Role of Base G_{-2} of Pre-tRNA^{fMet} in Cleavage Site Selection by *Escherichia coli* RNase P in Vitro

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ABSTRACT: In this study, a protocol for the purification of fully active *Escherichia coli* RNase P holoenzyme from a strain overproducing both the C5 protein and the M1 RNA components is described. A total of 0.8 mg of homogeneous enzyme, with a 1:1 protein/RNA subunit stoichiometry, was recovered from a 1 L bacterial culture. In addition, a convenient and reliable method based on capillary gel electrophoresis was developed to measure initial rates of pre-tRNA maturation by RNase P. Using these tools, the kinetic parameters of cleavage by RNase P of various mutants of pre-tRNA^{fMet} showing maturation defects in vivo [Meinnel and Blanquet (1995) *J. Biol. Chem. 270*, 15906−15914] were investigated in vitro and the locations of cleavage sites were determined from the length of the various products of the reaction. The nucleotide at position −2 of pre-tRNA^{fMet} is shown to be important only in the selection of the cleavage site, whereas it has no role in the efficiency of the reaction. It is concluded that base G_{-2} acts as an antideterminant by preventing an alternative cleavage by RNase P. In addition, the presence of G_{-2} alone is enough to fully compensate for the lack of a G at position +1 of pre-tRNA^{fMet}.

The 5′ maturation of tRNAs and some other RNAs depends on the action of a single and ubiquitous ribonucleoprotein, RNase P (EC 3.1.16.5; see refs *1*−*3* for general reviews). In bacteria, the RNase P holoenzyme consists of a large RNA and a small basic protein in a 1:1 subunit stoichiometry. For instance, the *Escherichia coli* enzyme is composed of a 377 base RNA moiety (M1 RNA) and a 14 kDa protein subunit (C5 protein) encoded by the *rnpB* and *rnpA* genes, respectively.

In the absence of the C5 protein, the RNA component is capable of cleaving pre-tRNA substrates. M1 RNA behaves, therefore, as a catalytic RNA in vitro. Surprisingly, although its product is not strictly required for the catalysis in vitro, the *rnpA* gene is essential for *E. coli* growth (*4*). Indeed, in vitro catalysis by the M1 RNA alone must occur in the presence of high salt concentrations to compensate for the absence of the protein moiety. Under these conditions, the catalytic efficiency of pre-tRNA maturation is still, however, 20−70-fold decreased compared to that of the holoenzyme (*5*, *6*). So far, the exact function of the C5 protein in the catalytic process is not understood fully. As is the case in other analogous systems involving a ribozyme and a protein component (see for instance refs *7*−*10*), most studies generally refer to the idea that the protein moiety might act like a chaperone assisting the proper folding of the M1 component, preventing or resolving misfolded RNA structures (*6*, *11*, *12*). A role of the C5 protein in the facilitation of the release of the product and in the selection of the cleavage site was also proposed (see ref *12* and references cited therein). In addition, the protein cofactor could extend the range of substrates of the enzyme (*13*). Clearly, kinetic and specificity analyses of the RNase P maturation reaction therefore require the use of the holoenzyme. However, such studies have been hampered by the apparent difficulty in obtaining a homogeneous holoenzyme from a bacterial cell extract (*14*−*16*). Usually, the RNase P holoenzyme is prepared by in vitro reconstitution experiments with M1 RNA obtained by in vitro transcription (*17*) and overexpressed and purified C5 protein (*18*).

To understand the enzymatic mechanism of RNase P, most studies have focused on the elucidation of the structural determinants in the precursor tRNAs that govern recognition and cleavage site selection. This led to a definition of the minimal substrate requirements (*19*, *20*). Among these requirements, the composition and length of the amino acid acceptor stem are major determinants in the selection of the cleavage site (*21*). In addition, the identity of nucleotides at and around the cleavage site, especially base G_{+1}, which was suggested to act as a guiding nucleotide, was shown to be involved in the process of cleavage site recognition (*22*−*26*). In this context, maturation of *E. coli* pre-tRNA^{fMet} merits attention because this species differs from most other tRNAs by the absence of a G_{+1} and the occurrence of a C_{+1}/A_{+72} mismatch at the top of the acceptor stem. In a previous paper, we established that a G at position −2 in the 5′-flanking sequence of pre-tRNA^{fMet} compensated in vivo for the absence of G_{+1} in cleavage site selection (*27*).

To investigate the mechanism of recognition and hydrolysis of pre-tRNA^{fMet} by RNase P in vitro, we developed a new protocol for purification of the holoenzyme using an *E. coli* strain in which both the RNA and protein components of RNase P are overexpressed. In addition, to circumvent the use of radioactive material, which is a major drawback

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of the usual analytical methods involving RNAs, an alternative strategy allowing the use of unlabeled RNA and involving capillary gel electrophoresis (CGE)[1] was assessed. CGE proved convenient and reliable at all steps of the study of RNase P, including the preparation of the pre-tRNA sample, the kinetic analysis, and the control of the yield of the purification steps. Using this new assay, we studied the kinetics and mechanism of maturation of a set of pre-tRNA^{fMet} mutants bearing substitutions in their 5′-flanking sequences.

MATERIALS AND METHODS

Materials. All chemicals were purchased from Merck. Enzymes were purchased from Boehringer-Mannheim and Pharmacia. Buffers used were those indicated by the suppliers. Oligonucleotides were synthesized on a Pharmacia Gene Assembler II and purified by anion-exchange chromatography (Mono Q, Pharmacia) as described (*28*).

Pre-tRNA^{fMet} Synthesis and Purification. The secondary structure of pre-tRNA$^{fMet}_{Y}$ is shown in Figure 1. tRNA numbering is as follows: (i) Base +1 refers to the first base at the 5′ end of the mature tRNA; (ii) the bases belonging to the 5′-flanking sequence are numbered from −1 to −8, base −1 being immediately upstream from base +1.

