Purification, Cloning, and Properties of the 16S RNA Pseudouridine 516 Synthase from *Escherichia coli*†

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Received March 22, 1995⊗

ABSTRACT: Pseudouridine (Ψ) is commonly found in both small and large subunit ribosomal RNAs of prokaryotes and eukaryotes. In *Escherichia coli* small subunit RNA, there is only one Ψ, at position 516, in a region of the RNA known to be involved in codon recognition [Bakin et al. (1994) *Nucleic Acids Res. 22*, 3681−3684]. To assess the function of this single Ψ residue, the enzyme catalyzing its formation was purified and cloned. The enzyme contains 231 amino acids and has a calculated molecular mass of 25 836 Da. It converts U516 in *E. coli* 16S RNA transcripts into Ψ but does not modify any other position in this RNA. It does not react with free unmodified 16S RNA at all, and only poorly with 30S particles containing unmodified RNA. The preferred substrate is an RNA fragment from residues 1 to 678 which has been complexed with 30S ribosomal proteins. The yield varied from 0.6 to 1.0 mol of Ψ/mol of RNA, depending on the preparation. Free RNA(1−678) was inactive, as was RNA(1−526) and the RNP particle made from it. 23S RNA and $tRNA^{Val}$ transcripts were also inactive. These results suggest that Ψ formation *in vivo* occurs at an intermediate stage of 30S assembly. The gene is located at 47.1 min immediately 5′ to, and oriented in the same direction as, the bicyclomycin resistance gene. The gene was cloned behind a $(His)_6$ leader for affinity purification. Virtually all of the overexpressed protein was found in inclusion bodies but could be purified to homogeneity on a Ni^{2+}− containing resin. Over 200 mg of pure protein could be obtained from a liter of cell culture. Amino acid sequence comparison revealed the existence of a gene in *Bacillus subtilis* with a similar sequence, and Ψ sequence analysis established that *B. subtilis* has the equivalent of Ψ516 in its small subunit rRNA. On the other hand, no common sequence motifs could be detected among this enzyme and the two tRNA Ψ synthases which have been cloned up to now.

Although the importance of ribosomal RNA (rRNA) in ribosome function is now accepted (Nierhaus et al., 1993), the role of the modified nucleotides of rRNA is less well-established. In particular, although more than four decades have passed since pseudouridine [Ψ; 5-(β-D-ribofuranosyl)-uracil], the first and most abundant of these modified nucleosides, was discovered [references cited in Bakin and Ofengand (1993)], its still unexplained occurrence in rRNA remains a subject for conjecture about how it might contribute to rRNA structure and function (Lane et al., 1992, 1995). Until very recently, there were two obstacles to ascertaining the role of Ψ in rRNA. The first was assigning its exact location in prokaryotic and eukaryotic rRNAs (Maden, 1990), and the second was preventing formation of specific Ψ residues in order to assess the consequences of their absence in rRNA. Previously, precise localization of Ψ was an arduous task, but with the introduction of a fast scanning technique (Bakin & Ofengand, 1993) this problem has now largely been solved (Bakin & Ofengand, 1993, 1994, 1995; Bakin et al., 1994a,b). The problem of blocking formation of a particular Ψ residue can be approached in two ways. One way is to mutagenize the parent U residue, preferably to C, so that Ψ cannot be formed. This method suffers from the disadvantage that a C residue may not be the equivalent of U for reasons other than an inability to form Ψ. The other, and in our view preferable, way is to block Ψ synthesis by inactivating the gene for the enzyme responsible for the conversion, leaving the parent U residue in the RNA. Since this approach requires that the Ψ synthase involved be purified and cloned, it has the added advantage of allowing study not only of the enzymology of Ψ formation but also of the regulation of the synthesis of the synthase.

We have previously reported the location of the single Ψ residue in *E. coli* 16S RNA to be at position 516 in the so-called "530 loop" (Figure 1). This is a functionally important segment of the molecule involved in the fidelity of codon recognition [references cited in Santer et al. (1993)]. In order to determine the functional significance of this single Ψ residue, we have searched for and found the enzyme responsible for its synthesis. The enzyme has been purified, cloned, overexpressed in *Escherichia coli*, and partially characterized.

MATERIALS AND METHODS

Materials. [5-^{3}H]UTP and [α-^{32}P]dATP were from Amersham. RNasin was from Promega. Restriction enzymes and T4 RNA ligase were from New England Biolabs. T7 RNA polymerase was from Ambion, Inc. Plasmid pET-15b, the BL21/DE3 and Novablue strains of *E. coli*, His-Bind resin, and thrombin were obtained from Novagen, Inc. pCR-

† Supported in part by a grant (MRC-MT-1226) from the Medical Research Council of Canada to B.G.L.

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⊗ Abstract published in *Advance ACS Abstracts,* June 15, 1995.

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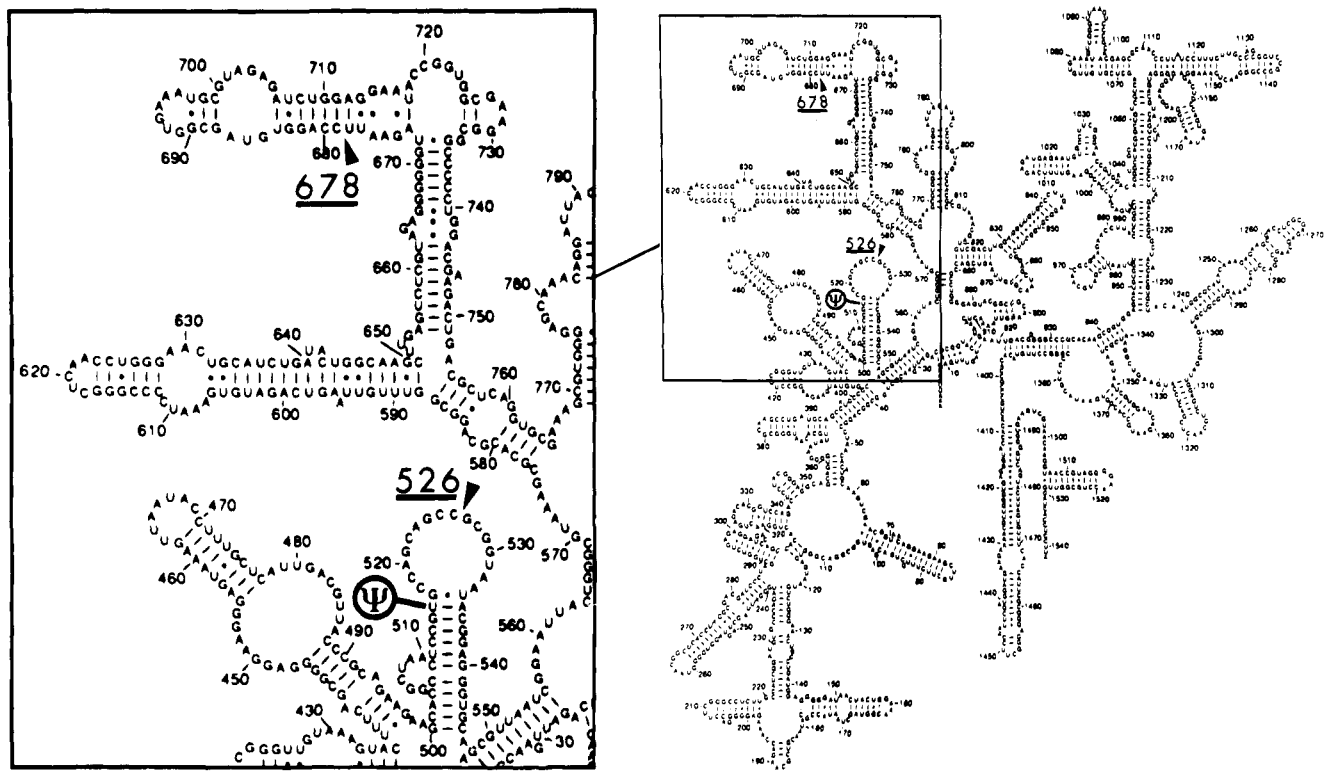

FIGURE 1: Location of Ψ residues and RNA fragment termination sites in *E. coli* 16S RNA. The 16S RNA secondary structure (Stern et al., 1989) is on the right side. The expanded view on the left side shows the position of Ψ516 (Bakin et al., 1994b) as well as the termination positions for the two 16S RNA transcript fragments used in this work.

