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Directed hydroxyl radical probing of the rRNA neighborhood of ribosomal protein S13 using tethered Fe(II)

GABRIELE M. HEILEK[1] and HARRY F. NOLLER
Center for the Molecular Biology of RNA, Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064, USA

ABSTRACT

Directed hydroxyl radical probing was used to probe the rRNA neighborhood around protein S13 in the 30S ribosomal subunit. The unique cysteine at position 84 of S13 served as a tethering site for attachment of Fe(II)-1-(*p*-bromoacetamidobenzyl)-EDTA. Derivatized S13 (Fe-C84-S13) was then assembled into 30S ribosomal subunits by in vitro reconstitution with 16S rRNA and a mixture of the remaining 30S subunit proteins. Hydroxyl radicals generated from the tethered Fe(II) resulted in cleavage of the RNA backbone in two localized regions of the 3′ major domain of 16S rRNA. One region spans nt 1308–1333 and is close to a site previously cross-linked to S13. A second set of cleavages is found in the 950/1230 helix. Both regions have been implicated in binding of S13 by previous chemical footprinting studies using base-specific chemical probes and solution-based hydroxyl radical probing. These results place both regions of 16S rRNA in proximity to position C84 of S13 in the three-dimensional structure of the 30S ribosomal subunit.

Keywords: crosslinking; hydroxyl radical probing; ribosomal protein S13

INTRODUCTION

The absence of a high-resolution three-dimensional structure for the ribosome presents a significant barrier to a detailed mechanistic understanding of the process of translation. As an alternative, a number of indirect approaches have been undertaken to learn about spatial relationships between the different macromolecular components within the ribosome. Chemical and photochemical crosslinking methods have been used to identify protein–RNA or RNA–RNA proximities (Brimacombe et al., 1990). Chemical footprinting, using either base-specific chemical probes (Stern et al., 1989) or solvent-based hydroxyl radical cleavage (Powers & Noller, 1995), has been used to localize sites of protein–RNA interaction. Unfortunately, crosslinking methods are labor-intensive, and depend on fortuitous juxtaposition of chemical groups that can react with the crosslinking agent, whereas interpretation of chemical footprinting results suffers from the inherent problem of distinguishing between direct and indirect protection. Because of these limitations, there remains a need to extend the range of methodology for biochemical analysis of ribonucleoprotein (RNP) structure.

Recently, we have pursued development of a new approach to the study of RNA–protein proximities, using hydroxyl radicals generated from a defined position on a single protein in an RNP complex (Heilek et al., 1995). In this method, Fe(II) is chelated to a linker [1-(*p*-bromoacetamidobenzyl)-EDTA (BABE)] (DeRiemer et al., 1981) that is covalently attached to a unique cysteine residue in a specific ribosomal protein, which is incorporated into RNP particles or 30S ribosomal subunits. Hydroxyl radicals are then generated from the bound Fe(II) via the Fenton reaction (Imlay et al., 1988), resulting in cleavage of the RNA sugar–phosphate backbone. Cleavage is observed roughly within a 10-Å radius from the site of production of the radicals (Dreyer & Dervan, 1985; Tullius & Dombrowski, 1985); since the acetamidobenzyl tether is itself about 12 Å in length, the overall probing radius is predicted to be of the order of 22 Å from the position of the sulfur of the derivatized cysteine residue. This level of resolution is useful and appropriate for studies on ribosomes, which have overall dimensions of about

Reprint requests to: Harry F. Noller, Center for the Molecular Biology of RNA, Sinsheimer Laboratories, University of California, Santa Cruz, Santa Cruz, California 95064, USA; e-mail: harry@nuvolari.ucsc.edu.
[1] Present address: Tularik, Inc., 270 E. Grand Avenue, South San Francisco, California 94080, USA; e-mail: gabs@rapids.tularik.com.

250 Å, and whose morphology has been studied at a resolution of about 20 Å (Frank et al., 1995).

This study describes efforts to map the rRNA environment surrounding the unique Cys 84 of ribosomal protein S13 in the 30S ribosomal subunit of *Escherichia coli*. S13 was derivatized with Fe(II)-BABE, and reconstituted with 16S rRNA in the presence of a full complement of 30S ribosomal proteins lacking S13. The generation of hydroxyl radicals in situ from the tethered Fe(II) resulted in cleavage of the 16S rRNA chain in two localized regions of the 3′ major domain, which have been implicated previously in binding of S13 by footprinting (Powers et al., 1988; Powers & Noller, 1995) and crosslinking (Osswald et al., 1987) approaches.