To synthesize pre-tRNA^{fMet} precursors, plasmid pBStRNA$^{fMet}_{Y}$ (*27*) was used as the template in 1 mL PCR reactions using two oligonucleotides, T_1 (5′-TAATACGACTCACTATAGTTTCAGGCGCGGGGTGG) and T_2 (5′-TGGTTGCGGGGGCCGGATTTGAA). T_1 introduced the T_7 RNA polymerase promoter just upstream of the first nucleotide of pre-tRNA$^{fMet}_{Y}$ (*27*), and T_2 was complementary to the CCA terminal end of tRNA^{fMet} up to the 3′ adenosine. Mutant substrates were obtained by using oligonucleotides carrying base substitutions at position −2 and/or +1 of the pre-tRNA (underlined G and C in oligonucleotide T_1) or at position +72 (underlined T in oligonucleotide T_2). The PCR products were purified with Qiagen plasmidprep 20 columns. The purified amplified DNA fragments (50 nM) were incubated for 3 h at 37 °C in the presence of 40 mM Tris-HCl (pH 8.1), 6 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 0.01% Triton X-100, 1 mM each of the four NTPs, and 16 000 U T_7 RNA polymerase in a final volume of 10 mL. The pre-tRNA^{fMet} runoff transcripts (85-mer) were purified on a MonoQ column (1.6 × 5 cm; Pharmacia) as previously described (*29*). A linear gradient from 0.25 to1.25 M NaCl (1.2 M/h; 0.25 mL/min) in Tris-HCl (pH 8.1) was used to separate the full-length products from free nucleotides and abortive transcripts (major products; see also Figure 2). In vitro transcription of precursors labeled with [α-^{32}P]UTP (at a final concentration of 1 Ci/mmol in the reaction) was performed as described above, except that UTP concentration was set at 0.5 mM, in a final volume of 0.15 mL. After phenol extraction and ethanol precipitation, transcripts were purified by electrophoresis on a denaturating 8% polyacrylamide gel. Bands were detected by autoradiography, excised from the gel, and eluted overnight in elution buffer [0.5 M CH_3COONH_4, 0.01 M $(CH_3COO)_2Mg$, 0.1% SDS, 0.1 mM EDTA].

CGE. CGE was performed on a P/ACE system 5500 (Beckman Instruments, Fullerton, CA) equipped with a UV detector set at 254 nm for RNA analysis or with a diode array detector when a protein sample was analyzed. Separation of RNA fragments was achieved using an eCAP ssDNA 100 capillary of 20 cm effective length. Replaceable gels and buffers were those provided in the eCAP ssDNA 100-R kit. Samples were electrokinetically injected (10 kV, 2−8 s) and separated at a constant field strength of 300 V/cm (8.1 kV for a 27 cm capillary) for 50 min at 30 °C.

Purification of the E. coli RNase P Holoenzyme to Homogeneity. JM101Tr cells carrying the pUCrnpAB plasmid (*27*) were used to inoculate four flasks of 2xTY medium (0.5 L) containing 50 μg/mL ampicillin and 0.5 mM IPTG. Cultures were grown overnight at 37 °C, harvested by centrifugation, and resuspended in 40 mL of 50 mM Tris (pH 7.5), 250 mM NH_4Cl, and 10 mM $MgCl_2$ (buffer A). The sample was sonicated, and cell debris was removed by centrifugation. The supernatant was precipitated with 80% ammonium sulfate and centrifuged again. The pellet was redissolved in 10 mL of buffer A, dialyzed against the same buffer to remove ammonium sulfate, and finally applied onto a gel filtration column (Superose 6; 1.6 × 50 cm; Pharmacia) equilibrated in buffer A. The column was eluted at 0.2 mL/min. Fractions containing the RNase P activity were pooled (15 mL), diluted to 40 mL, and applied onto an anion-exchange column (Q-Hiload; 1.6 cm × 10 cm; Pharmacia) equilibrated in 50 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 100 mM NH_4Cl (buffer B). A linear ammonium chloride gradient (0.14 M/h; 2.5 mL/min) was used to elute the sample. The fractions containing RNase P activity (40 mL) were pooled, concentrated with Centriprep 3 concentrators (Amicon) to 10 mL, and loaded onto a Superdex 200 column (60 × 1.6 cm; Pharmacia). The sample was eluted with buffer B at a flow rate of 0.5 mL/min. The fractions containing RNase P activity (18 mL) were pooled and reapplied to the same column to get rid of nucleic acid contaminants of high molecular weight. The column was eluted with buffer A. The fractions containing the homogeneous enzyme were concentrated, dialyzed extensively against buffer B containing 55% glycerol, and stored at −30 °C. A molar extinction coefficient at 260 nm of 2.8×10^6 M^{-1} cm^{-1}, corresponding to a 377 base RNA (120.6 kDa), was used to determine the concentration of the RNase P holoenzyme, knowing that the contribution of the C5 protein component to the absorbance at 260 nm is negligible (4760 M^{-1} cm^{-1}).

RNase P Assay. Various concentrations (7−250 nM) of pre-tRNA$^{fMet}_{Y}$ or its mutant derivatives were prepared in buffer B to give a final volume of 20 μL. Reactions were started by the addition of RNase P (0.01 pmol usually). The sample was incubated for 5−100 min at 37 °C, and the reaction was eventually quenched by adding 1 volume of 20 mM EDTA. Samples were then ethanol precipitated and centrifuged, and the pellet was resuspended in 10 μL of H_2O. Capillary electrophoresis was used to separate the various components of the reaction mixture.

Determination of the Cleavage Sites. Gel-purified pre-tRNAs labeled with [α-^{32}P]UTP were incubated for up to 40 min at 37 °C with RNase P, and the reaction was stopped by adding 1 volume of loading buffer (8 M urea, 10 mM EDTA, 10% glycerol, 0.05% bromophenol blue, and 0.05%

[1] Abbreviations: CGE, capillary gel electrophoresis; EDTA, ethylenediaminetetraacetate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction.

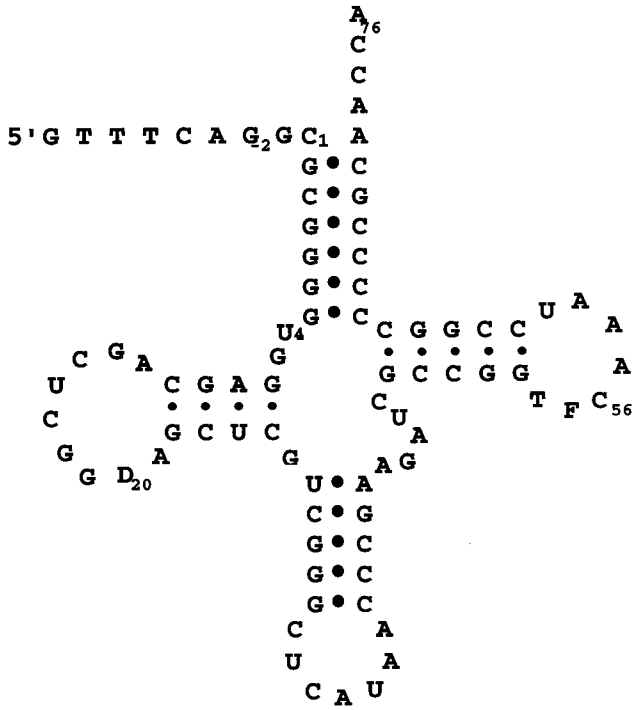

FIGURE 1: Secondary structure of pre-tRNA^{fMet}. The secondary structure of pre-tRNA^{fMet} shown in the cloverleaf representation is depicted. The numbering of each base is indicated (see also Materials and Methods).

