown). To exclude

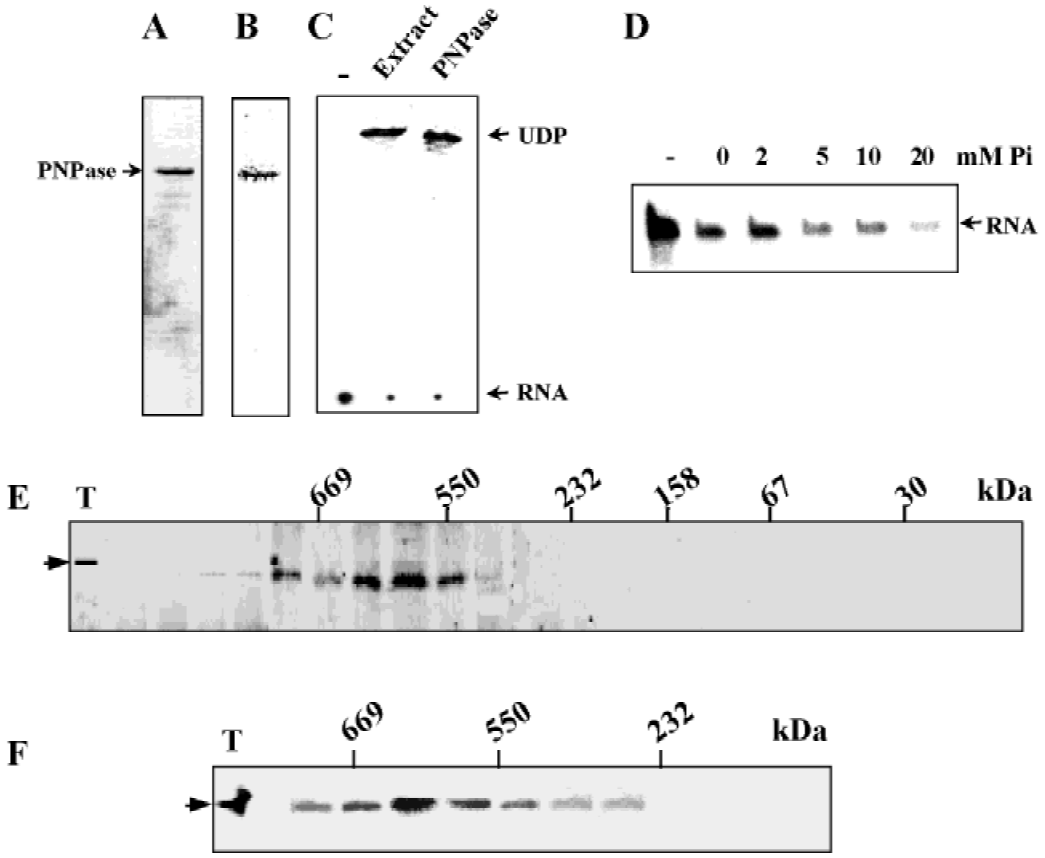

FIGURE 4. Purified recombinant PNPase fractionates on a size exclusion column at 580–600 kDa. **A:** Chloroplast PNPase was overexpressed and purified from bacterial cells using affinity chromatography (Lisitsky & Schuster, 1999). **A** shows a silver-stained gel of the purified protein. **B:** An autoradiograph of a UV crosslinking of the recombinant protein to the *petD* 3′-UTR probe is shown. **C:** The radiolabeled *petD* 3′-UTR probe was incubated without proteins (−), soluble chloroplast protein extract (Extract), or recombinant purified PNPase (PNPase). Following incubation for 16 h, the resulting products were analyzed by TLC chromatography and autoradiography as described (Lisitsky & Schuster, 1999). UDP was identified by a comparison with the migration characteristic of radioactive UDP in the same TLC system. **D:** The recombinant PNPase was incubated with the same RNA for 1 h in the presence of the indicated concentrations of P_i. The lane marked (−) represents a no-protein control. Following incubation, the RNA was purified and analyzed by denaturing acrylamide gel electrophoresis and autoradiography. **E:** The purified protein was applied to a Superdex 200 column using the same buffer and separation conditions as described for the fractionation of the chloroplast proteins in Figure 1. Fractions were analyzed by SDS-PAGE and silver-staining. **F:** To ensure that the purified recombinant chloroplast PNPase was not associated with bacterial RNAs, the protein was extracted from the SDS-PAGE gel, renatured, and fractionated on the size exclusion column. Every two fractions were pooled and analyzed by immunoblotting using specific antibodies. T: Total proteins of the fraction applied to the column. The migration peaks of the protein markers that were fractionated on the same column are indicated at the top.

