The location of protein S8 and surrounding elements of 16S rRNA in the 70S ribosome from combined use of directed hydroxyl radical probing and X-ray crystallography

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ABSTRACT

Ribosomal protein S8, which is essential for the assembly of the central domain of 16S rRNA, is one of the most thoroughly studied RNA-binding proteins. To map its surrounding RNA in the ribosome, we carried out directed hydroxyl radical probing of 16S rRNA using Fe(II) tethered to nine different positions on the surface of protein S8 in 70S ribosomes. Hydroxyl radical-induced cleavage was observed near the classical S8-binding site in the 620 stem, and flanking the other S8-footprinted regions of the central domain at the three-helix junction near position 650 and the 825 and 860 stems. In addition, cleavage near the 5′ terminus of 16S rRNA, in the 300 region of its 5′ domain, and in the 1070 region of its 3′-major domain provide information about the proximity to S8 of RNA elements not directly involved in its binding. These data, along with previous footprinting and crosslinking results, allowed positioning of protein S8 and its surrounding RNA elements in a 7.8-Å map of the Thermus thermophilus 70S ribosome. The resulting model is in close agreement with the extensive body of data from previous studies using protein–protein and protein–RNA crosslinking, chemical and enzymatic footprinting, and genetics.

Keywords: ribosome structure; RNA–protein interaction; rRNA; S8

INTRODUCTION

Ribosomal proteins are believed to be examples of some of the founding proteins in molecular evolution and provide powerful model systems for studying protein–RNA interactions. Among them, the small subunit protein S8 is one of the most extensively studied. It is a primary binding protein that binds independently to the central domain of 16S rRNA and is required for proper assembly of the 30S ribosomal subunit (Nomura et al., 1969; Schaup et al., 1971). According to the 30S assembly map (Mizushima & Nomura, 1970; Held et al., 1974), S8 is required for subsequent incorporation of proteins S5 and S12, but, curiously, seems not to influence the assembly of central domain-binding proteins such as S15, S6, S18, and S11.

The RNA binding site for S8 was initially localized to the 620 stem (helix 21) of 16S rRNA by RNase protection studies (Schaup et al., 1973; Ungewickell et al., 1975; Zimmermann et al., 1975; Muller et al., 1979; Zimmermann & Singh-Bergmann, 1979). Additional support for this assignment came from chemical crosslinking (Wower & Brimacombe, 1983; Urlaub et al., 1997) and footprinting (Mougel et al., 1987) experiments. The RNA-recognition features for S8 were further localized to the middle of the 620 stem around the single nucleotide bulge at A595 and the 2-nt bulge at U641–A642 by modification-interference and mutagenesis studies (Thurlow et al., 1983; Gregory & Zimmermann, 1986; Wower et al., 1992; Mougel et al., 1993; Allmang et al., 1994; Wu et al., 1994). In vitro selection of S8-binding variants from a pool of RNAs in which the S8-binding region was randomized generated a major class of S8 aptamers in which the phylogenetically conserved G597–C643 base pair and
bulged A642 appeared in the context of a secondary structure resembling the natural one (Moine et al., 1997), providing additional support for the importance of these features in RNA recognition by S8.

Measurements of the binding affinities of S8 for minimal RNA-binding-site homologs reinforced the view that the region of 16S rRNA contained within the 588–603/635–651 sequences contained the sole determinants for S8 binding (Mougel et al., 1993; Wu et al., 1994). However, chemical and enzymatic footprinting studies showed protection by S8 of parts of 16S rRNA that lie outside the 620 helix (Fig. 1), suggesting the possibility of additional contacts between S8 and the central-domain RNA (Svensson et al., 1988; Powers & Noller, 1995). Interaction of S8 with these additional elements, including the three-helix junction at the convergence of helices 20, 21, and 22, the 820 stem (helix 25), and the flanking 860 region (helix 26a), are consistent with a more extensive role for S8 in influencing the organization of the central domain RNA, and help to explain its established importance for 30S subunit assembly.

