Single Protein Omission Reconstitution Studies of Tetracycline Binding to the 30S Subunit of *Escherichia coli* Ribosomes†

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ABSTRACT: In previous work we showed that on photolysis of *Escherichia coli* ribosomes in the presence of [^{3}H]tetracycline (TC) the major protein labeled is S7, and we presented strong evidence that such labeling takes place from a high-affinity site related to the inhibitory action of TC [Goldman, R. A., Hasan, T., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1983) *Biochemistry 22*, 359-368]. In this work we use single protein omission reconstitution (SPORE) experiments to identify those proteins that are important for high-affinity TC binding to the 30S subunit, as measured by both cosedimentation and filter binding assays. With respect to both sedimentation coefficients and relative Phe-tRNA^{Phe} binding, the properties of the SPORE particles we obtain parallel very closely those measured earlier [Nomura, M., Mizushima, S., Ozaki, M., Traub, P., & Lowry, C. V. (1969) *Cold Spring Harbor Symp. Quant. Biol. 34*, 49-61], with the exception of the SPORE particle lacking S13. A total of five proteins, S3, S7, S8, S14, and S19, are shown to be important for TC binding, with the largest effects seen on omission of proteins S7 and S14. Determination of the protein compositions of the corresponding SPORE particles demonstrates that the observed effects are, for the most part, directly attributable to the omission of the given protein rather than reflecting an indirect effect of omitting one protein on the uptake of another. A large body of evidence supports the notion that four of these proteins, S3, S7, S14, and S19, are included, along with 16S rRNA bases 920-1396, in one of the major domains of the 30S subunit. Our results support the conclusion that the structure of this domain is important for the binding of TC and that, within this domain, TC binds directly to S7.

Tetracycline (TC)[1] binds to a single high-affinity site on the *Escherichia coli* 70S ribosome ($K_D \sim 1$-20 μM) that has been localized to the 30S subunit as well as to a large number of low-affinity sites on both the 30S and 50S subunits (Strel'tsov et al., 1975; Tritton, 1977; Goldman et al., 1983; Epe & Whalley, 1984; Chopra, 1985; Epe et al., 1987). There is good evidence that the major inhibitory effect of tetracycline on protein synthesis, the blocking of aminoacyl-tRNA binding to the A site, is a direct consequence of its binding to the high-affinity site (Goldman et al., 1983; Epe et al., 1987). Tet(O) is a plasmid-encoded protein that confers resistance to TC. Recent work (Manavathu et al., 1990) on the characterization of Tet(O)-mediated resistance to TC has shown that although the poly(U)-dependent polyphenylalanine synthetic activity of ribosomes isolated from TC-resistant cells is only weakly inhibited by TC, the affinity of such ribosomes for TC is essentially the same as that of ribosomes isolated from TC-sensitive cells. This result suggests that TC inhibition of aminoacyl-tRNA binding to ribosomes isolated from TC-sensitive cells is due to an allosteric effect of TC binding rather than reflecting a direct steric overlap of the TC and tRNA binding sites. We speculate that Tet(O) resistance arises from an interference with the transmission of this allosteric effect.

A focus of research carried out in our laboratory has been the determination of the ribosomal proteins that are at the TC high-affinity binding site. In earlier work we took advantage of the photolability of tetracycline by employing the unmodified, ^{3}H-labeled molecule as a photoaffinity label and showed that the major protein labeled is S7, as identified by both PAGE and immunoprecipitation analyses. In addition, we obtained strong evidence that such labeling took place from the high-affinity binding site (Goldman et al., 1983). In the work presented in this paper we employ the alternative approach of single protein omission reconstitution (SPORE), originally pioneered by Nomura and his co-workers (Nomura et al., 1969), to identify those proteins that are important for high-affinity TC binding.

Experimental Procedures

Materials

The following buffers were used: TKM10, 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 10 mM $MgCl_2$; TKM20, 50 mM Tris-HCl (pH 7.6), 50 mM KCl, 20 mM $MgCl_2$; TM20, 30 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$; REC20U, 30 mM Tris-HCl (pH 7.4), 20 mM magnesium acetate, 500 mM ammonium chloride, 6 M urea, 6 mM β-mercaptoethanol; REC20, REC20U without urea; buffer C, 50 mM methylamine (pH 6.3), 6 M urea, 0.5 mM dithiothreitol, 16.7% HPLC-grade acetonitrile; buffer D, buffer C with 1.0 M NaCl. All pH measurements were made at 23 °C.

