Mutagenesis of Ribosomal Protein S8 from *Escherichia coli*: Expression, Stability, and RNA-Binding Properties of S8 Mutants†

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ABSTRACT: Protein S8, a 129 amino acid component of the *Escherichia coli* ribosome, plays an essential role in the assembly of the 30S ribosomal subunit and in the translational regulation of the *spc* operon by virtue of its capacity to bind specifically to rRNA and mRNA. To study structure–function relationships within the protein, we have constructed a vector for its high-level expression in vivo and developed efficient methods for its purification. Under our conditions, S8 accumulates to a level of 35% of the cellular protein and can be prepared at a purity of over 98% using either HPLC or a combination of ion-exchange and gel-filtration chromatography. The unique cysteine residue at position 126 was replaced by alanine or serine by oligonucleotide-directed mutagenesis, and the two mutant proteins, CA126 and CS126, were expressed and isolated. The effects of the mutations on the RNA-binding ability, secondary structure, and stability of S8 were assessed. CD spectra indicated that wild-type S8 and the two mutant proteins have very similar secondary structures at 25 °C. In addition, both mutants are metabolically stable in vivo as inferred from pulse–chase labeling and immunoprecipitation experiments. However, while CA126 exhibits the same affinity for RNA and the same susceptibility to urea and thermal denaturation as wild-type S8, CS126 is severely impaired in its ability to interact with RNA and displays a dramatic reduction in conformational stability. Our results suggest that Cys126 is unlikely to play a specific role in RNA recognition but that it is an integral part of the RNA-binding domain of protein S8.

Modern genetic techniques provide unprecedented opportunities for manipulating the structure and function of proteins in an effort to understand their biological properties. Cloned protein genes can be altered at will, and the corresponding polypeptides can be synthesized either on a small scale in vitro via coupled transcription–translation systems (Zubay, 1977) or in large quantities in vivo through the use of specialized vector–host systems (Studier et al., 1990). Surprisingly, these approaches have not yet been applied extensively to the study of structure–function relationships in ribosomal proteins. Nonetheless, several recent reports demonstrate the potential of such techniques for the analysis of protein–RNA and protein–protein interactions during ribosome assembly, the role of ribosomal proteins in protein biosynthesis, and the translational regulation of ribosomal protein synthesis (Donly & Mackie, 1988; Romero et al., 1990; Rutgers et al., 1991; Yeh et al., 1992; Wower et al., 1992).

Site- or region-directed mutagenesis, for example, has established the importance of the C-terminal portion of *Escherichia coli* protein S20 in S20–16S rRNA interaction (Donly & Mackie, 1988). In these studies, a number of S20 deletion mutants were synthesized in vitro and tested for their binding to the small-subunit rRNA. In a similar manner, both deletions and point mutations were used to identify a 62-residue segment within protein L25 from *Saccharomyces cerevisiae* that enables it bind to 25S rRNA (Rutgers et al., 1991). Regions of *S. cerevisiae* protein L1a that are necessary for its association with 5S rRNA have also been analyzed with the aid of L1a mutants translated in vitro (Yeh et al., 1992). A different approach, which took advantage of the in vivo overexpression of mutants of *E. coli* protein L2, a component of the peptidyltransferase center, revealed that polypeptides lacking a seven amino acid sequence near the C terminus were incorporated into 50S ribosomal subunits that were unable to assemble protein L16 or to function in protein synthesis (Romero et al., 1990).

We have recently isolated over 40 mutants of protein S8 (Wower et al., 1992), a polypeptide component of the *E. coli* ribosome that plays an essential part in 30S subunit assembly (Held et al., 1974) and mediates autogenous control of *spc* mRNA translation (Yates et al., 1980; Dean et al., 1981). Both activities are attributable to the interaction of S8 with either 16S rRNA or *spc* mRNA (Gregory et al., 1988). Cells expressing mutant S8 from plasmid-borne *rpsH* genes were identified by their fast growth on solid or liquid media, indicating that they were no longer subject to the inhibition of growth that accompanies moderate overexpression of the wild-type *rpsH* gene (Dean et al., 1981; Wower et al., 1992). The fast-growth phenotype presumably results from the inability of the S8 mutants to bind, and translationally regulate, *spc* operon mRNA. Because S8 recognizes virtually the same structural features in both *spc* mRNA and 16S rRNA (Gregory et al., 1988), the mutants were also expected to be defective in their interaction with rRNA. Although the mutations occurred throughout the polypeptide chain, most were confined to evolutionarily conserved sequences within its N-terminal, central, and C-terminal portions. As the majority of the mutant proteins were metabolically stable, we have been able to isolate a number of them for an analysis of their structure and RNA-binding capacities.

We presently describe the construction of a vector for the high-level expression of wild-type and mutant S8 from the bacteriophage T7 promoter in vivo (Studier et al., 1990). Under our conditions, S8 accumulates to a level of over 35% of the total cellular protein and could be prepared at a purity greater than 98% by simple chromatographic procedures. We took

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advantage of this system to isolate two S8 mutants in which the unique cysteine residue at position 126, previously reported to be essential for the interaction of the protein with RNA (Mougel et al., 1986), was changed to serine or alanine. The RNA-binding properties and physical characteristics of the mutant proteins are reported here.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, T4 DNA polymerase, T4 DNA ligase, and calf intestine alkaline phosphatase were obtained from New England Biolabs, International Biotechnologies, Inc. or Boehringer Mannheim. Modified T7 DNA polymerase (Sequenase 2.0) and T7 RNA polymerase were the products of U.S. Biochemical Corp. DNase I (Type II), and the protease inhibitors PMSF[1] and benzamidine were purchased from Sigma. BSA (fraction V) was from Pierce. RNasin ribonuclease inhibitor was supplied by Promega. SeaPlaque low-melting-point agarose was from FMC BioProducts, and IPTG was from 5 Prime–3 Prime, Inc. Oligonucleotides for mutagenesis were prepared by the DNA Synthesis Facility at the University of Massachusetts, Amherst. [α-^{35}S]dATP ($\geq$400 Ci/mmol) and [α-^{32}P]UTP (3000 Ci/mmol) were provided by Amersham and New England Nuclear, respectively. Elutip-d columns were from Schleicher and Schuell. Magna nylon filters (0.45 μM) were obtained from Fisher Scientific, and nitrocellulose membranes (HAWP) were purchased from Millipore. The Synchropak RP-P C_{18}-silica column was supplied by Symchrom, Inc. Sephacryl HR-100 gel and CM-Sephadex C-25 ion-exchange medium were from Pharmacia/LKB. Casamino acids, Bacto-Tryptone, and yeast extract were the products of Difco.