RESULTS

To test for accessibility of cysteine 84 of S13, we reacted purified protein S13 with [^{14}C]-iodoacetic acid, which mimics the nucleophilic displacement reaction that is used for linking Fe(II)-BABE to the ribosomal protein. Under conditions used for derivatization with Fe(II)-BABE, 0.32 mol of [^{14}C]-iodoacetate were incorporated per mol of S13. S13 was conjugated with Fe(II)-BABE, and the ability of the modified protein (Fe-C84-S13) to assemble into 30S subunits was tested by chemical footprinting with kethoxal. Derivatized and nonderivatized S13 gave similar RNA footprints (Powers et al., 1988), consistent with proper assembly of Fe-C84-S13 (data not shown). Sedimentation profiles for subunits reconstituted from either TP30 (a mixture of total extracted 30S ribosomal proteins), a mixture of all 20 purified proteins (Σ R30S), or Fe-C84-S13 plus a mixture of the other 19 proteins (C84-S13 R30S), were similar to that of native 30S subunits (Fig. 1), although for both the TP30 R30S and C84-S13 R30S, a small shoulder is evident on the leading edge of the 30S peak (Fig. 1). The biological activity of the reconstituted 30S particles was assayed by poly-[U]-dependent binding of tRNA^{Phe}. The activity of Fe-C84-S13 30S subunits is somewhat lower (9% versus 13%) than that of subunits reconstituted from the full set of unmodified proteins (Table 1). The decreased activity of Fe-C84-S13-containing 30S subunits is possibly indicative of interference of the Fe(II)-BABE with 30S subunit tRNA binding site (see below).

The RNA neighborhood of Fe-C84-S13 in reconstituted 30S subunits was then probed by initiating hydroxyl radical formation, identifying the sites of cleavage of 16S rRNA by primer extension of RNA extracted from the modified 30S subunits. A series of synthetic DNA oligonucleotide primers permits scanning of the entire 16S rRNA chain, except for the 3′ terminal 40 nt. Cleavage of the RNA backbone was observed exclusively in two regions of the 3′ major domain of 16S rRNA (Fig. 2, lane 2). One region comprises nt 953–958 and 1228–1231, which are located on opposite strands of the 950/1230 helix (Fig. 3). The second set of backbone cleavages is found at positions 1306–1312 and 1331–1332, in the two strands of the 1310 hairpin helix and its non-canonically paired extension. No other Fe-C84-S13-dependent cleavages were detected.

Control experiments were performed, in which an eightfold molar excess of unmodified S13 was added