HPLC-grade trifluoroacetic acid (Pierce), HPLC-grade acetonitrile (Fisher), ultrapure sucrose (Schwarz-Mann), and tRNA^{Phe} (Boehringer-Mannheim) were used without further

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[1] Abbreviations: AUFS, absorbance units at full scale; IE-HPLC, ion-exchange high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; SPORE, single protein omission reconstitution; TC, tetracycline; TP30, total protein from 30S subunits; TP70, total protein from 70S ribosomes.

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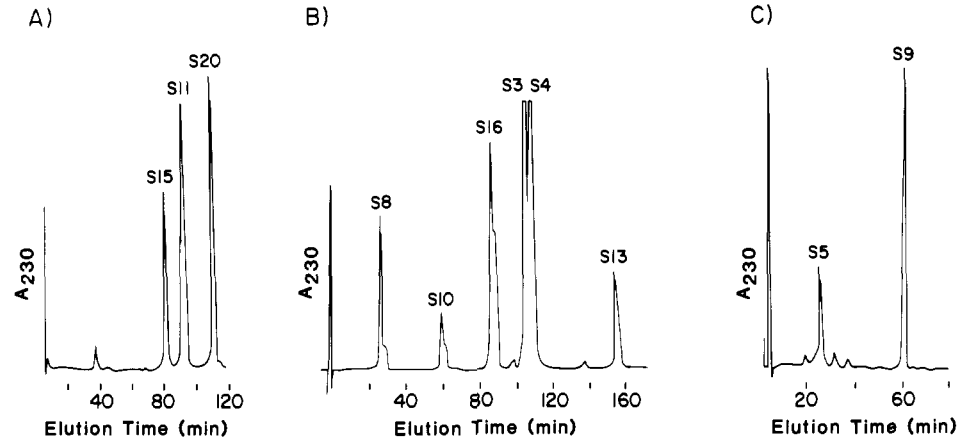

FIGURE 1: IE-HPLC of protein pools prepared by RP-HPLC. Panel A: proteins S11/S15/S20, from 23 nmol of 30S subunits, linear gradient of 0–50% buffer D in 120 min. Panel B: proteins S3/S4/S8/S10/S13/S16, from 14 nmol of 30S subunits, linear gradient of 0–40% buffer D in 180 min. Panel C: proteins S5/S9, from 9 nmol of 30S subunits, linear gradient of 0–50% buffer D in 120 min. For each chromatogram buffer C was the starting buffer, the flow rate was 1 mL/min, and AUFS was 0.32.

purification. Reagent grade urea (Sigma) was purified by treatment of 8 M solutions with 4 g of Norit A (Fisher) and 10 g of Amberlite MB-3 (Thomas Scientific) per liter to decolorize and remove cyanate, respectively. [^{3}H]TC (0.5 Ci/mmol) was purchased from New England Nuclear and purified prior to use, as necessary, by RP-HPLC (Hasan & Cooperman, 1985). All other chemicals were reagent grade. Spectropor 3 dialysis tubing (Thomas Scientific) was soaked in REC20U buffer (15 min) prior to use.

70S ribosomes, 30S subunits, and 16S rRNA were prepared from *E. coli* Q13 cells as previously described (Kerlavage & Cooperman, 1986).

Preparation and Purification of 30S Proteins. 30S proteins (TP30) were acetic acid extracted as previously described (Kerlavage & Cooperman, 1986). A combination of RP-HPLC and IE-HPLC was employed to prepare pure samples of proteins S3–S21. Acetone-precipitated proteins were first redissolved in 0.1% TFA or REC20U prior to injection on RP-P (Synchrom) reverse-phase columns. Chromatography was carried out as previously described (Olah et al., 1988). Proteins S6, S7, S12, S14, S17, S19, and S21 required no further purification. Three groups of coeluting proteins (S11/S15/S20, S3/S4/S8/S10/S13/S16, and S5/S9) were collected and further purified by IE-HPLC on a TSK SP-5-PW column (75 mm × 7.5 mm, Bio-Gel) following Capel et al. (1988). An LKB HPLC system consisting of a 2150 pump containing titanium tubing and a 2152 solvent controller was used, giving the results shown in Figure 1. The individual protein peaks were collected, concentrated by using a UM-2 (Amicon) concentrator, and desalted by reinjection on a preparative RP-P column and elution with a linear gradient of 15–45% acetonitrile in 0.1% trifluoroacetic acid. This second RP-P step was also useful for resolving S3 from S4 (Figure 1B) and for preparing S18 free from S15 contamination (Olah et al., 1988).