xylene cyanol). The reaction products were separated on a 12% polyacrylamide gel containing 7 M urea. The lengths of the 5′-cleavage products were determined using an RNA ladder prepared as follows: a 19-mer RNA oligonucleotide was 5′-end-labeled with [γ-^{32}P]ATP in the presence of T4 polynucleotide kinase and purified on a denaturing 12% polyacrylamide gel. The radioactive band was excised and eluted in elution buffer. After ethanol precipitation, the appropriate quantity of material was subjected to alkaline hydrolysis by incubation at 85 °C for 30 min in 20 μL of 50 mM $NaHCO_3$ (pH 9.0) and 1 mM EDTA and then mixed with an equal volume of loading buffer.

RESULTS

Use of CGE To Assay for RNase P Activity. Recently, capillary electrophoresis on acrylamide gels (CGE) has emerged as a powerful separation method, suitable for nucleic acids analyses (*30*). To determine whether CGE could be used to study the kinetics of maturation of a precursor tRNA by RNase P, we used pre-tRNA$^{fMet}_{Y}$, bearing an eight nucleotide long 5′ flank, as a model substrate (Figure 1). Purified tRNA^{fMet} and unlabeled pre-tRNA^{fMet} prepared from large-scale in vitro transcriptions were subjected to CGE as described under Materials and Methods. Electrophoresis for 50 min proved necessary to elute either species. The two species, when injected together, separated easily with retention times increased by 2 min for the pre-tRNA compared to the matured tRNA. Depending on the gel template, variation could be observed for the retention times of both species (45 ± 1 min for pre-tRNA^{fMet}). As expected, the integration of the absorbance area associated with various tRNA samples was directly proportional to the injected concentration. Finally, CGE proved to be a powerful method

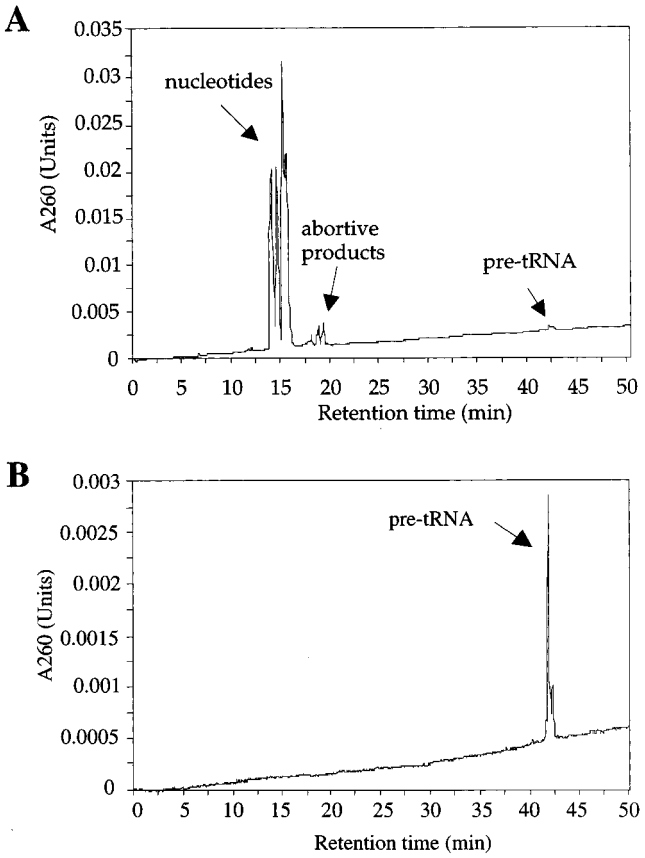

FIGURE 2: Analysis of the pre-tRNA^{fMet} runoff transcripts by CGE: (A) crude runoff product; (B) purified pre-tRNA^{fMet}. The shoulder corresponds to the $n + 1$ species (see text). Pre-tRNA^{fMet} was synthesized by runoff in vitro transcription and purified as described under Materials and Methods. The various separated species of the mixture are indicated by arrows.

for assessing the quality of purification of pre-tRNA species produced by in vitro transcription from nonincorporated nucleotides and abortive transcription products. As shown in Figure 2A, most of the resulting material from an in vitro transcription reaction corresponded to unincorporated nucleotides and abortive products. The anion-exchange purification step freed the sample of this contaminating material (Figure 2B). In addition to the transcription product (85 nucleotides for pre-tRNA^{fMet}), the final product contained the $n + 1$ species, which is always produced by runoff transcription (*31*). However, the occurrence of a supplementary nucleotide on a full-length pre-tRNA was shown not to interfere with the rate of hydrolysis by RNase P (*24*). Finally, since the pre-tRNA was the only species contributing to the measured absorbance in the electropherogram, this experiment allowed us to derive directly the concentration of the purified pre-tRNA^{fMet} sample from the measurement of its absorbance at 260 nm.