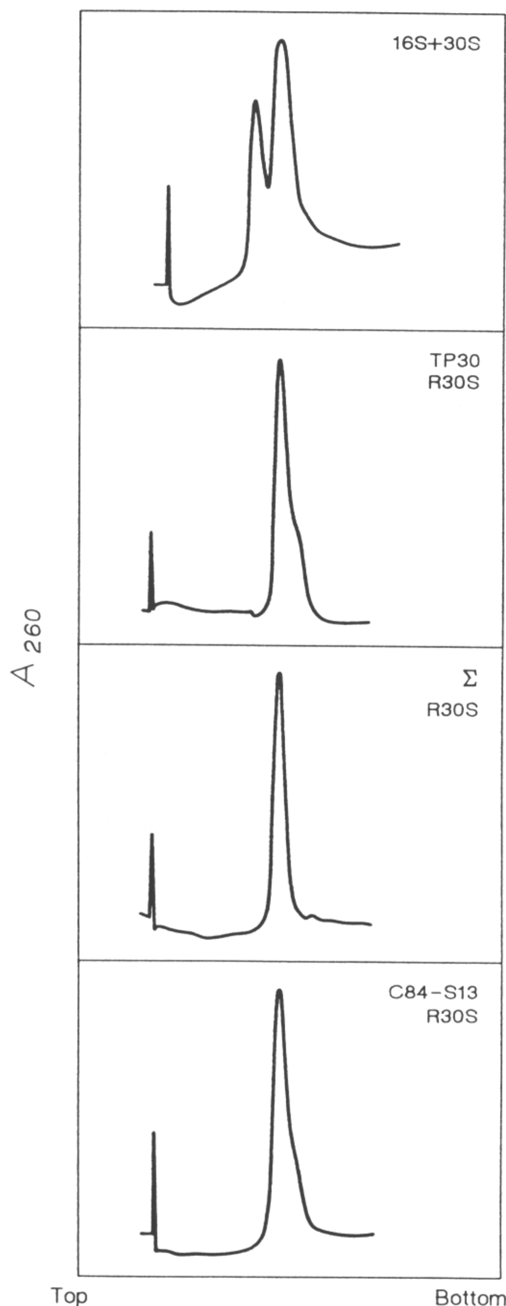

FIGURE 1. Sedimentation analysis of 30S subunits reconstituted from 16S rRNA and a mixture of individual purified ribosomal proteins plus unmodified protein S13 (Σ R30S) or S13 derivatized with Fe(II)-BABE at position 84 (C84-S13 R30S). Gradients containing 16S rRNA plus native 30S subunits (16S + 30S) and 30S particles reconstituted from TP30 protein mixture (TP30 R30S) are shown as markers.

TABLE 1. tRNA binding activity of 30S subunits reconstituted with Fe(II)-BABE-derivatized protein S13.[a]

	$tRNA^{Phe}$ bound (pmol)	Activity (%)
Native 30S subunits	1.10	(100)
Subunits reconstituted from 16S rRNA and the following protein mixtures:		
TP30	0.98	92
Σ-13 mix + untreated S13	0.14	13
Σ-13 mix + Fe-C84-S13	0.10	9

[a] Native or reconstituted 30S particles (5 pmol) were incubated with 5 pmol of 5′-[^{32}P]-$tRNA^{Phe}$ and 5 μg of poly[U], in 75 μL of 20 mM $MgCl_2$, 110 mM KCl, and 80 mM Hepes, pH 7.7, for 15 min at 37 °C, and 10 min on ice. Bound tRNA was measured by filter binding (Moazed & Noller, 1986). TP30, mixture of total r-proteins extracted from 30S subunits; Σ-13 mix, stoichiometric mixture of individually purified 30S r-proteins lacking S13.

to the 30S reconstitution reaction. If the observed cleavages originate from Fe-BABE-S13 bound to the S13 binding site of the 30S subunit, they would be expected to be diminished in the presence of excess S13. As is seen in Figure 2 (lane 3), the hydroxyl radical cleavages are essentially abolished under these conditions, providing further evidence for correct assembly of the derivatized S13. Further control experiments, in which mock radical initiation was performed on 30S subunits reconstituted with unmodified S13 (lane 1), unmodified TP30 (lane 4), or on natural 30S subunits (lane 5), show that the RNA cleavages are dependent on the presence of Fe-C84-S13 and are not due, for example, to adventitiously bound transition metals.

DISCUSSION

These experiments provide information about the RNA neighborhood around position 84 of ribosomal protein S13. Hydroxyl radicals generated from Fe(II) tethered to this site on S13 cause cleavage of two localized regions in the 3′ major domain of 16S rRNA. One region comprises nt 953–958 and 1228–1231, which are located in the two strands of the 950/1230 helix (Fig. 3). This finding confirms base-specific chemical footprinting results reported previously (Powers et al., 1988), which indicated interaction, either direct or indirect, between S13 and this feature of 16S rRNA. The cleavage pattern shows a 3′ stagger along the two helical strands, further suggesting that S13 may interact with the minor groove of this rRNA helix.

The other site of cleavage is the irregular stem at positions 1306–1312 and 1331–1332. These data overlap and extend earlier findings obtained by base-specific footprinting (Powers et al., 1988) and solvent-based hydroxyl radical probing (Powers & Noller, 1995) (Fig. 3). The cleavages at position 1332/1333 are located within a few nucleotides of a previously identified photochemical crosslink with S13 obtained using methyl *p*-azidophenyl acetimidate (Osswald et al., 1987; Fig. 3). In close proximity to the hydroxyl radical cleavages at nt 1332–1333 is nt 1338, which has been implicated in