Although proteins S1 and S2 are well resolved from the other 30S proteins by RP-HPLC (Kerlavage et al., 1983a) they are present in only limited amounts on our washed 30S ribosomes. Larger amounts of these proteins were obtained by RP-HPLC of acetic acid extracts of 70S ribosomes. Typically, TP70 from 7.4 nmol of 70S ribosomes was applied to a preparative (i.d. 10 mm) RP-P column. The eluting solvent had a constant 0.1% trifluoroacetic acid, and the gradient steps had the following percentages of acetonitrile and flow rates: 20–37% in 5 min at 3.0 mL/min; 37–40% in 20 min at 3.0 mL/min; 40–48% in 60 min at 3.6 mL/min; 48–75% in 5 min at 4.0 mL/min. Protein S2 eluted at 24 min and protein S1 at 57 min (Buck, 1988).

Protein amounts were estimated by the integrated peak areas (IPAs) at 214 nm of the peaks from the first or second RP-P column, corrected for contributions from amino acid side chains (Buck et al., 1989), or by Bradford (1976) analysis. Protein identifications were based on the earlier work of Kerlavage et al. (1982, 1983a, 1984), on ratios of A_{280}/A_{214} obtained by dual-wavelength detection of RP-HPLC peaks, and on one-dimensional PAGE analysis (Goldman et al., 1983). Protein purity was checked by analytical RP-HPLC and one-dimensional PAGE analysis.

Methods

Reconstitution of 30S Subunits and Single Protein Omission Reconstitution (SPORE) Particles. Partial reconstitution experiments were performed by adding molar excesses of S1 and S2 and 2 A_{260} units of isolated 30S subunits in 300 μL of REC20 buffer and heating at 40 °C for 20 min, followed by standard density gradient centrifugation in a VTi80 rotor (Goldman et al., 1983). Total reconstitution of 30S subunits and SPORE particles from 16S rRNA and HPLC-purified ribosomal proteins was carried out essentially as described earlier (Kerlavage & Cooperman, 1986). Protein pools for total reconstitution experiments were constructed by combining equivalent stoichiometric amounts of pure proteins. Proteins S1 and S2 were only included in total reconstitution experiments designed to give SPORE particles lacking S2 and S1, respectively (Table I). Typically, protein pools (3.0–4.5 nmol in 200–300 μL) were placed in Spectropor 3 dialysis tubing and dialyzed at 4 °C, against first REC20U and then REC20 buffer. Following dialysis they were combined with 10–20 A_{260} units of 16S RNA in TM20 buffer. The total volume was 1.0 mL, and the final protein and RNA concentrations were 1.4 μM and 0.78 μM, respectively. Reconstitution, subunit pelleting, and sucrose density gradient centrifugation (in TKM10 buffer), using a VTi50 rotor, proceeded as previously described (Kerlavage & Coopermann, 1986; Olah et al., 1988).

TC Binding Assays. (A) Cosedimentation. [^{3}H]TC binding to 30S subunits was measured by cosedimentation in a sucrose density gradient, essentially as described earlier (Goldman et al., 1983), by combining 0.25 nmol of [^{3}H]TC with 3 A_{260} units of either 30S subunits or SPORE particles that had been previously heat-activated at 40 °C for 20 min in a total volume of 300 μL. This mixture was layered on top of 1 mL of a 7.5% sucrose layer above a 36-mL 15–30% sucrose density gradient

Table I: Properties of SPORE Particles[a]