that the typical PNPase elution characteristic resulted from an association with bacterial RNA molecules or proteins, we performed two control experiments. First, any potential RNA remaining in the 580–600-kDa PNPase fraction was digested with RNase A. Subsequent size-exclusion chromatography did not alter the elution profile of the PNPase complex (data not shown). Second, following fractionation by SDS-PAGE, the PNPase protein band was electroeluted from a gel, renatured, and again subjected to size-exclusion chromatography. Immunoblot detection revealed the same elution peak at 580–600 kDa (Fig. 4F). Unlike the results obtained with the purified or the recombinant PNPase, in this case, the elution profile revealed trailing of the PNPase towards the 300–230-kDa region of the column (Fig. 4F). This can be explained by an incomplete renaturation of the PNPase population electroeluted from the SDS-PAGE, probably resulting in incorrectly folded PNPase monomers that are incapable of forming a homo-multimeric complex. Therefore, a weak trailing of the PNPase elution from the Superdex 200 column towards the low molecular weight range can be observed. Despite of the incorrect folding, the renatured PNPase was active in RNA binding as observed in UV-crosslinking analyses (data not shown). Together, the results presented so far support the conclusion that chloroplast PNPase can form a homo-multimer complex that does not include RNase E or other proteins that are associated with the "degradosome" complex in *E. coli*.

Chloroplast proteins do not bind to a PNPase affinity column

When *E. coli* RNase E was isolated from total-cell proteins by affinity chromatography or immunoprecipitation, the other proteins previously identified as components of the degradosome copurified in this fractionation (Carpousis et al., 1994; Kaberdin et al., 1998). Copurification of several proteins was also observed when the total proteins of *E. coli* were fractionated on an affinity column prepared with *E. coli* PNPase (Fig. 5, lanes 3 and 4). To determine if proteins copurify with chloroplast PNPase, affinity chromatography was performed using recombinant chloroplast PNPase tagged with six histidine residues and bound to an NTA-agarose column. We did not fractionate chloroplast PNPase using immunoprecipitation because our antibodies did not perform efficiently in immunoprecipitation assays. When chloroplast proteins were passed through the NTA-agarose-PNPase affinity column followed by elution of any bound proteins with 1 M KCl, no proteins could be detected in the eluate (Fig. 5, lane 1). Next, chloroplast PNPase was eluted from the NTA-agarose column using 0.5 M imidazole to determine if proteins were bound to PNPase that had not dissociated during the elution with 1 M KCl. The chloroplast PNPase was the major protein in the imidazole fraction together with some very low abundant proteins (Fig. 5, lane 2). These proteins were most likely proteolysis products of PNPase, as they reacted with the PNPase antibody (not shown). We used the same conditions with *E. coli* PNPase coupled to NTA-agarose in an experiment in which we chromatographed proteins isolated from an *E. coli* strain that lacks PNPase. Under these conditions, and unlike what we observed with the chloroplast proteins, several *E. coli* proteins that bound the PNPase affinity column could easily be detected (Fig. 5, lanes 3 and 4), indicating that the experimental conditions are suitable for protein–protein interactions involving PNPase. Together, these results provide strong evidence that chloroplast PNPase does not form a heteromeric degradosome protein complex.