Script was from Stratagene. T4 DNA ligase and shrimp phosphatase were from U.S. Biochemical Corp. Nuclease-free bovine serum albumin was from BRL. Yeast inorganic pyrophosphatase and Norit A washed with HCl were obtained from Sigma. Deoxyoligonucleotide primers were prepared as described previously (Bakin & Ofengand, 1993). DEAE Sepharose CL6B and MonoS FPLC columns were from Pharmacia. Protein standards were from Bio-Rad (161-0304) or Novagen, Inc. (69149-1). The RNA ladder (0.16−1.77 kb) was from BRL.

Buffers. Buffer A is 10 mM Hepes, pH 8.0, 10 mM $MgCl_2$, 5 mM mercaptoethanol, and 0.1 mM EDTA. Buffer B is 20 mM Hepes, pH 8.0, 20 mM NH_4Cl, 5 mM mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. Buffer C is buffer B but at pH 7.8. Buffer D is 20 mM Hepes, pH 8.0, 20 mM NH_4Cl, 5 mM $MgCl_2$, 5 mM mercaptoethanol, 0.1 mM EDTA, and 10% glycerol. Buffer E is 20 mM Hepes, pH 8.0, 100 mM NH_4Cl, 5 mM mercaptoethanol, 0.1 mM EDTA, and 6 M urea. Buffer LB is 50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, and 0.1% bromophenol blue. RD buffer is 20 mM Hepes, pH 7.5, 100 mM NH_4Cl, 15 mM $Mg(OAc)_2$, and 5 mM mercaptoethanol. Binding buffer is 20 mM Hepes-HCl, pH 7.9, 0.5 M NaCl, and 5 mM imidazole. Elution buffer is binding buffer with the imidazole concentration raised to 1.0 M.

Pseudouridine Formation Assay. Reactions contained 50 mM Hepes, pH 7.5, 100 mM NH_4Cl, 1 mM $Mg(OAc)_2$ except as indicated, 50−100 nM [5-^{3}H]uracil-containing RNP(1−678) or as indicated, 5 mM DTT, 400 units/mL RNasin, and enzyme. Incubation was at 37 °C. Reactions were stopped by addition of 95-μL aliquots to 1.0 mL of 12% Norit A in 0.1 N HCl. Samples were mixed and allowed to stand at room temperature for 5 min and then centrifuged to remove the charcoal, and the supernatant was passed through an Acrodisc filter assembly (0.2 μm, cat. no. 4192, Gelman Sciences). In the earlier experiments, an 0.5-mL sample was counted and the value multiplied by 2. Later, the entire supernatant was removed, and the charcoal pellet was washed two times with 1.0 mL of 0.1 N HCl, each time passing the supernatant wash through the same filter assembly as a rinse. The washes and supernatant were combined and counted. Two washes were sufficient to elute 97% of the recoverable ^{3}H. Both methods gave essentially the same results but the latter was more reproducible. One unit of activity is that amount of enzyme catalyzing the release of 1 pmol of ^{3}H to the supernatant in 30 min at 37 °C.

RNA Transcripts. The rRNA transcript of full-length 16S RNA(1−1542) was prepared by linearization of pWK1 (Krzyzosiak et al., 1988) and transcription in 40 mM Hepes, pH 7.8, 20 mM $MgCl_2$, 40 mM NaCl, 4 mM spermidine, 10 mM DTT, 5 mM each of ATP, CTP, UTP, and GTP, 2 units/mL inorganic pyrophosphatase, 1000 units/mL RNasin, 29 nM linearized plasmid, 5000 units/mL T7 RNA polymerase, and 300 μCi/mL [5-^{3}H]UTP at 37 °C for 7−10 h. For synthesis of fragment 1−526 and 1−678 by runoff transcription, pWK1 was linearized with *Sst*II and *Eco*RI, respectively. 23S RNA transcripts were prepared by linearization of pCW1 as previously described (Weitzmann et al., 1990) and transcription as described above for pWK1 except with 22 nM plasmid. All RNA samples were purified by phenol extraction, ethanol precipitation, and gel filtration except for 16S RNA(1−526), which was additionally purified by gradient centrifugation (Weitzmann et al., 1993).

RNP Particles. The assembly of the 16S RNA 1−526 and 1−678 fragments with 30S proteins were done according to Weitzmann et al. (1993) except that the RNA(1−678) was

not purified on a sucrose gradient prior to reconstitution. The resultant RNP particles were recovered from the sucrose gradient by Centricell 20 concentration with >90% recovery. The particles were stored at −170 °C in RD buffer. The particles are referred to as RNP(1−526) and RNP(1−678). *In vitro* assembly of 30S ribosomes were done as described by Cunningham et al. (1990).

Purification of the 16S Ψ516 Synthase. One hundred grams of *E. coli* MRE600 frozen cell paste (Grain Processing Corp.), harvested in mid-log phase and washed, was thawed at 4 °C with 30 mL of buffer A. After removal of the liquid by centrifugation, the cells were suspended in 215 mL of buffer A plus 10% glycerol and disrupted by sonication. Ribosomes and cell debris were removed by centrifugation at 35 000 rpm for 3 h in a Spinco Ti45 rotor. To the supernatant (S200) was added $^1/_7$ volume of 20% streptomycin sulfate adjusted to pH 7.5, and the mixture was stirred at 4 °C for 30 min. The streptomycin supernatant (216 mL) was recovered by centrifugation and precipitated with ammonium sulfate (120.7 g). The mixture was adjusted to pH 7−7.5 with NH_4OH and stirred overnight at 4 °C. The precipitate was collected by centrifugation, and the pellet was dissolved in 30 mL of buffer B and dialyzed against the same buffer to remove ammonium sulfate.

The dialyzed sample was loaded at 0.5 mL/min on a 1.5- × 35-cm column of DEAE Sepharose CL6B equilibrated with buffer C and eluted with a linear gradient of 1 mM/mL NH_4Cl in buffer C. Synthase activity eluted at 350 mM NH_4-Cl. Peak fractions were pooled, concentrated, and changed into buffer C by filtration through an Omega cell (Filtron) with an overall activity recovery of 40% of the input to the column. The sample (10 mL) was then loaded on an 8-mL MonoS column equilibrated in buffer C and eluted at 1 mL/min with a linear gradient of 3.33 mM/mL NaCl in buffer C. The enzyme eluted at 330 mM NaCl with a recovery of 35% of the input activity. Pooled samples were concentrated and changed into buffer C by filtration through a Centricell 20 (Polysciences, Inc.) membrane, glycerol was added to 50% final concentration, and the samples were stored at −20 °C.