protein omitted	sedimentation coefficient: this work	sedimentation coefficient: Nomura et al. (1969)	relative Phe-tRNA^{Phe} binding: this work[b]	relative Phe-tRNA^{Phe} binding: Nomura et al. (1969)	relative TC binding[c]: cosedimentation	relative TC binding[c]: filter binding
S1	30S ± 0.0 (5)	30S		0.97	0.91 ± 0.08 (5)	1.00 ± 0.05 (4)
S2	30S ± 0.0 (3)	30S		0.78	0.90 ± 0.07 (3)	1.00 ± 0.05 (4)
S3	25S ± 0.5 (3)	26S	0.18 ± 0.02	0.25	0.24 ± 0.04 (3)	0.22 ± 0.01 (4)
S4	24S ± 2.0 (2)[d]	20–25S	0.63 ± 0.04	0.29	0.41 ± 0.06 (2)	0.36 ± 0.03 (4)
S5	29S ± 0.5 (4)	28S	0.70 ± 0.02	0.64	0.91 ± 0.08 (4)	0.85 ± 0.05 (3)
S6	30S ± 0.0 (5)	30S	0.77 ± 0.04	0.81	0.70 ± 0.07 (5)	0.50 ± 0.02 (2)
S7	24S ± 1.0 (5)	20–25S	0.14 ± 0.01	0.27	<0.05 (5)	0.08 ± 0.01 (4)
S8	23S ± 0.5 (2)	24S	0.07 ± 0.01	0.03	0.18 ± 0.03 (2)	0.25 ± 0.02 (3)
S9	26S ± 0.5 (2)	25S	0.28 ± 0.02	0.39	0.50 ± 0.06 (3)	0.59 ± 0.04 (4)
S10	28S ± 0.3 (3)	28S	0.28 ± 0.03	0.04	0.30 ± 0.05 (3)	0.56 ± 0.03 (4)
S11	29S ± 0.5 (4)	28S	0.18 ± 0.02	0.25	0.82 ± 0.07 (6)	0.58 ± 0.04 (2)
S12	30S ± 0.0 (3)	30S	0.98 ± 0.03	0.98	0.79 ± 0.07 (3)	0.50 ± 0.03 (2)
S13	29S ± 0.3 (2)	30S	0.07 ± 0.01	1.04	0.50 ± 0.06 (2)	0.97 ± 0.03 (2)
S14	28S ± 0.2 (3)	28S	<0.02	0	<0.05 (3)	0.02 ± 0.01 (4)
S15	30S ± 0.0 (3)		0.55 ± 0.03		0.82 ± 0.05 (3)	0.58 ± 0.05 (2)
S16	25S ± 0.4 (2)[d]	22S[e]	0.11 ± 0.01	0.13[e]	0.31 ± 0.05 (2)	0.47 ± 0.02 (2)
S17	28S ± 0.5 (5)	22S[e]	0.29 ± 0.02	0.13[e]	0.92 ± 0.07 (5)	0.97 ± 0.03 (2)
S18	30S ± 0.0 (6)	30S	0.49 ± 0.04	0.49	0.70 ± 0.05 (3)	1.00 ± 0.04 (2)
S19	28S ± 0.5 (3)	27S	<0.02	0.25	<0.05 (3)	0.42 ± 0.02 (4)
S20	30S ± 0.3 (3)	30S	0.73 ± 0.06	0.98	0.76 ± 0.06 (3)	0.43 ± 0.03 (2)
S21	30S ± 0.0 (5)	30S	0.59 ± 0.04	0.68	0.80 ± 0.06 (5)	nd

[a] S1 and S2 were omitted in forming SPORE particles lacking S3–S21. Number of determinations given in parentheses. Ranges are average deviations. [b] Average of two determinations. 1.00 corresponds to 0.75–0.80 tRNA/30S after correcting for a background of 0.03 tRNA/30S. [c] Relative to native 30S subunits. In the cosedimentation assay, 1.00 corresponds to 0.07–0.09 bound TC/30S (Goldman et al., 1983) after background subtraction of 0.01–0.02 TC/30S. In the filter binding assay, 1.00 corresponds to 0.81–0.84 TC/30S after background subtraction of 0.02–0.03 TC/30S. [d] Two peaks were observed; the S value given is for the major peak. [e] S16 and S17 were jointly omitted.

made up in TKM10 buffer. Centrifugation proceeded for 135 min at 50 000 rpm at 4 °C in a Beckman VTi50 rotor.

(B) Filter Binding. Varying amounts of [^{3}H]TC were added to a reaction mixture containing 10 mM Tris-HCl (pH8.0), 20 mM $MgCl_2$, 200 mM NaCl, 6 mM β-mercaptoethanol, 0.1 mM Na_2EDTA, and 26 pmol of 30S subunits or SPORE particles (78 pmol/A_{260} unit) in a total volume of 100 μL. The reaction mixture was incubated for 15 min at 37 °C with vortexing and then filtered over HAWP nitrocellulose filters (0.45-μm pore size, Millipore) and rinsed three times with 7 mL of cold buffer. The filters were dried and the bound radioactivity was determined (Hillen et al., 1982). The assay was linear in 30S subunits up to 50 pmol/reaction mixture. Binding to SPORE particles was tested at a uniform concentration of 0.33 μM [^{3}H]TC.

Phe-tRNA^{Phe} Binding Assay. [^{3}H]Phe-tRNA^{Phe} binding to control and reconstituted 30S subunits and SPORE particles was assayed essentially as described previously (Goldman et al., 1980). Final concentrations in the binding assay (total volume 100 μL), made up in TKM20 buffer, were as follows: 30S subunits or SPORE particles, 0.26 μM; 50S subunits, 0.26 μM; poly(U), average length 200–300 bases, 0.24 mg/mL; bulk *E. coli* tRNA, 0.24 mM. Following preincubation for 10 min at 37 °C, [^{3}H]Phe-tRNA^{Phe} (0.15 mM) was added and incubation was continued for 10 min before quenching and Millipore filtration.