To gain a more comprehensive understanding of the rRNA environment surrounding protein S8 within the ribosome, we carried out directed hydroxyl radical probing (Heilek et al., 1995) of the 16S rRNA backbone from specific locations on the surface of S8. Fe(II) was tethered to nine different positions on S8 via the linker 1-(p-bromoacetamidobenzyl)-EDTA (BABE), and the derivatized proteins were assembled into 70S ribosomes for the probing experiments. Each of the nine different positions gave a characteristically distinct probing pattern. Cleavage of the RNA chain was observed near the previously described S8 footprinting sites, including the three-helix junction, 820 stem, and 860 region, in addition to its classical binding site in the 620 helix. Moreover, the tethered probes targeted RNA features near the 5′ terminus of 16S rRNA, in the 1100 region of the 3′ major domain and in the 300 region of the 5′ domain. This information, together with previous footprinting and crosslinking results, allowed us to model the position of S8 and its surrounding RNA in a 7.8-Å electron-density map of the Thermus thermophilus 70S ribosome (Cate et al., 1999). Our findings for the 70S ribosome are similar, but not identical, to the results of recent modeling studies based on a 5.5-Å crystal structure of the T. thermophilus 30S subunit (Clemons et al., 1999). Virtually all of the available experimental data on S8–16S rRNA interaction are accounted for by the model.

RESULTS

Construction, purification, and Fe(II) derivatization of S8 mutant proteins

We used the cysteine-reactive linker 1-(p-bromoacetamidobenzyl)-EDTA (BABE) to tether Fe(II) to S8 via unique cysteine residues introduced by site-directed mutagenesis. Escherichia coli ribosomal protein S8 has a single buried, nonconserved cysteine that we replaced with alanine to obtain a cysteine-free S8 (S8-C126A), which served as a starting construct for the introduction of mutant cysteines. Exposed, phylogenetically variable side chains were chosen, based on the X-ray crystal structure of T. thermophilus S8 (Nevskaya et al., 1998) and an alignment of 37 S8 sequences, in order to minimize potential disruption of S8 structure or its interactions with the ribosome. Cysteine residues were introduced into S8-C126A at nine positions (19,

**FIGURE 1.** S8-dependent protection of 16S rRNA from (A) base-specific chemical probes (Svensson et al., 1988) and (B) free hydroxyl radicals (Powers & Noller, 1995). Helix numbers are shown in italics. Dot sizes indicate extent of protection.
28, 46, 54, 61, 67, 73, 86, and 107; according to the E. coli numbering system) distributed over its surface (Fig. 2). The mutant proteins were overexpressed, purified by FPLC cation-exchange chromatography, and derivatized with Fe(II)-BABE. To confirm the extent of Fe(II)-BABE derivatization, we monitored the reactivity of each mutant cysteine with a thiol-specific fluorescent coumarin reagent (7-diethylamino-3-((4’-iodoacetyl)-amino)phenyl)-4-methylcoumarin; DCIA) before and after derivatization. DCIA analysis showed that the cysteine residues of all of the mutant proteins were accessible and efficiently derivatized (data not shown).

Mutant S8 proteins bind to 16S rRNA and assemble into 30S subunits and 70S ribosomes

To determine whether the various mutant S8 proteins bind normally to 16S rRNA, we carried out RNA-footprinting experiments using the base-specific reagent dimethyl sulfate (DMS), and compared the results to the footprints on 16S rRNA previously observed for wild-type S8 (Svensson et al., 1988). The expected protections of A574, A583, A640, A642 (Fig. 3), and other regions of 16S rRNA (data not shown) were observed for each mutant with slight variability in the extent of the protections. These results indicate that the S8 mutants bind appropriately to 16S rRNA.

In vitro assembly of 30S subunits in the absence of S8 results in a 24S particle (Nomura et al., 1969), allowing us to use sedimentation analysis to monitor incorporation of the Fe(II)-derivatized S8 proteins. We reconstituted 30S subunits with one of the Fe(II)-derivatized S8 proteins, the remaining complement of 19 recombinant 30S ribosomal proteins, and 16S rRNA (Culver & Noller, 1999). All of the reconstituted 30S subunits displayed sedimentation values similar to that of natural 30S subunits (Fig. 4A). In addition to forming 24S particles, we observed that subunits reconstituted in the absence of S8 are unable to associate with 50S subunits to form 70S ribosomes (data not shown). All of the 30S subunits reconstituted with the derivatized S8 proteins associated with natural 50S subunits (Fig. 4B), and the resulting 70S ribosomes were isolated by sucrose gradient sedimentation and used in the following probing experiments.

