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Expression, purification and characterization of the recombinant ribonuclease P protein component from *Bacillus subtilis*

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ABSTRACT

Ribonuclease P is a ribonucleoprotein complex that catalyzes the essential 5′ maturation of all precursor tRNA molecules. The protein component both alters the conformation of the RNA component and enhances the substrate affinity and specificity. To facilitate biochemical and biophysical studies, the protein component of *Bacillus subtilis* ribonuclease P (RNase P) was overproduced in *Escherichia coli* using the native amino acid sequence with the initial 20 codons optimized for expression in *E.coli*. A simple purification procedure using consecutive cation exchange chromatography steps in the presence and absence of urea was developed to purify large quantities of P protein without contaminating nucleic acids. The identity of the recombinant protein as a cofactor of RNase P was established by its ability to stimulate the activity of the RNA component in low ionic strength buffer in a 1:1 stoichiometry. Circular dichroism studies indicate that P protein is a combination of α-helix and β-sheet secondary structures and is quite stable, with a T_m of 67°C. The described methods facilitated the large scale purification of homogeneous, RNA-free P protein required for high resolution crystallographic analyses and may be useful for the preparation of other RNA binding proteins.

INTRODUCTION

Ribonuclease P (RNase P) is essential for tRNA maturation, converting a primary precursor tRNA (pre-tRNA) transcript to a functional tRNA molecule. This enzyme is responsible for specific cleavage of the 5′ leader sequence from all cellular pre-tRNAs (reviewed in 1–4) as well as precursors of 4.5 S RNA (5) and 10 Sa RNA (6). RNase P, a ubiquitous enzyme found in all cells of bacteria, archaea and eucarya, is a ribonucleoprotein complex (3). In eubacteria, RNase P consists of a large RNA of ~400 nt (encoded by the *rnpB* gene) and a small basic protein of ~120 amino acids (encoded by the *rnpA* gene). The RNA component alone (P RNA) is an efficient and accurate catalyst *in vitro* in the presence of high concentrations of monovalent and divalent cations (7,8); however, the protein subunit is essential for physiological activity (9,10). In contrast, no catalytic activity has yet been reported for non-bacterial enzymes in the absence of the protein component.

Extensive biochemical characterization of the RNA component combined with phylogenetic analyses and crosslinking data have established the secondary structure of the RNA component and have been used to model the tertiary structure of RNase P RNA and its interaction with pre-tRNA (reviewed in 1–4,11–13). In contrast, the function and structure of the protein component has only recently been studied extensively (14–21). A recent kinetic study comparing the cleavage activity and substrate affinity of RNase P holoenzyme with P RNA alone at identical salt concentrations (10 mM $MgCl_2$, 100 mM NH_4Cl) indicates that the main role of the protein component is to specifically enhance affinity for the pre-tRNA substrate without increasing affinity for the tRNA product (19,21). Additionally, the protein component may influence the substrate specificity (22–24) and stabilize the folded structure of RNase P RNA (11,14,17,25).

The amino acid sequence of the RNase P protein does not indicate the presence of any previously identified RNA binding motifs, such as the ribonucleoprotein (RNP) motif, the K-homology (KH) motif or the double-stranded RNA binding domain (dsRBD) (26,27). Additionally, alignment of the protein sequences from 20 bacterial sources (28) reveals no substantial consensus sequences except for an ~18 residue putative RNA recognition motif (RNR motif) containing a central $KX_{4-5}AX_2RNX_2$(K,R)-RX_2(R,K) segment flanked by additional basic residues (20). However, these diverse bacterial RNase P proteins can be functionally equivalent (7,29–31). To explore these interesting properties and to facilitate biochemical and biophysical characterization of the protein component of *Bacillus subtilis* RNase P (P protein) and the holoenzyme, it is essential to have a convenient source of large quantities of pure P protein. Although the protein components of *Escherichia coli* and *B.subtilis* have been used for biochemical studies, so far only the *E.coli* protein (C5 protein) has been overproduced (14,32). Recently, the protein component of RNase P from the cyanobacterium *Synechocystis* has also been expressed in *E.coli* (31). The low abundance of RNase P protein in the cell and the difficulty of purifying this enzyme from the natural source (33) prompted us to develop a method to overexpress the native sequence of the

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B.subtilis RNase P protein component. The *rnpA* gene was amplified from genomic DNA using PCR and this fragment was subcloned into a T7 polymerase-based expression vector (34). Using this expression system we have produced functionally active *B.subtilis* P protein in *E.coli* (80 mg/l culture), developed methods to purify this protein to homogeneity and initiated preliminary biophysical characterizations.