Results

Proteins S1 and S2 Are Not Critical for TC Binding to 30S Particles. The washed 30S subunits we prepare typically contain very low amounts of S1 and S2. Nomura et al. (1969) have previously demonstrated, and we have confirmed, that neither of these proteins is important for reconstitution of 30S particles (Table I). Nomura et al. (1969) further showed that omission of S1 has no effect on poly(U)-dependent Phe-tRNA^{Phe} binding to a reconstituted 30S particle and that omission of S2 has only a minor effect (Table I). In the present work we have used partial reconstitution experiments (Table II) to demonstrate that large increases in the S1 and S2 contents of partially reconstituted subunits are not accompanied by appreciable changes in TC binding. On the basis of these results, total reconstitutions designed to give SPORE particles lacking, individually, proteins S3–S21 were performed in the absence of S1 and S2.

Table II: Effect of Added S1 and S2 on TC Binding[a]

S1 added (pmol)[a]	S1/30S[b]	relative TC bound[c]
none	0.03	1.00[c]
31	0.15	0.88 ± 0.05
62	0.24	0.90 ± 0.07
78	0.38	1.00 ± 0.06
S2 added (pmol)[a]	**S2/30S[b]**	**relative TC bound**
none	0.10	1.00[c]
31	0.19	0.90 ± 0.05
47	0.39	0.87 ± 0.04
62	0.51	0.93 ± 0.05

[a] Additions to 15.6 pmol of washed 30S subunits. [b] S1 or S2 content of 30S particles purified by sucrose density centrifugation from partial reconstitution reaction mixture. [c] Determined by cosedimentation. The absolute value for washed 30S subunits (no additions) was 0.07–0.09 TC/30S.

Properties of SPORE Particles. SPORE particles were characterized by their sedimentation coefficients, by poly(U)-dependent Phe-tRNA^{Phe} binding, and by TC binding, as summarized in Table I. The first two properties were previously measured by Nomura et al. (1969). The general agreement is quite good between the earlier and current results. Both sets of results clearly show that single omission of a majority of S proteins substantially decreases Phe-tRNA^{Phe} binding. The only new results are that S15 is not required for 30S formation but that its omission decreases Phe-tRNA^{Phe} binding by about 50% and that S16 is more important for 30S formation than is S17. In addition, we find that omission of S10 has a less marked (though still considerable) effect on Phe-tRNA^{Phe} binding than did the earlier workers.

There is, however, one major point of disagreement. We find protein S13 to be essential for Phe-tRNA^{Phe} binding, whereas Nomura et al. (1969) claim that it is unimportant. Perhaps S13 is essential for Phe-tRNA^{Phe} binding only in the

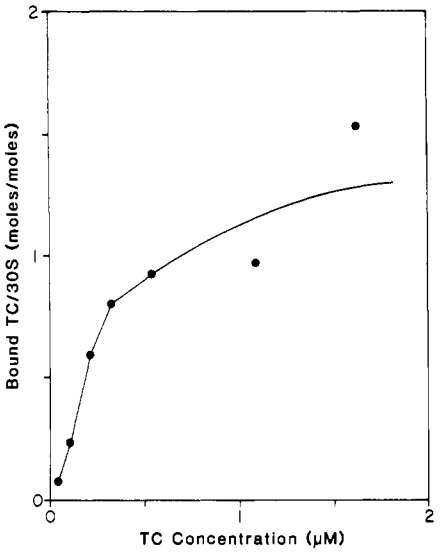

FIGURE 2: [^{3}H]TC binding to 30S subunits detected by the filter binding assay.

Table III: RP-HPLC Column Yields for 30S Ribosomal Proteins

protein	column yield[a] (%)	column yield[b] (%)	protein	column yield[a] (%)	column yield[b] (%)
S1	53 ± 4	53	S12	72 ± 3	84
S2	50 ± 2	50	S13	78 ± 3	75
S3	86 ± 5	75	S14	85 ± 2	82
S4	73 ± 4	71	S15	84 ± 2	69
S5	88 ± 4	78	S16	72 ± 2	71
S6	82 ± 2	78	S17	74 ± 4	86
S7	78 ± 2	71	S18	84 ± 4	64
S8	64 ± 4	71	S19	85 ± 3	84
S9	95 ± 2	78	S20	84 ± 3	75
S10	54 ± 4	71	S21	82 ± 2	91
S11	79 ± 4	78			

[a] This work, based on three analyses; linear gradient, 20–45% ACN/0.1% TFA, 270 min. Yields were determined by comparing peak areas of reinjected samples to original areas, as in Kerlavage et al. (1983b). [b] From Kerlavage et al. (1983b). Average deviation ±6%.

absence of proteins S1 and S2. Nevertheless, we wonder if this possible inconsistency is related to the anomalous results that have accumulated regarding the placement of S13 within the 30S subunit, as reviewed recently by both Moore et al. (1986) and Brimacombe et al. (1988).