Construction of a High-Level Expression Vector for the rpsH Gene. The *rpsH* gene was placed under the control of the ϕ*10* T7 RNA polymerase promoter as follows. Plasmid pZNO28 (Wower et al., 1992) was first modified by the introduction of a *Nde*I restriction site that overlapped the initial AUG codon of the *rpsH* gene using the oligonucleotide-directed mutagenesis technique of Kunkel (1985) as described by Sambrook et al. (1989). The mutagenic primer was annealed to single-stranded pZNO28 DNA obtained from *E. coli* CJ236 (*dut*$^-$ *ung*$^-$) after infection with helper phage R408 (Russel et al., 1986). After second-strand synthesis with Sequenase in the presence of the four dNTPs and ligation with T4 DNA ligase, the closed circular DNA was introduced by transformation into *E. coli* XL1-B cells (Bullock et al., 1987). Clones were screened by restriction analysis, resulting in the isolation of plasmid pZNO28*Nde*I. In the next step, the single *Sph*I restriction site was removed from pET-3a (Studier et al., 1990) by digesting the plasmid with *Sph*I and filling in the overhanging ends with T4 DNA polymerase. The linear DNA was then isolated by agarose-gel electrophoresis and recircularized with T4 DNA ligase to yield plasmid pET-3aΔ*Sph*I. Finally, to place the *rpsH* gene adjacent to the ϕ10 promoter, plasmids pZNO28*Nde*I and pET-3aΔ*Sph*I were each cleaved with *Nde*I and *Eco*RI, and the products were fractionated by electrophoresis on a 0.6% (w/v) low-melting-point agarose gel. Bands containing the backbone fragment of pET-3aΔ*Sph*I and the 760-bp fragment of pZNO28*Nde*I, which spans the entire *rpsH* gene, were excised, melted at 65 °C, mixed, and ligated together to produce plasmid pET-*rpsH*. All plasmids were maintained in *E. coli* strain XL1-B. Routine recombinant DNA procedures were carried out essentially as described by Sambrook et al. (1989).

Mutagenesis of the rpsH Gene. The TGC triplet encoding Cys at position 126 of protein S8 was changed to TCC or GCC, encoding Ser or Ala, respectively, by oligonucleotide-directed mutagenesis of pZNO28 as described above (Kunkel, 1985; Sambrook et al., 1989). The mutagenic primers corresponded to the segment of the sense-strand DNA between codons 123 and 129 of the *rpsH* gene. Prospective mutants were identified by nucleotide sequence analysis of single-stranded DNA from the recombinant plasmids using the dideoxynucleotide chain termination method (Sanger et al., 1977) in conjunction with Sequenase and [α-^{35}S]dATP. To ensure that the DNA contained only the desired mutations, the entire *rpsH* gene was sequenced using the primers previously described (Wower et al., 1992). Approximately 15% of the clones exhibited unique codon changes, yielding plasmids pZNO28Ser126 and pZNO28Ala126. The metabolic stability of mutant S8 proteins, expressed from the pZNO28 derivatives in *E. coli* XL1-B cells, was assessed by specific immunoprecipitation and gel electrophoresis (Wower et al., 1992).

The mutant *rpsH* genes were substituted for the wild-type *rpsH* gene in plasmid pET-*rpsH*. Plasmid pET-*rpsH* was cleaved with *Sph*I and *Kpn*I, and the backbone fragment was isolated by electrophoresis on a low-melting-point agarose gel, dephosphorylated using calf intestine alkaline phosphatase, and purified by passage through an Elutip-d column. The 600-bp *Sph*I–*Kpn*I fragments containing the *rpsH* genes from pZNO28Ser126 and pZNO28Ala126 were purified separately on a low-melting-point agarose gel and ligated to the pET-*rpsH* backbone to yield plasmids pET-*rpsH*Ser126 and pET-*rpsH*Ala126.

Overexpression of Protein S8. Plasmid pET-*rpsH* and its mutant derivatives were introduced into *E. coli* BL21 (DE3)/pLysS (Studier et al., 1990) by transformation. The cells were grown to mid-log phase (OD_{600} 0.4–0.6) in YT broth (8 g/L Bacto-Tryptone, 5 g/L yeast extract, and 5 g/L NaCl) containing 200 μg/mL ampicillin and 30 μg/mL chloramphenicol. T7 RNA polymerase, encoded in the host chromosome, was then induced by the addition of IPTG to a final concentration of 1.0 mM, leading to the transcription of the *rpsH* gene and overproduction of protein S8. The level of S8 synthesis at various times after induction was monitored by subjecting cell extracts to SDS–PAGE (Laemmli & Favre, 1973) followed by staining with Coomassie brilliant blue.

