Reconstitution of *Escherichia coli* 50S Ribosomal Subunits Containing Puromycin-Modified L23: Functional Consequences†

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ABSTRACT: In previous work we have shown that both puromycin [Weitzmann, C. J., & Cooperman, B. S. (1985) *Biochemistry 24*, 2268–2274] and *p*-azidopuromycin [Nicholson, A. W., Hall, C. C., Strycharz, W. A., & Cooperman, B. S. (1982) *Biochemistry 21*, 3809–3817] site specifically photoaffinity label protein L23 to the highest extent of any *Escherichia coli* ribosomal protein. In this work we demonstrate that L23 that has been photoaffinity labeled within a 70S ribosome by puromycin (puromycin–L23) can be separated from unmodified L23 by reverse-phase high-performance liquid chromatography (RP-HPLC) and further that puromycin–L23 can reconstitute into 50S subunits when added in place of unmodified L23 to a reconstitution mixture containing the other 50S components in unmodified form. We have achieved a maximum incorporation of 0.5 puromycin–L23 per reconstituted 50S subunit. As compared with reconstituted 50S subunits either containing unmodified L23 or lacking L23, reconstituted 50S subunits containing 0.4–0.5 puromycin–L23 retain virtually all (albeit low) peptidyl transferase activity but only 50–60% of mRNA-dependent tRNA binding stimulation activity. We conclude that although L23 is not directly at the peptidyl transferase center, it is sufficiently close that puromycin–L23 can interfere with tRNA binding. This conclusion is consistent with a number of other experiments placing L23 close to the peptidyl transferase center but is difficult to reconcile with immunoelectron microscopy results placing L23 near the base of the 50S subunit on the side facing away from the 30S subunit [Hackl, W., & Stöffler-Meilicke, M. (1988) *Eur. J. Biochem. 174*, 431–435].

The *Escherichia coli* ribosome is the subject of extensive studies directed toward the goal of constructing a structure–function map in which specific proteins and RNA regions are located within the ribosome structure and assigned specific roles in the overall process of protein synthesis [for excellent recent collections of articles, see Hardesty and Kramer (1986) and Noller and Moldave (1988)]. Photoaffinity labeling, with its intrinsic capability of defining the components of ligand binding sites, has contributed importantly toward this goal (Cooperman, 1987, 1988). However, the inherent difficulty of unambiguously demonstrating that labeling of a particular ribosomal component has taken place at a functionally important site has limited the usefulness of this approach. This difficulty is exacerbated by the generally low yields of photoaffinity-labeling reactions. Recently, we addressed this difficulty by reconstituting a 30S subunit in which a single protein was replaced by a photoaffinity-labeled form of that protein while all other 30S components were unmodified and then by measuring the functional effects of such replacement (Kerlavage & Cooperman, 1986). This work took advantage of the ease and high resolution offered by high-performance liquid chromatography (HPLC)[1] for the separation and preparation of ribosomal proteins and the reconstitutability of the 30S subunit. We here extend this approach to a photoaffinity-labeled protein in the 50S subunit.

The antibiotic puromycin is a structural and functional analogue of the 3′-end of aminoacylated tRNA. We have been engaged for some time in studying the photoaffinity labeling of *E. coli* ribosomes by puromycin as a probe of the peptidyl transferase center as well as of other sites of binding of the 3′-end of aminoacylated tRNA. The most pertinent results from our earlier studies for the work to be described below are as follows: (1) Puromycin photoincorporates into a large number of ribosomal components, including rRNA, but photoincorporation into two proteins, in particular L23 and S14, proceeds in relatively high yield and from sites of comparatively high affinity. The site leading to L23 photoincorporation is located on the 50S subunit, and that leading to S14 photoincorporation is on the 30S subunit (Jaynes et al., 1978; Grant et al., 1979; Weitzmann & Cooperman, 1985). (2) *p*-Azidopuromycin, a functional, photolabile analogue of puromycin, also photoincorporates into a large number of ribosomal components (Nicholson et al., 1982a,b; Hall et al., 1988). L23 is again the protein labeled site specifically to the highest extent, although the labeling of proteins L15 and L18/L22 also appears significant. (3) Photoaffinity labeling carried out at high concentrations of puromycin or *p*-azidopuromycin and, in the case of puromycin, a large light dose leads to very high levels of covalent incorporation (3–12 per ribosome) and corresponding losses in the activity of the modified ribosome toward both mRNA-dependent peptidyl transferase and tRNA binding.