The major new results of this work concern the TC binding of SPORE particles. The cosedimentation assay has been described previously (Goldman et al., 1983). Results from the filter binding assay with native 30S particles are shown in Figure 2 and are consistent with a model involving one tight site with a dissociation constant of ~1 μM and an undetermined number of weaker sites, in agreement with previous studies of TC binding to ribosomes, as discussed above. In control experiments using the filter binding assay, TC was found not to bind to any of the 30S proteins, added free in solution, at concentrations of 0.25–0.50 μM.

Filter binding assays with SPORE particles were carried out at a TC concentration at which the tight site dominates overall binding. With only a few exceptions (vide infra), the two assays for TC binding give similar results. The five SPORE particles having the lowest TC binding are those lacking S3, S7, S8, S14, and S19. Three of these five (−S3, −S7, and −S8) have low (<26S) sedimentation coefficients. The SPORE particle lacking S19 is the only one of the five that shows substantially different behavior in the two binding assays, displaying virtually no binding in the sedimentation assay but retaining considerable binding in the filter assay. This may reflect a larger effect of S19 on the rate constant for TC dissociation than on the equilibrium constant for TC association. A similar explanation could account for the significant, though less marked, differences in the results of the two TC-binding assays for the SPORE particles lacking S10, S13, and S20.

Protein Compositions of SPORE Particles Lacking S3, S7, S8, S14, or S19. Analytical RP-HPLC was performed on acetic acid extracts of native and reconstituted (S3–S21) 30S subunits and on SPORE particles lacking S3, S7, S8, S14, and S19. The protein compositions of these particles were estimated from the integrated peak areas (Buck et al., 1989) and from the estimated yields of the eluted proteins [Table III—the earlier results of Kerlavage et al. (1983b) are presented for comparison], giving the results presented in Table IV. Although calculation of the absolute copy numbers is subject to some uncertainty (a full consideration of this topic will be presented elsewhere[2]), the relative values listed in the last five

Table IV: Protein Composition of 30S Subunits and SPORE Particles

			SPORE particle composition[b]				
protein	native 30S[a]	reconstituted 30S[a]	−S7	−S14	−S19	−S3	−S8
S3	0.73	0.65	0.70 ± 0.03	0.97 ± 0.03	1.00 ± 0.02	<0.10	0.31 ± 0.07
S4	0.96	1.44	0.90 ± 0.03	0.79 ± 0.03	0.80 ± 0.03	0.75 ± 0.02	0.69 ± 0.02
S5	0.55	0.55	1.00 ± 0.04	0.95 ± 0.04	0.78 ± 0.06	0.76 ± 0.01	0.73 ± 0.01
S6	0.96	1.00	0.76 ± 0.03	0.67 ± 0.01	1.00 ± 0.01	0.38 ± 0.02	0.56 ± 0.01
S7	0.86	0.96	<0.05	0.70 ± 0.02	0.80 ± 0.02	0.65 ± 0.02	0.63 ± 0.02
S8	0.48	0.64	0.88 ± 0.01	0.89 ± 0.01	0.97 ± 0.03	0.89 ± 0.04	<0.10
S9	0.69	0.71	0.77 ± 0.03	0.73 ± 0.04	0.72 ± 0.05	0.92 ± 0.07	0.68 ± 0.04
S10	0.56	0.69	0.37 ± 0.07	0.43 ± 0.03	0.67 ± 0.03	0.58 ± 0.04	0.55 ± 0.04
S11	0.99	1.01	0.74 ± 0.03	0.93 ± 0.01	0.97 ± 0.01	0.54 ± 0.02	0.75 ± 0.05
S12	1.08	0.98	0.91 ± 0.01	0.81 ± 0.02	0.90 ± 0.02	0.60 ± 0.01	0.46 ± 0.01
S13	1.31	1.30	0.51 ± 0.02	0.54 ± 0.01	0.71 ± 0.03	0.50 ± 0.03	0.42 ± 0.02
S14	1.02	0.91	0.80 ± 0.01	<0.05	0.50 ± 0.02	0.85 ± 0.01	0.68 ± 0.02
S15	0.90	1.02	0.91 ± 0.01	0.97 ± 0.02	0.98 ± 0.02	0.99 ± 0.03	0.90 ± 0.03
S16	1.15	1.29	0.85 ± 0.04	0.78 ± 0.02	0.83 ± 0.04	0.36 ± 0.04	0.85 ± 0.05
S17	1.07	1.22	0.94 ± 0.05	0.80 ± 0.01	0.92 ± 0.03	0.83 ± 0.02	0.82 ± 0.03
S18	0.88	1.03	0.92 ± 0.03	0.60 ± 0.04	0.70 ± 0.03	0.96 ± 0.02	0.94 ± 0.02
S19	1.05	0.97	0.70 ± 0.02	0.82 ± 0.03	<0.05	0.74 ± 0.02	0.80 ± 0.01
S20	0.76	0.82	1.00 ± 0.01	0.91 ± 0.05	0.90 ± 0.02	0.95 ± 0.04	0.99 ± 0.01
S21	0.70	1.00	0.97 ± 0.01	0.79 ± 0.01	0.81 ± 0.01	0.98 ± 0.02	0.82 ± 0.02