MATERIALS AND METHODS

Materials

Bacillus subtilis strain 168 was a generous gift from Dr Norman Pace (University of Berkeley, CA). Restriction endonucleases, calf intestinal alkaline phosphatase, T4 polynucleotidyl kinase and T4 DNA ligase were from New England Biolabs, Life Technologies or Boehringer Mannheim. CM-Sepharose and nucleoside triphosphates were from Pharmacia Biotech. Radiochemicals [γ-^{32}P]ATP and [α-^{35}S]dATP were purchased from ICN Radiochemicals. T7 RNA polymerase was purified from the overexpressing strain provided by W.Studier (35). Oligonucleotides were synthesized by the DNA Synthesis Facility at Duke University. Plasmid preparation, bacterial transformation, enzymatic manipulation of DNA and PCR DNA amplification were performed according to standard procedures (36). The *E.coli* strains JM83 and BL21(DE3)pLysS (34,37) were used for cloning and overexpression respectively.

PCR amplification and cloning of the *rnpA* gene

Genomic DNA was isolated from *B.subtilis* strain 168 as described previously (38). The *rnpA* gene coding for the protein component of RNase P was generated by PCR amplification using genomic DNA as a template. Two complementary 66mer oligonucleotides encoding the beginning of the gene were chemically synthesized (see Fig. 1) containing an *Nco*I site at the 5′-end and half of a *Pme*I site at the 3′-end, phosphorylated and annealed (heating to 95°C followed by slow cooling to room temperature). The remainder of the gene (61–360 nt) was amplified from the *B.subtilis* genomic DNA using a 5′ primer (5′-GAAAGTGTTTAAACATGGG, *Pme*I site underlined) and a 3′ primer (5′-CATGCCATGGTTATTTGGAGGAAGATTTCTT-ATATAATGAAG, with a stop codon followed by an *Nco*I restriction site, underlined). Twenty five cycles of PCR amplification using *Pfu* DNA polymerase (2.5 U) were carried out in a 50 µl reaction containing 20 mM Tris–HCl, pH 8.75, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 100 µg/ml BSA, 20 µM dNTPs, 100 ng each 5′ and 3′ primers and 5 ng genomic DNA template. Each cycle consisted of 94°C for 30 s, 42°C for 45 s and 72°C for 45 s, followed by a final extension at 72°C for 10 min. The amplified DNA fragment was digested with *Pme*I and ligated to the annealed oligonucleotide fragment. The resulting fragment was digested with *Nco*I and then cloned into the pET-8c vector (39) at the *Nco*I site. The nucleotide sequence and the orientation of the *rnpA* gene was confirmed by dideoxy DNA sequencing (40) using Sequenase 2.0 (Amersham). The resulting plasmid was designated pPWT1.

Expression and purification of P protein

Escherichia coli BL21(DE3)pLysS cells (34,37) were transformed with plasmid pPWT1. A single colony was inoculated into 10 ml LB medium containing 100 µg/ml ampicillin and 17 µg/ml chloramphenicol and grown overnight at 37°C. The overnight culture was diluted (1:100) into 1 l LB medium containing the same antibiotics and incubated at 37°C until the OD_{600} was 0.7–0.8. Expression of P protein was induced by addition of 0.4 mM isopropylthio-β-D-galactopyranoside (IPTG) and the culture was incubated for an additional 4–5 h at 37°C. The cells were harvested by centrifugation at 4°C (4000 *g*, 20 min) and resuspended in 50 ml lysis buffer [50 mM Tris–HCl, pH 8.0, 5 mM EDTA and 10% glycerol plus one tablet of Complete™ protease inhibitor (Boehringer Mannheim)] per liter of culture broth. The cells were then lysed by passing the cell suspension twice through a French pressure cell at 12 000 p.s.i.

Purification of P protein in urea (Method 1)

An equal volume of 2× buffer A (1× buffer A, 50 mM sodium acetate, pH 6.5, 5 mM EDTA, 0.1 M NaCl and 5 M urea) was added to the cell free lysate and incubated at room temperature for 1 h. The resulting lysate was diluted 2-fold with buffer A and applied to a CM-Sepharose column (5 × 5 cm) equilibrated with buffer A. The column was washed extensively with buffer A until the A_{260} was <0.05. The P protein was then eluted from the column with buffer A containing 1 M NaCl. The presence of P protein was assayed by SDS–PAGE (41) and subsequent staining with Coomassie Brilliant Blue R-250. The fractions containing P protein were pooled and dialyzed three times against a 20-fold excess volume of buffer B (50 mM sodium acetate, pH 6.5 and 5 mM EDTA) containing 0.1 M NaCl. The dialysate was applied to a second CM-Sepharose column (1 × 2.5 cm) equilibrated with buffer B and washed with the same buffer containing 0.25 M NaCl until the A_{280} was <0.05. The P protein was eluted with a linear NaCl gradient (0.25–1.0 M, 50 ml each) in buffer B. After SDS–PAGE analysis, the fractions containing pure P protein were pooled, dialyzed against 10 mM Tris–HCl, pH 8.0, and stored at –20°C in the presence of 50% glycerol.