On the basis of these results we have undertaken experiments using the reconstitution approach to answer the question of whether the loss of ribosomal functions mentioned above can be directly linked to photoincorporation into proteins S14 and L23. Because the experiment is technically much easier

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[1] Abbreviations: AUFS, absorbance units at full scale; *hν*-L23, protein L23 that has been prepared from 70S ribosomes photolyzed in the presence of tetracycline; HPLC, high-performance liquid chromatography; L23*, protein L23 that has been photoaffinity labeled within a 70S ribosome by puromycin; *N*-AcPhe, *N*-acetylphenylalanine; RP-HPLC, reverse-phase high-performance liquid chromatography; TP50, total protein from 50S subunits.

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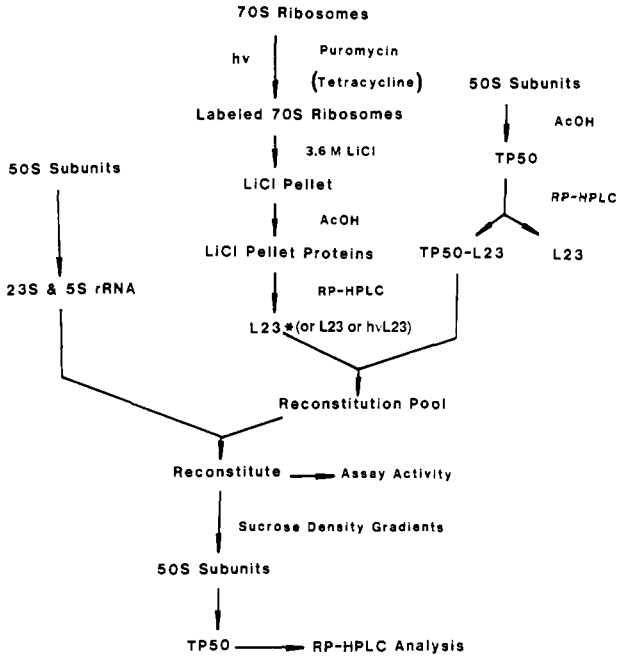

FIGURE 1: 50S subunit reconstitution with [^{3}H]puromycin-modified L23 (L23*).

to carry out with puromycin–S14 than with puromycin–L23, the S14 experiment was completed first, resulting in the demonstration that 30S subunits containing puromycin–S14 in place of S14 lose the ability to bind Phe-tRNA^{Phe} in a poly(U)-dependent manner (Kerlavage & Cooperman, 1986). The results of this experiment led to the conclusion that the perturbation caused by puromycin–S14 was not restricted to the region of 3′-terminus binding but rather was propagated more widely in the tRNA binding locus, weakening binding to both the A and P sites. In the work reported below we extend this effort by measuring the functional properties of a 50S subunit reconstituted with puromycin–L23 (L23*) in place of L23, following the overall scheme shown in Figure 1.

Experimental Procedures

Buffers. The following buffers were used: PT, 70 mM Tris·HCl (pH 7.14), 22 mM $Mg(OAc)_2$, and 750 mM KCl; REC4, 20 mM Tris·HCl (pH 6.84), 4 mM $Mg(OAc)_2$, 400 mM NH_4Cl, and 4 mM β-mercaptoethanol; REC4/U, REC4 buffer made up in 6 M urea; RNA, 10 mM Tris·HCl (pH 6.94), 10 mM $Mg(OAc)_2$, 50 mM NH_4Cl, and 4 mM β-mercaptoethanol; TENX, 110 mM Tris·HCl (pH 7.04), 4 M NH_4Cl, 4 mM $Mg(OAc)_2$, and 4 mM β-mercaptoethanol; TKM10, 50 mM Tris·HCl (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$; TKM20, 50 mM Tris·HCl (pH 7.6), 100 mM KCl, and 20 mM $MgCl_2$; TM4, 10 mM Tris·HCl (pH 6.94), 4 mM $Mg(OAc)_2$, and 4 mM β-mercaptoethanol. All buffers were autoclaved, with β-mercaptoethanol and urea added just before use. Values of pH reported were measured at 22 °C. At 0 °C, pH values of Tris buffers were 0.66 unit higher than at 22 °C.