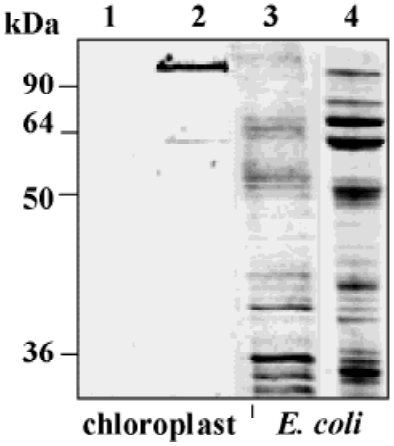

FIGURE 5. Chloroplast proteins do not bind to an affinity column with chloroplast PNPase. His-tagged chloroplast PNPase was expressed in *E. coli* and coupled to an affinity column with NTA-agarose as column matrix. Soluble chloroplast proteins were applied to the column in Buffer E containing 60 mM KCl, followed by extensive washing with the same buffer. Elution of any proteins bound to PNPase was performed with buffer E containing 1 M KCl (lane 1). PNPase and any proteins that had remained bound to PNPase were then eluted with 0.5 M imidazole (lane 2). The eluted proteins were precipitated and analyzed by SDS-PAGE and silver-staining (lanes 1 and 2). As a control for protein complex (re)association conditions, a purified *E. coli* PNPase was used for a similar PNPase affinity column. A soluble protein extract of *E. coli* cells lacking PNPase was fractionated as described above for the chloroplast proteins. Under these conditions, *E. coli* proteins efficiently associate with PNPase and can be eluted from the column with 1 M KCl (lane 3) and 0.5 M imidazole (lane 4). No proteins associated with the chloroplast PNPase under the same conditions.

RNase E-like proteins from photosynthetic organisms lack the C-terminal domain of the *E. coli* protein that serves as the platform for binding degradosome proteins

E. coli RNase E is a large, 1,061 amino acid long endoribonuclease (Cohen & McDowall, 1997; Coburn & Mackie, 1999; Grunberg-Manago, 1999) that provides the platform for the assembly of the degradosome complex (Vanzo et al., 1998; Coburn et al., 1999). The N-terminal domain of the protein (amino acids 1–498), which comprises the catalytic site of RNase E, is sufficient for complementation of *rne* loss-of-function mutants (Vanzo et al., 1998; Coburn et al., 1999). Interestingly, the *E. coli cafA* gene encodes another endonuclease, termed RNase G (Li et al., 1999; Tock et al., 2000), which has strong homology to the N-terminal catalytic domain of RNase E (Fig. 6). RNase G has not been identified as a subunit of the degradosome complex. The C-terminal domain of RNase E was found to be necessary for protein–protein interactions and serves as a platform for assembly of the degradosome (Vanzo et al., 1998). We searched the available databases to identify all sequences that encode proteins related to RNase E. The homology of the proteins we identified was restricted to the N-terminal catalytic domain of *E. coli* RNase E, and no homology was observed in the C-terminal sequences of the proteins (Fig. 6). Moreover, when the *Synechocystis* RNase E was overexpressed in *E. coli*, it was not associated with the degradosome (Kaberdin et al., 1998). Similar results were obtained with expression of genes in *E. coli* encoding RNase E from *Nephroselmis* and *Arabidopsis* (V. Liveanu, V.R. Kaberdin, & G. Schuster, in prep.). Together with the sequence alignment data shown in Figure 6, these results support the biochemical fractionation data and suggest that PNPase in chloroplast and cyanobacteria is not assembled into an *E. coli*-like degradosome complex.