We then investigated whether CGE could also be used to follow the kinetics of hydrolysis of pre-tRNA^{fMet} by RNase P. A typical experiment, shown in Figure 3, indicated the utility of this analytical method for separation of RNAs. Interestingly, the fact that we had removed the abortive RNA products allowed us to detect the second product of the maturation reaction, that is, an eight base long RNA fragment (Figure 3A). After quantitative analysis, a time course analysis of pre-tRNA^{fMet} maturation to near-completion was plotted. Initial velocities could easily be measured, proving

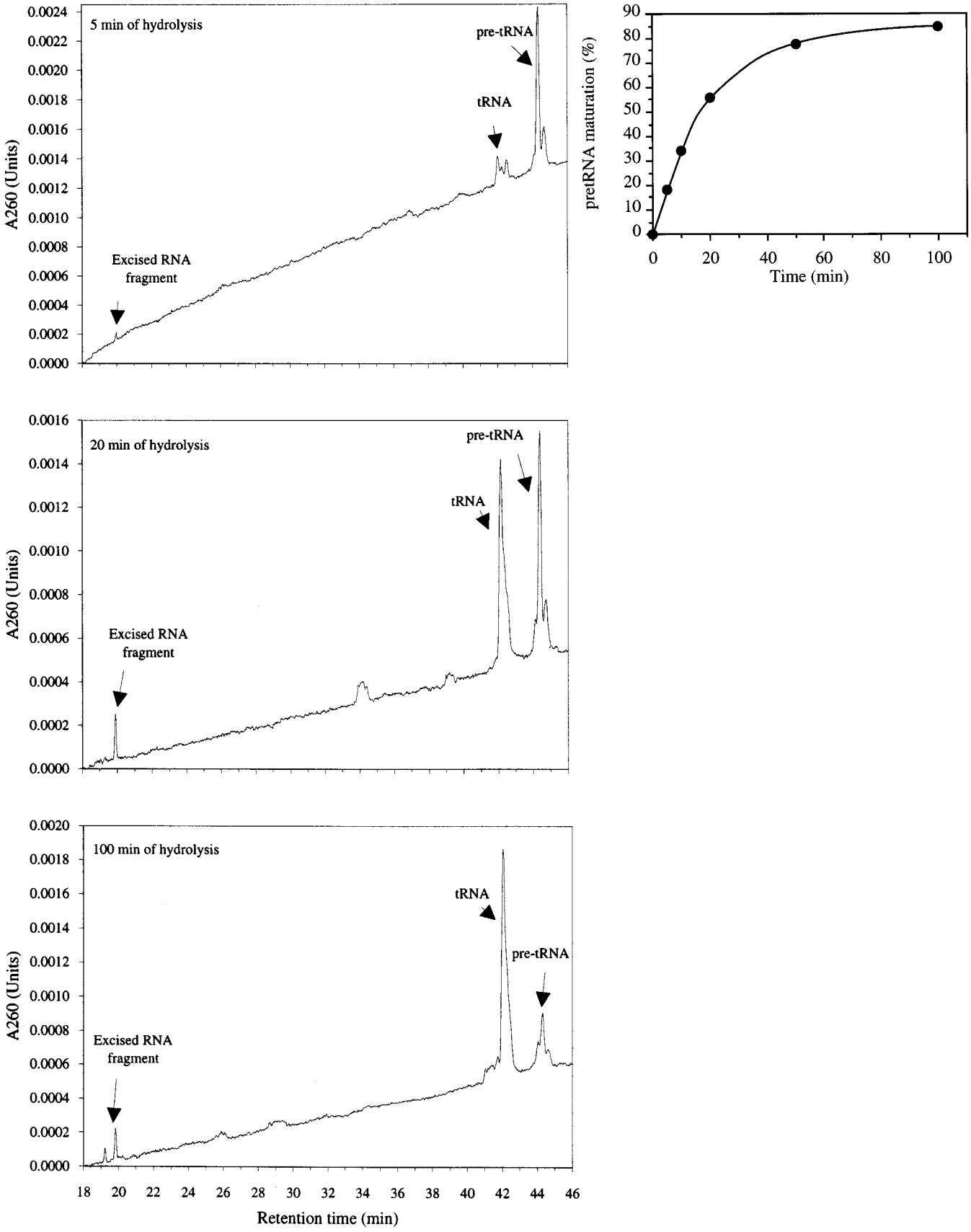

FIGURE 3: Kinetic analysis of the maturation of pre-tRNA^{fMet} by capillary electrophoresis. Pre-tRNA^{fMet} (116 nM) was incubated with 0.6 nM RNase P. Aliquots were removed at the indicated time, stopped with EDTA, and ethanol precipitated, and the resulting sample was analyzed by capillary electrophoresis as indicated under Materials and Methods. (A, left) Electropherograms at three incubation times are shown. The various separated RNA species are indicated by arrows. (B, right) The areas corresponding to the tRNA and pre-tRNA peaks were integrated. Molar coefficients of 6.3×10^5 and 5.7×10^5 M^{-1} cm^{-1}, respectively, were used to convert the A_{254} values into moles of RNA. The percentage of matured product was calculated as the ratio of the number of moles of tRNA^{fMet} to the sum of moles of pre-tRNA^{fMet} + moles of tRNA^{fMet}. This percentage was plotted as a function of time. A time course of maturation to near completion is shown.

Table 1: Purification of RNase P to Homogeneity[a]

purifn step	amt of macro-molecules[b] (A_{260} unit)	total enzyme act.[c] (units)	specific enzyme act. (units/ A_{260} unit)	yield (%)	rel purifn
crude extract	3441	388	0.11	100.0	1.0
Superose 6	1320	358	0.27	90	2.5
Q-Hiload	698	203	0.29	52	2.6
concentration	640	188	0.29	48	2.6
Superdex 200 (1)	70	131	1.87	34	17.0
Superdex 200 (2)	34	102	3.00	27	27.3

[a] See the details of the purification under Materials and Methods. [b] One A_{260} unit corresponds to the amount of macromolecules in a 1 mL solution yielding an absorbance of 1 at 260 nm in a 1 cm optical path. [c] One enzyme unit is defined as the amount of enzyme capable of hydrolyzing 1 nmol of pre-tRNA^{fMet}/min in the standard assay at a concentration of 150 nM of substrate.

that CGE was a good method for assaying RNase P activity (Figure 3B).