Cloning and Overexpression of the 16S RNA Ψ516 Synthase Gene. The putative gene was amplified and prepared for insertion into pET-15b by polymerase chain reaction (PCR). The N-terminal primer extended from −10 to +24 where the A of the initiating AUG is +1, with changes at −1 to create an *Nde*I site adjacent to the initiating AUG. The C-terminal primer, in the reverse orientation, extended from +673 to +705, where the last sense nucleotide is 693, and contained mismatches at 697, 698, 699, and 701 in order to create a *Bam*HI site. After PCR, the reaction mixture was concentrated by membrane filtration (Amicon Microcon 100) and the amplified product was subcloned into pCR-Script. Plasmid DNA was isolated, the gene was cut out with *Nde*I and *Bam*HI, and the insert was purified by agarose gel electrophoresis. The pET vector was digested with *Nde*I and *Bam*HI, and the trimmed vector was purified by gel electrophoresis and incubated with the gene insert in a ligation mixture containing 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 25 μg/mL nuclease-free bovine serum albumin, 10 mM DTT, 1 mM ATP, 500 units/mL T4 DNA ligase, 5 μg/mL vector, and 5 or 20 μg/mL insert for 20 h at 16 °C. Transformation of Novablue cells was done by standard methods and yielded 9 clones out of 25 tested with the correct insert in the pET vector. Plasmids of two clones were transferred into BL21/DE3 cells.

For overexpression, the transformed BL21/DE3 cells were grown in M9ZB (Studier et al., 1990) at 30 or 37 °C to an A_{600} of 0.6. IPTG (1 mM) was added and cells were grown at 30 or 37 °C to an A_{600} of 1.1−1.7. Cells were recovered and quick-frozen on dry ice in aliquots. For analysis of the whole cell contents, one aliquot was thawed in $^1/_{10}$ the original culture volume of buffer LB, heated to 100 °C for 5 min, and then chilled. Other aliquots were disrupted by sonication in $^1/_8$ the original culture volume of buffer D plus 1 mM phenylmethanesulfonyl fluoride and centrifuged at 15000*g* to obtain the S15 supernatant and pellet fractions. The pellet was dissolved in $^1/_{24}$ the original culture volume of buffer E. The S15 supernatant was centrifuged at 45 000 rpm for 3 h in a Spinco Ti60 rotor to obtain the S200 supernatant and the ribosomal pellet. The top $^3/_4$ of the supernatant was taken as the S200 extract. The ribosomal pellet was suspended in $^1/_{40}$ the original culture volume of 20 mM Hepes, pH 8.0, and 1 M NH_4Cl, incubated at 0 °C for 2 h, and then centrifuged at 200000*g* for 14 h. The supernatant was taken as the ribosomal high-salt wash (HSW), and the ribosomal pellet was dissolved in buffer E.

Affinity Purification of the Synthase. The S15 supernatant from a 300-mL cell culture was dialyzed versus binding buffer immediately before application to a 2.5-mL column of His-Bind resin. Conditions of preparation and operation of the column were as described in the pET System Manual, 4th ed., Novagen, Inc. Upon addition of elution buffer, the tagged protein was released. The A_{280}-containing fractions were pooled and dialyzed against buffer B. The S15 pellet from the same culture was solubilized in buffer E, dialyzed versus binding buffer plus 6 M urea, and applied to a 2.5-mL His-Bind column equilibrated in the same buffer. Gel electrophoresis of the passthrough showed that >90% of the recombinant protein was retained by the column (Figure 6C, lane B). Elution was with elution buffer plus 6 M urea. The pooled A_{280}-containing fractions were dialyzed against buffer E with decreasing concentrations of urea from 6 to 3 M at 1.0 M intervals and then from 3 to 0 M at 0.5 M intervals for 1 h each. Both protein solutions were adjusted to contain 50% glycerol and stored at −20 °C.

Polyacrylamide Gel Electrophoresis. SDS gels were 12% polyacrylamide and contained 0.375 M Tris-HCl, pH 8.8, and 0.1% SDS. The 5% stacking gel contained 0.127 M Tris-HCl, pH 6.8, and 0.1% SDS. Samples were heated at 95 °C for 5 min in buffer LB and then quenched on ice before loading. Gels were stained either with Coomassie Blue or by using the silver stain reagent kit and protocol from Bio-Rad Laboratories, Inc.

Protein Sequencing. Glycerol was removed from the purified enzyme by dialysis and the protein was precipitated with 9 volumes of cold acetone at −20 °C overnight and then 1 h at −70 °C. The precipitate was dissolved in buffer LB plus 3.5 M urea and electrophoresed as above. Samples were electroblotted onto a PVDF membrane (Millipore Corp.) following standard procedures (Matsudaira, 1987). N-Terminal sequencing was carried out as described previously (Denman et al., 1989a). We thank Kurt Hollfelder and Yu-Ching Pan of the Department of Protein Biochemistry, Hoffmann-La Roche, Inc., for the sequence analysis.

Protein Determinations. Protein content was assayed by a modified Bradford procedure (Bio-Rad protein assay, cat.

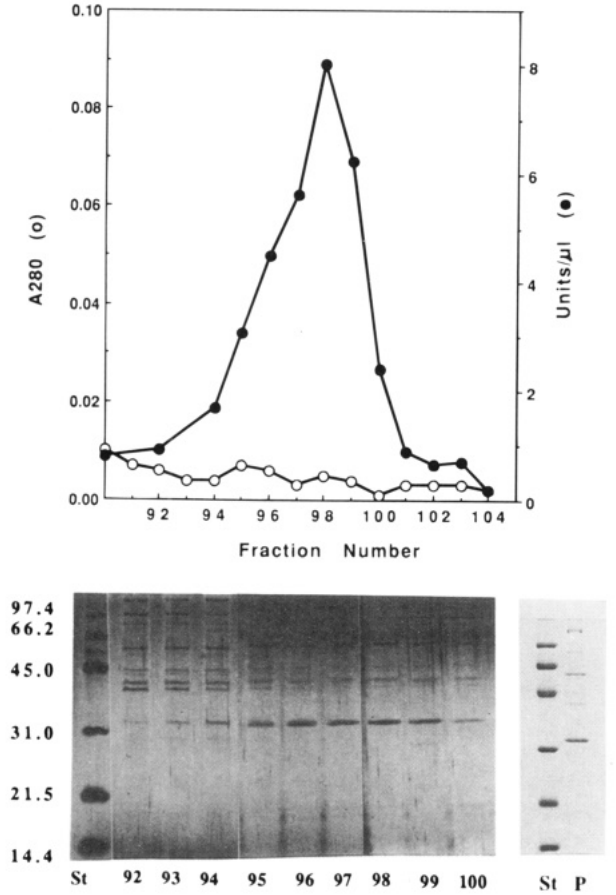

FIGURE 2: Final step of purification of 16S RNA Ψ516 synthase on MonoS. Upper panel, chromatography of the enzyme on an FPLC MonoS column was done as described in Materials and Methods. Lower panel, column fractions as indicated were electrophoresed in SDS gels. St, MW standards with values as indicated. P, pooled fractions 94−101, which were used for the subsequent experiments.

no. 500-0006), using bovine serum albumin as a standard.