Purification of Protein S8. For small-scale preparations of protein S8, 150-mL cultures were harvested 2 h after induction by centrifugation at 5000*g* for 10 min. The cell pellet was washed once with buffer containing 10 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, and 18 mM β-mercaptoethanol and resuspended in the same buffer in the presence of the protease inhibitors PMSF (2 mM) and benzamidine (1 mM). β-Mercaptoethanol was omitted in the purification of the S8 mutants, CA126 and CS126, since they no longer contained cysteine. The cells were then lysed by sonication for 5 min in an ice bath and incubated with 10 μg/mL DNase I for 20 min at 0 °C. When cells were grown at 37 °C, S8 accumulated in insoluble inclusion bodies, which could be separated from the cytoplasm by centrifugation at 10000*g* for 30 min. The inclusion bodies were resuspended in 3 mL of fresh 6 M urea containing 50 μM PMSF, 100 μM benzamidine, and 18 mM

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; TFA, trifluoroacetic acid; BSA, bovine serum albumin; SDS–PAGE, 15% polyacrylamide gel electrophoresis in the presence of 0.1% SDS; CD, circular dichroism.

β-mercaptoethanol and solubilized by gentle stirring overnight at 4 °C. The extract was then clarified by centrifugation at 12000*g* for 20 min.

A 750-μL aliquot of the urea extract was loaded onto a Synchropak RP-P C_{18} silica column (6.5-μm silica, 300-Å pore, 4.1 × 250 mm). Proteins were eluted at room temperature using a linear gradient of 25–60% acetonitrile in 0.1% TFA and detected by monitoring the eluant at 214 and 280 nm. All solvents were degassed by filtration through 0.45-μm nylon filters prior to use. Peak fractions were lyophilized and the proteins were dissolved in 6 M urea. The proteins present in these fractions were analyzed by SDS–PAGE.

For large-scale preparations, inclusion bodies were isolated from 3.6 L of cell culture and solubilized as described above in 50 mL of buffer A (50 mM NaOAc, pH 5.6, 6 M urea, 18 mM β-mercaptoethanol, 50 μM PMSF, and 100 μM benzamidine). After clarification, the extract was applied to a CM-Sephadex C-25 ion-exchange column (2.5 × 51 cm), and the proteins were eluted at 4 °C with a 0–0.4 M LiCl gradient in buffer A. Fractions containing S8 were identified by SDS–PAGE, pooled, concentrated using an Amicon ultrafiltration apparatus with a YM5 membrane, and applied to a Sephacryl HR-100 gel filtration column (2.5 × 120 cm). The proteins were eluted with buffer A and identified by SDS–PAGE.

The purity of isolated S8 was monitored by SDS–PAGE and/or two-dimensional gel electrophoresis. In the latter procedure, the first dimension was performed according to the method of Mets and Bogorad (1974) and the second dimension was as described by Kaltschmidt and Wittmann (1970). The concentration of the protein was determined by the Bradford method using BSA (fraction V) as a standard (Bradford, 1976).

Preparation of ^{32}P-Labeled RNA. Fragments of 16S rRNA containing the S8 binding site were transcribed by T7 RNA polymerase from pEX0, a derivative of plasmid pT7-1 containing a 384-bp *Sca*I–*Sal*I fragment encoding nucleotides 435–821 of mature 16S rRNA (Gregory et al., 1988). Runoff transcripts of 299 nucleotides were produced by treating approximately 2 μg of pEX0 with *Bgl*II and incubating the linearized plasmids with 500 μM each of the four ribonucleoside triphosphates, 50 μCi of [α-^{32}P]UTP, 40 units of T7 RNA polymerase, and 40 units of RNasin ribonuclease inhibitor in a buffer containing 40 mM Tris-HCl (pH 7.5), 10 mM NaCl, 6 mM $MgCl_2$, 10 mM dithiothreitol, and 50 μg/mL BSA at 37 °C for 1.5 h. The incubation mixture was then extracted with phenol/chloroform (1:1 v/v) and the RNA was precipitated with ethanol. The transcripts were purified by electrophoresis in 5% denaturing polyacrylamide gels (acrylamide/*N*,*N*′-methylenebis[acrylamide] 19:1, w/w) in TBE buffer (100 mM Tris-HCl, 100 mM boric acid, pH 8.3, and 2.5 mM EDTA) containing 8 M urea and visualized by autoradiography. The radioactive band was excised and minced, and the transcripts were extracted in a buffer containing 0.5 M NH_4OAc, 0.01 M $Mg(OAc)_2$, 0.1 mM EDTA, and 0.1% SDS, precipitated with ethanol, and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA).

Assay of S8–rRNA Interaction. The rRNA transcripts (0.01–1 pmol) were incubated at 40 °C for 1 h in TMK buffer (50 mM Tris–acetate, pH 7.6, 20 mM $Mg(OAc)_2$, 350 mM KCl, and 5 mM β-mercaptoethanol) in the presence of 0.2 unit/μL RNasin prior to the assay. Highly purified wild-type and mutant S8, at concentrations of either 1.6 or 3.2 μM, were incubated separately in TMK buffer containing 40 μg/mL BSA at 40 °C for 1 h. A fixed amount of ^{32}P-labeled rRNA transcript was then mixed with different amounts of wild-type or mutant S8. The protein–rRNA mixtures were incubated for 10 min at 40 °C, followed by 15 min at 0 °C, and filtered through nitrocellulose membranes with gentle suction. Filters were washed twice with 150 mL of cold TMK buffer. Protein-bound rRNA fragments were retained on the filter while unbound rRNA passed through into filtrate. The percentage of the input ^{32}P retained on the filters was determined by Čerenkov counting in a Beckman scintillation counter.