Purification of the RNase P Holoenzyme to Homogeneity. To study the kinetics of maturation of pre-tRNA^{fMet}, we attempted to obtain homogeneous RNase P holoenzyme preparations using a single bacterial strain that overproduced both components. In previous work (*27*), we reported the construction of a high-copy-number pUC derivative, pUCrnpAB, which expressed both *rnpA* and *rnpB* genes. Determination of the RNase P activity in JM101Tr-pUCrnpAB crude cell extract compared to that in an extract of JM101Tr-pUC18 cells showed an overexpression of RNase P on the order of 15-fold (data not shown). Taking advantage of conditions already defined for the purification of the chromosomally encoded RNase P holoenzyme (*16*) combined to the aforementioned CGE-based activity assay, we undertook to set the conditions of a rapid purification of RNase P. This could be achieved in four chromatographic steps, and the enzyme could be purified quickly to apparent homogeneity in a couple of days (Table 1). The rapidity of the purification proved to be important as it was noticed that any delay during this step could lead to a partial cleavage of the M1 RNA. The step of anion-exchange chromatography showed the advantage of removing most of the proteins, since they eluted at a much lower ionic strength than RNase P. The further two steps of molecular sieving on Superdex 200 proved very efficient in freeing the enzyme from nucleic acids of higher molecular weight. On the second column, the peak containing the activity fit perfectly with the detected absorbance at 260 nm, which showed a symmetrical profile. The elution volume was that of a molecule of 150 000 Da, suggesting that the purified species did indeed correspond to the RNase P holoenzyme. The specific activity of the sample was of the order of that described for RNase P holoenzyme preparations obtained by reconstitution experiments. A yield of 0.8 mg/L of bacterial culture was routinely obtained, and no significant loss of activity was noted for the duration of the purification (Table 1). The preparation could be stored at −30 °C for several months and retain full catalytic activity. We also purified the M1 RNA to homogeneity according to the same method, starting with the pUCrnpB plasmid (*27*), which overexpresses only the RNA component of RNase P. It displayed the same chromatographic behavior as the holoenzyme during the purification (results not shown). However, the specific

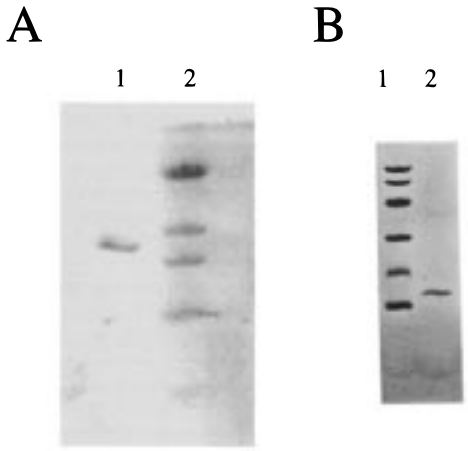

FIGURE 4: PAGE analysis of the two components of the purified RNase P holoenzyme. Purified RNase P was analyzed by SDS−PAGE. (A) 200 ng of purified RNase P was analyzed on a 12.5% SDS−PAGE (lane 1) and stained with ethidium bromide. A fluorogram is shown. Lane 2 corresponds to low molecular weight markers from Boehringer (200 000, 116 300, 97 400, and 66 200). (B) 8 μg of the purified holoenzyme was analyzed on a 20% SDS−PAGE (Phast-system; Pharmacia), stained with Coomassie blue and applied in lane 2. Lane 1 corresponds to high molecular weight markers from Pharmacia (97 000, 67 000, 43 000, 30 000, 20 100, and 14 400).

cleavage activity of pre-tRNA^{fMet} by the M1 RNA alone was 20-fold lower than that determined with the holoenzyme preparations. The fact that the activation effect caused by the binding of the C5 protein was similar to that already described (*6*) indicated that the protein moiety was functional in our holoenzyme preparations.

Characterization of the Purified RNase P Holoenzyme. The purity of the RNase P holoenzyme preparations was assessed by SDS−PAGE and found to be >95%. A single band could be detected by ethidium bromide staining as shown in Figure 4A. On an 8 M urea−6% polyacrylamide gel stained with ethidium bromide, the RNA moiety was calculated to have a length of 375 ± 15 bases, using pBr322-*Hinf*I hydrolyzed DNA as the molecular weight standard (data not shown). This indicated that the RNA component had the expected size (377 bases) and that the additional bases of the precursor species were fully processed (see refs *32*−*34*). Regarding the protein moiety, a band migrating like a 17 ± 2 kDa protein (Figure 4B) and in a 1:10 mass ratio with respect to the above RNA species could be seen by SDS−PAGE stained with Coomassie blue. Such behavior corresponded to that of the C5 protein component (*15*). Moreover, a CGE experiment on an SDS−polyacrylamide capillary indicated that the actual size of the protein was 14 000 ± 1 000, a value in keeping with the actual molecular weight (14 000) of the C5 component (data not shown). The stoichiometry of the C5 protein with respect to the RNA component was determined by measuring the protein concentration of the purified RNase P sample using bovine serum albumin as the standard (*35*) and the RNA concentration from the absorbance at 260 nm. The obtained value of 1.1 ± 0.1 is in good agreement with the values determined by gel retardation experiments (*36*). To conclude, the overproduction vector as well as the purification strategy that was used in this work led to a holoenzyme that appeared to be homogeneous and identical to the non-overproduced species.

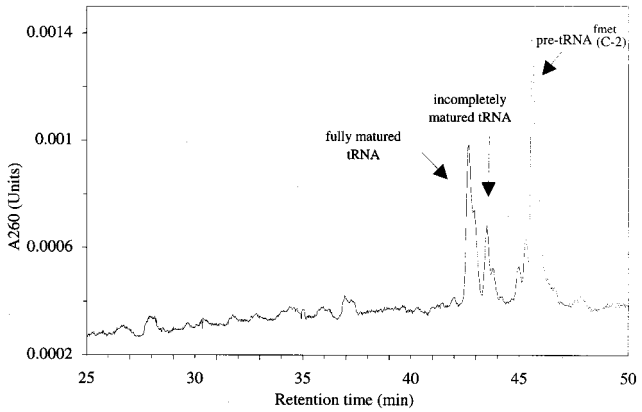

FIGURE 5: Analysis of the cleavage reaction of pre-tRNA^{fMet}(C_{-2}) by RNase P. Pre-tRNA^{fMet}(C_{-2}) (30 nM) was incubated with 1 nM RNase P for 10 min at 37 °C. The reaction was stopped with EDTA and ethanol precipitated, and the resulting sample was analyzed by capillary electrophoresis as indicated under Materials and Methods. The various separated species of the mixture are indicated by arrows.