RESULTS

Purification of the 16S RNA Ψ516 Synthase. The assay chosen to measure Ψ synthesis was the release of ^{3}H from the 5-position of the uracil ring as U is converted to Ψ (Cortese et al., 1974), the U residues being incorporated into a suitable polynucleotide by *in vitro* transcription. When a [5-^{3}H]uridine-labeled full-length 16S RNA transcript was used to screen cell extracts for activity, none was detected. Reasoning that the substrate for the enzyme might be a ribonucleoprotein, the extracts were rescreened with 30S ribosomes made *in vitro* by reconstitution of the [5-^{3}H]-uridine-labeled 16S RNA transcript with 30S proteins. With this latter substrate, activity was detected. The activity was primarily located in the S200−streptomycin supernatant fraction and eluted as a discrete peak from a DEAE column. The peak fraction reacted in a time-dependent manner with the substrate, yielding at the plateau 0.8 mol of ^{3}H released/mol of substrate. Since only one Ψ was known to be present in mature 16S RNA (Bakin et al., 1994b), and the only other modified uridine in 16S RNA, m^{3}U, would not be expected to release a proton from the 5-position during biosynthesis, we tentatively ascribed this activity to the Ψ516 synthase. Further purification on a MonoS column (Figure 2) identified a polypeptide whose staining intensity paralleled the enzyme

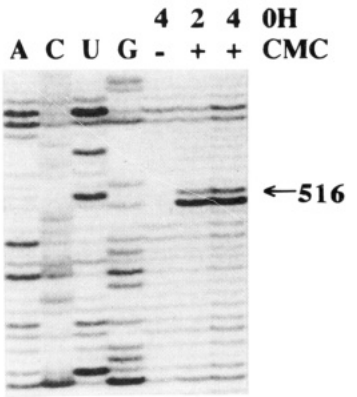

FIGURE 3: Reverse transcription analysis of the site of Ψ formation. RNA extracted from 30S subunits which had been reacted with enzyme to a level of 0.8 mol of ^{3}H released/mol of 30S were treated with (+) or without (−) CMC followed by 2- or 4-h incubation at pH 10.4 (OH). The methodology was according to Bakin and Ofengand (1993). A, C, U, and G, sequencing lanes using the *in vitro* transcript. The arrow shows the position of Ψ516.

activity. The molecular weight of the protein from the gel analysis of Figure 2 was 32.5 kDa. Treatment of the substrate with the pooled enzyme of Figure 2 followed by sequence analysis for Ψ (Figure 3) confirmed that the enzyme was indeed a Ψ synthase and that it was specific for U516 (positions 320−590 were screened). Control analyses which were not treated with the enzyme did not show any Ψ formation (data not shown).

Substrate Specificity of the Enzyme. The experiments described so far were done with a single preparation of [^{3}H]-30S particles. Subsequent testing of several 30S preparations failed to show significant reaction with the enzyme. It was then noted that the 30S preparation that was a substrate had a sedimentation profile indicative of a poorly assembled particle. Suspecting that either a "loosened" 30S or a subparticle might be an active substrate, we prepared two sizes of subparticles. The first one, RNP(1−526), consisted of a transcript corresponding to 16S RNA fragment 1−526 (Figure 1) reconstituted with four 30S proteins and has been previously described (Weitzmann et al., 1993). The second one, RNP(1−678), consisted of the 1−678 transcript of 16S RNA (Figure 1) reconstituted in the presence of a full set of 30S proteins under the same conditions used to make the RNP(1−526) particle. The size of this RNA was dictated by the location of the next downstream restriction site in the pWK1 plasmid (Krzyzosiak et al., 1988).

The RNA(1−678) fragment gave a single band running somewhat faster than the 780-nucleotide marker RNA upon analysis by denaturing gel electrophoresis (Denman et al., 1989a), indicating that the size was correct. Upon velocity sedimentation after reconstitution, a well-defined peak (26S by reference to a ^{32}P-labeled 30S marker added to the same tube) was obtained. This value was higher than the expected value of 21S calculated by a linear extrapolation based on RNA size from the 16S value for RNP(1−526) determined previously (Weitzmann et al., 1993). Nevertheless, denaturing gel electrophoresis of RNA extracted from the isolated particle did not show any molecules larger than the original 1−678 fragment. The higher S value for RNP(1−678) could perhaps be due to a higher protein content and/or a more condensed particle than RNP(1−526).

In any case, the RNP(1−678) particles proved to be the only effective substrate for the 16S RNA Ψ516 synthase

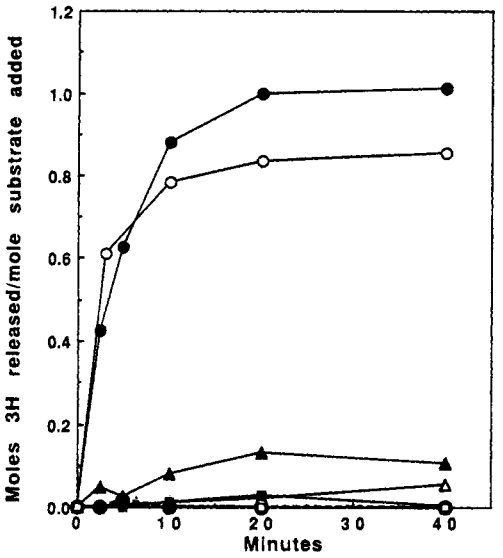

FIGURE 4: Specificity of the 16S RNA Ψ516 synthase. [5-^{3}H]-Uracil-containing RNP(1−678) [○ and ●], reconstituted 30S [△ and ▲], RNP(1−526) [□ and ■], and RNA(1−678) [○ and ●] were prepared and pseudouridine formation was measured as release of ^{3}H as described in Materials and Methods. Substrate concentrations were 80 nM. Solid symbols, 1.1 mM Mg^{++}; open symbols, 10 mM Mg^{++}.

AGGCAAAATCACACACATG CGA CTT GAT AAA TTT ATC GCA CAG CAA
M R L D K F I A Q Q
1 10

CTC GGC GTT.....AGC GTC GTC TAA
L G V ..... S V V
11 231

FIGURE 5: Gene sequence of 16S RNA Ψ516 synthase. Upper line shows the gene sequence with the deduced amino acid sequence below. In the nucleotide sequence, the Shine−Dalgarno sequence, initiation codon, and termination codon are underlined. The amino acid sequence underlined was obtained by experimental analysis of the purified protein. The total number of amino acid residues calculated from the ORF is indicated.

among the three RNP particles and five RNAs which were tested. As shown in Figure 4, both at 1.1 and 10 mM Mg^{2+}, RNP(1−678) was an efficient substrate, releasing 0.8−1.0 mol of ^{3}H/mol of substrate, whereas RNP(1−526) was completely inactive. The 30S particles were slightly active at 1.1 mM Mg^{2+} but not at 10 mM Mg^{2+}, consistent with the previous observation that a "loosened" 30S had some activity. The free RNA(1−678) was inactive. RNA(1−526), 16S RNA, 23S RNA, and $tRNA^{Val}$ transcripts were also inactive (data not shown). These results are consistent with the natural substrate being an RNP at some intermediate stage of assembly into a 30S ribosome.

Identification of the Gene. N-Terminal amino acid sequencing of the gel-purified protein band shown in Figure 2 yielded the sequence underlined in Figure 5. A search of the Genbank data base located a putative open reading frame (P26 ORF) containing this sequence (Figure 5). There was an exact match for the first 13 residues, including the N-terminal methionine. The gene is a previously described open reading frame located immediately 5′ to, and oriented in the same direction as, the bicyclomycin resistance gene (Genbank Accession No. X63703) at 47.1 min on the *E. coli* chromosome (Bentley et al., 1993). The gene codes for a weak three-base Shine−Dalgarno sequence 13 nucleotides from the initiating AUG. This length of spacer is at the upper edge of the range which has little inhibitory effect on translational efficiency (Gold, 1988).