Probing the 16S rRNA environment of S8

The Fenton reaction was initiated to generate hydroxyl radicals from the tethered Fe(II). Positions of cleavage of the 16S rRNA backbone were detected by primer extension (Fig. 5), and the results are summarized in Figure 6. Strengths of cleavages are classified as strong, medium, or weak relative to intensities of adjacent sequencing lanes (see Materials and methods). Each prob-
The long 620 stem (helix 21), which contains the classical S8 binding site, was cleaved strongly from Fe-C86-S8 and Fe-C107-S8 at the bottom of the C-terminal domain (Fig. 2), and more weakly from Fe-C73-S8 (Fig. 5A,B). Nucleotides around the three-helical junction formed at the convergence of the 620, 750, and 755 helices (helices 21, 22, and 20, respectively) were cleaved medium to strongly from positions 19, 28, 54, and 61 of S8 (Fig. 5A–D). Three of the probes (at positions 28, 54, and 61) targeted the connecting strand around position 653, which is the site of crosslinking with Lys55 of E. coli S8 (Urlaub et al., 1997).

The 820 helix (helix 25), distinct from the classical 16S rRNA-S8 binding site, is strongly protected by S8 from free hydroxyl radicals (Powers & Noller, 1995), but has not so far been shown to contribute to its binding affinity. Proximity of the 820 helix to S8 was confirmed in these studies by directed hydroxyl radical cleavage in the nearby 830 and 850 regions of 16S rRNA from positions 19, 61, and 67 in the N-terminal domain (Fig. 5E). Fe-C61-S8 also strongly cleaved the 850 region of the 840 stem (helix 26).

Additional cleavage from S8 was detected outside its footprinted region of 16S rRNA, identifying features of the RNA that are near S8, but not directly connected to its binding elements. Two probing positions, 73 and 86, targeted the 565 region near the junction of the three major domains of 16S rRNA (Fig. 5B). Both the 5′ end and the 300 region in the 5′ domain were strongly cleaved from position 86 and weakly from position 73 (Fig. 5F,G). Finally, the three-way junction around positions 1075 and 1100 (helices 35–37) in the 3′ major domain was targeted from positions 19 and 67 located at the top of the N-terminal domain (Figs. 2, 5H).

**Modeling the position and orientation of S8 and its surrounding RNA elements**

Using these probing data as well as earlier footprinting and crosslinking information, we modeled the position and orientation of protein S8 and the surrounding elements of 16S rRNA in the 7.8-Å electron-density map of the 70S ribosome (Cate et al., 1999). Because of the significantly higher electron density of RNA relative to that of protein, RNA and protein can often be distin-
guished by raising or lowering the contour level of the electron-density map (see below; Fig. 8). We first located the long 620 helix, based on previous modeling studies (H.F. Noller, unpubl. results), that then led to identification of S8. The directed hydroxyl radical probing data confirmed these assignments, and allowed us to identify other elements in contact with, or in proximity to S8.

Protein S8 is visible as a lower electron-density mass contacting the minor groove of the 620 stem halfway along its length, at the location of its classical RNA binding site, where it has been crosslinked and footprinted. We established the general orientation of S8 from the directed hydroxyl radical probing results. Previous calibration experiments (Joseph et al., 1997) constrain the probe-target distance to within approximately 25 Å for strong, 35 Å for medium, and 45 Å for weak cleavages. Fe(II) probes tethered to positions 86 and 107 of S8 cleave nucleotides in the 620 stem around the 630 internal loop and at the classical binding site around position 640, respectively (Fig. 7a). In addition, the probe at position 86 cleaves the 300 loop, an RNA feature that is footprinted by protein S16, which is located in the body of the subunit. These results place the α3 corner of the delta-shaped S8 (Fig. 2) next to its binding site in the 620 stem (Fig. 7a). The second corner of S8, at loop 5 between β2 and β3, was placed at the base of the 16S rRNA 620 stem at the three-way junction near nt 650 (Fig. 7a,b). This positioning was constrained by the 2-iminothiolane crosslink between