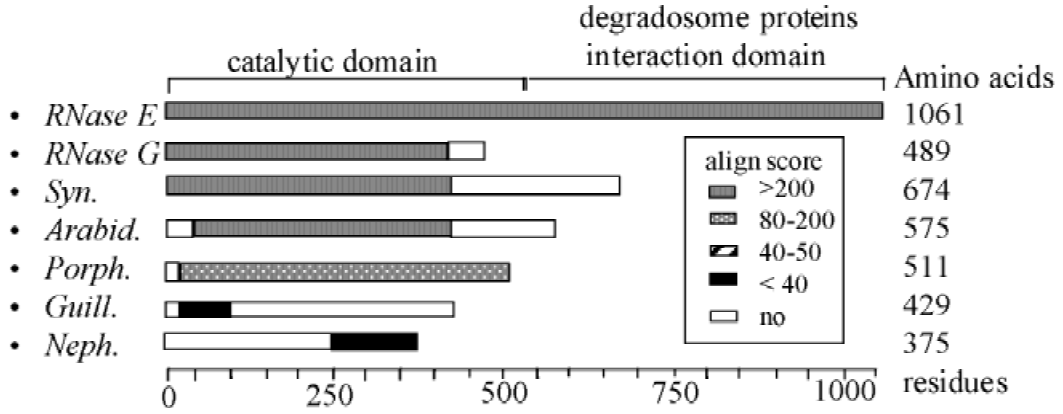

FIGURE 6. Alignment of several RNase E-like proteins from different organisms. The proteins are: 1: *E. coli* (Proteobacteria) RNase E; 2: *E. coli* RNase G; 3: *Synechocystis* PCC6803 (Cyanobacteria); 4: *Arabidopsis thaliana* (higher plant); 5: *Porphyra purpurea* (Rhodophyta); 6: *Guillardia theta* chloroplast (Cryptophyta); and 7: *Nephroselmis olivacea* chloroplast (Chlorophyta). The proteins were aligned for maximum homology using the BLAST program. The score of homology to the *E. coli* RNase E is presented. The N-terminal domain of the *E. coli* RNase E is sufficient for catalytic activity. The C-terminus of *E. coli* RNase E, which is required for the assembly of the degradosome, shares no homology with the RNase E-like proteins from the photosynthetic organisms.

DISCUSSION

Chloroplast PNPase shares significant biochemical properties and amino acid sequence similarity with *E. coli* PNPase, which has been implicated in processing and degradation of mRNAs (reviewed in Hayes et al., 1999). Moreover, chloroplast PNPase can be isolated as a 580–600-kDa protein complex that is similar in molecular mass to the 460–500-kDa *E. coli* "degradosome," a complex of PNPase with other associated proteins (Hayes et al., 1996; Carpousis et al., 1999). It was therefore reasonable to assume that chloroplast PNPase is also part of a multimeric complex of proteins with different functions. We have now purified the chloroplast 580–600-kDa PNPase complex and demonstrate that it contains only the chloroplast PNPase protein. Therefore, the chloroplast PNPase can assemble into a homo-multimer complex of 580–600 kDa. Because we have not found chloroplast PNPase in other complexes of similar or different molecular masses, it is unlikely that this enzyme is assembled with other proteins into an *E. coli*-like degradosome complex.

Recently, the crystal structure of PNPase from *Streptomyces antibioticus* was determined (Symmons et al., 2000). This enzyme forms a structure in which three proteins are assembled into a complex that forms an RNA channel, suggesting a possible mode of processivity in which RNA degradation is perhaps facilitated by structural changes in the RNA as it passes through the channel. A similar association of two trimer complexes was suggested for the *S. antibioticus* PNPase (Symmons et al., 2000). During our work, we addressed the question of the stoichiometry of the PNPase homo-multimeric complex by comparing the molecular weight of the PNPase determined under native and denaturing conditions using the same methods. In gel filtration chromatography, PNPase elutes under dissociating conditions (20 mM DTT) at a molecular weight of approximately 180 kDa (data not shown). A similar experiment was performed with blue native-PAGE. Upon addition of DTT and urea to the samples, the PNPase complex of 550 kDa (Fig. 3A, lanes 3 and 4) could be dissociated to a PNPase monomer that migrates at a position close to the 200-kDa marker protein in this gel system. In both cases, the stoichiometry, defined as native molecular weight divided by denatured molecular weight, is close to 3, indicating a trimeric complex (3.2 for gel filtration and 2.75 for BN-PAGE). However, the accuracy of these methods for molecular weight determination is limited. More reliable information about the stoichiometry of chloroplast PNPase will be available only after the enzyme has been crystallized.

In *E. coli*, a subset of the PNPase population is associated with RNase E, the RNA-helicase Rhl B, and the glycolytic enzyme enolase in a high molecular weight complex that was termed "degradosome" based on its nucleolytic properties (Carpousis et al., 1999; Coburn & Mackie, 1999; Grunberg-Manago, 1999; Regnier & Arraiano, 2000; Steege, 2000). Current models suggest that degradation of mRNAs in *E. coli* and the chloroplast is initiated by endonucleolytic cleavage followed by polyadenylation by poly(A)-polymerase in *E. coli* and PNPase in the spinach chloroplast (Yehudai-Resheff et al., 2001). Exonucleolytic digestion of the polyadenylated cleavage products then proceeds in the 3′ to 5′ direction (Carpousis et al., 1999; Coburn & Mackie, 1999; Hayes et al., 1999; Schuster et al., 1999; Regnier & Arraiano, 2000). Thus, the association of exo- and endoribonucleases in a single complex could increase the efficiency of the degradation process and prevent

accumulation of the endonucleolytic cleavage products. In addition, RNA-helicase in vitro facilitated PNPase activity by removing secondary structures (Py et al., 1996; Coburn et al., 1999).