Purification of P protein without urea (Method 2)

All purification steps were carried out at 4°C. Sodium chloride was added to the cell-free lysate to a final concentration of 1 M and the lysate was clarified by centrifugation (20 000 *g*, 30 min). The resulting supernatant was fractionated further by ammonium sulfate precipitation. Solid ammonium sulfate (0.314 g/ml, 50% ammonium sulfate) was added slowly to the lysate with gentle stirring followed by incubation on ice for 1 h. The precipitate was collected by centrifugation (17 000 *g*, 15 min) and discarded. Additional solid ammonium sulfate (0.21 g/ml, 80% ammonium sulfate) was added to the lysate. After incubating on ice for 1 h, the precipitate was collected by centrifugation (17 000 *g*, 15 min) and the supernatant was discarded. The pellet was dissolved in 2.5 ml buffer B containing 0.5 M NaCl and applied to a Sephadex G-50 gel filtration column (2.5 × 48.5 cm) equilibrated with the same buffer. Elution of the protein from the column was performed at a flow rate of 9 ml/h (2 ml fractions) and the fractions were analyzed by SDS–PAGE. Fractions containing P protein were pooled and diluted with an equal volume of 50 mM sodium acetate, pH 6.5. The diluted fractions were applied to a CM-Sepharose column (1 × 2.5 cm) and chromatographed as described above. The purified P protein was dialyzed and concentrated using Centriprep-3 and stored in aliquots at –80°C.

RNA preparation

The RNA component of *B.subtilis* RNase P and pre-tRNA^{Asp} (with a 33 nt 5′ leader sequence) were prepared by *in vitro* transcription from the linearized plasmids pDW 25 (*Dra*I digest) and pDW 152 (*Bst*NI digest), respectively, using T7 RNA polymerase and purified by denaturing polyacrylamide gel electrophoresis as described previously (42). The RNA concentration was determined by absorbance at 260 nm using the extinction coefficients previously determined by alkaline hydrolysis of the RNA (42). For RNase P RNA, $\varepsilon_{260} = 3.9 \times 10^6$/M/cm and for pre-tRNA^{Asp}, $\varepsilon_{260} = 9.9 \times 10^5$/M/cm.

Reconstitution assay

RNase P activity was monitored by catalysis of the cleavage of a 5′ leader sequence from pre-tRNA^{Asp} (42) in low salt buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$) and high salt buffer (50 mM Tris–HCl, pH 8.0, 100 mM $MgCl_2$ and 800 mM NH_4Cl) in the presence or absence of P protein. Typically, P RNA and pre-tRNA^{Asp} were denatured by incubation at 95°C for 3 min, followed by addition of an equal volume of 2× buffer (low or high salt) and pre-incubation at 37°C for 15 min. To form holoenzyme, the P protein was added to the P RNA after the first 5 min pre-incubation. Reactions were initiated by addition of enzyme to pre-tRNA^{Asp} and incubation was continued at 37°C. For the stoichiometry experiments, the initial rate (≤10% cleavage) of the reaction was measured. At various times the reaction was quenched by adding an equal volume of stop mix (10 M urea, 200 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol). Pre-tRNA^{Asp} was separated from the mature tRNA and 5′ leader sequence on an 8% polyacrylamide gel containing 7 M urea and the products were visualized and quantified using a PhosphorImager (Molecular Dynamics).

Protein quantitation

Protein concentrations were determined either by the dye binding method (43) using bovine serum albumin as standard or by measuring the absorbance at 280 nm. In the latter case, the extinction coefficient at 280 nm in 5 M guanidine hydrochloride has been calculated as 5120/M/cm, based on the amino acid composition of four tyrosines (1280/M/cm/tyrosine) (44). Protein concentrations determined by the dye binding method differed by <5% from those based on the extinction coefficient.

Circular dichroism (CD) spectrum

CD spectra were collected on an Aviv CD spectrophotometer, model 17DS. The protein sample (10 µM purified P protein in 20 mM Na_2HPO_4 buffer, pH 6.5) was repetitively scanned at least five times from 320 to 190 nm in 1 nm increments with a 2 nm bandwidth and a 1 s averaging time. The raw CD signal (in millidegrees), after subtracting the blank signal of the cuvette and buffer, was converted to mean residue ellipticity [θ] using equation **1**, where C is the protein concentration (mM), $n = 118$ (number of residues in the protein) and l is the path length (cm) (45). Thermal melts of the protein were monitored at 222 nm from 4 to 90°C with the temperature raised by 1°C increments, an equilibration time of 30 s and an averaging time of 75 s. The transition curve for thermal denaturation was analyzed using a two-state transition model and the linear extrapolation method. The CD signal (a_{obs}) was fitted as a function of temperature (T) directly using equations **2** and **3** (Gibbs–Helmholtz equation) (46,47) where: a_N and m_N, a_U and m_U are the y-intercept and slope of the pre- and post-transitional baselines respectively; ΔG_U is the free energy of unfolding at any temperature; R is the gas constant (1.987 cal/deg/mol); T is the temperature (in K); T_m is the mid-point of the thermal transition; ΔH_m is the change in enthalpy that accompanies the observed unfolding reaction; ΔC_p is the change in heat capacity accompanying the unfolding reaction. The basic assumption in this analysis is that ΔC_p is independent of temperature. This equation allows a non-linear least squares computer fit (Kaleidagraph; Synergy Software) to all the data and calculation of realistic errors (48). Moreover, the calculated values of T_m and ΔH_m are identical to values obtained from a plot of ΔG (calculated from K_{app}) versus temperature and a van't Hoff plot ($\ln K_{app}$ versus $1/T$).