**FIGURE 5.** Directed hydroxyl radical cleavage of 16S rRNA from Fe(II)-S8 in 70S ribosomes detected by primer extension. A,G: sequencing lanes. All other lanes are from 70S ribosomes containing S8-C126A (cysteine-free S8), Fe-C19-S8, Fe-C28-S8, Fe-C46-S8, Fe-C54-S8, Fe-C61-S8, Fe-C67-S8, Fe-C73-S8, Fe-C86-S8, or Fe-C107-S8. Primers used for analysis were 673 (A,B), 795 (C,D), 880 (E), 232 (F), 480 (G), and 1199 (H). Bars at the right indicate regions of 16S rRNA cleavage.
Lys55 of *E. coli* S8 and U653 of 16S rRNA (Urlaub et al., 1997) and the results of directed hydroxyl radical probing from Fe-C28-S8 and Fe-C54-S8. Two other iminothiolane crosslinks between S8 and 16S rRNA within the nucleotide sequences 593–597 and 629–633 (Wower & Brimacombe, 1983) are consistent with the model, although the crosslinked positions of S8 are not known. The third corner of S8, at loop 6 between β4 and β5, is constrained by directed probing from Fe-C19-S8 and Fe-C67-S8. Both of these probing positions target the 1075/1100 region of 16S rRNA, which is located at the head of the subunit, constraining the orientation of this corner of S8 toward the top of the subunit (Fig. 7b). No other orientation of S8 is able to satisfy all of these constraints simultaneously.

At low contour levels, characteristic features of S8, including α-helices and β-sheets are recognizable in the 7.8-Å electron-density map (Fig. 8a). Fit of the published crystal structure of *T. thermophilus* S8 (Nevskaya et al., 1998) with the electron-density map was optimized by slightly shifting the position of its C-terminal domain (~3–4 Å) with respect to its N-terminal domain. The compound 620 helix containing the classical S8 binding site is particularly well resolved as a long rod of density with RNA helical dimensions and clearly distinguishable major and minor grooves (Fig. 8b). It originates from a three-way junction at the back of the platform on the left-hand side of the 30S subunit, extending to the right across the back of the subunit, curving gently around S8. The helix terminates in the 620 hairpin loop, which makes interactions in the body of the subunit (not shown) consistent with the observed footprint by protein S16 in the 610/630 internal loop and in the hairpin loop (Stern et al., 1988a; Powers & Noller, 1995).

This docking arrangement places the classical S8 binding region of 16S rRNA in contact with the C-terminal domain of S8, which has been implicated in RNA binding from earlier studies (cited in Wu et al., 1993). Protection of specific bases from chemical probes and protection of minor groove backbone riboses from free hydroxyl radical probing in the 620 helix are also accounted for by this arrangement (Fig. 7c). In addition, position 93 of *E. coli* S8, which has been crosslinked to

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**FIGURE 6.** The location of hydroxyl radical cleavages in 16S rRNA from Fe(II)-S8 in 70S ribosomes. Cleavage strength is defined as strong (large circle), medium (medium circle), or weak (small circle) as described in Materials and methods. Large numbers indicate the Fe(II) tethering positions.
the C-terminal Lys 166 of protein S5 (Allen et al., 1979), directly faces the predicted location of S5 (Capel et al., 1987; Oakes et al., 1990; Heilek & Noller, 1996; Clemens et al., 1999).

As mentioned above, free hydroxyl radical footprinting studies have implicated a second feature of 16S rRNA, the 820 helix (helix 25), in interactions with S8 (Fig. 1B; Powers & Noller, 1995). Directed probing from...
positions 19 and 61 helped to confirm the placement of the 840 stem and its adjacent 860 stem-loop region (Fig. 7b), constraining the location of the flanking 820 helix. All three RNA elements were fitted to the electron density wrapping around the N-terminal domain at the left side of S8 (Fig. 8b). This arrangement is further confirmed by the location of the base of the 820 stem near the junction of the three major domains of 16S rRNA, from which the Lodmell-Dahlberg switch helix (helix 27) emerges (Cate et al., 1999; Clemons et al., 1999).

The S8-dependent free hydroxyl radical footprint on the 820 stem (Powers & Noller, 1995) is best explained by a combination of protein–RNA and RNA–RNA interactions. The α1 helix in the N-terminal domain of S8 is aligned with the minor groove face of the 820 helix of 16S rRNA at a distance of about 6–7 Å between their respective backbones, such that side chains from α1 could make contact with the backbone of the 820 helix, particularly on its 3' strand between positions 875–877. Protection of the base of the 820 helix, which cannot make direct contact with S8, can be accounted for by RNA–RNA interaction with the 584–587/754–757 helix (helix 20) of 16S rRNA, involving contact between the respective minor grooves of the two helices. This interaction also explains S8-dependent protection of the 754–757 strand from free hydroxyl radicals.