Other Materials. Reagent-grade urea (Sigma) was purified by treatment of 8 M solutions with ion-exchange resin (10 g/L Amberlite MB-3, Thomas Scientific) and activated charcoal (4 g/L Norit A, Fisher) and then filtered through a sterile 0.2-μm filter. HPLC solvents were degassed by two filtrations under vacuum and were kept under He pressure. Dialysis tubing (SpectraPor 3, M_r cutoff 3000) and clamp-type closures were obtained from Spectrum Medical Industries. Dialysis tubing was soaked in sterile water and then in the buffer of choice before use. Centricon centrifugal ultrafiltration devices were from Amicon. Glassware was heated to 190 °C for $>\sim 1$ h, and plasticware was treated with 0.2% diethyl pyrocarbonate and autoclaved. HPLC-grade acetonitrile (Fisher), HPLC-grade trifluoroacetic acid (Pierce), tRNA^{Phe} (Boehringer-Mannheim), [^{14}C]phenylalanine (500 Ci/mol, Amersham), and [^{3}H]phenylalanine (44 000 Ci/mol, Amersham) were used without further purification.

[^{3}H]Puromycin was prepared as described by Kerlavage et al. (1985) from [8-^{3}H]puromycin aminonucleoside (Amersham) and stored at −20 °C in 50% ethanol for up to 30 days. [^{3}H]Phe-tRNA^{Phe} and [^{14}C]Phe-tRNA^{Phe} were prepared as described previously (Goldman et al., 1983). *N*-Acetyl[^{3}H]-Phe-tRNA^{Phe} and *N*-acetyl[^{14}C]Phe-tRNA^{Phe} were prepared from the parent-charged tRNA with acetic anhydride (Nicholson et al., 1982b). *E. coli* (strain Q13) 70S ribosomes were prepared as described by Jaynes et al. (1978) as method T, and subunits were separated with a zonal rotor as described by Goldman et al. (1983). As needed, 30S subunits were repurified in a VTi50 rotor (Beckman) as described by Goldman et al. (1983).

Photoincorporation of [^{3}H]Puromycin into 70S Ribosomes and Protein Extraction. Photolysis of solutions containing [^{3}H]puromycin (0.5 mM) and 70S ribosomes (100 A_{260} units/mL), in the presence or absence of tetracycline (0.1 mM), for 30 min or 20 h, respectively, was carried out as described previously (Kerlavage & Cooperman, 1986). The 70S ribosomes were recovered by precipitation with 2 volumes of 9:1 ethanol–β-mercaptoethanol immediately on termination of photolysis. The pellets were resuspended in TKM10 buffer and precipitated twice more with 2 volumes of ethanol (1% β-mercaptoethanol) to remove free puromycin. The final pellet was redissolved in a minimum volume of TKM10, to a concentration of 500–800 A_{260}/mL, and stored frozen at −80 °C.

The suspension of labeled 70S particles from photolyses were extracted with 3.6 M LiCl as previously described (Kerlavage & Cooperman, 1986) and the pellet redissolved in a minimum volume (<1 mL/400 A_{260} units) of REC4 buffer. Aliquots of approximately 250 A_{260} units of this resuspended pellet were extracted with 0.1 volume of 1 M $MgCl_2$ and 1 volume of acetic acid for 5–15 min at 0 °C and centrifuged for 15 min at 15 000 rpm in an SS34 rotor at 4 °C. The supernatant was applied directly to an RP-HPLC column.

RP-HPLC Preparation and Purification of Ribosomal Proteins. Preparations and purifications of individual proteins or groups of proteins were carried out by RP-HPLC with the system described previously (Kerlavage et al., 1984) except that the LC15B detector was sometimes replaced by an LC95 variable-wavelength detector. Both 4.1 × 250 mm (analytical) and 9 × 250 mm (preparative) Synchropak RP-P (C18) columns were used [particle size, 6.5 μm; pore size, 300 Å (Synchrom)]. Solvent A was 0.1% (w/v) trifluoroacetic acid in water, pH 2.15; solvent B was acetonitrile made 0.1% (w/v) in trifluoroacetic acid [the trifluoroacetic acid was actually added as a 1% (w/v) aqueous solution, pumped in at a constant 10% of the total flow rate].