Circular Dichroism Measurements. CD spectra were measured with an Aviv Model 62DS spectrometer. A rectangular cuvette with a 1-mm path length was used in all measurements. Protein solutions (0.1–0.3 mg/mL) were prepared in PKF buffer (1.0 mM potassium phosphate, pH 7.4, and 300 mM KF). β-Mercaptoethanol (5 mM) was added to the buffer when wild-type S8 was analyzed. S8 concentrations determined by the Bradford method with BSA as a standard were multiplied by a correction factor of 0.94. This factor was determined by comparing the S8 concentration derived using BSA as a standard with the absolute concentration obtained from dry-weight measurements. The molar extinction coefficient of S8 at 280 nm was found to be 5762 M^{-1} cm^{-1} by dry-weight measurement. Spectra were recorded at 1-nm intervals between 180 and 240 nm at 25 °C, with an averaging time of 10 s at each wavelength, and were corrected by subtracting the background ellipticity due to buffer alone. The mean molar residue ellipticity, [Θ], for all wavelengths was calculated according to

$$[\Theta] = \theta M_0 / lc$$

where θ is the measured ellipticity in millidegrees, M_0 is the mean residue molecular weight, l is the path length in millimeters, and c is the protein concentration in milligrams per milliliter (Schmid, 1989).

For urea denaturation, samples were prepared in PKF buffer with appropriate concentrations of urea and were incubated at room temperature for at least 30 min prior to measurement. Spectra were recorded between 215 and 230 nm under the conditions described above. To test the reversibility of urea denaturation, wild-type S8 and CA126 were unfolded by incubation in PKF buffer containing 7 M urea for 1 h. The solution was then adjusted to lower urea concentrations by dilution and incubated for 30 min, and its CD spectrum measured. In all cases, the final protein concentration was 0.1 mg/mL.

Thermal denaturation of protein S8 in PKF buffer was monitored by the change in ellipticity at 222 nm. Spectra were recorded at 2 °C intervals between 10 and 85 °C, with an equilibration period of 1 min at each temperature. The reversibility of thermal unfolding was assessed by cooling the sample and remeasuring the ellipticity at 222 nm.

RESULTS

Overexpression of Protein S8. Many techniques for the investigation of structure–function relationships in proteins require that the proteins be available in large amounts and at high purity. To construct a vector for the overproduction of *E. coli* ribosomal protein S8 in vivo, we made use of the conditional expression system developed by Studier et al. (1990). The *rpsH* gene, which encodes protein S8, was isolated from plasmid pZNO28*Nde*I and inserted into plasmid pET-3a under the control of the strong φ10 T7 RNA polymerase

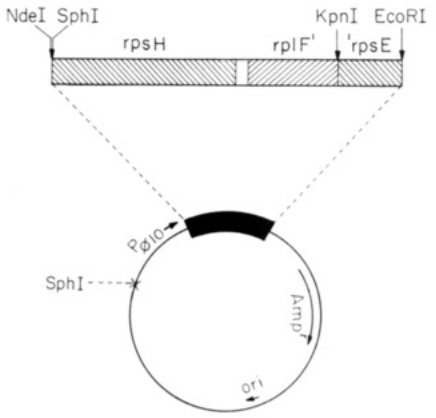

FIGURE 1: Construction of plasmid pET-*rpsH*. Symbols are as follows: *rpsH*, S8 gene; *rplF'*, L6 gene, truncated at its 3′ end; *'rpsE*, S5 gene, truncated at its 5′ end; $P_{\phi 10}$, T7 promoter and translation initiation region for T7 gene 10 protein; Amp^r, ampicillin resistance gene; ori, origin of replication. Unique recognition sites for *Nde*I, *Sph*I, *Kpn*I and *Eco*RI are also indicated.

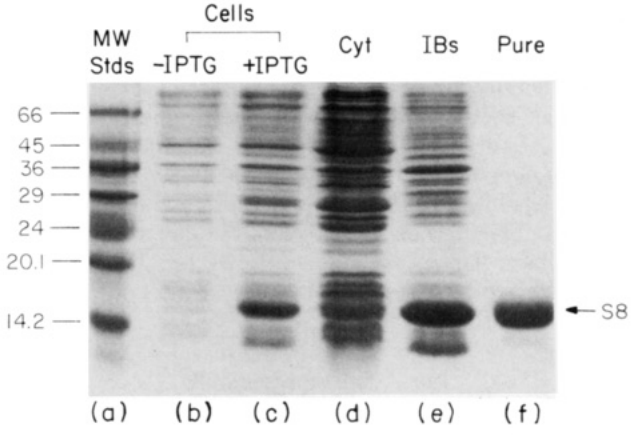

FIGURE 2: Overexpression and purification of wild-type protein S8. Proteins were separated by SDS–PAGE and stained with Coomassie brilliant blue. (a) Molecular mass standards (kilodaltons). (b) Cell extract before induction with IPTG. (c) Cell extract after induction with IPTG. (d) Cytoplasm after sedimentation of inclusion bodies. (e) Solubilized inclusion bodies. (f) Protein S8 after purification by reverse-phase HPLC.

promoter and the efficient translation signal of the the bacteriophage T7 gene 10 protein (Figure 1). The resulting plasmid, pET-*rpsH*, was introduced by transformation into *E. coli* strain BL21 (DE3)/pLysS, a λ lysogen in which expression of the T7 RNA polymerase gene is regulated by the *lacUV5* promoter. After induction of the T7 polymerase gene by IPTG, the protein composition of bacteria carrying pET-*rpsH* was analyzed by SDS–PAGE. As shown in Figure 2, an intense band corresponding to protein S8 in molecular weight was observed in extracts from induced, but not uninduced, cells. This component was estimated to comprise about 35% of the total cellular protein by densitometry of the stained gel.

Purification of Protein S8. Techniques were developed to isoalte S8 in both small and large amounts. A small-scale procedure was adequate for most of the RNA-binding and biophysical studies described here, as they required only a few hundred micrograms of purified S8. When the bacterial cultures were grown at 37 °C, the overproduced S8 aggregated in the form of inclusion bodies, while at 25–30 °C, most of the S8 was found in soluble form in the cell extract. As inclusion bodies can be readily sedimented by centrifugation, this method was used as the first step in the isolation of S8.