**DISCUSSION**

Using a combination of chemical probing results and the 7.8-Å X-ray crystallographic map of the 70S ribosome (Cate et al., 1999), we have modeled the position and orientation of ribosomal protein S8 and its surrounding 16S rRNA elements. Virtually all of the numerous constraints generated from directed hydroxyl radical probing (Fig. 6) and free hydroxyl radical footprinting (Fig. 1B; Powers & Noller, 1995) of the RNA are satisfied by the model, as are the two crosslinks for S8 for which precise positions are known (Wower & Brimacombe, 1983; Urlaub et al., 1997). Agreement of the model with the directed hydroxyl radical probing data can be seen in Figure 9; all of the probe-target distances are within the predicted ranges for strong,
medium, and weak cleavages. In addition to its classical binding site contacts in the 620 stem (helix 21) around positions 595 and 640, S8 interacts with the 820 stem (helix 25) and the three-helix junction at the base of the 620 stem, as predicted from the free hydroxyl radical footprinting studies. Our model for the S8–16S rRNA region in the 70S ribosome is similar, but not identical, to one that was recently proposed by Clemens et al. (1999) based on a 5.5-Å resolution X-ray map of the T. thermophilus 30S subunit. We compared the two models by several different least-squares superpositions of the proteins modeled into the two different maps. The lowest r.m.s.d. values between the complete set of modeled protein positions (S4, S5, S6, S7, S8, S15, S17, and S20; Clemens et al., 1999; Noller et al., unpubl.) were obtained using superposition of protein S8, proteins S5 plus S8, or the separate N- and C-terminal domains of S8. In all cases, the overall r.m.s.d. values for the resulting nucleotide positions for the S8 region RNA are between 9 Å and 11 Å; however, this is probably an overestimate of the actual discrepancy, because of the approximate numbering designations of the nucleotides in the Clemens et al. (1999) model (Protein Data Bank ID number 1QD7). Visual comparison shows that the positions of helices 20, 21, 22, 25, and 26 are rather similar in the two models (within 3–6 Å of each other). In contrast, the position of helix 26a is significantly different. In the 70S model, the position of helix 26a is rotated about 90° relative to that of the 30S subunit. In the absence of a direct comparison of the two electron-density maps, we cannot be certain whether this difference is the result of changes in the conformation of 16S rRNA resulting from the formation of 70S couples, or differences in interpretation of the electron density.

The RNA wraps around two of the three edges of the delta-shaped structure of T. thermophilus S8 (Fig. 7a,b,c). The 620 stem (helix 21) runs parallel to the lower edge of S8; its classical binding site at positions 595/640 contacts the C-terminal domain near its α3 corner, while the base of the stem and elements of the three-way junction interact with the N-terminal domain of S8 at its loop 5 corner. The 820 and 840 stems (helices 25 and 26) flank the back side of the N-terminal domain where α1 interacts with the 820 stem. Most, if not all, of the contacts are with the minor groove surfaces of the RNA. The protein–RNA contacts are consistent with the regions of Bacillus stearothermophilus S8 predicted to bind RNA based on patches of basic and aromatic residues identified in the crystal structure (Davies et al., 1996).

S8 also appears to interact with other ribosomal proteins. The right-hand edge of S8 faces S5, where an S5–S8 crosslink has been identified between positions 166 of S5 and 93 of S8 (Allen et al., 1979). The resulting purified S5–S8 covalent dimer has been shown to restore activity to 30S subunits depleted for S5 and S8 in reconstitution experiments (Lutter & Kurland, 1973), providing strong evidence for interaction between S5 and S8. A blob of density contacting the right-hand side of β4–β5 at the top of the N-terminal domain of S8 (Fig. 8a) is connected to the C-terminal end of S5 in the 7.8-Å electron-density map (not shown). This unassigned density probably represents the C-terminal segment that was disordered in the crystal structure of protein S5 (Ramakrishnan & White, 1992). In addition, the upper corner of S8 at loop 6 is continuous with a low-density feature coming from the head of the subunit that is likely to belong to one of the 3′-domain proteins. Thus, virtually all but the solvent face of S8 is involved in either protein–RNA or protein–protein interactions.