The gene sequence reported by Bentley et al. (1993) differs from that in Genbank Accession No. U00008 at two sites. The former sequence has C159-G160 counting the A of the initiating ATG as 1, whereas the latter sequence has GC at the same site. This results in an amino acid change from valine to leucine but does not change the reading frame. The former sequence has a stop codon after amino acid residue K167, whereas the latter sequence, as a result of an insertion of G after C491, again counting from the A of the initiating ATG, shifts the reading frame and only encounters a stop after V231. As this yields a molecular weight for the protein more consistent with our measured value, we have assumed that the latter sequence is the correct one. As shown in Figure 5, this sequence codes for a 231 amino acid protein with a calculated molecular mass of 25 836 Da.

Cloning of the Gene, Overexpression, and Affinity Purification. The P26 ORF was cloned into pET-15b by standard methods. Two clones, 1 and 2, containing the correct-sized insert were selected. As shown in Figure 6a, induced cells, but not uninduced ones, containing the insert produced a large amount of a protein of about 33 kDa in size while no such band was visible in the pET control, whether induced or uninduced. The overexpressed protein appeared to form inclusion bodies since the majority was found in the S15 pellet fraction (Figure 6B, lane B). The smaller amount of protein in the S15 supernatant fraction (lane A) appeared to be mainly associated with ribosomes (compare lanes C−E). The above results were obtained with cells grown at 37 °C. Since in some cases more overexpressed protein stays soluble in *E. coli* when cells are grown at 30 °C, a parallel experiment was conducted at this temperature. Judging from the relative intensities of the gel bands in analyses like those of Figure 6A,B, growth at 30 °C did not increase the proportion of overexpressed protein in the S15 supernatant.

The P26 ORF gene was cloned such that its translation product should contain a $(His)_6$ sequence as part of a leader peptide in order to facilitate affinity purification of the protein specified by the cloned gene on a Ni^{2+}-containing resin column. Ψ synthase activity in such an affinity-purified protein would constitute definitive proof that the gene cloned is the gene for the synthase. Since the S15 pellet was insoluble in buffer D, affinity purification of this fraction was conducted in the presence of 6 M urea. Figure 6C shows that the Ni^{2+}-column effectively removed all of the contaminating protein from the solubilized S15 pellet fraction, resulting in a single protein band with a monomer molecular mass of 36.0 kDa. Since the 5′ tag increases the molecular mass by 2179 Da, the expected value for the recombinant protein, 34.7 kDa, based on the experimental value of 32.5 kDa (Figure 1), is in reasonable agreement with what was found. Urea was then removed by stepwise dialysis to allow slow renaturation of the protein. The protein remained soluble by this procedure and possessed enzymatic activity (see below). The S15 supernatant was also purified by affinity chromatography but without the need for urea solubilization (data not shown).

The Ψ Synthase Enzymatic Activity Resides in the Affinity-Purified Protein. Treatment of [5-^{3}H]uracil-containing RNP-(1−678) with this protein yielded 0.9 mol of ^{3}H released/mol of substrate, whereas 30S ribosomes were only slightly

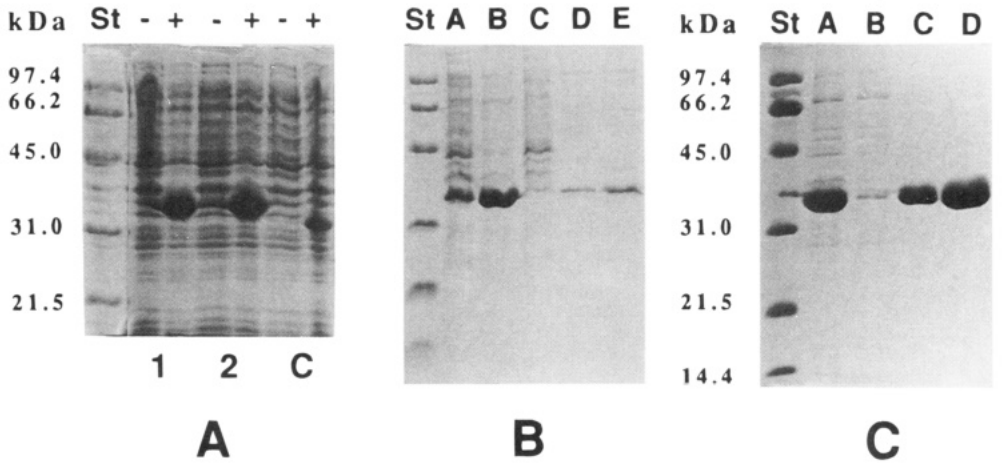

FIGURE 6: Overexpression, cellular localization, and affinity purification of the 16S RNA Ψ516 synthase gene product. Panel A, overexpression. Cells grown at 37 °C were transformed with plasmid from clones 1, 2, or C (the pET vector lacking the gene insert), harvested either before (−) or after (+) induction with IPTG, and lysed by boiling in SDS as described in Materials and Methods. Panel B, cellular localization. Cells were broken by sonication and fractionated into an S15 supernatant and a pellet. The supernatant was further fractionated into an S200, a ribosomal 1 M NH_4Cl ribosomal wash (HSW), and ribosomes. Lane A, S15 supernatant; lane B, solubilized S15 pellet; lane C, S200; lane D, HSW; lane E, solubilized ribosomes. Panel C, affinity purification. Lane A, S15 pellet solubilized in 6 M urea; lane B, flowthrough of the column load; lane C, 1 μg of the affinity-purified recombinant protein containing the $(His)_6$ tag after removal of urea; lane D, 3 μg of the sample in lane C. In each panel, extracts from approximately equivalent amounts of cells were loaded except for lanes C and D in panel C. St, molecular weight standards whose values are as indicated.

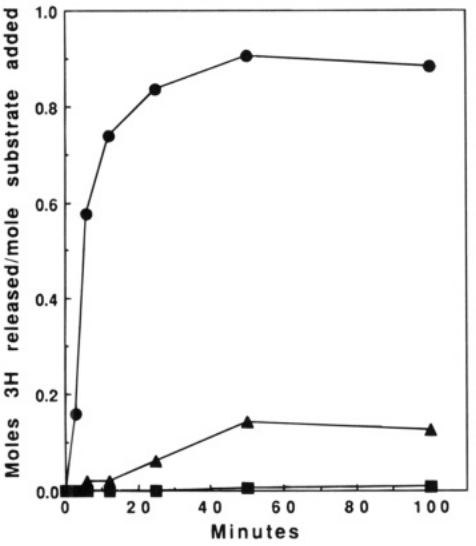

FIGURE 7: Specificity of the recombinant Ψ516 synthase. [5-^{3}H]-Uracil-containing RNP(1−678) (●), reconstituted 30S (▲), and 16S RNA (■) were assayed with the affinity-purified recombinant enzyme as described in the legend to Figure 4 at 1.1 mM Mg^{++} and 80 nM substrate.