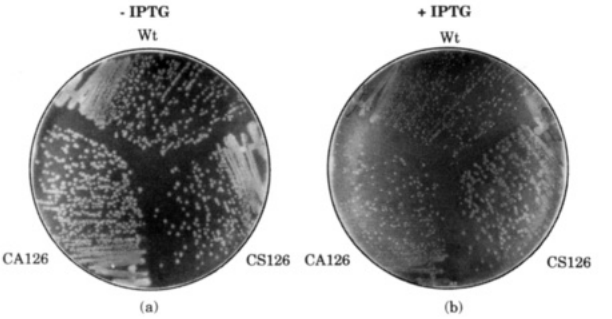

FIGURE 3: Growth of strains expressing wild-type S8, CS126, and CA126 on solid medium. XL1-B cells harboring plasmids pZNO28, pZNO28Ser126, and pZNO28Ala126 were streaked on nutrient agar containing ampicillin in the (a) absence or (b) presence of IPTG and incubated for 20 h at 37 °C. The *Sph*I–*Kpn*I fragments from the mutagenized plasmids were excised and recloned prior to growth assays.

Earlier studies indicated that urea-treated S8 can be renatured in an appropriate buffer without loss of function (Muto et al., 1974; Mougel et al., 1986). Therefore, the isolated inclusion bodies were solubilized in 6 M urea by overnight stirring at 4 °C. More than 60% of the protein in the urea extracts was S8 (Figure 2). The protein was further purified by chromatography on an analytical reverse-phase HPLC column. Wild-type S8 was eluted at 43.8–46.0% acetonitrile in 0.1% TFA. Three separate peaks, containing intact S8, a degraded fragment of S8, and unidentified proteins of high molecular weight, were usually observed. The isolated S8 was at least 98% pure as demonstrated by densitometric scans of SDS–polyacrylamide gels such as that presented in Figure 2, lane f. The yield was about 16 mg/L of cell culture. Large-scale purification procedures were designed to prepare S8 for NMR investigations which will be reported separately. In this case, S8 can be prepared at a purity of over 98%, and with a yield of about 20 mg/L of cell culture by a combination of traditional ion-exchange and gel-filtration chromatography (see Experimental Procedures).

Mutants of Protein S8 Containing Ser and Ala at Position 126. To assess the role of Cys126 in S8–rRNA interaction, the corresponding codon was changed to that for Ser or Ala by oligonucleotide-directed mutagensis of the *rpsH* gene in plasmid pZNO28*Nde*I. When the plasmids were expressed in *E. coli* XL1-B, cells harboring pZNO28Ala126 formed small colonies on solid growth medium, equivalent in size to cells transformed with wild-type plasmid, whereas those expressing pZNO28Ser126 formed large colonies (Figure 3). The mutant *rpsH* genes were then introduced into plasmid pET-3a as described for wild-type *rpsH* to yield pET-*rpsH*Ser126 and pET-*rpsH*Ala126. Expression of these two plasmids in *E. coli* BL21 (DE3)/pLysS led to the synthesis of the altered proteins, CS126 and CA126, in abundance (Figure 4). The chromatographic properties of these two variants were essentially indistinguishable from those of wild-type S8.

Under nonreducing conditions, wild-type S8 readily dimerizes via its single cysteine residue, which interferes with many kinds of physicochemical investigations. One objective of our work was to isolate S8 mutants that lacked the ability to dimerize. Analysis of CS126 and CA126 by nonreducing SDS–PAGE revealed that neither of the mutant proteins showed any propensity toward dimer formation (not shown).

RNA-Binding Capacity of Protein S8 and Its Mutant Derivatives. The affinity of wild-type S8 for its rRNA binding site was compared with that of CS126 and CA126 by the nitrocellulose filter assay (Spierer et al., 1978). In the present

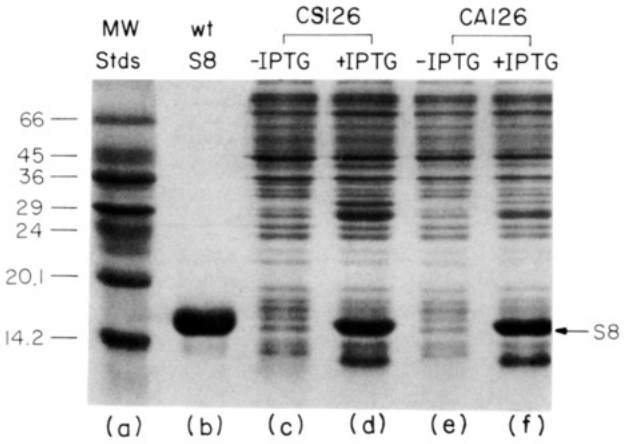

FIGURE 4: Overexpression of S8 mutants CS126 and CA126. Cells were prepared as described in Experimental Procedures and fractionated by SDS–PAGE. (a) Molecular mass standards (kilodaltons). (b) Wild-type S8. (c and d) Extracts of BL21(DE3)/pLysS cells harboring plasmid pET-*rpsH*Ser126, before and after induction with IPTG. (e and f) Extracts of BL21(DE3)/pLysS cells harboring plasmid pET-*rpsH*Ala126, before and after induction with IPTG.