Influence of the Base Composition of Pre-tRNA on Cleavage Site Selection by RNase P. Previous in vivo studies have shown that in the case of *E. coli* pre-tRNA^{fMet}, the 5′-flanking sequence and, in particular, base G_{-2}, play a crucial role in directing the cleavage by RNase P. Indeed, when tRNA^{fMet} was overproduced from a multicopy expression vector, the absence of G_{-2} caused accumulation of immature tRNA species in vivo with, depending on the flanking sequence, one to three extra bases at the 5′ side (*27*). The availability of purified RNase P allowed us to investigate the mechanism of hydrolysis by RNase P of incorrectly matured pre-tRNA^{fMet}. First, the nucleotide at position −2 was systematically substituted by A, U, or C, and the kinetics of RNase P cleavage of the derivatives were analyzed using the CGE-based assay. As can be seen in Figure 5, substitution of base G_{-2} by a C led to an intermediate product displaying incomplete maturation. This immature species did not accumulate at the steady state and behaved as a further substrate of RNase P, since a time course analysis of pre-tRNA^{fMet}(C_{-2}) hydrolysis to completion led to the disappearance of incompletely matured tRNA (result not shown). In the course of the maturation reaction of the U_{-2} derivative, tRNA species with extra bases at the 5′ end could also be observed. However, when an A was introduced at position −2, the cleavage reaction proceeded directly to the fully mature tRNA (result not shown). These results are in good agreement with results obtained in vivo showing that, in the context of the pre-tRNA$^{fMet}_{Y}$ 5′-leader sequence, replacement of G_{-2} by U or C, but not by A, led to the accumulation of incorrectly processed tRNA^{fMet}. A G at position −2 was, however, strictly required in the context of other leader sequences (*27*).

The mode of analysis, based on CGE, of the cleavage products of the RNase P reaction did not allow the precise determination of the cleavage sites corresponding to incompletely matured pre-tRNA^{fMet}. Therefore, the cleavage sites were determined from the mobility of the 5′-cleavage products labeled with [α-^{32}P]UTP on polyacrylamide−urea gels as described under Materials and Methods. Figure 6 shows an analysis of the cleavage products generated by RNase P of the wild-type precursor (pre-tRNA$^{fMet}G_{-2}$) and that of one of the mutants (pre-tRNA$^{fMet}U_{-2}$). Maturation

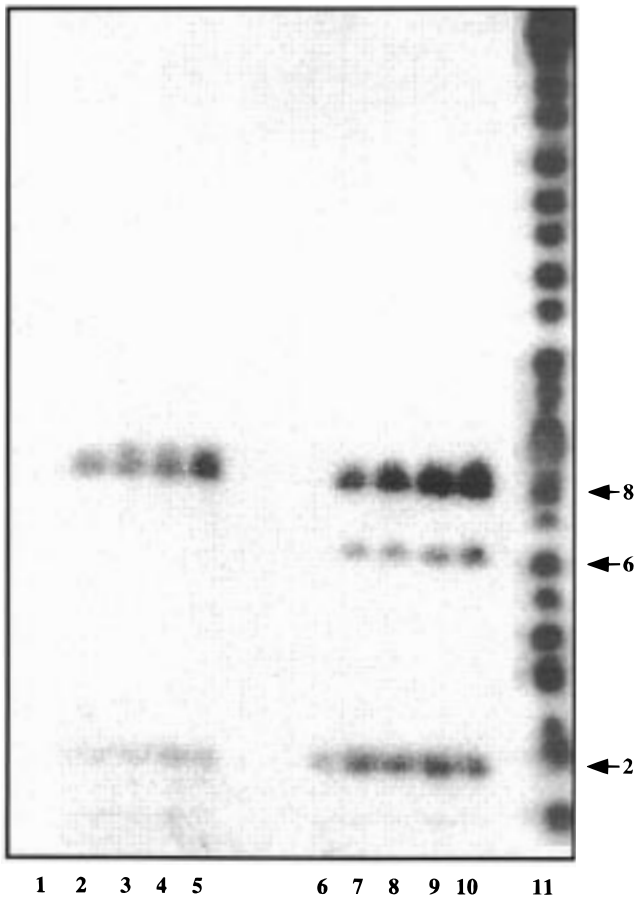

FIGURE 6: Determination of the cleavage sites of pre-tRNA^{fMet} by RNase P. 50 nM wild-type pre-tRNA^{fMet} labeled with [α-^{32}P]UTP (lanes 1−5) or its mutant derivative pre-tRNA^{fMet}(U_{-2}) (lanes 6−10) was incubated at 37 °C with 0.6 or 2 nM RNase P, respectively. Aliquots were withdrawn at various times, stopped with gel loading buffer, and analyzed on a denaturing 12% polyacrylamide gel as indicated under Materials and Methods. (Lanes 1 and 6) No enzyme added; (lanes 2 and 7) 5 min of incubation; (lanes 3 and 8) 10 min; (lanes 4 and 8) 20 min; (lanes 5 and 10) 40 min; (lane 11) RNA ladder. The lengths of the 5′-cleavage products are indicated on the side.

of the wild-type precursor resulted in a unique 5′-cleavage product that was eight nucleotides long. This indicated that cleavage had occurred, as expected, between positions −1 and +1, thereby generating the fully matured tRNA (Figure 6, lanes 1−5). In contrast, cleavage of the U_{-2} derivative showed three bands that increased as a function of time (lanes 6−10). The major band migrated at the same position as the product from the wild-type precursor, and the minor bands were six and two nucleotides long, respectively. This result showed that two RNase P cleavage sites are present in pre-tRNA^{fMet}(U_{-2}): a first cleavage site between nucleotides −1 and +1, generating the mature tRNA, and a second site between positions −3 and −2, generating a two-base 5′-extended tRNA. Substitution of nucleotide G_{-2} by a C resulted in cleavage at positions −2 and +1, whereas the mutant A_{-2} was cleaved only between nucleotides −1 and +1 (results not shown). Taken together, these results suggest that the identity of the nucleotide at position −2 is important for recognition of the cleavage site and that a purine at this position primarily acts by impeding an alternative cleavage site.

Influence of the Base Composition of Pre-tRNA on Catalytic Parameters of the Maturation Reaction. The

Table 2: Kinetic Parameters of RNase P Cleavage Reaction[a]

substrate	cleavage site	K_m (nM)	k_{cat} (min^{-1})	k_{cat}/K_m [(M^{-1} min^{-1}) $\times 10^{-6}$]
pre-$tRNA^{fMet}_Y$	+1	34 ± 9	8 ± 1	235 ± 30
pre-$tRNA^{fMet}_Y(A_{-2})$	+1	30 ± 10	7 ± 1	233 ± 49
pre-$tRNA^{fMet}_Y(U_{-2})$	+1	25 ± 7	3.0 ± 0.3	120 ± 24
pre-$tRNA^{fMet}_Y(C_{-2})$	+1	24 ± 12	1.5 ± 0.2	33 ± 5
pre-$tRNA^{fMet}_Y(C_{-2})$	−2	92 ± 18	3.8 ± 0.5	41 ± 3
pre-$tRNA^{fMet}_Y(G_{+1}C_{+72})$	+1	35 ± 8	6.5 ± 1.0	186 ± 27
pre-$tRNA^{fMet}_Y(C_{-2}G_{+1}C_{+72})$	+1	10 ± 4	1.0 ± 0.1	100 ± 27
pre-$tRNA^{fMet}_Y(U_{-2}G_{+1}C_{+72})$	+1	15 ± 2	1.4 ± 0.1	93 ± 9