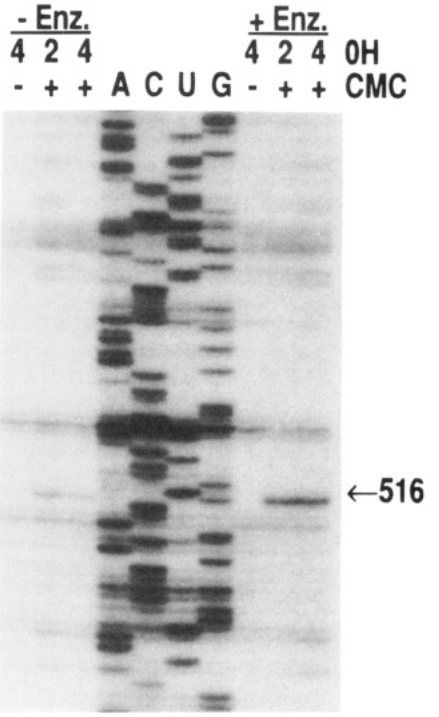

FIGURE 8: Reverse transcription analysis of the site of Ψ formation by the recombinant enzyme. RNA extracted from RNP(1−678) which had been reacted with enzyme to completion (see Figure 7) was treated as in Figure 3. The arrow shows the position of Ψ516.

active and 16S RNA was totally inactive (Figure 7). This result is the same as that obtained in Figure 4 with the natural enzyme. To confirm that Ψ516 was being formed, sequencing was performed (Figure 8). Although only residues 452−538 are shown in the figure, the sequence from ca. 120 to 590, or 70% of the 1−678 fragment, was screened. Clearly Ψ516 was formed only when the substrate was treated with the affinity-purified protein. From these two results, it is clear that the affinity-purified protein is in fact the Ψ516 synthase and that the gene has been identified.

The availability of two affinity-purified enzyme fractions, one which had been denatured and renatured and one which was "native", made it possible to determine the effectiveness of the denaturation−renaturation procedure for this enzyme. As shown in Table 1, the specific activities of the two fractions were virtually identical. Therefore, at least for this enzyme, the renaturation procedure used was completely effective.

From the amount of purified protein eluted from the affinity resin, both the yield of protein and the distribution of the enzyme between the S15 pellet and supernatant fractions could be determined. When the cells were grown at 37 °C to an A_{600} of 1.1, the amount of purified protein derived from the S15 pellet fraction was 39 mg/L of cell culture. In a second preparation in which the cells were grown to an A_{600} of 1.7, the S15 pellet fraction yielded 227 mg of pure protein/L of cell culture, while that derived from the S15 supernatant of the same culture was 3 mg of enzyme/

Table 1: Specific Activity of Affinity-Purified Recombinant Enzyme with and without Urea Denaturation and Renaturation[a]

recombinant enzyme	specific activity [units/(mg of protein) × 10^{-3}]
without denaturation/renaturation[b]	104
with denaturation/renaturation[c]	98

[a] Enzyme preparations were assayed as described in Materials and Methods with 200 nM RNP(1−678) at 1.1 mM Mg^{2+}. [b] The S15 supernatant of induced cells grown at 37 °C was affinity-purified on the His-Bind resin and assayed. [c] The S15 pellet dissolved in urea was affinity-purified on the His-Bind resin and then freed of urea by dialysis as described in Materials and Methods before assay.

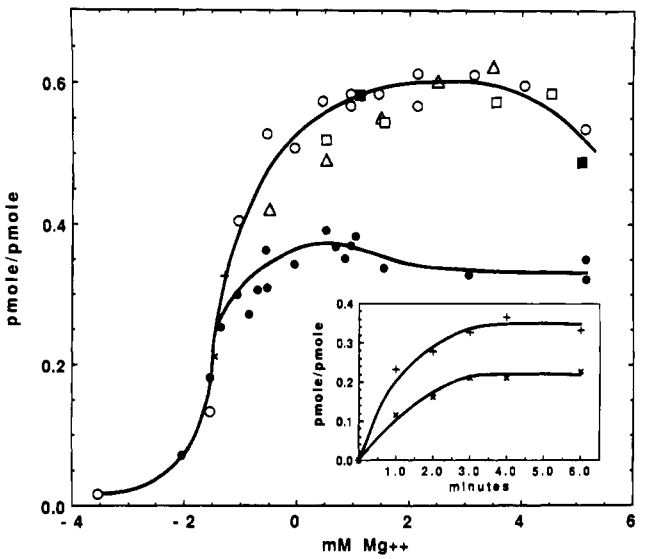

FIGURE 9: Magnesium dependence of the reaction with RNP(1−678) and with 30S subunits. RNA(1−678), ○, △, □, +, ×, ■; 30S subunits, ●. Samples contained 2.2 mM Mg^{++} from the substrate. Lower Mg^{++} values were obtained by EDTA addition and calculated on the assumption that each mole of EDTA would bind 2 mol of Mg^{++}. Amounts of EDTA in excess of the Mg^{++} present are shown as negative values of Mg^{++}. Thus addition of 2.1 mM EDTA is shown as −2 mM Mg^{++} (see text). Reaction was with 100 nM substrate and 3.4 μg of the recombinant enzyme in 100 μL for 0.5 min (□), 1.75 min (△), 3 min (○, ●, +, ×), or 10 min (■) at 37 °C. Inset: Rate of reaction at −1.29 (+) and −1.45 (×) mM Mg^{++}. The symbols correspond to those on the main figure.

L, or 1.3%. This value is consistent with a visual estimation of the enzyme band intensities in the S15 supernatant and pellet fractions after SDS gel electrophoresis (data not shown). The percent of enzyme in the S15 supernatant of the first preparation was clearly higher (Figure 6B) but considering the almost 6-fold increase in the pellet fraction of the second preparation, the absolute amounts in the S15 supernatant of the two preparations may not be dissimilar. The large increase in the amount of enzyme produced in the second preparation may be a result of growth to a higher cell density in that preparation.

Mg^{2+} Dependence of the Ψ516 Synthase. Because the two known Ψ synthases for tRNA, the *E. coli* anticodon arm Ψ synthase (Green et al., 1982) and the *E. coli* Ψ55 synthase (Nurse, et al., 1995), do not need added Mg^{2+}, the requirement for Mg^{2+} by this enzyme was examined. It was already shown in Figure 4 that 1.1 mM Mg^{2+} was sufficient for reaction, but the effect of a more complete removal of Mg^{2+} was not known. Figure 9 shows the effect of removal of Mg^{2+} on the reactivity of RNP(1−678) and 30S particles. Because the samples themselves contributed 2.2 mM Mg^{2+} to the final reaction, EDTA was added to reduce the Mg^{2+} concentration below that value. For calculation purposes, we assumed that 1 mol of EDTA would deplete the reaction of 2 mol of Mg^{2+}, although we recognize that the true value is probably less. Thus the addition of 1.1 mM EDTA is assumed to reduce the net Mg^{2+} concentration to 0. The addition of more EDTA is indicated on the figure as negative values of Mg^{2+}. For example, a plotted value of −2 mM Mg^{2+} means the addition of 2.1 mM EDTA (1.1 mM to bring the net Mg^{2+} to 0, and 1 mM equivalent to the removal of 2 mM Mg^{2+}). As calculated by this method, at ca. −1.5 mM Mg^{2+}, there was a precipitous drop in the amount of ^3H release both with the RNP(1−678) and with the 30S particles. This was not simply due to a decrease in the rate of the reaction. As shown in the inset, the maximum amount of reaction was already reached at the chosen time (3 min) of reaction. Similar results were obtained at the other Mg^{2+} concentrations, since approximately the same extent of reaction was obtained when the time of reaction was varied from 0.5 to 10 min. From these results, it appears that Mg^{2+} is needed to maintain the active conformation of the RNP-(1−678) and, by extension, the 30S particles. This being the case, it was not possible to determine if Mg^{2+} was also needed by the enzyme.