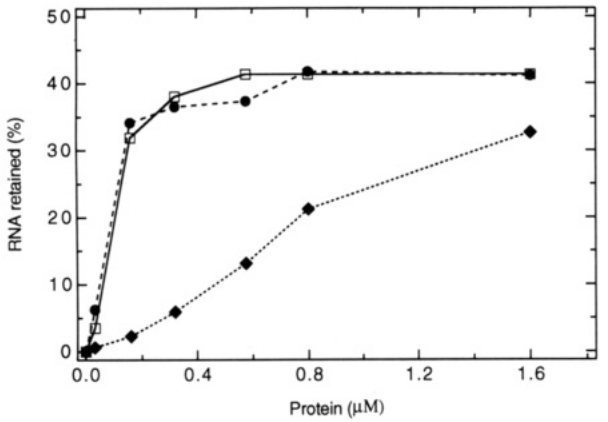

FIGURE 5: Binding curves for the interaction of wild-type and mutant S8 with rRNA. A 299-nucleotide transcript spanning the S8 binding site from *E. coli* 16S rRNA was transcribed in vitro and incubated with increasing amounts of protein. Background retention of RNA on the filter was subtracted before plotting. Wild-type S8 (●); CA126 (□); CS126 (◆).

experiments, the S8 binding site was synthesized in vitro as part of a 299-nucleotide transcript encompassing positions 435–708 of the 16S rRNA (Gregory et al., 1988). Varying amounts of protein were incubated with a fixed amount of radioactively labeled RNA and rapidly filtered through nitrocellulose membranes. Whereas the protein and protein–rRNA complexes bound to the membranes, free rRNA passed through into the filtrate. The amount of complex retained on the filters was quantitated by scintillation counting.

Binding curves for wild-type and mutant S8 are presented in Figure 5. Background retention of rRNA in the absence of S8, which was normally in the range of 5–8% of the total rRNA, was subtracted from the data before plotting. Saturation was usually observed when 30–40% of the rRNA was associated with protein. Incomplete saturation may result from degradation of the rRNA transcripts, inability of some transcripts to fold properly, or the failure of a fraction of the S8–rRNA complexes to be retained on the filter (Gregory et al., 1988). Apparent association constants were calculated from the protein concentration necessary to half-saturate the rRNA. Each value was derived from the average of at least three experiments. The K'_a values for the interaction of wild-type S8, CS126, and CA126 are presented in Table I. While CA126 binds to the rRNA fragment as well as wild-type S8, the affinity of CS126 is reduced by almost one order of magnitude.

Analysis of Protein S8 by Circular Dichroism. The secondary structures of wild-type S8 and the two mutants, CA126 and CS126, were studied by circular dichroism. The proteins were dissolved in PKF buffer instead of TMK buffer since KF and potassium phosphate have a higher transparency in the far-UV region than KCl and Tris–acetate. However, both wild-type and mutant proteins were found to have identical spectra in TMK and PKF buffers between 205 and 260 nm (not shown). This suggests that there is no significant difference in the structure of protein S8 in the two buffers. The CD spectra of wild-type and mutant S8, depicted in Figure 6, are nearly superimposable, indicating that their secondary structures are very similar at room temperature.

The conformational stabilities of wild-type S8, CA126 and CS126 were investigated by monitoring changes in mean molar residue ellipticity at 222 nm ($[\Theta]_{222}$) during urea and thermal denaturation. A representative denaturation curve for CA126 in the presence of increasing urea concentration is depicted in Figure 7 (top). In the case of wild-type S8 and CA126, urea-induced unfolding was determined to be reversible (see Figure 7, top). The urea denaturation curves for the three proteins were analyzed according to a two-state folding/unfolding mechanism. In these circumstances the unfolded fraction of protein, F_U, can be calculated as

$$F_U = (Y_F - Y)/(Y_F - Y_U) \quad (1)$$

where Y is the observed value of $[\Theta]_{222}$ and Y_F and Y_U are the values of $[\Theta]_{222}$ characteristic of the folded and unfolded protein conformations, respectively. Y_F and Y_U were derived from a least-squares analysis of data for pre- and posttransition states (see Figure 7, top). Figure 7 (bottom), in which F_U is plotted against urea concentration for wild-type and mutant S8, shows that CS126 is considerably more sensitive to denaturation by urea than wild-type S8 or CA126.

The free energy change for the transition, ΔG, can be estimated by

$$\Delta G = -RT \ln [(Y_F - Y)/(Y - Y_U)] \quad (2)$$

Since ΔG decreased linearly with increasing urea concentration for all three proteins, the data can be fit to the following equation by least-squares analysis:

$$\Delta G = \Delta G(H_2O) - m[\text{urea}] \quad (3)$$

where $\Delta G(H_2O)$ is the free energy change in the absence of urea and m is the rate of decrease of ΔG with increasing urea concentration. $\Delta G(H_2O)$ was obtained by assuming that the dependence of ΔG on urea concentration can be extrapolated linearly to zero urea concentration. The urea concentration at the midpoint of the unfolding curve, $[\text{urea}]_{1/2}$, is equal to $\Delta G(H_2O)/m$ since at that point ΔG is zero. The values of $\Delta G(H_2O)$, m, and $[\text{urea}]_{1/2}$ for wild-type S8, CS126, and CA126 are presented in Table I.

Thermal denaturation of wild-type and mutant S8 yielded T_m values for wild-type S8, CA126, and CS126 of 57, 56, and 47 °C, respectively (Table I). In contrast with urea denaturation, thermal denaturation of S8 was not reversible. Both urea and thermal denaturation experiments therefore indicate that wild-type S8 and CA126 are substantially more stable than CS126. At the same time, all three proteins were found to be metabolically stable in vivo. Almost all of the labeled amino acids incorporated into the polypeptides during a 0.5-

Table I: Characteristics of Wild-Type S8 and Its Mutant Derivatives

protein	RNA-binding ability K'_a (M^{-1})	urea denaturation $\Delta G(H_2O)$ (kcal mol^{-1})	urea denaturation m (cal mol^{-1} M^{-1})	urea denaturation $[urea]_{1/2}$ (M)	thermal denaturation T_m (°C)	metabolic stability in vivo[a]
wild type	1.0×10^7	2.25	877	2.57	57	stable
CA126	9.1×10^6	2.06	856	2.41	56	stable
CS126	1.3×10^6	1.18	733	1.61	47	stable

[a] Metabolic stability was assessed as described by Wower et al. (1992).