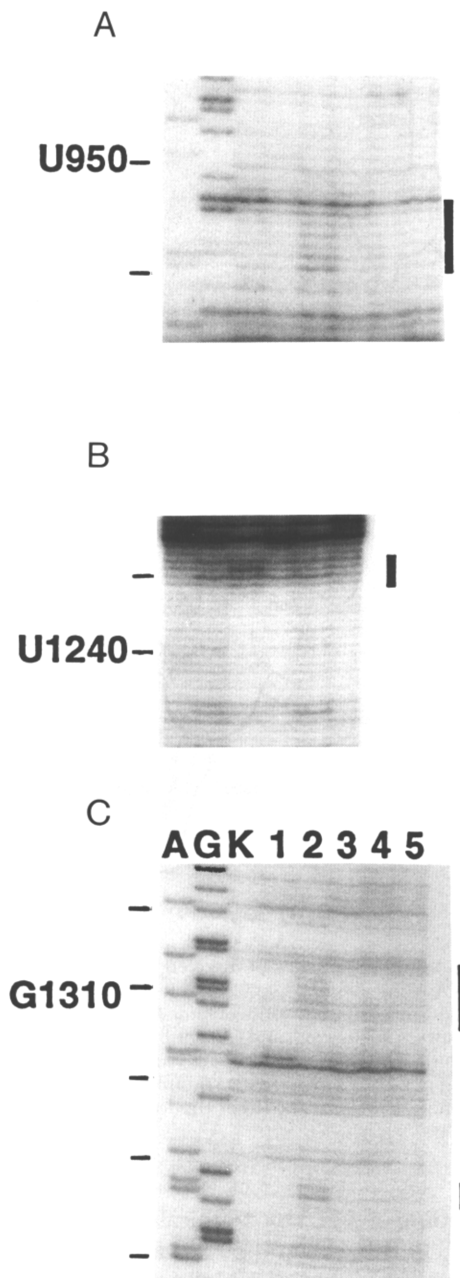

FIGURE 2. Cleavage of 16S rRNA by directed hydroxyl radical probing of 30S subunits reconstituted with Fe-C84-S13. Sites of cleavage were localized by primer extension (Stern et al., 1988a). Cleavages are shown for (**A**) the 950 region, detected using the 1046 primer; (**B**) the 1249 region, using the 1391 primer; and (**C**) the 1300 region, detected using the 1490 primer. A, G, sequencing lanes; K, unmodified 16S rRNA; samples in lanes 1–5 were treated with H_2O_2 and ascorbate. Lane 1, 30S reconstituted with nonderivatized S13; lane 2, 30S reconstituted with Fe-C84-S13; lane 3, as for lane 2, but reconstituted in the presence of an eightfold molar excess of unmodified S13; lane 4, 30S reconstituted with TP30; lane 5, native 30S subunits.

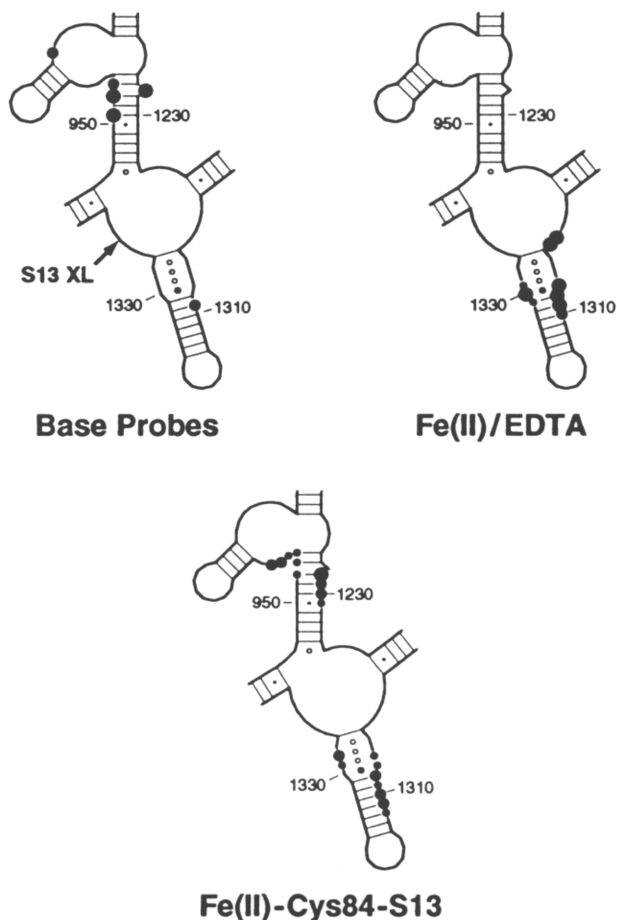

FIGURE 3. Summary of sites of directed hydroxyl radical cleavage in 16S rRNA using 30S ribosomal subunits reconstituted with Fe(II)-C84-S13 (bottom). S13 footprints obtained by base-specific chemical probing (Powers et al., 1988) and solvent-based hydroxyl radical footprinting using Fe^{2+}/EDTA (Powers & Noller, 1995) and the site of crosslinking of S13 using methyl *p*-azidophenyl acetimidate (S13 XL; Osswald et al., 1987) are shown at the top.