However, the recent observation that *E. coli* cells that express RNase E from which the C-terminal domain was deleted are viable (Lopez et al., 1999; Ow et al., 2000) questioned the necessity of the degradosome complex for the degradation and processing of RNA in *E. coli* under in vivo conditions. This observation, together with the results reported here that RNA processing and degradation in the chloroplast does not require a degradosome-like complex, suggests the possibility that in *E. coli*, the degradosome complex has a specific rather than a general role in the processing and degradation of RNA.

Our results show that PNPase as the major exoribonuclease in chloroplasts is assembled into a homomultimer complex. Although the molecular mechanism of RNA degradation seems to be very similar in the chloroplast and *E. coli*, two significant differences were recently observed. In addition to the lack of the degradosome complex reported in this work, it was recently shown that chloroplasts lack poly(A)-polymerase. Thus, polyadenylation of RNA during degradation is performed by PNPase, which can function both as a polymerase and a phosphorylase (Yehudai-Resheff et al., 2001). PNPase also has polyadenylation activity in *E. coli* cells that lack poly(A)-polymerase (Mohanty & Kushner, 2000).

At present, it is difficult to reconcile why RNA degradation in *E. coli* involves a degradosome complex and poly(A)-polymerase while chloroplasts lack these functions. One possible reason could be significant differences in the half-life of mRNAs, which in *E. coli* can be as short as a few minutes (Regnier & Arraiano, 2000), whereas in chloroplasts, mRNAs are stable for many hours (Klaff & Gruissem, 1991; Mullet, 1993; Hayes et al., 1999). Further studies on the molecular mechanisms of RNA degradation in bacteria and organelles will provide answers to these questions.

MATERIAL and METHODS

Purification of the PNPase from chloroplasts

Purification of the PNPase was performed as previously described with modifications (Gruissem et al., 1986; Hayes et al., 1996; Baginsky & Gruissem, 2001). Chloroplasts were isolated from 5-week-old hydroponically grown spinach plants and purified by centrifugation in Percoll gradients. Soluble chloroplast proteins were isolated as previously described (Gruissem et al., 1986) and dialyzed against Buffer E (20 mM HEPES/KOH, pH 7.9, 60 mM KCl, 12.5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, and 5% glycerol). Proteins were first purified by ion-exchange chromatography on EconoQ (Bio-Rad). Bound proteins were eluted with a linear KCl gradient (60 mM–1 M) in Buffer E. Fractions were analyzed for RNA-binding proteins by UV crosslinking (Baginsky & Gruissem, 2001) and PNPase-containing fractions were pooled and dialyzed against Buffer E containing 5 mM KCl. RNA-binding and single-strand DNA-binding proteins from the EconoQ fraction were affinity purified by chromatography on single-strand DNA (ssDNA) cellulose (3.5 mg ssDNA/g cellulose; Sigma). Bound proteins were eluted with a linear KCl gradient in Buffer E (5 mM–1.0 M) and analyzed for RNA-binding proteins as described (Baginsky & Gruissem, 2001). Fractions containing PNPase were pooled, dialyzed against Buffer E (60 mM KCl) and subjected to size exclusion chromatography on a size-calibrated Superose 6 column (Pharmacia) in Buffer E containing 60 mM KCl. Individual fractions were analyzed by gel electrophoresis and silver staining of the gel (Merril et al., 1983), and RNA-binding activities and the presence of PNPase were verified by immunodetection using appropriate antibodies. In some experiments, fractionation of chloroplast soluble proteins or purified PNPase was performed using superdex 200 size exclusion column (Figs. 1 and 4). Size calibration of both columns was based on the following marker proteins: thyreoglobulin, 669 kDa; RubP-carboxylase, 550 kDa; ferritin, 443 kDa; aldolase, 150 kDa; chymotrypsinogen A, 29 kDa.

Purification of a 67-kDa protein reacting with an *E. coli* RNase E antibody

The chloroplast soluble protein extract was dialyzed in Buffer E and fractionated on a Superdex 200 size exclusion column in Buffer E containing 60 mM KCl. The fractions containing PNPase were pooled, applied to a MonoQ column, and bound proteins were eluted with a linear KCl gradient developed in Buffer E. PNPase eluted at a salt concentration of 330 mM KCl, together with a 67-kDa protein that cross-reacted with an RNase E antibody. The PNPase and the 67-kDa protein containing fractions were dialyzed and used for protein identification. The chloroplast PNPase was further purified to homogeneity as described before (Lisitsky et al., 1997; Yehudai-Resheff et al., 2001).