$$[\theta] = 100(\text{signal})/Cnl \quad \mathbf{1}$$

$$a_{obs}(T) = \{(a_N + m_N T) + (a_U + m_U T)\,(e^{-\Delta G_u/RT})\}/(1 + e^{-\Delta G_u/RT}) \quad \mathbf{2}$$

$$\Delta G_u(T) = \Delta H_m[1 - (T/T_m)] + \Delta C_p[T - T_m - T\ln(T/T_m)] \quad \mathbf{3}$$

Analytical techniques

Mass spectrometric analysis of the purified sample was done at the Mass Spectrometry Facility, Duke University Medical Center. The N-terminal sequence of the recombinant P protein was determined by Edman degradation at Harvard Microchemistry Facility, The Biological Laboratories, Harvard University.

RESULTS AND DISCUSSION

To carry out biophysical experiments designed to probe the functional and structural role of P protein in RNase P-catalyzed cleavage of pre-tRNA, we cloned and overproduced *B.subtilis* P protein in *E.coli* using a T7 RNA polymerase expression system (34). We then purified the P protein using two different procedures (Methods 1 and 2) and demonstrated that the P protein readily refolds to an active structure capable of stimulating the activity of the RNA component. This strategy provides milligram quantities of homogeneous recombinant P protein necessary for structural studies using X-ray crystallography (20) and NMR spectroscopy (R.A.Venters, C.Henkels, S.Niranjanakumari and C.A.Fierke, unpublished data).

Cloning of the *rnpA* gene

Construction of the pPWT1 plasmid harboring the *B.subtilis rnpA* gene is diagramatically shown in Figure 1. Based on the available *rnpA* gene sequence (49), which codes for the P protein of *B.subtilis*, primers were designed to amplify a 300 bp fragment (spanning amino acids 21–119) of the *rnpA* gene using PCR. An oligonucleotide coding for the first 20 amino acids was synthesized to alter the sequence of 13 of 20 rare *E.coli* codons (Fig. 1) in order to enhance the expression of P protein. This oligonucleotide was designed based on computer analysis of the sequence by DNA Strider (a program for DNA and protein sequence analysis designed and written by Christian Marck, Gif-Sur-Yvette, France). The rare codons were replaced with codons that are used frequently in proteins expressed at high levels in *E.coli* (50). The