[a] Values were determined from iterative nonlinear least-squares fits of the Michaelis−Menten equation to the experimental value (*45*).

catalytic parameters of purified RNase P in the maturation reaction of pre-$tRNA^{fMet}$ were measured using the CGE-based assay. As shown in Table 2, a K_m value of 34 ± 9 nM, a k_{cat} of 8 ± 1 min^{-1}, and a k_{cat}/K_m of $(235 \pm 30) \times 10^6$ M^{-1} min^{-1} were determined for wild-type pre-$tRNA^{fMet}$. These values are similar to those previously described for other pre-tRNA systems assayed with the RNase P holoenzyme, such as pre-$tRNA^{His}$, pre-$tRNA^{Tyr}$, pre-$tRNA^{Phe}$, or pre-RNA4.5S, which showed K_m values of 10−200 nM, k_{cat} values between 5 and 35 min^{-1}, and k_{cat}/K_m values of 190−900 $\times$ 10^6 M^{-1} min^{-1} (*6*, *19*, *21*, *37*). Substitution of nucleotide G_{-2} by any of the other studied bases had no effect on the K_m and only slightly affected the rate of the cleavage reaction at the +1 position (Table 2). The catalytic parameters of cleavage of pre-$tRNA^{fMet}(C_{-2})$ at the −2 position were also determined under the same conditions. For this substrate, the efficiencies of cleavage at either position −2 or +1 were very similar to one another (Table 2).

One of the special features of *E. coli* $tRNA^{fMet}$, accounting for its specialized role in translation initiation, is the occurrence of a C_{+1}/A_{+72} mispairing. Guanosine at position +1 was previously suggested to be important for cleavage by RNase P by acting as a guiding nucleotide (*24*). Indeed, in a previous paper it was shown that, in the case of pre-$tRNA^{fMet}$, absence of G_{-2} could be compensated by substitution of nucleotide C_{+1} by a G (*27*). To investigate the role of base pairing at positions +1/+72 in the kinetics of pre-$tRNA^{fMet}$ maturation, we studied the catalytic parameters of a pre-$tRNA^{fMet}$ derivative bearing a G_{+1}/C_{+72} base pairing in addition to a G, a C, or a U at position −2 (Table 2). Restoration of base pairing at the top of the acceptor stem did not significantly affect K_m or k_{cat} values, indicating that the effects of RNase P cleavage determinants are not cumulative. It should be noted that, whatever the nature of the base at position −2, cleavage always occurred at the correct site when G_{+1}/C_{+72} was present (data not shown).

DISCUSSION

In this work, we have shown that the RNase P holoenzyme can be easily and quickly purified to homogeneity, directly from a bacterial crude extract using a vector overproducing both components. Although no special care was taken for balancing the expression of the two components, the holoenzyme was obtained with a 1:1 subunit stoichiometry at the end of the purification. A protocol yielding a homogeneous holoenzyme appears advantageous compared to the usual preparation protocols in which the holoenzyme is obtained by in vitro reconstitution experiments (*18*). Indeed, a major drawback of the latter method lies in the difficulty of reaching completion and determining the actual subunit stoichiometry during the reconstitution step (*36*). It was also reported that only a fraction (25−50%) of the C5 protein was active for M1 RNA recognition, most probably due to its denaturing purification protocol involving urea, that an alternative low-affinity mode of binding to the RNA moiety occurred with a weaker dissociation constant, and finally that the two components aggregated at high protein concentration (*14*, *18*, *36*). The ease and rapidity of our purification protocol facilitates the purification of large amounts of homogeneous holoenzyme, making structural studies feasible, such as crystallization trials.

Furthermore, we developed an improved and more convenient assay of RNase P activity, based on CGE. The main advantage of this method is that it allows the use of unlabeled RNA substrates which can be prepared in large amounts, purified, and then stored for further use. The high resolving power of CGE, coupled to the sensitivity of the on-line UV detector, made quantitative analyses possible. In addition, full automation of the process made it feasible to analyze up to 20 samples overnight. More generally, we believe that CGE is a valuable method for anyone interested in the analysis of RNA molecules.

Having developed these methods, we could investigate the cleavage reaction of a set of mutants of pre-$tRNA^{fMet}$ bearing substitutions in their 5′-flanking sequences. In a previous study, we reported that the absence of a G at position −2 of pre-$tRNA^{fMet}$ caused improper maturation to occur in vivo, when the tRNA was overproduced from a multicopy expression vector. Half of the recovered $tRNA^{fMet}$ retained an extension at the 5′ side (*27*). The effect of base −2 was independent of the nature of base −73. Three alternative mechanisms accounting for the accumulation of immature species could be proposed: (i) Whatever the nature of the base at position −2, maturation of pre-$tRNA^{fMet}$ would always proceed through an initial cleavage upstream of the 5′ end of mature tRNA. A trimming activity of RNase P on the enzyme−pre-tRNA complex would lead to the fully matured tRNA. In the absence of the correct flanking sequence, a decrease in the efficiency of the latter step would cause a premature dissociation of the enzyme−substrate complex, thereby producing abnormal, accumulating intermediary reaction products. (ii) In the second mechanism, base G_{-2} would be the major initial signal directing the first cleavage at position +1. In the absence of such a signal, an alternative cleavage site (at position −2) would be preferred over the proper one. In this model, the fully matured products would originate from a further cleavage at site +1. (iii) The third possibility would be that both cleavage sites (at positions −2 and +1) coexist in pre-$tRNA^{fMet}$. The role of nucleotide G_{-2} would be to preclude improper cleavage at position −2. In the absence of G_{-2}, cleavage would occur either at −2 or at +1, depending on the relative efficiencies of the reactions. However, in vivo analyses (*27*) could not distinguish between these possibilities. In this work, we studied, in vitro, the kinetics of cleavage of mutants of pre-$tRNA^{fMet}$ and identified the cleavage sites by determining the lengths of the different products. RNase P maturation of wild-type pre-$tRNA^{fMet}$ produced a single 5′-cleavage

product of eight nucleotides (see Figure 6), thereby excluding the first mechanism. When G_{-2} was replaced by C or U, two 5′-cleavage species were observed: a major, eight nucleotide long product and a minor one of six nucleotides. These results indicate that cleavage occurred at either position −2 or +1, in favor of the third mechanism. In addition, the relative amounts of the small products during the course of the reaction (Figure 6) indicated that the cleavages occurred at similar rates.