Protein analysis and identification by mass spectrometry

Proteins from liquid chromatography fractions were identified using mass spectrometry (MS). Solution samples were treated with trypsin (Promega, Madison, Wisconsin; 2 μg, room temperature overnight) directly without further sample processing. The digestion was quenched by addition of glacial acetic acid to produce the final sample. Approximately 1/100 of the solution sample was subjected to mass analysis. Gel samples were digested in gel using a modification of Wilm et al. (1996). Briefly, gel slices were reduced, alkylated (50 mM DTT, 51 °C 1 h, 100 mM iodoacetamide, room temperature, 45 min), and equilibrated in buffer (100 mM ammonium bicarbonate, pH 8). Gel slices were then dehydrated and swelled in digestion buffer (12.5 ng/μL trypsin, 100 mM ammonium bicarbonate, pH 8) on ice for 45 min. Excess digestion buffer was removed, 10 μL 100 mM ammonium bicarbonate was added, and the digest was allowed to proceed overnight at room temperature. Extraction was accomplished by alternating three hydration (100 mM ammonium bicarbonate) and

dehydration (50% acetonitrile, 5% formic acid) steps, followed by two more hydration/dehydration (100% acetonitrile) steps. The pooled final extract was lyophilized to ~5 μL and diluted with 20 μL 0.1% acetic acid. Approximately one-fifth of the extract was used for mass analysis.

Microsequencing of the samples was accomplished using nano-HPLC micro-ESI MS (HP 1100 separation system, Agilent, Palo Alto, California; LCQ ion trap MS, Finnigan, San Jose, California) as described previously (Martin et al., 2000). HPLC gradients were 0–70% (linear) acetonitrile in 0.1 M acetic acid in 30 min for gel samples, 0–70% (linear) in 1 h for solution samples. The ion trap MS was set to operate in automatic data-dependent acquisition mode using five MS/MS scans for each MS scan. MS instrument settings were as described in Shabanowitz et al. (2000). TurboSEQUEST (Finnigan, San Jose, California), a database searching algorithm, was used to identify the MS/MS data. Manual spectral interpretation was used as necessary to verify database hits or identify novel peptide sequences. Additionally, individual proteins purified by gel electrophoresis were also identified using MS. Following gel destaining (one- or two-dimensional), the proteins were carboxymethylated "in gel" using 100 mM iodoacetamide. The gel was then further destained in 50% acetonitrile with 100 mM ammonium bicarbonate, cut into small pieces, and dried. The gel was rehydrated with 10 mM ammonium bicarbonate, pH 7.4, containing modified trypsin (Promega). After overnight incubation at 37 °C, the resulting peptides were eluted from the gel pieces with 60% acetonitrile and 0.1% TFA, and analyzed by MS/MS. The peptides were resolved by HPLC on a 1 × 150-mm C-18 column (Vydac), eluted with a linear gradient of 4 to 65% acetonitrile in 0.025% TFA, at 1%/min and a flow rate of 40 nL/min. The flow was postcolumn split: about 30% of the sample was microsprayed directly from the HPLC column into an electrospray ion trap mass spectrometer (LCQ, Finnigan, San Jose, California), and 70% was collected manually for a biological assay. MS analysis was carried out in the positive ion mode, using a full MS scan followed by MS/MS on the most dominant ion selected from the first MS scan. MS and MS/MS data were analyzed using TurboSEQUEST. If the protein was not identified in this approach, two or three peptides were sequenced on a Peptide Sequencer (Procise, Perkin Elmer).