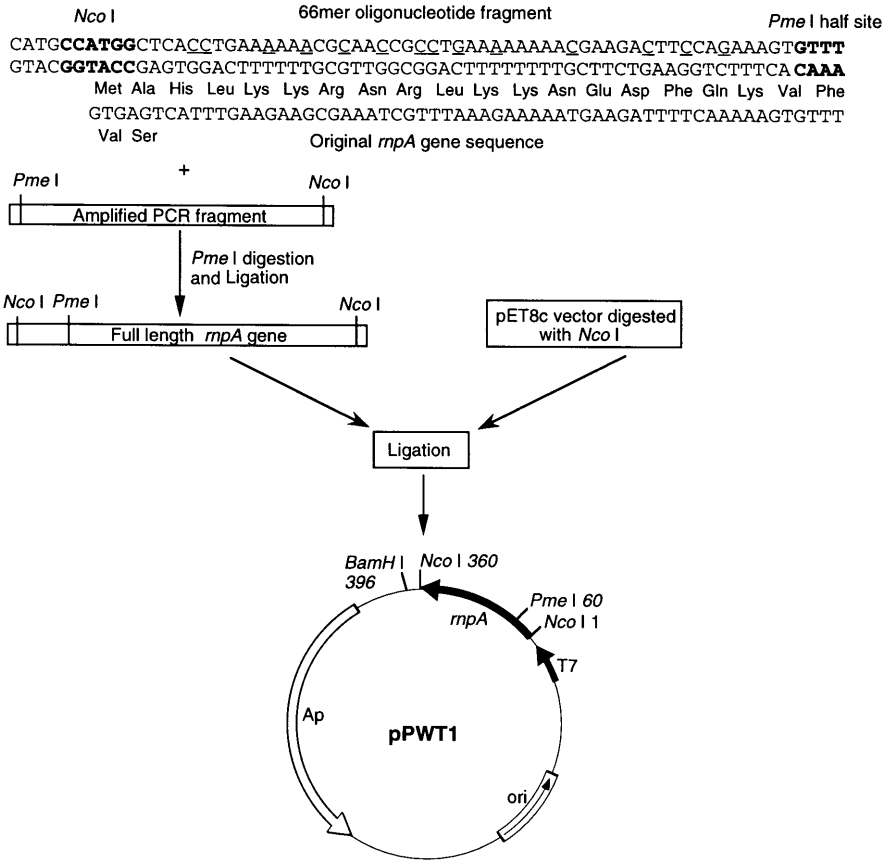

Figure 1. Diagram describing the construction of the recombinant expression plasmid, pPWT1, containing the *B.subtilis rnpA* gene behind a T7 RNA polymerase promoter. The underlined nucleotides represent the altered codons for optimization to *E.coli* codon usage.

full-length gene (360 bp) was obtained by ligating the annealed oligonucleotide fragment and the PCR-amplified fragment and was then inserted into the *Nco*I site downstream of the T7 promoter of the pET-8c vector (34) (for details see Materials and Methods). Since ligation at the *Nco*I site is symmetrical, both the sense and antisense orientation of the gene are possible. The correct orientation was identified by *Pme*I and *Bam*HI restriction endonuclease double digestion, which should give a 400 or 220 bp DNA fragment for the sense or antisense orientations respectively. The sequence of the gene in the resulting plasmid, pPWT1, was confirmed by dideoxy sequencing (40).

Overexpression of P protein in *E.coli*

In the cloning strategy described, transcription of the *rnpA* gene is under the control of a bacteriophage T7 promoter and the translated P protein contains no additional amino acids. To investigate growth conditions for maximal protein production, the P protein was assayed by PAGE after lysing the cells with the endogenous T7 lysozyme and treating with DNase I. No P protein production is observed in *E.coli* BL21(DE3)pPWT1 cells after addition of IPTG (Fig. 2, compare lanes 1 and 2), while the BL21(DE3)pPWT1 cells containing the pLysS plasmid produce a high level of P protein (Fig. 2, compare lanes 3, 4 and 6). The overexpressed protein band migrates at the predicted molecular weight of P protein (14 kDa). This dependence of P protein

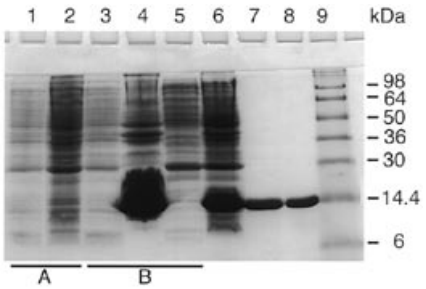

Figure 2. Expression and purification of P protein. SDS–PAGE was carried out on a 15% polyacrylamide gel under reducing conditions (41) and the protein was detected by Coomassie Brilliant Blue R-250 stain. In (A) and (B) the bacterial pellet was resuspended in lysis buffer, lysed with a Dounce homogenizer and incubated with DNase I for 20 min at 37°C. The supernatant and pellet were separated by centrifugation. The samples were boiled in sample buffer (50 mM Tris–HCl, pH 6.8, 0.25% SDS, 0.1 M DTT and 10% glycerol) for 5 min. (**A**) BL21(DE3)/pPWT1: lane 1, uninduced; lane 2, induced with 0.4 mM IPTG for 5 h at 37°C. (**B**) BL21(DE3)pLysS/pPWT1: lane 3, uninduced; lane 4, induced with 0.4 mM IPTG for 5 h at 37°C and the bacterial pellet processed as described above; lane 5, as lane 4 except that the supernatant is shown here; lane 6, supernatant from cells lysed using a French press; lanes 7 and 8, P protein purified by Methods 1 and 2 respectively; lane 9, molecular weight markers (Novex).

production on the presence of the pLysS plasmid suggests that the heterologous gene product may be toxic to *E.coli* cells, since the T7 lysozyme expressed from the pLysS plasmid inhibits T7 RNA polymerase and reduces the basal level of expression in the pre-induction period (37). Although high production is observed under these conditions, almost all of the P protein is present in the pellet after low speed centrifugation (Fig. 2, compare lanes 4 and 5), suggesting that the P protein may aggregate *in vivo* when produced in large quantities. Alteration of the induction conditions, including varied concentrations of IPTG (0.1–2 mM), altered time (0.5–8 h) and lower temperatures (30 and 22°C), did not increase either the yield or the solubility of P protein. However, when the cells are lysed using a French press, the quantity of soluble P protein increases (Fig. 2, lane 6). Therefore, the appearance of P protein in the pellet could be due to adherence of the positively charged P protein to cellular nucleic acids rather than formation of inclusion bodies. Hence, mechanical disruption enhances solubilization.