Preparation of Puromycin-Labeled L23 (L23).* [^{3}H]Puromycin-labeled L23 (L23*) was prepared by RP-HPLC of acetic acid extracts of 3.6 M LiCl pellets derived from puromycin-labeled 70S ribosomes. The extracts, prepared as described above, were immediately applied to an analytical column. The proteins were eluted and collected in 13 × 100 mm polypropylene test tubes set in dry ice (Figure 2A). Fractions were evaporated to dryness in a Savant Speedvac without thawing and were promptly removed for storage at

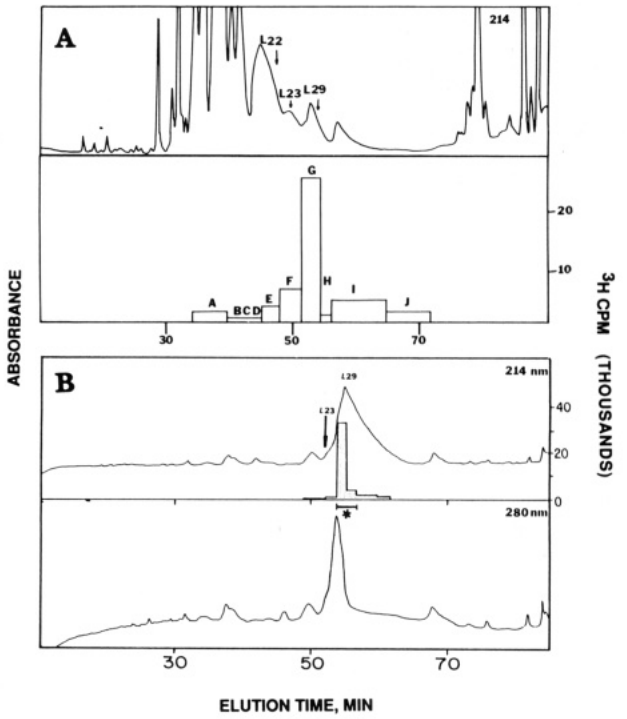

FIGURE 2: Purification of [^{3}H]puromycin-modified L23 from photoaffinity-labeled 70S ribosomes. (Panel A) RP-HPLC of proteins extracted from the 3.6 M LiCl pellet of 200 A_{260} units of 70S ribosomes labeled by [^{3}H]puromycin in the presence of tetracycline. Fraction G was collected for further purification. (Panel B) Rechromatography of fraction G (above) and the corresponding fractions from two other chromatographic runs. Bracketed fractions were used in further experiments as L23*. Upper trace shows absorbance at 214 nm (0.045 AUFS), and lower trace shows absorbance at 280 nm (0.0125 AUFS). The elution position of authentic L23 is indicated. For both chromatographies, the column was preequilibrated at 17% solvent B, and then the following gradient was employed: 17–32.8% B in 20 min, curve 0.2; 32.8–33.3% B in 15 min, linear; 33.3–33.9% B in 20 min, linear; 33.9–44% B in 20 min, linear; 44–60% B in 5 min, linear. For both panels, the flow rate was 0.7 mL/min, and a 4.1-mm internal diameter column was used.

−80 °C. Samples were redissolved in 0.5 mL of REC4/U per fraction, the pH was adjusted to 7.0–7.2 with 1 M Tris, and aliquots were removed for radioactivity determination. The appropriate fractions were pooled and concentrated with Centricon ultrafiltration devices (Amicon) to a volume of 0.3–0.7 $\mu L/A_{260}$ of extracted 70S ribosomes. A second RP-HPLC step, using the same methods of sample workup, afforded a purer sample of L23* (Figure 2B). As may be seen, this material is largely free of unmodified L23 although it is substantially contaminated with L29 (identified both by its elution position and by its characteristic lack of absorbance at 280 nm—L29 contains neither tyrosine nor tryptophan).

Assignment of the radioactive peak in Figure 2B as L23* is based on its elution position from the RP-HPLC column, the prior work of Grant et al. (1979) identifying the major photolabeled protein as L23 by both two-dimensional PAGE and specific immunoprecipitation analyses, and two-dimensional PAGE analysis of L23* using added L23 and TP30 as markers. Such analysis showed a single major radioactive region that was slightly retarded with respect to L23 in both dimensions, exactly as seen previously (Jaynes et al., 1978; Grant et al., 1979).

L23* demonstrated considerable lability toward loss of radioactivity on storage in acidic solution as well as on long-term storage at −70 °C. The sample handling procedures described in this section and the use of L23* within 2 weeks of its preparation limited such losses to ≤7%.

Preparation of L23 and hν-L23. L23 and *hν*-L23 were prepared by selection of the appropriate fractions from RP-HPLC separations of acetic acid extracts of unmodified 50S subunits and of LiCl pellets of 70S ribosomes photolyzed in the presence of tetracycline (but in the absence of [^{3}H]puromycin) respectively. Protein *hν*-L23 has the same retention on RP-HPLC as does unmodified L23.