P-site binding of tRNA by chemical footprinting (Moazed & Noller, 1986, 1990) and by modification-interference experiments (von Ahsen & Noller, 1995). The decreased activity in poly-U-dependent tRNA binding of 30S subunits reconstituted with Fe-C84-S13, compared with that of subunits reconstituted with unmodified S13 (Table 1), could be explained by interference of the chelating reagent with tRNA–ribosome interaction.

Placement of S13 in the 30S subunit has been based on a number of different approaches. Immunoelectron microscopy (Tischendorf et al., 1974) and neutron diffraction (Capel et al., 1987) place S13 toward the very top of the 30S subunit, in the so-called "head" region of the particle, which contains the 3′ major domain of 16S rRNA (Stern et al., 1988b). Accordingly, S13 has been identified as a member of the subgroup of r-proteins that assemble into a ribonucleoprotein particle containing only the 3′ domain of 16S rRNA (Samaha et al., 1994). Other studies have reported proximity of S13 to the anticodon arm and central fold of $tRNA^{Phe}$ bound at the ribosomal P site (Wower et al., 1990; Podkowinski & Gornicki, 1991). S13 has been crosslinked to a number of other ribosomal proteins within the "head" of the 30S subunit, including S7 and S11 (Bollen et al., 1975). Contact between proteins S13 and S19 is supported by a well-characterized intermolecular crosslink (Pohl & Wittmann-Liebold, 1988) as well as formation of a specific heterodimer between these two proteins (Dijk et al., 1977). A recent version of a model for the folding of 16S rRNA in the 30S subunit (H.F. Noller, T. Powers, G.M. Heilek, S. Mian, & B. Weiser, unpubl.) is shown in Figure 4. Because the structure of S13 is presently unknown, it is represented by a dotted sphere (Fig. 4, top). The sites of directed hydroxyl radical cleavage by Fe-C84-S13 can all be positioned within range of S13, while satisfying all of the numerous other constraints used in predicting the folding of the RNA. Further application of this approach should lead to a

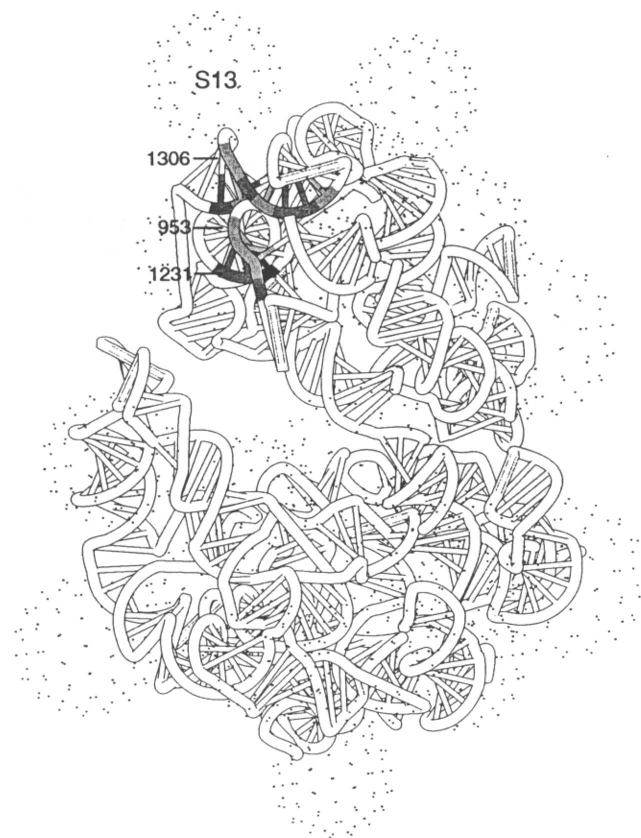

FIGURE 4. Model for the folding of 16S rRNA in the 30S ribosomal subunit (H.F. Noller, T. Powers, G.M. Heilek, S. Mian, & B. Weiser, unpubl.), viewed from the solvent side. The position of ribosomal protein S13, as determined by neutron diffraction (Capel et al., 1987), is indicated at the top, and positions of strong, medium, or weak hydroxyl radical cleavage by Fe-C84-S13 are indicated by dark, medium, or light shading of the RNA backbone, respectively. Locations of nt 953, 1231, and 1306 are indicated. Computer rendering was done using the program XRNA (B. Weiser & H.F. Noller, unpubl.).