Purification of P protein

The P protein was purified by both a denaturing and non-denaturing protocol to compare the yield, purity and activity of the protein component. In Method 1, P protein was purified using two consecutive CM-Sepharose columns. The basic isoelectric point of P protein (~10.2, calculated from the amino acid composition) indicated that cation exchange chromatography (CM-Sepharose) should be useful for purifying this protein. For the first column, binding between P protein and nucleic acids was disrupted by the addition of a denaturant, urea, to the cell-free lysate and the column was washed extensively to remove all of the nucleic acids before the protein was eluted. This step was essential for the separation of P protein from cellular nucleic acids; P protein eluted from this column was >90% pure and devoid of nucleic acids, as determined by an A_{260}:A_{280} ratio of 0.4–0.5. The function of urea may be mainly to alter the properties of the bound nucleic acids, since P protein likely does not completely denature under these conditions (data not shown). The urea is subsequently easily removed by extensive dialysis. The second CM-Sepharose column was performed in the absence of urea and was necessary to remove the remaining impurities. This step both purifies (to >98% pure) (Fig. 2, lane 7) and concentrates P protein, since nearly 50 mg P protein can bind to 1 ml gel material.

In order to rule out the possibility that the properties of the P protein were altered by this purification method, a second procedure to purify the soluble P protein in the absence of urea was developed. In this case, extensive purification was achieved using ammonium sulfate precipitations followed by gel filtration and cation exchange chromatography (Fig. 2, lane 8). During cell lysis, the solubility of P protein was enhanced by inclusion of 1 M NaCl, which disrupted interactions with cellular nucleic acids. P protein was then fractionated by two ammonium sulfate precipitations and a Sephadex G-50 column, which removed high molecular weight contaminants and residual ammonium sulfate. Further purification of P protein on a CM-Sepharose column resulted in two peaks, one eluting at 0.4–0.5 M NaCl and the other at 0.6–0.7 M NaCl. The A_{260}:A_{280} ratio of the first peak was >1.6, suggesting significant contamination with nucleic acids, while the ratio of the second peak was 0.4, indicative of the absence of nucleic acids.

Yield of P protein

The yield and the percent protein recovery for each purification method are given in Table 1. The yield of purified P protein obtained by Method 1 was 75–80 mg/l culture (~13% of the total protein), while Method 2 gave a substantially decreased yield at 13–15 mg/l (~2%). The major losses of protein in Method 2 occurred during the ammonium sulfate precipitations and the gel filtration column, likely due to a number of factors, including: (i) precipitation of P protein in the cell lysate; (ii) incomplete precipitation of P protein at 80% ammonium sulfate; (iii) removal of high molecular weight aggregrates of P protein and/or nucleic acids in the Sephadex G-50 column. Despite the differences in the overall yield, both methods provide pure P protein and are improvements over previous methods for the production of recombinant RNase P protein, which resulted in either low yields (<2 mg/l) (14) or a protein with a His tag at the N-terminal end (~16 mg/l) (32). Although the latter preparation has been shown to be functionally active, the six positively charged additional histidines could affect interaction of P protein with the RNA component. Optimization of the first 20 amino acid codons in the *B.subtilis* P protein gene to the *E.coli* codon usage likely enhances high level expression, as has been observed for other proteins (51,52). Because of the simplicity and high yield, Method 1 is an ideal choice for purification of large amounts of P protein, particularly since the refolded protein behaves identically to the soluble native protein (shown later). This strategy may also be applicable to purification of other RNase P proteins or RNA binding proteins.

Table 1. Purification of *B.subtilis* P protein

Fraction	Total protein (mg)[a]	Yield (%)
Method 1		
Cell-free lysate	615	100
Denatured CM-Sepharose	130	21.1
Native CM-Sepharose	79	12.8
Method 2		
Cell-free lysate	620	100
Ammonium sulfate precipitation	125	20
Sephadex G-50 gel filtration	17	2.7
Native CM-Sepharose	13	2.1

[a]Total protein was estimated by the Bradford dye binding assay (43).

Purity and characterization of the P protein

The P protein purified by either Method 1 or 2 was >98% pure as indicated by SDS–PAGE (Fig. 2, lanes 7 and 8) and mass spectrometry analyses (data not shown). On 15% SDS–PAGE the P protein migrated at an apparent molecular weight of 14 kDa, which was further confirmed by electrospray ionization mass spectrometry. The N-terminal sequence analysis established that the first 10 amino acids are Ala-His-Leu-Lys-Lys-Arg-Asn-Arg-Leu-Lys, as coded by the pPWT1 construct. This is identical to the N-terminal sequence reported for the *B.subtilis* RNase P protein component (49) except that the second amino acid is alanine instead of serine (Fig. 1). Both N-terminal sequencing and mass spectrometry reveal the absence of methionine at the N-terminus, which is likely removed by the methionylamino-peptidase of *E.coli* in a post-translational processing step. This enzyme exhibits high activity on polypeptides containing alanine