Another result of this experiment was the demonstration that different preparations of RNP(1−678) and 30S vary in their fraction of active substrate. Whereas in Figures 4 and 7 the RNP(1−678) yielded 0.9−1.0 mol of ^3H release/mol of RNP, this preparation did not exceed 0.6. Moreover, while the 30S was poorly reactive (ca. 0.15 mol/mol) at 1.1 mM Mg^{2+} in Figures 4 and 7, in Figure 9 values of about 0.35 were obtained. The decrease in 30S reactivity with increased Mg^{2+} (Figure 4) was still detected, however, although to a lesser extent. With this preparation, the fraction of active particles decreased from 0.34 at 5 mM Mg^{2+} to 0.26 at 10 mM and 0.21 at 15 mM Mg^{2+} (data not shown). The RNA(1−678) uncomplexed with protein was also tested in 5 mM EDTA. It was completely inactive (data not shown), just as it was in the presence of Mg^{2+} (Figure 4).

Comparison of the Amino Acid Sequence of the Synthase with Other Known Proteins. A search of the GenBank database using the TFASTA module of the GCG Sequence Analysis Software suite of programs (Devereux et al., 1984) revealed the existence of a gene in *Bacillus subtilis*, located in the *spoVA−serA* region, whose deduced amino acid sequence had similarity to the Ψ516 synthase. According to BESTFIT analysis (Devereux et al., 1984), there was a 30% identity and an additional 27% high similarity. The possibility that this gene could code for the equivalent enzyme in *B. subtilis* prompted us to ask if the equivalent of Ψ516 existed in this organism. Reverse transcription analysis of *B. subtilis* 16S RNA showed clearly the presence of Ψ517, at the same site as Ψ516 in *E. coli* (Figure 10). Although we cannot say if this Ψ is the only one in *B. subtilis* 16S RNA, the reverse transcription screen did not reveal any others between residues 360 and 557. Since *B. subtilis* must possess an enzyme for the synthesis of Ψ517, we suggest that the *B. subtilis* ORF whose deduced amino acid sequence is similar to the Ψ516 synthase is the corresponding gene. We are attempting to verify this by overexpression of the gene in *E. coli*.

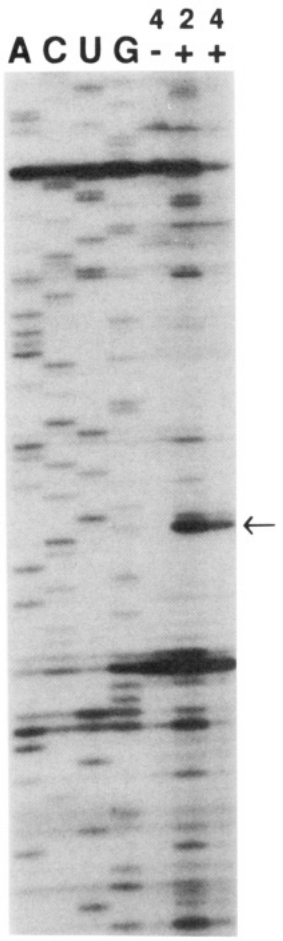

FIGURE 10: Reverse transcription analysis of *B. subtilis* 16S RNA for the presence of Ψ. Total RNA extracted from cells by phenol was fractionated by 1 M NaCl precipitation and treated with (+) or without (−) CMC followed by 2- or 4-h incubation at pH 10.4 as indicated. The methodology was as in the legend to Figure 3. A, C, U, and G, sequencing lanes using untreated RNA. Arrow marks the stop at G518 due to Ψ formation at U517.

Figure 11 shows a comparison of the *E. coli* Ψ516 synthase gene sequence (*rsuA*) with those of the only other two cloned Ψ synthases that are known, the *truA* (also known as the *hisT*) gene product, which converts U residues in the anticodon arm of tRNA into Ψ (Arps et al., 1985; Kammen et al., 1988), and *truB*, whose gene product forms Ψ55 in tRNA (Nurse et al., 1995). Despite the common catalytic reaction carried out by these enzymes, virtually no amino acid sequence correspondence could be detected.

DISCUSSION

Substrate Specificity. In this work, we have described the purification and cloning of an enzyme which site-specifically forms Ψ in *E. coli* 16S RNA. This is the first description of a Ψ synthase for any ribosomal RNA and only the third example of a cloned Ψ synthase from any RNA despite the ubiquitious occurrence of Ψ in tRNA, rRNA, and sn(o)-RNA. The enzyme only formed Ψ at position 516 in 16S RNA. Although this was not rigorously proved by direct analysis of the entire 16S RNA molecule, the fact that approximately unit stoichiometry of ^{3}H release was obtained with the RNP(1−678) particle (Figures 4 and 7) argues strongly that all of the Ψ formation occurred in the first 678 nucleotides. Residues 120−590, or 70% of those nucleotides, were directly screened. Moreover, in native 16S RNA, Ψ516 is the only Ψ in the entire 16S RNA molecule (Bakin et al., 1994b). Taken together, these facts provide strong evidence that the enzyme described in this work is site-specific.

The enzyme did not react with the RNA fragment 1−678 until it was complexed with 30S protein(s). Either the enzyme directly interacts with one or more of the bound 30S proteins as well as with the RNA, or the 30S protein(s) put the RNA fragment into a conformation which is recognized by the enzyme. In this regard, it is striking that RNP(1−526) was not a substrate. It is possible that the base-paired structure in which Ψ516 is embedded is necessary, since it is missing in this particle. Alternatively, a protein present in RNP(1−678) but absent from RNP(1−526) could be necessary. The reactivity of RNP(1−678) and 30S varied both with the preparation and with Mg^{2+} concentration. Whereas the preparations used for Figures 4 and 7 yielded 0.9−1.0 mol of ^{3}H released/mol of RNP, the preparation used for Figure 9 which was made in ostensibly the same way only yielded 0.6 mol/mol. Since the exact nature of the elements in the RNP which are recognized by the enzyme are not known, it need not be surprising that subtle differences in the method of preparation could result in this difference. Similar variations with respect to susceptibility to the Ψ516 synthase were also observed in the 30S preparations. The effect of varying the Mg^{2+} concentration was of two types. At high Mg^{2+} concentrations, the fraction of active substrate decreased by 25% (RNP) or 40% (30S), suggestive of a Mg^{2+}-dependent condensation of the particles which limits access by the synthase. As the Mg^{2+} concentration was lowered, the active fraction remained constant until a critical value was reached, at which point both particles appeared to switch from an active to an inactive conformation. The occurrence of the switch at the same Mg^{2+} concentration for both particles implies involvement of a closely similar structure. The lower overall level of reactivity of the 30S particles compared to RNP(1−678) may be viewed as the result of a general inhibitory effect of the rest of the 30S structure, possibly by physically limiting access of the enzyme or by perturbing the active substrate structure. Since free RNA was inactive and 30S was not a good substrate at the more physiological Mg^{2+} concentrations (5 mM Mg^{2+} or greater), while the RNP(1−678) was still quite active, the most reasonable interpretation is that Ψ516 formation occurs at some intermediate stage of 30S assembly.

Does the enzyme react with any other RNA molecules? Neither 23S RNA nor $tRNA^{Val}$ transcripts were reactive. Moreover, when the nine known Ψ sites in 23S RNA (Bakin & Ofengand, 1993; Bakin et al., 1994a) were examined for any sequence homology to that around Ψ516, none was found. The same was true for all known Ψ sites in *E. coli* tRNAs. Thus, there is no reason to believe that this enzyme is anything but a Ψ synthase specific for only one site in 16S RNA.