[a] Copy numbers per 30S particle. Values reported are the average of four (−S3), five (−S7, −S14, −S19), or six (−S8) determinations. [b] Relative to the reconstituted 30S particle.

Table V: Characterization of SPORE* Particles

omitted protein	protein(s) Sx added in excess[a]	copy no. Sx in SPORE[d]	copy no. Sx in SPORE*	TC binding[c] SPORE[d]	TC binding[c] SPORE*
S19	S14	0.45	1.30	<0.05	0.30
S14	S18[b]	0.62	1.65	<0.05	0.225
	S19[b]	0.80	1.13		

[a] 3.6-fold excess over 16S rRNA. [b] Added in excess together. [c] By cosedimentation. [d] From Table IV.

columns are both reproducible and precise. The values presented show how omission of one protein affects the incorporation of other proteins into a SPORE particle. These results make it clear that the largest losses of other proteins result from omission of proteins S3 and S8. This result is expected for S8, which binds directly to 16S RNA early in the assembly of the 30S subunit, but is somewhat unexpected for S3, which is a late assembly protein (Held et al., 1974). In the three SPORE particles (–S7, –S14, or –S19) having the lowest TC-binding activity, omission of any one of proteins S7, S14, or S19 generally decreases the uptake of any of the other two by only 20–30%, with the exception that omission of S19 leads to a 50% decrease in the uptake of S14. Interestingly, the uptake of both S10 and S13 is significantly decreased (60% and 50%, respectively) in SPORE particles lacking either S7 or S14.

SPORE Particles.* SPORE particles formed in reconstitution mixtures containing excess stoichiometries of particular proteins (denoted SPORE* particles) were also purified by sucrose density gradient centrifugation and characterized with respect to protein content and TC binding. The results of two such experiments are presented in Table V. The first experiment shows partial restoration of TC binding when the effect of omitting S19 on the uptake of S14 is overcome by adding excess S14 to the reconstitution mix. A similar effect is seen when excess S18 and S19 are added in reconstituting particles lacking S14.

Discussion

The goal of the present work is to use SPORE experiments to determine which, if any, 30S proteins are crucial for TC binding to its high-affinity site on 30S subunits and to compare the results of these experiments with the results of our earlier photoaffinity labeling experiments showing that S7 is the major protein photoaffinity-labeled by [^{3}H]TC in a site-specific fashion (Goldman et al., 1983). In this earlier work, the principal lines of evidence for the significance of S7 labeling were as follows: first, that photoincorporation of [^{3}H]TC was decreased in the presence of nonradioactive TC, demonstrating that photoincorporation takes place from a saturable site; second, that photoincorporation of TC into S7 proceeded to a much greater extent than photoincorporation of its 4-epimer (E-TC), paralleling the stereospecificity of both high-affinity binding to the 70S ribosome and antibiotic activity (Summ & Christ, 1967); and third, that photoincorporation of TC into S7 was relatively insensitive to quenching by β-mercaptoethanol, consistent with labeling from a high-affinity site. Despite this evidence, the conclusion that S7 forms part of the high-affinity TC binding site was subject to the inherent limitations of the affinity labeling approach: first, that TC might have moved from its principal site of noncovalent binding prior to incorporating covalently, and second, that even if S7 were present at the noncovalent binding site of TC, the functional importance of S7 for TC binding was unclear.