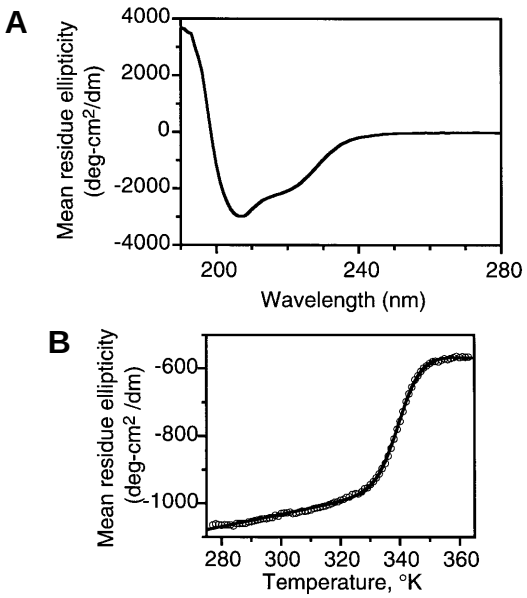

Figure 3. CD spectrum of P protein. (**A**) CD spectrum of P protein (10 μM) taken in 20 mM NaH_2PO_4 buffer, pH 6.5. Data are plotted as molar ellipticity versus wavelength. The protein sample was scanned from 320 to 190 nm in 1 nm increments. (**B**) CD thermal melt of P protein (5 μM) from 4 to 90°C in 1°C increments. The signal was monitored at 222 nm. The solid line shows a non-linear least squares fit (Kaleidagraph; Synergy Software) of the data using equations **2** and **3** with the following values: a_N = –3250 ± 150 cm²/dm, m_N = 3.9 ± 0.4 cm²/dm/deg, a_U = –1470 ± 130 cm²/dm, m_U = 0.9 ± 0.4 cm²/dm/deg, T_m = 339.5 ± 0.5 K, ΔH_m = 64 000 ± 6000 cal/mol and ΔC_p = 1640 ± 220 cal/mol/deg.

or serine in the second position (53). The molecular mass for the N-terminal processed P protein is predicted to be 13.981 kDa, which agrees with the observed value of 13.990 ± 0.010 kDa determined by mass spectrometry. Additionally, mass spectrometry revealed that the purified P protein has a homogeneous chemical structure.

Solubility and stability of P protein

The purified P protein is highly soluble; no precipitation is observed up to 40 mg/ml P protein in either 10 mM Tris–HCl, pH 7.0 or 100 mM KPO_4, pH 7.0. Furthermore, the recombinant P protein is very stable; protein stored at –20°C for 3 years or 4°C for 6 months has the same ability to activate P RNA as a freshly purified preparation. This is in contrast to the *E.coli* protein, which has to be stored in the presence of 7 M urea to reduce precipitation (14). To obtain information about the secondary structure of P protein, we measured the CD spectrum of recombinant P protein (Fig. 3A). This spectrum indicates that the protein is not highly helical and is suggestive of a combination of α-helix and β-sheet secondary structures, consistent with the X-ray crystal structure of the P protein (20). At high temperature, the shoulder observed in the CD spectrum at 222 nm decreases significantly. Therefore, to estimate the thermal stability of P protein we measured the temperature dependence of the appearance of unfolded protein, as indicated by an increase in the mean residue ellipticity at 222 nm (Fig. 3B). The single cooperative transition indicates that this protein preparation does not contain significant concentrations of slowly equilibrating species of different stabilities. Furthermore, these data demonstrate that the P protein is quite stable, with a T_m of 66.5 ± 0.5°C, ΔH_m of 64 ± 6 kcal/mol and ΔC_p of 1.6 ± 0.2 kcal/mol/deg. The values of T_m and ΔH_m are well constrained, while the value of ΔC_p depends on the denatured

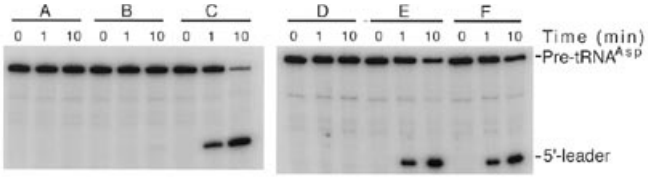

Figure 4. Stimulation of P RNA activity by P protein. Pre-tRNA cleavage activity was assayed by incubating 25 nM P RNA with 250 nM 5′-end-labeled pre-tRNA^{Asp} in low salt buffer (50 mM Tris–HCl, pH 8.0, 5 mM $MgCl_2$) or high salt buffer (50 mM Tris–HCl, pH 8.0, 100 mM $MgCl_2$ and 800 mM NH_4Cl) in the presence or absence of P protein (25 nM). The reactions were incubated at 37°C for the time indicated and an aliquot was quenched with an equal volume of stop mix containing 10 M urea and 200 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. The reaction products were separated on an 8% polyacrylamide gel containing 7 M urea and quantified using a PhosphorImager. The following conditions are shown: (**A**) P protein in low salt buffer; (**B**) P RNA in low salt buffer; (**C**) holoenzyme in low salt buffer; (**D**) P protein in high salt buffer; (**E**) P RNA in high salt buffer; (**F**) holoenzyme in high salt buffer.