In contrast to the close detailed fit with the free hydroxyl radical footprinting data, the base-specific protections (Fig. 1A) are only partially explained by the model. The bases around positions 575, 812, and 860 are out of range of direct contact with S8, and are more likely to be protected as a result of S8-dependent RNA–RNA interactions, including the phylogenetically and genetically established tertiary interaction between G570 and C866 (Gutell et al., 1985, 1986). Even the protection of the three bases A595, A640, and A642 in the 595/640 region of the 620 stem appears to be the result of stabilization of RNA–RNA interactions by S8, rather than protein–RNA contact. Protection of A640 can be explained by stabilization of its Watson–Crick pair with U598 by S8, whereas A595 is sterically inaccessible to the DMS probe because of tertiary folding of the RNA, according to an NMR structure of the S8-binding region (Kalurachchi et al., 1997; Kalurachchi & Nikonowicz, 1998). S8 clearly contacts the minor groove surface of its classical RNA-binding region, in agreement with the NMR structure, but differing from a model derived from chemical probing and stereochemical con-

![FIGURE 9. Distribution of probe-target distances for weak, medium, and strong directed hydroxyl radical cleavages measured from the S8-16S rRNA model. Cleavage intensities were scored as defined in Materials and methods.](image-url)
Phylogenetic sequence conservation of protein S8 can be attributed to its interactions with 16S rRNA (Table 1). Helix α1 runs nearly parallel to the 3' strand of the 820 helix of 16S rRNA (positions 874 to 878), within about 6–8 Å between their respective backbones. The side-chains of the conserved Asp8, Thr11, Arg12, Arg14, and Asn15 on the RNA-proximal face of α1, as well as Arg79 in loop 7, are close enough to make contacts with the RNA backbone. In contrast, the RNA sequence in this region is variable, except for conservation of a pyrimidine at position 875, whose 2-keto group could provide a minor groove recognition feature. The helical axis of α1 points to the junction between helices 20 and 21, suggesting a possible electrostatic interaction between its permanent dipole and the phosphate backbone around position 587. Similarly, the dipole of α2 is oriented toward the RNA backbone around positions 589–590. Residues Ser29 and Lys30 are within contact distance of this same part of the RNA backbone. Near the classical binding region in the 620 helix, a cluster of conserved amino acid side-chains is juxtaposed with the RNA. The conserved Tyr85 faces the RNA minor groove at the G597–C643 base pair, one of the few strongly conserved features of the S8 RNA binding site, and could participate in a stacking interaction facilitated by irregularities in the RNA helix generated by the two bulges in the strands flanking the conserved G-C pair. Several other conserved residues including Arg83, Ser104, Ser106, and Glu123 are also close enough to interact with the core of the binding-site region. The general orientation of S8 with respect to its classical binding site and the stacking of Tyr85 in the vicinity of base A642 is in agreement with a model for S8–RNA interaction proposed by Kaluarachchi and Nikonowicz (1998). Genetic studies have identified mutants of S8 that disrupt its interaction with RNA (Wower et al., 1992). Among these mutations, six map to conserved, exposed residues (Ser29, Lys30, Arg79, Tyr85, Ser106, and Glu123) that we predict will make direct contact with 16S rRNA (Table 1).

Some understanding of the role of S8 in ribosome assembly can be inferred from the emerging structure of the S8 region of the 30S subunit. During transcription of 16S rRNA, the first secondary structure element of the central domain that is able to fold into its mature conformation is the 620 hairpin loop, which could anchor the central domain to the 5' domain via its interactions with protein S16 (Stern et al., 1988a; Powers et al., 1993; Powers & Noller, 1995). Completion of the long 620 stem would then allow S8 to bind to its classical binding site around position 640. Upon completion of transcription of the central domain, S8 would then be able to bind the 820 stem, fixing the locations of the two extremities of the domain. Interaction between S8 and S5 would further help to orient the central domain relative to the 5' end of 16S rRNA near the point of convergence of its three major domains. Thus, despite the limited size of its primary binding site in the 620 stem, S8 may play a central role in coordinating the assembly of the 30S subunit.