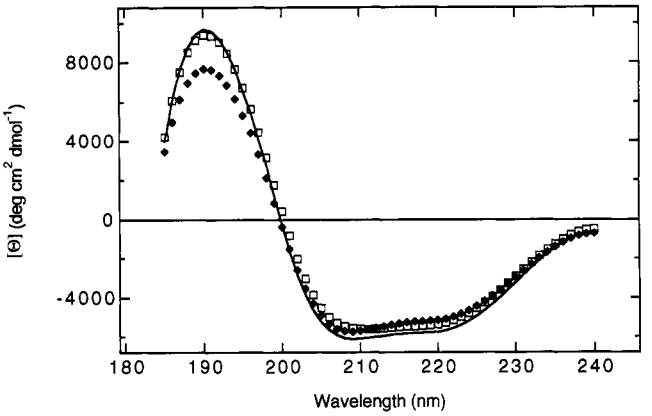

FIGURE 6: CD spectra of wild-type S8, CA126, and CS126. CD measurements were made in PKF buffer as described in Experimental Procedures. Wild-type S8 (—); CA126 (□); CS126 (◆).

min pulse can be immunoprecipitated with S8-specific antibodies after a 2-h chase (Table I).

DISCUSSION

Studies of the biological and physicochemical properties of *E. coli* ribosomal proteins are greatly facilitated by the availability of large amounts of highly purified material. In the past, it was necessary to isolate individual ribosomal proteins from a complex mixture of more than 50 polypeptides which, together with the 16S, 23S, and 5S rRNAs, comprise the *E. coli* ribosome. In the present report, we show that 30S-subunit protein S8 can be produced at a level representing 35% of the total cellular protein when expressed from a bacteriophage T7 promoter in the pET-3a–BL21(DE3) plasmid–host system of Studier et al. (1990). During construction of the S8 expression vector, pET-*rpsH*, pET-3a was modified so that the wild-type S8 gene could be easily replaced by mutant S8 genes generated in plasmid pZNO28 (Wower et al., 1992).

The excess S8 synthesized by the transformed BL21(DE3) cells was found in two different forms, depending on the growth temperature. Between 25 and 30 °C, most of the S8 remained in soluble form, whereas at 37 °C, the protein was sequestered in insoluble inclusion bodies. In the former case, the protein can be further purified without exposure to urea if so desired. However, the presence of S8 in inclusion bodies proved to be advantageous in that they can be conveniently separated from the bulk of the cellular proteins by sedimentation, which we therefore adopted as the initial purification step. Two schemes were developed to isolate protein S8 from inclusion bodies, each of which yielded preparations that were over 98% pure. Although they both involved the use of urea, we show that the resulting material regained its normal RNA-binding capacity and secondary structure after removal of urea. In the first procedure, reverse-phase HPLC was used for the rapid purification of small amounts of S8 (1–5 mg) for RNA-binding assays and optical measurements. The second procedure,

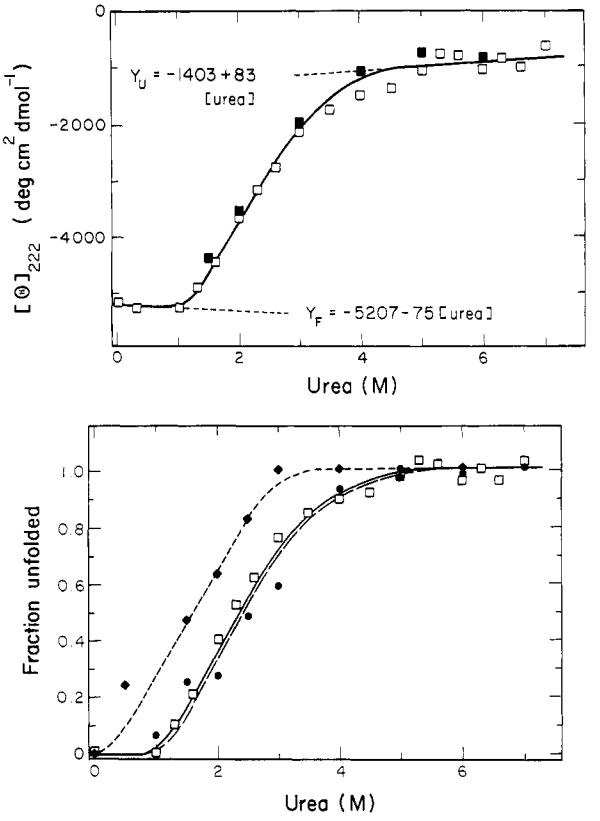

FIGURE 7: Denaturation and renaturation of wild-type and mutant S8. (Top) Mean molar residue ellipticity of CA126 at 222 nm ($[\Theta]_{222}$) at 25 °C is plotted as a function of urea concentration. Denaturation (□); renaturation (■). Coincidence of the denaturation and renaturation curves indicates that CA126 was completely renatured upon removal of urea. The dashed lines for the pre- and posttransition states are described by the equations $Y_F = -5207 - 75[urea]$ and $Y_U = -1403 + 83[urea]$, derived from least-squares analysis of data for the two states. Y_F and Y_U were used to calculate F_U and ΔG of protein according to eq 1 and 2 (see text). Similar results were obtained for wild-type S8 and CS126, although the latter protein was not tested for renaturation. (Bottom) F_U as a function of urea concentration at 25 °C for wild-type S8 (●), CA126 (□), and CS126 (◆).

which entailed conventional ion-exchange and gel-filtration chromatography, was developed to provide larger amounts of protein (50–100 mg) for NMR analysis and other biophysical experiments. Both methods should be applicable to the purification of other ribosomal proteins expressed in the pET-3a–BL21(DE3) plasmid–host system.