well-defined low-resolution model for the 30S ribosomal subunit.

MATERIALS AND METHODS

Ribosomes, 30S ribosomal subunits, 16S rRNA, and ribosomal protein S13 were isolated as described previously (Craven et al., 1969; Hardy et al., 1969; Moazed et al., 1986). Preparation of BABE was synthesized as described (DeRiemer et al., 1981) and generously provided by C. Meares. Fe(II)-BABE complex was formed by mixing 2 μL of freshly prepared 50 mM $FeSO_4$ with 18 μL of 11 mM BABE in 100 mM NaOAc, pH 6.0, followed by incubation at room temperature for 30 min. Conjugation of S13 with Fe(II)-BABE or with [^{14}C]-iodoacetate was conducted by mixing 10 μL of an 8.3-mg/mL solution of S13 (in 80 mM K-Hepes, pH 7.7, 1 M KCl, and 6 mM β-mercaptoethanol) with 10-μL of a 10-mM solution of Fe(II)-BABE (in 100 mM NaOAc, pH 6.0) or 10 μL of a 10-mM solution of [^{14}C]-iodoacetate (Amersham; 5.93 mCi/mmol) in a buffer containing 1 M KCl, 80 mM Hepes, pH 7.7, and 0.01% Nikkol (Nikko Chemicals Co., Ltd., Tokyo, Japan), in a total volume of 100 μL, followed by incubation at 37 °C for 15 min. Separation of the modified S13 from excess reagent was performed by loading the reaction mixture onto Microcon3 vials (Amicon) and spinning at 12,000 rpm for 30 min in an Eppendorf centrifuge, followed by two washes, spun at the same speed and time.

Reconstitution of 30S ribosomal subunits containing Fe-C84-S13 or unmodified S13 was conducted, in a typical experiment, by adding 20 pmol of *E. coli* 16S rRNA (in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 300 mM KCl) to buffer A (80 mM K-Hepes, pH 7.7, 20 mM $MgCl_2$, and 0.01% Nikkol), followed by addition of 1.8 μL of an 88-μM solution of S13 or Fe-C84-S13 and 10 μL containing 80 pmol of Σ-S13 (an equimolar mixture of all 30S ribosomal proteins except S13) to allow formation of complete subunits (all proteins were stored at −80 °C in 80 mM K-Hepes, pH 7.7, 1 M KCl, and 6 mM β-mercaptoethanol). The KCl concentration was then adjusted to 330 mM and the final reaction volume to 50 μL, and the mixture incubated at 40 °C for 1 h, followed by 10 min on ice. Reconstituted 30S subunits were purified by sedimentation in a SW41 rotor for 18 h at 35,000 rpm at 4 °C, using a 10–40% sucrose gradient in 50 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, and 100 mM KCl. In these experiments, the activity observed for subunits reconstituted from Σ mixture was lower than usual (13%, relative to natural subunits as compared with the 30–40% activity values that are often found). This is likely due to low activity of one or more individual proteins used in making up the Σ-S13 mixture.

Hydroxyl radical formation was initiated by addition of 1 μL of 250 mM ascorbic acid and 1 μL of 2.5% H_2O_2 to 25 μL of a 200 nM solution of Fe-C84-S13 30S subunits, followed by incubation for 10 min at 4 °C. The reaction was terminated by addition of 1/10 volume 3 M NaOAc, pH 5.2, 1 μL of a 10 mg/mL solution of glycogen, and 2.5 volumes ethanol. Modified RNA was extracted and analyzed by primer extension as described (Stern et al., 1988a). Poly-[U]-dependent binding of 5′-[^{32}P]-tRNA^{Phe}, performed as described (Moazed & Noller, 1986), was used as an assay for the activity of reconstituted 30S subunits.

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