### MATERIALS AND METHODS

#### Materials

Preparation of 16S rRNA and 50S subunits were as described (Moazed & Noller, 1986). Buffer A is 80 mM K+-HEPES (pH 7.6), 330 mM KCl, 20 mM MgCl₂, and 0.01% Nikkol. Buffer B is 20 mM Tris-HCl (pH 7.0 at 4°C), 20 mM KCl, 6 M urea, and 6 mM βME. Buffer D is 80 mM K+-HEPES (pH 7.6), 1 M KCl, and 5 mM βME. Buffer E is 20 mM K+-HEPES (pH 7.6), 20 mM KCl, and 6 mM βME. Buffer H is 80 mM K+-HEPES (pH 7.6), 20 mM MgCl₂, and 0.01% Nikkol. Buffer 1 is 80 mM K+-HEPES (pH 7.6), 1 M KCl, and 0.01% Nikkol. Buffer 2 is 25 mM Tris-HCl (pH 6.8 at 25°C), 6 M urea, 1% SDS, 10% glycerol, 0.001% bromophenol blue, and 1% βME. Buffer 3 is 0.3 M NaOAc (pH 5.5), and 1 M βME. Buffer 4 is 0.3 M NaOAc (pH 5.5), 0.5% SDS, and 5 mM EDTA (pH 8.0). Buffer 5 is 50 mM K+-HEPES (pH 7.8), 100 mM KCl, and 20 mM MgCl₂. Buffer 6 is 80 mM K+-HEPES (pH 7.6), 100 mM KCl, 20 mM MgCl₂, and 0.01% Nikkol.

#### Mutagenesis, expression, and purification of S8

The gene-encoding ribosomal protein S8 was previously cloned from *E. coli* MRE600 genomic DNA (Culver & Noller, 1986).
1999). A combined Nde1-BamH1 restriction enzyme digest of the S8 clone in pET24b (Novagen) was ligated into identically digested pET21b (Novagen). The naturally occurring cysteine at position 126 was changed to alanine by site-directed mutagenesis (Kunkel, 1985), and cysteine residues were introduced into the cysteine-free construct at nine non-conserved positions (19, 28, 46, 54, 61, 67, 73, 86, and 107). Mutations were confirmed by dyeoxy sequencing (Sanger et al., 1977). Each mutant S8 protein was expressed in E. coli BLR(DE3) by growth in LB plus ampicillin (100 µg/mL) at 37 °C to an A550 of 0.4–0.9, followed by induction with IPTG (1 mM final concentration) and continued growth for 4 h. Cells were pelleted by centrifugation at 5,000 rpm, washed in buffer E, and frozen at −20 °C. Cells were lysed by sonication in buffer E, and inclusion bodies containing recombinant S8 were pelleted by spinning at 7,000 rpm for 15 min at 4 °C in a Beckman J2-21M centrifuge (JA20 rotor), then solubilized by dissolving in buffer B. Proteins were purified by FPLC chromatography at 4 °C using a Resource S cation-exchange column (Pharmacia) and eluted with a 120-mL salt gradient from 20 mM to 118 mM KCl in buffer B. All mutant S8 proteins eluted at approximately 80 mM KCl. Purified proteins were dialyzed into buffer D, aliquoted, and frozen in liquid nitrogen.

Derivatization of S8 proteins

Derivatization of cysteine-containing S8 mutant proteins was done essentially as described (Heilek et al., 1995). Fe(II)-BABE (100 nmol) was incubated with S8 (3 nmol) in buffer 1 in a volume of 100 µL at 37 °C for 30 min. Excess Fe(II)-BABE was removed by ultrafiltration in Microcon 3 concentrators (Amicon) at 4 °C and 10,000 rpm, followed by four 400-µL washes with buffer 1. Mock derivatization of the cysteine-free S8 protein (S8 C126A) was included as a control for possible derivatization of noncysteine residues.

The extent of Fe(II)-BABE derivatization of each S8 mutant protein was estimated by reactivity with the thiol-specific fluorescent coumarin reagent 7-diethylamino-3-((4-(iodoacetyl)-amino)phenyl)-4-methylcoumarin (DCIA; Molecular Probes). Nondervatized and Fe(II)-BABE-derivatized S8 proteins (500 pmol) were incubated with DCIA (4 nmol) at 30 °C for 15 min in buffer 1 in a volume of 25 µL. The reaction was quenched by the addition of 7 µL of buffer 2, resolved on a 15% SDS polyacrylamide gel, and visualized by transillumination at 365 nm.

Footprinting of S8 on 16S rRNA

Footprinting of the S8 mutant proteins on 16S rRNA with the base-specific probe DMS, which modifies the N-1 of A and the N-3 of C, was done as described (Powers & Noller, 1994). Natural 16S rRNA (20 pmol) was preincubated at 42 °C for 15 min in buffer 6, combined with 120 pmol of S8 (wild-type or mutant) protein and the KCl concentration adjusted to 330 mM in a 50-µL volume. S8–16S rRNA complexes were formed by incubating at 42 °C for 30 min and then on ice for 10 min. DMS was diluted 1:14 (v/v) in 95% ethanol, and 2.5 µL were added to each reaction. Modification of 16S rRNA was done at 37 °C for 8 min, then stopped by the addition of 50 µL of buffer 3. The 16S rRNA was precipitated by adding 250 µL of 95% ethanol, then dissolved in 200 µL of buffer 4, extracted three times with phenol and twice with chloroform, and reprecipitated with 600 µL of 95% ethanol. The 16S rRNA was dissolved in 30 µL of ddH2O and stored at −20 °C. Changes in the reactivity of 16S rRNA with DMS because of the binding of S8 were detected by primer extension with reverse transcriptase (Stern et al., 1988b).