Our present results also show that the abnormally long 3′ product generated by an initial cleavage at position −2 can subsequently serve as a substrate of RNase P for cleavage at +1. No accumulation of this product was observed in vitro, and complete maturation was obtained. This was quite surprising since, in contrast, immature $tRNA^{fMet}$ species were observed in vivo when mutant pre-$tRNA^{fMet}$ was overproduced (*27*). This phenomenon could be reverted, however, by an overproduction of RNase P. To explain this result, we propose that there are two alternative binding modes of pre-$tRNA^{fMet}$ to RNase P, causing cleavage at either position −2 or +1. Recently, chemical probing of the M1 RNA structure, as well as cross-linking studies performed with various substrates, have indeed led to the proposal that there is more than one mode of binding of a tRNA precursor in the active site of RNase P (*38*−*40*). These experiments suggest that, after a first cleavage, for a second maturation event to occur, either the enzyme−substrate complex undergoes a major conformational change to make the second site available or, more likely, the release of the primary product is required before rebinding to the second site of the enzyme can occur. If, after an initial cleavage at position −2, dissociation indeed occurs and since immature $tRNA^{fMet}$ species behave as competent substrates for methionyl-$tRNA^{fMet}$ formyltransferase and methionyl-tRNA synthetase (*27*), these species could easily be processed in vivo by these much more efficient enzymes (with k_{cat} values 2 orders of magnitude higher than that of RNase P) before rebinding to RNase P. As a result of the addition of formylmethionine to their acceptor ends, the immature tRNA species could subsequently be sequestered by initiation factor IF2 for further putative use in translation initiation. This would explain the stability of these species at steady state in vivo. An increase of the cellular RNase P concentration, upon overproduction of the enzyme, would draw the equilibrium toward the rebinding of immature pre-$tRNA^{fMet}$ to the enzyme, thereby allowing complete maturation of pre-$tRNA^{fMet}$.

Determination of the catalytic parameters of the cleavage of pre-$tRNA^{fMet}(C_{-2})$ at positions −2 and +1 shows that the efficiencies of cleavage at either site are very similar (see Table 2). This further explains why both immature and mature species are produced in comparable amounts in vivo. Similar catalytic parameters for cleavage at two different sites have already been demonstrated for some model tRNA precursors that can also be cleaved at two positions (*41*). In this context, the mechanism by which wild-type pre-$tRNA^{fMet}$ (i.e., bearing a G at position −2) is exclusively cleaved between nucleotides −1 and +1 needed to be addressed. We show that substitution of base G_{-2} of pre-$tRNA^{fMet}$ does not significantly affect the catalytic parameters of the maturation reaction at position +1. It seems therefore unlikely that the small decrease of cleavage efficiency determined for the C_{-2} or U_{-2} derivatives can account for the appearance of a second site of cleavage, as observed with these mutants. The fact that substitution of G_{-2} does not lead to a significant decrease of the catalytic cleavage efficiency by RNase P suggests that base G_{-2} does not directly participate in the mechanism of cleavage at the correct position but rather that it only prevents the cleavage at the wrong site. If, as we propose above, two binding modes exist for pre-$tRNA^{fMet}$, then this could be achieved by preventing the binding to the alternative site, the one leading to the wrong cleavage. For instance, G_{-2} could directly participate in a molding of the active center which is not compatible with the cleavage at −1. In this regard, one might consider base G_{-2} as an antideterminant, acting to prevent undesirable reactions from occurring. A similar mechanism might occur in the case of pre-$tRNA^{His}$, where it has been shown that the G_{-1}/C_{+73} base pair is an important determinant for correct cleavage at position −1. Substitution of C_{+73} resulted in cleavage at either position −1 or +1(*21*, *26*). Thus, the role of base C_{+73} might be to prevent cleavage at position +1. The concept of an antideterminant has already been proposed in the case of several reactions involving tRNAs, such as formylation of $tRNA^{fMet}$ or aminoacylation of $tRNA^{Asp}$. In the latter case, the post-transcriptional methylation of nucleotide G_{37} of $tRNA^{Asp}$ was shown to prevent very efficiently mischarging by arginyl-tRNA synthetase (*42*, *43*). In the case of formylation of $tRNA^{fMet}$, it has been demonstrated that introduction of a GC or CG base pair at position +1/+72 of the tRNA precludes the formylation of an aminoacyl-tRNA (*44*). Because most elongator *E. coli* tRNAs possess the G_{+1}/C_{+72} base pair, it has been suggested that this alone could explain their inability to be used as substrates by methionyl-$tRNA^{fMet}$ formyltransferase. Interestingly, whereas G_{+1}/C_{+72} behaves as an antideterminant toward formylation, G_{+1} was suggested to guide cleavage of various precursor tRNAs by RNase P (*24*). Indeed, in the case of pre-$tRNA^{fMet}$, it has been shown that, in the absence of G_{-2}, correct maturation could be restored by substituting nucleotide C_{+1} (present in wild-type $tRNA^{fMet}$) by a G (*27*). In this paper, we show that substitution of C_{+1}/A_{+72} by G_{+1}/C_{+72} does not improve the catalytic parameters of the cleavage reaction. This result indicates that G_{-2} alone is enough to compensate for the absence of a G at position +1 of pre-$tRNA^{fMet}$. In addition, it suggests that G_{+1}, present in most *E. coli* tRNAs, might play a role similar to that of G_{-2} in preventing binding and cleavage by RNase P at an alternative site. In this regard, experiments carried out with small RNA model substrates have shown that aberrant cleavages occurred when a pyrimidine was present at position +1, whereas a purine at this position directed proper cleavages to occur (*20*). Moreover, studies dealing with pre-$tRNA^{Tyr}_{Su3}$ (bearing both bases G_{-2} and G_{+1}) have shown that substitution of both nucleotides resulted in aberrant cleavages, whereas one substitution at either position did not alter the location of the cleavage site (*24*). The mechanism by which G_{-2} or G_{+1} prevents any incorrect cleavage from occurring remains to be elucidated. For this, knowledge of the three-dimensional structure of the pre-tRNA−RNase P complex would be required.

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