Preparation of Protein Pools for Reconstitution. Ribosomal proteins for reconstitutions were extracted from 250–500 A_{260} units of 50S subunits with acetic acid and precipitated with acetone as described by Nierhaus and Dohme (1979), quickly redissolved in 1–3 mL of REC4/U, and immediately applied to a preparative RP-P column, repeated injections being used as needed. Proteins were applied to a column that had been preequilibrated with 15% solvent B and eluted with the following gradient: 15–33.5% B in 25 min, curve 0.2; 33.5–35% B in 25 min, linear; 35–41% B in 10 min, linear; 41–60% B in 10 min, linear. The flow rate was 1.5 mL/min. Fractions were collected, evaporated to dryness as described above, and stored briefly (<12 h) at −80 °C. When all fractions were dry, they were all dissolved in REC4/U and pooled as appropriate, giving a final concentration of >100 A_{260} equiv units/mL. The pH of each pool was adjusted to 6–6.5 with 1 M Tris base. Pools were stored at −80 °C in REC4/U. The protein concentration of the pool was assayed by HPLC analysis of an aliquot as described below. Preparations deficient in one or more proteins were either discarded or supplemented with a preparation of the missing protein(s) isolated from other HPLC runs, as described under Reconstitutions.

Quantification of Proteins by Peak Area. Concentrations of proteins in protein pools that were used in reconstitution were determined by applying a measured aliquot to an RP-HPLC column and comparing the peak areas at 214 nm to the areas obtained from chromatograms of total protein extracted from measured quantities of 50S subunits, as determined by assuming 1 A_{260} unit equals 39 pmol of 50S subunits (Jaynes et al., 1978). Such areas were found to increase linearly with the amount of 50S subunits extracted (Buck et al., 1989). This method depends on the assumption that the yield of a given protein on an RP-P column is reasonably constant, which we have independently shown to be generally valid.

RNA Preparation. The RNA pool for reconstitutions was prepared from 50S subunits exactly as described by Nierhaus and Dohme (1979) except that the storage buffer was replaced by RNA buffer.[2]

Reconstitutions. The 50S reconstitution procedure described by Nierhaus and Dohme (1979), as modified more recently (Schulze & Nierhaus, 1982), was adapted for use with the L23* preparations described above. Close attention was paid to the total volume of the reconstitution mix, as the success of reconstitution depended on not permitting too high a dilution. TP50–L23 pools, supplemented as required with other protein pools (L22/29, L27, L20/L4/L10), were tested in a series of reconstitutions to determine an optimal protein/RNA ratio, as defined by measuring peptidyl transferase activity (see below). In different experiments this ratio typically varied from 1.5 to 1.8. For TP50–L23 pools that required supplementation, similar optimization experiments were carried out by adding varying amounts of the supplementary proteins. Following addition of the appropriate L23 species, protein pools were combined, and REC4/U was added to bring the volume of each pool to 0.7–0.8 of the planned final protein pool

[2] M. Stark, personal communication.

volume. The protein pools were then dialyzed for 4–8 h against 500 volumes of REC4/U, for 55 min against 500 volumes of REC4, and for 35 min against 500 volumes of REC4. The contents of the dialysis bags were removed, each bag was washed with 0.1 volume of REC4 that was combined with the dialyzed solution, and each protein pool was immediately added to a reconstitution mixture. The reconstitution mixture contained (in order of addition) 3–5 A_{260} units of bulk RNA from 50S subunits, 0.05 volume of TENX buffer, 0.45 volume of TM4 buffer, the dialyzed protein pool, and REC4 buffer to make up the total volume. Final RNA concentration was 5 A_{260} units/mL. Reconstitution mixtures were incubated for 20 min at 40 °C, the Mg^{2+} concentration was then raised from 4 to 20 mM by addition of 1 M $Mg(OAc)_2$, and incubation was continued at 50 °C for 90 min. Samples were next centrifuged (2 min in an Eppendorf microfuge) to remove protein aggregates, and aliquots of the supernatants were assayed for peptidyl transferase and tRNA binding stimulation activity. The remaining supernatants were applied to sucrose density gradients for isolation of 50S subunits.