baseline, which is not well determined in this experiment. Nonetheless, this value is consistent with the average value of ΔC_p per residue of 14.2 ± 2.5 cal/mol/deg/residue determined for a number of small proteins (54).

Activity of recombinant P protein

In order to determine whether the recombinant P protein retains its function as a cofactor in RNase P-catalyzed pre-tRNA cleavage, we measured the effect of the protein component on the activity of RNase P RNA. In low salt buffer (5 mM $MgCl_2$), RNase P RNA alone is a poor catalyst (Fig. 4B), however, the recombinant P protein enhances cleavage activity by >100-fold (Fig. 4C), while the P protein by itself has no detectable RNase P or nuclease activity (Fig. 4A and D). Therefore, the recombinant P protein does function as a cofactor of RNase P RNA. In high salt buffer (100 mM $MgCl_2$ and 800 mM NH_4Cl) P protein does not significantly stimulate the activity of P RNA (Fig. 4F); the monovalent and divalent cations enhance the activity of RNase P RNA under these conditions (Fig. 4E), as previously observed (7,15). P protein purified by either method behaved identically in stimulating the activity of RNase P RNA, indicating that the protein purified using Method 1 retains the active conformer.

Stoichiometry of P protein binding to P RNA

RNase P RNA forms an equimolar complex with the protein component under physiological conditions (14). To determine the stoichiometry for the recombinant P protein we incubated varying concentrations of P protein (0–250 nM) with a fixed concentration of P RNA (25 nM) that is significantly higher (>50-fold) than the dissociation constant for the protein, so that complex formation is thermodynamically favorable. Since the cleavage activity of the holoenzyme is ~100-fold higher than P RNA at 5 mM Mg^{2+}, we assayed formation of the holoenzyme as an increase in the initial rate of pre-tRNA cleavage. As shown in Figure 5, the initial rate for cleavage under these conditions is linearly dependent on the protein concentration until stoichiometric (25 ± 2 nM) P protein is added. Increasing the protein concentration to a 2-fold excess did not affect the activity of P RNA; however, addition of high concentrations of protein (5-fold excess) inhibits RNase P activity 4-fold (data not shown), likely due to non-specific interactions of the protein with either pre-tRNA or P RNA (14).

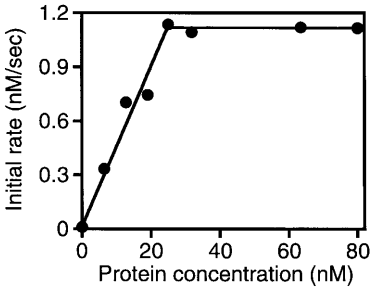

Figure 5. Stoichiometry of P protein and P RNA complex. The initial rate for pre-tRNA^{Asp} (250 nM) cleavage was measured in low salt buffer at 25 nM P RNA, as described in the legend to Figure 4, and increasing concentrations of P protein (0–250 nM). Lines drawn through the initial and final titration points intersect at 25 nM P protein (with an error of 8%). This stoichiometric titration shows that 25 nM P protein is sufficient to completely activate 25 nM P RNA, indicating that the functional holoenzyme is a 1:1 complex of P protein and P RNA.

This experiment demonstrates both that the stoichiometry of the active complex is one molecule of RNA and one molecule of recombinant P protein and that the majority of the P protein molecules in this preparation can form active holoenzyme. Furthermore, for reconstituted holoenzyme the initial rate is 1.12 ± 0.03 nM/s, equal to a turnover number of 0.045 ± 0.01/s, since the pre-tRNA concentration is above the K_m for the holoenzyme (19). This value is comparable with the k_{cat} measured under similar conditions for RNase P holoenzyme reconstituted with P protein partially purified from natural sources (7). Therefore, these data demonstrate that the recombinant P protein is functionally uniform and forms highly active holoenzyme when reconstituted with P RNA.

CONCLUSION

The preparation of highly purified functionally active P protein in large quantities has been essential for detailed analysis of the interaction of the P protein and RNA components in the holoenzyme, as well as biophysical studies, such as NMR and X-ray crystallography (20). These analyses, combined with three-dimensional models of the RNA component (11–13), should provide an enhanced picture of the three-dimensional structure of the holoenzyme and its possible modes of interaction with pre-tRNAs and other substrates.

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