Reconstitution of 30S ribosomal subunits and formation of 70S ribosomes

Reconstitution of 30S subunits with purified recombinant 30S proteins was carried out essentially as described (Culver & Noller, 1999). Natural 16S rRNA (40 pmol) was incubated at 42 °C for 15 min in buffer H, then combined with 280 pmol of each primary 30S-binding protein (S4, S7, S8, S15, S17, and S20), the KCl concentration was adjusted to 330 mM, and the reaction incubated at 42 °C for 20 min. Next, 280 pmol of each secondary 30S-binding protein (S5, S6, S9, S11, S12, S13, S16, S18, and S19) were added to the reaction that was again adjusted to 330 mM KCl and incubated at 42 °C for 20 min. Finally, 280 pmol of each tertiary 30S-binding protein (S2, S3, S10, S14, and S21) were added, and the reaction adjusted to 330 mM KCl and incubated an additional 20 min at 42 °C in a final volume of 100 µL. Excess proteins were removed by ultrafiltration in Microcon 3 concentrators at 4 °C and 2,000 rpm for 10 min, followed by three washes with 100 µL of buffer A. Natural 50S subunits (30 pmol) were associated with the reconstituted 30S subunits in a volume of 75 µL with the KCl concentration adjusted to 100 mM, and incubated at 37 °C for 30 min to form 70S ribosomes that were isolated by 10%–40% sucrose gradient sedimentation in buffer 5.

Directed hydroxyl radical probing

Directed hydroxyl radical probing of 16S rRNA from Fe(II)-derivatized S8 was done as described (Heilek et al., 1995). Briefly, 10–20 pmol of 70S ribosomes were incubated with 2 µL of 250 mM ascorbate and 2 µL of 1.25% hydrogen peroxide on ice for 10 min in 100 µL of buffer 6. The reaction was stopped by the addition of 10 µL of 40 mM thiourea. The rRNA was precipitated with 0.3 M NaOAc and 2.5 vol of 95% ethanol, resuspended in 200 µL of buffer 4, and extracted three times with phenol and twice with chloroform. The rRNA was reprecipitated with 600 µL of 95% ethanol, resuspended in 30 µL of ddH2O, and stored at −20 °C. The precise location of cleavage of the 16S rRNA backbone was detected by primer extension with reverse transcriptase (Stern et al., 1988b). Cleavage intensities were scored visually, according to whether the primer extension stops were strong (at least twice as strong as adjacent dyeoxy sequencing bands), weak (less than half as strong as adjacent sequencing bands), or medium (in between strong and weak) (Joseph et al., 1997).

Computer modeling

Protein S8 and elements of 16S rRNA were modeled in the 7.8-Å electron-density map of the T. thermophilus 70S ribosome (Cate et al., 1999) using the program O (Jones & Kjeldgaard, 1997). Helical regions of the T. thermophilus 16S rRNA...
secondary structure (Gutell & Fox, 1988) were rendered as pseudomotif models based on A-form RNA helices, in which each nucleotide is represented by a single atom at the phosphorus position (Stern et al., 1988a). Single-stranded loops and connecting strands were not explicitly modeled. Protein S8 was modeled into the density using coordinates from the X-ray crystal structure of T. thermophilus S8 (Nevskaya et al., 1998) (Protein Data Bank accession code 1an7). Optimal fitting of S8 was done by modeling its N- and C-terminal domains (residues 3–80 and 81–138, respectively) separately. Structure figures were drawn using the program O (Jones & Kjeldgaard, 1997) or RIBBONS (Carson, 1997).

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REFERENCES

Structure of the S8 region of the 70S ribosome

The location of protein S8 and surrounding elements of 16S rRNA in the 70S ribosome from combined use of directed hydroxyl radical probing and X-ray crystallography.

L Lancaster, G M Culver, G Z Yusupova, et al.

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