Little is known about the structural features of ribosomal proteins that participate in specific interactions with rRNA. In the case of S8, several studies suggest that the C-terminal portion of the polypeptide plays an important role in its association with 16S rRNA. The first evidence implicating the C-terminal region stemmed from experiments in which S8 was found to be protected from carboxypeptidase digestion when complexed with rRNA (Daya-Grosjean et al., 1974). More recently, the involvement of the C-terminal sequence

in S8 function has been demonstrated by directed mutagenesis of the S8 gene (Wower et al., 1992). In particular, the RNA-binding activity of two truncated forms of S8, which lacked either nine or 30 amino acids from the C terminus, was found to be severely curtailed (our unpublished results). Furthermore, chemical modification of the unique Cys residue at position 126 by *N*-ethylmaleimide or *p*-(chloromercuri)-benzenesulfonate almost completely abolished the binding of S8 to 16S rRNA (Mougel et al., 1986).

As reported here, we have employed site-specific amino acid replacement to further investigate the importance of Cys126 in S8–16S rRNA interaction. At the same time, we wished to eliminate the potential for S8 dimerization that we observed at high protein concentrations under nonreducing conditions and which is therefore a serious impediment to biophysical analysis. We reasoned that Ala might serve as a suitable replacement for Cys126 as it occurs at the equivalent position of a number of S8 homologs from plant chloroplasts (Markmann-Mulisch & Subramanian, 1988). Ser, though far more hydrophilic, was selected because it is similar in both size and steric properties to Cys. While the substitution of Ser for Cys at position 126 led to a 7–8-fold decrease in the affinity of S8 for its binding site, the presence of Ala at the same position had no detectable effect on the association constant. Thus, in contrast to the inference drawn from the chemical modification studies (Mougel et al., 1986), Cys126 is not essential for the RNA-binding activity of S8. These results are in accord with the effects of the mutant proteins on cell growth when overexpressed in vivo. Because CA126 binds normally to rRNA, its presence reduces the growth rate to approximately the same extent as excess wild-type S8, presumably via its interaction with, and translational repression of, *spc* operon mRNA (Wower et al., 1992). Conversely, the presence of excess CS126 had little influence on growth rate as its low affinity for rRNA renders it ineffective in *spc* operon regulation.

The secondary structures of wild-type and mutant S8 at room temperature were judged to be very similar from their CD spectra. We estimate that wild-type S8 contains 11% α-helix, 58% β-sheet, and 31% random coil according to the PROSEC program (Aviv) based on the method of Chang et al. (1978). The CD data for the S8 mutants yielded comparable values. Our predictions differ significantly from earlier estimates, which indicate that S8 is composed of 31–43% α-helix, 21–32% β-sheet, and 24–48% random coil (Lemieux et al., 1974; Dijk et al., 1986). The accuracy of these structural predictions depends strongly upon the precision with which the protein concentration, and hence the mean molar residue ellipticity, is determined. We calculate that an error of ±10% in protein concentration would lead to values for wild-type S8 in the range of 10–13% α-helix, 56–60% β-sheet, and 30–31% random coil. Although we cannot account for the disparity between our results and those of previous studies, the preponderance of β-sheet conformation is compatible with the observations of Gratzer and co-workers, who showed on the basis of optical rotatory dispersion and infrared spectroscopy that an unfractionated mixture of *E. coli* ribosomal proteins contained substantial amounts of β-structure but very little α-helix (McPhie & Gratzer, 1966; Cotter & Gratzer, 1969). It is noteworthy in this regard that a wide variety of eukaryotic proteins that interact with RNA share a common RNA-binding surface known as the RNA recognition motif which consists of a four-stranded antiparallel β-sheet (Kenan et al., 1991). Similar structural motifs in ribosomal protein S5 are believed to be at or near the sites at which this protein contacts 16S rRNA (Ramakrishnan & White, 1992). Our own predictions of S8 secondary structure, based on the algorithms of Garnier et al. (1978) and Chou and Fasman (1978), suggest that this protein contains a number of segments of β-structure that could form an analogous RNA-binding domain. We note that Cys126 is located in one of the predicted β-strands.

While the wild-type and mutant forms of protein S8 are metabolically stable in vivo, they can be differentiated by their susceptibility to urea and thermal denaturation in vitro. Both wild-type S8 and the CA126 mutant exhibit very similar conformational stabilities and both can be renatured reversibly and, apparently, completely after unfolding in urea. The restoration of S8 to its normal state following the removal of urea demonstrates that the exposure of ribosomal proteins to this denaturant during purification does not necessarily lead to irreversible changes in their physical or functional properties. On the other hand, the CS126 mutant is much more readily denatured than the other two proteins. Its melting temperature is almost 10 °C lower and the $\Delta G(H_2O)$ associated with its unfolding in urea is reduced by about 1 kcal/mol. The physical characteristics of the wild-type and mutant proteins thus correlate well with their RNA-binding properties. CA126 exhibits the same stability to denaturation and the same affinity for its rRNA binding site as wild-type S8, whereas CS126 displays reductions in both conformational stability and its capacity to associate with rRNA.

Our results suggest that the amino acid at position 126 is an integral part of the RNA-binding domain of S8 but that it is unlikely to play a specific role in RNA recognition since neither the stability nor the RNA-binding function of the protein changes significantly when Cys is replaced by Ala. By contrast, the substitution of Cys126 by Ser appears to destabilize the tertiary folding of the polypeptide chain. In the latter case, the strongly hydrophilic side chain of Ser may disrupt hydrophobic interactions that stabilize the C-terminal sequence of the protein, perhaps by hydrogen-bonding to neighboring amino acid residues. Ala, on the other hand, may be tolerated at position 126 because it is only slightly less hydrophobic than Cys (Kyte & Doolittle, 1982; Engelman et al., 1986). For much the same reason, the loss of RNA-binding activity associated with the modification of Cys126 by bulky substituents such as *N*-ethylmaleimide or *p*-(chloromercuri)benzenesulfonate (Mougel et al., 1986) may result from structural perturbations within the RNA-binding domain rather than direct interference with protein–rRNA contacts.

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