Native-PAGE analysis of protein complexes

Subunit composition of protein complexes was also analyzed by blue-native PAGE (Schaegger & von Jagow, 1991). This method is based on a charge shift in a protein complex by binding to a negatively charged Coomassie dye. Fifty microliters of the chloroplast protein fraction were incubated with 12.5 μL of a buffer containing 300 mM Tris-HCl, pH 7, 10 mM DTT, 5 mM EDTA, 3.75% Serva Blue G dye for 20 min at room temperature. Fractions were subsequently loaded onto a native polyacrylamide gel [5% acrylamide (30/0.8), 0.5 M Tris-HCl, pH 7] and electrophoresed at 70 V for 3 h. The electrophoresis buffer contained 25 mM Tris and 192 mM glycine; the cathode buffer also contained 0.02% Serva Blue G. Protein complexes that were visible as a dark blue band on a blue background were excised from the gel. The gel slice was incubated in SDS sample buffer (4% SDS, 5% 2-mercaptoethanol, 60 mM Tris-HCl, pH 6.8) at 30 °C for 20 min and subsequently subjected to SDS-PAGE. Proteins were visualized by silver staining or analyzed by protein blot analysis and immunodetection with a PNPase specific antibody (Hayes et al., 1996). Denaturation of protein complexes prior to BN-PAGE analysis was achieved by adding 1.6 M urea (from an 8 M urea stock solution) and 10 mM DTT (from a 1 M stock solution) to the 50-μL fractions. Following the addition of DTT and urea, samples were treated as described for the native analysis (see above). For the protein blot analysis, the native gel was equilibrated in electrotransfer buffer (40 mM glycine, 50 mM Tris, and 0.04% SDS) for 15 min at room temperature before electroblotting in a semidry blotting apparatus (Biorad).

PNPase affinity column chromatography

For expression of PNPase in *E. coli*, the cDNA without the chloroplast transit peptide was cloned into the expression vector pet20 (Novagen Inc.). Expression and purification were performed according to the manufacturer's protocol with the addition of a purification step on a MonoQ column (Pharmacia) following the NTA-agarose affinity column. The recombinant protein was analyzed for RNA-binding and degradation activity as described before (Lisitsky & Schuster, 1999; Yehudai-Resheff et al., 2001). For further purification by extraction of the PNPase protein from SDS-PAGE gels, the protein was electroeluted from the gel and renatured as described (Cormack et al., 1993). Chloroplast and bacterial PNPases, with the addition of six histidines at the C-terminus, were expressed in bacteria and purified as described above. Soluble bacterial proteins were prepared from 50 mL of overnight culture of ENS134-3 cells (pnp::Tn5; Lopez et al., 1999). The cells were broken using a French-press, incubated for 1 h at 4 °C with 20 U of DNase I and 100 μg of RNase A and cleared by centrifugation. One hundred micrograms of the *E. coli* PNPase was incubated with 20 mg of bacterial soluble proteins for 1 h at 4 °C, followed by the addition of 0.5 mL NTA-agarose for one more hour of incubation. The material was applied to a column that was extensively washed with buffer E. The proteins bound to the PNPase were eluted with buffer E containing 1 M KCl, and the PNPase with the proteins remaining following this elution step were eluted with 0.5 M imidazole. The eluted proteins were precipitated with cold acetone and analyzed by SDS-PAGE. The chloroplast PNPase affinity column was constructed in the same way using the chloroplast PNPase and chloroplast soluble proteins.

Production of PNPase and RNase E-specific antibodies

The production of specific antibodies to the chloroplast PNPase has been previously described (Hayes et al., 1996; Lisitsky et al., 1997). For the production of antibodies to the RNase E of *Synechocystis* sp., the corresponding amino acid coding region was amplified by PCR from genomic DNA and cloned into the pRSET expression vector (Invitrogen). The protein was overexpressed and purified on a NTA-agarose affinity column using the His_6-tag group attached to the protein according to the manufacturer's protocol. Immunization and antibody production was performed as previously described (Lisitsky et al., 1997)

Preparation of synthetic RNAs and in vitro processing/degradation analysis

Plasmids used for in vitro transcription of chloroplast *psbA* and *petD* mRNA 3′-end substrates have been previously described (Stern & Gruissem, 1987; Lisitsky et al., 1997). RNA was transcribed with T7 RNA polymerase (Lisitsky et al., 1994; Baginsky & Gruissem, 2001) and radioactively labeled with [α-^{32}P]UTP to a specific activity of 8×10^3 cpm/fmol for the degradation assays or 8×10^4 cpm/fmol for the UV cross-linking. The full-length transcription products were then purified on 5% denaturing PAGE (Baginsky & Gruissem, 2001) and used in processing and degradation assays. The products of the RNA degradation assays were analyzed either by 5% denaturing PAGE or by TLC chromatography (Lisitsky & Schuster, 1999). In the TLC assay, only nucleotides migrate on the TLC plate while the RNA and RNA fragments remain at the loading point.

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