In contrast to the earlier photoaffinity labeling experiments, the current SPORE experiments define those proteins whose presence is important for TC binding but do not distinguish whether such proteins directly form part of the TC site or are important for subunit assembly or induce long-range allosteric effects on TC binding. Thus, the loss of TC binding on omission of a particular protein might reflect either the loss of one or more other proteins that directly interact with TC in the native 30S subunit or a structural reorganization of the subunit that perturbs the TC binding site rather than demonstrating that the omitted protein contains all or part of the TC binding site. Because the affinity labeling and SPORE approaches have different inherent strengths and weaknesses, using them in conjunction provides a more powerful approach to defining the components of the TC site.

The SPORE results listed in Table I show that five proteins, S3, S7, S8, S14, and S19, are important for TC binding, using as a criterion that the corresponding SPORE particle has relative TC binding of <30% that of a native 30S subunit in at least one of the two binding assays; the largest effects are seen on omission of S7 and S14. Four of these proteins (all but S8) are linked together topographically by a variety of experimental results: (1) The classic 30S assembly map of Held et al. (1973, 1974) clearly shows an assembly scheme proceeding from S7 to S19 to S14 and via S10 to S3. (2) Limited nuclease digestion of 30S particles leads to isolation of a ribonucleoprotein particle containing proteins S7, S9, S10, S13, S14, and S19 and a fragment of 16S rRNA corresponding almost exactly to the 3′ major domain, comprising bases 920–1396 (Morgan & Brimacombe, 1973; Yuki & Brimacombe, 1975). (3) Wiener and Brimacombe (1987) have shown that extensive regions of the 3′ major domain of 16S RNA are protected from RNase A digestion in the presence of S7, S14, and S19 but are not protected in the presence of any of these proteins added singly, and they have suggested that these proteins, along with the protected regions of RNA and protein S9, cooperatively form a structural domain within the 30S subunit. Similarly, Powers et al. (1988a,b) have concluded from chemical footprinting analyses that proteins S3, S7, S14, and S19 all interact with rRNA in the 3′ major domain and that this domain is folded in an autonomous fashion. [Protein S8 does not interact with this domain of 16S rRNA. We attribute the loss of TC binding on S8 omission to the importance of this protein for 30S subunit assembly (Nomura et al., 1969; Held et al., 1973), since the corresponding SPORE particle has both a low sedimentation coefficient (Table I) and substantially reduced contents of several other S proteins (Table IV)]. (4) Immunoelectron microscopy results place all four proteins within the head region (Stöffler and Stöffler-Meilicke, 1984, 1986; Olson et al., 1988) of the 30S particle. Neutron diffraction data show S14 and S19 to be particularly close neighbors (Capel et al., 1987). This pair of proteins can also be cross-linked in intact 30S particles (Lambert et al., 1983).

The four proteins of interest are clearly too far away from one another on the 30S subunit to each be present at the single, high-affinity site for TC binding on the 30S subunit. Rather, we believe that the best interpretation of (a) our current results measuring TC binding to SPORE particles, (b) the earlier photoaffinity labeling and topographical results discussed above, and (c) the failure of TC to bind to 30S proteins free in solution is that the structural domain created by S7, S14, S19, and S3 binding to the 3′ major domain of 16S RNA is

[2] M. A. Buck, T. A. Olah, and B. S. Cooperman, manuscript in preparation.

important for high-affinity TC binding and that within this rather large structural domain TC binds directly to S7. This interpretation is also supported by the results with the SPORE* particles presented in Table V, showing that TC binding is not totally dependent on the presence of either S14 or S19. In addition, Moazed and Noller (1987) have obtained evidence for TC interaction with bases U-1052 and C-1054, which fall within the 3′ major domain of 16S rRNA.

We also considered the possibility that the effect of omitting S7 may be due to the reduction in uptake of one or more of the other S proteins important for TC binding rather than reflecting a direct interaction of TC with S7 itself. Several earlier workers have reported assembly experiments, in which only a few proteins at a time are added to 16S rRNA, showing a strong dependence of S19, S14, and S3 binding on the presence of S7 (Mizushima & Nomura, 1970; Green & Kurland, 1973; Powers et al., 1988a,b). However, as can be seen in Table IV, the SPORE particle lacking S7 retains substantial quantities of S3, S14, and S19 (70%, 80%, and 70%, respectively, when compared with the reconstituted 30S subunit), suggesting that the presence of other proteins compensates for the absence of S7 in the binding of these three proteins. Mizushima and Nomura (1970), using a one-dimensional PAGE analysis to quantify protein content, have also reported that the SPORE particle lacking S7 (which was then called P5) is quite deficient in proteins S14 and S19. We believe this difference from our results reflects the much higher ionic strength buffer (μ = 0.4) utilized by these workers in isolating the SPORE particle by sucrose gradient centrifugation, as compared with the TMK10 buffer (μ = 0.12) we employed.

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