Location in the Cell. When overexpressed at 37 °C, 99% of this protein was found in inclusion bodies in one preparation, and in a second one which yielded $^1/_6$ the amount of total overexpression, the majority of the protein was still found in the inclusion body fraction (Figure 6B). A clue to the location of the enzyme when present at normal levels in the cell comes from the distribution in the S15 supernatant. Most of this material was found associated with ribosomes even after a 1 M NH_4Cl wash (Figure 6B). This association

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      1                                                  50
truB  MSRPRRRGRD INGVLLLDKP QGMSSNDALQ KVKRIYNANR AGHTGALDPL
rsuA  .......... .......... .......... .......... ..........
truA  .......... .......... ......MSDQ QQPPVYKIAL GIEYDGSKYY

      51                                                 100
truB  ATGMLPICLG EATKFSQYLL DSDKRYRVIA RLGQRTDTSD ADGQIV..EE
rsuA  .......... ..MRLDKFIA QQLGVSRAIA GREIRGNRVT VDGEIV..RN
truA  GWQRQNEVRS VQEKLEKALS QVANEPITVF CAGRTDAGVH GTGQVVHFET

      101                                                150
truB  RPVTFSAEQL AAALDTFRGD I.....EQIP SMYSALKYQG KKLYEYARQG
rsuA  AAFKLLPEH. DVAYDGNPLA Q.....QHGP RYFMLNKPQG ...YVCSTDD
truA  TALRKDAAWT LGVNANLPGD IAVRWVKTVP DDFHARFSAT ARRYRYIIYN

      151                                                200
truB  IEVPREARPI TVYELL.... .FIRHEGNEL ELEIHCSKGT YIRTIIDDLG
rsuA  PDHP...... TVLYFLDEPV AWKLHAAGRL DID...TTGL VLMTDDGQWS
truA  HRLRPAVLSK GVTHFYEPLD AERMHRAAQC LLGENDFTS. .FRAVQCQSR

      201                                                250
truB  EKLGCGAHVI YLRRLAVSKY PVERMVTLEH LRE....... LVEQAEQQDI
rsuA  HRITSPRHHC EKTYLVTLES PVAD.DTAEQ FAK....... GVQLHNEKDL
truA  TPWRNVMHIN VTRHGPYVVV DIKANAFVHH MVRNIVGSLM EVGAHNQPES

      251                                                300
truB  PAAELLD... PLLMPMDSPA SDYPVVNLPL TSSVYFKNGN PVRTSGAPLE
rsuA  TKPAVLEVIT PTQVRLTISE GRYHQVKRMF AAV....GNH VVELHRERIG
truA  WIAELLAAKD RTLAAATAKA EGLYLVAVDY P........D RYDLPKPPMG

      301                                  336
truB  GLVRVTEGEN GKFIGMGEID DEGRVAPRRL VVEYPA
rsuA  GITLDADLAP GEYRPLTEEE IASVV..... ......
truA  PLFLAD.... .......... .......... ......
```

FIGURE 11: Sequence comparison of the 16S RNA Ψ516 synthase with all other known Ψ synthases. The sequences of the tRNA Ψ55 synthase (*truB*), the tRNA anticodon arm Ψ synthase (*truA*), also known as *hisT*, and this protein (*rsuA*) were compared using the PILEUP module of the GCG Sequence Analysis Software programs (Devereux et al., 1984). Bold letters show identity or high similarity in all three sequences. The numbers at the left and right margins are amino acid sequence numbers for the *truB* protein.

is supported by the apparent presence of a slight amount of activity in the 30S ribosomal protein fraction used to form RNP(1−678). Figure 8 showed that, even in the absence of treatment with enzyme, there was a faint band corresponding to Ψ516 when CMC was added but not in its absence. Free RNA(1−678) showed no such band when treated in the same way (data not shown). This result explains an earlier finding that synthetic 30S particles, i.e., 30S particles reconstituted from 16S RNA transcripts and 30S proteins, contained a low level of Ψ (ca. 0.2 mol/mol) which was not present in the RNA before reconstitution (Krzyzosiak et al., 1987).

Renaturation of the Enzyme. Although most of the enzyme was found in the inclusion body fraction, enough was obtained from the S15 supernatant to compare the specific activity of the native recombinant protein with that which had been urea-denatured and renatured. Under our conditions of renaturation, 94% of the activity was regained.

Gene and Protein Sequence. The gene was identified as a previously sequenced open reading frame just upstream of the bicyclomycin resistance gene. We propose the name *rsuA* for this gene (ribosomal small subunit pseudU formation or U modification). Comparison of this sequence to the two other Ψ synthase sequences known did not reveal any conserved primary sequence elements despite the fact that all three enzymes should have the same catalytic center. This may be because the unique nucleotide sequence which is recognized by the enzyme is near to the U to be modified so that the common amino acid elements of the catalysis site in the enzyme are obscured by the unique amino acid residues needed for recognition.

A search of the Genbank for homologs of this enzyme in other species revealed a potentially equivalent enzyme in the genome of *B. subtilis*. There was a 57% identity plus high similarity to the *E. coli* enzyme, and we showed that there was an equivalent to Ψ516 in the *B. subtilis* small subunit rRNA. Overexpression in *E. coli* and assay of the enzymatic activity of the product of the gene will be necessary to confirm the identity of the *B. subtilis* gene product.

The *rsuA* sequence does not use the AGA/G pair of codons for arginine, despite the presence of 16 Arg residues among 231 total amino acids. The use of these rare codons, particularly within the first 25 codons, is a characteristic of a group of essential genes for various cellular functions that appear to be globally regulated by the availability of the corresponding $tRNA^{Arg}$ (Chen & Inouye, 1994). If the *rsuA* gene is essential, it is not regulated in this manner.

Importance of the Enzyme. The significance of this enzyme for *E. coli* depends on the role of Ψ516 in ribosome

structure and/or function. On the one hand, Ψ516 is located at a very important position in the small subunit rRNA, being a closing nucleotide for the "530" loop, a region known to be involved in codon recognition [Santer et al. (1993, 1995) and Powers and Noller (1994) and references cited therein]. On the other hand, 30S subunits lacking this Ψ as well as all other modified bases are functional *in vitro* in all of the partial reactions of protein synthesis, including codon recognition (Denman et al., 1989b). It should be emphasized, however, that the assays were all carried out *in vitro* under optimized conditions and even in that situation, the unmodified 30S was only about 50% as active as native 30S in each of the partial reaction assays.

Conservation of the modification among species is another indicator of functional importance. Although Ψ516 in the Gram-negative *E. coli* is also found in the Gram-positive *B. subtilis*, it is not present in the small subunit RNA of yeast cytoplasmic ribosomes despite the presence of a total of 13 Ψ residues in that molecule (Bakin & Ofengand, 1995). Clearly, Ψ516 is not universally found.

Ψ516 may still be important or even essential for *E. coli* since *in vitro* protein synthesis assays need not mimic *in vivo* activity in all respects. Moreover, Ψ516 may be important for *in vivo* ribosome assembly, a process not tested in our previous work, or even for the decidedly nonphysiological *in vitro* assembly process, since the lack of modification of 16S RNA negatively affected *in vitro* assembly (Cunningham et al., 1991). In a similar vein, the lack of formation of a single specific Gm residue strongly inhibited *in vivo* assembly of the large subunit of yeast mitochondrial ribosomes (Sirum-Connolly & Mason, 1993). A potential role in assembly is suggested by the fact that the true substrate for this enzyme appears to be an intermediate in the assembly process. The special properties of Ψ and the ways in which they could be used functionally or structurally have been described previously (Lane et al., 1992, 1995; Bakin & Ofengand, 1993; Bakin et al., 1994a,b). Probably the most reliable way to determine the importance of Ψ516 in the metabolism of *E. coli* is to disrupt the gene for its formation. Such experiments are now in progress.

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BI950654Y