Peptidyl Transferase Assay. *N*-Acetyl[^{3}H]Phe-tRNA^{Phe} (2.5 pmol, 50 000 cpm) was added to 50 μL of PT buffer, 50 μL of reconstitution mixture supernatant (typically containing 10 pmol of 50S or 50S RNA) was added, the mixture was set in ice–water, and the reaction was initiated by addition of 50 μL of puromycin dihydrochloride, 1.0 mg/mL in ethanol (Hampl et al., 1981). Samples were quenched after 1 h with 100 μL of 0.2 M KOAc (adjusted to pH 5.0) saturated with $MgSO_4$ and extracted with 2.5 mL of ethyl acetate. An aliquot of the organic layer (2 mL) was mixed with 3 mL of the 25% Triton/toluene cocktail described previously (Jaynes et al., 1978) for liquid scintillation counting. Peptidyl transferase activity was linear with added 50S subunits up to 0.5 A_{260} unit provided that less than 60% of the total added radioactivity was transferred.

Stimulation of tRNA Binding. The ability of added 50S subunits to stimulate poly(U)-dependent tRNA binding in a mix originally containing only 30S subunits was carried out as previously described (Kerlavage & Cooperman, 1986) except that no ethanol was added to the assay. Final concentrations in the binding assay made up in TMK20 buffer were as follows: 30S subunits, 0.13 μM; poly(U), 0.24 mg/mL; [^{14}C]Phe-tRNA^{Phe}, 0.07 μM. Under these conditions, the tRNA binding increased linearly with 50S subunit concentration up to 0.08–0.10 μM, after which a plateau value was reached at 6.8-fold the level obtained in the absence of added 50S subunits. Routinely, aliquots of reconstitution mixtures giving a final concentration in the binding assay of 0.1 μM 50S rRNA were assayed for stimulation of tRNA binding activity. For 50S subunits the blank subtracted was that obtained on addition of the corresponding amount of boiled 50S subunits. For reconstitution mixes, the blank subtracted was that obtained on addition of TP50 alone, i.e., omitting rRNA from the reconstitution mix. Total volume of the assay solution was 0.1 mL.

Results

Preparation and Characterization of L23.* In generating L23* we exploited the known (Grant et al., 1979; Olson et al., 1982) specific stimulation by tetracycline of puromycin photoincorporation into L23 in an intact 70S ribosome. L23* could also be generated in the absence of tetracycline. However, in this case the large light dose needed to generate a significant level of labeling resulted in a sample of L23* that, rather than being able to be incorporated into reconstituting 50S subunits, actually inhibited reconstitution. The need to limit light dose so as to minimize structural damage to L23 had as a consequence that even with tetracycline stimulation the yield of L23*/70S ribosome never exceeded 0.12. This low yield, coupled with the limited efficiency of 50S reconstitution in our hands (vide infra), dictated a strategy for L23* purification affording the maximum recovery of L23* from labeled 70S ribosomes, consistent with such a preparation being free of unmodified L23. To avoid the considerable losses that occur on preparation of 50S subunits from 70S ribosomes by sucrose density gradient centrifugation (approximately 50%), L23* was prepared directly from labeled 70S ribosomes.

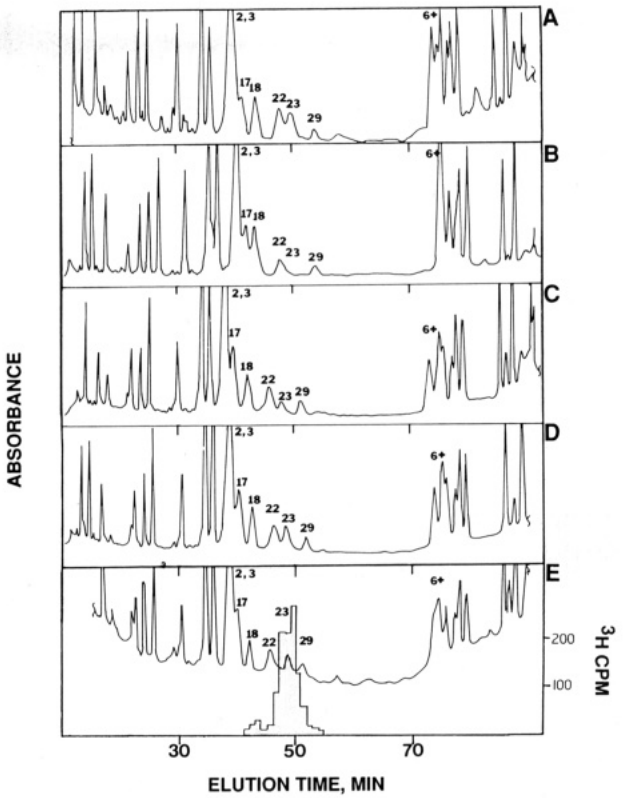

FIGURE 3: RP-HPLC analyses of TP50 pools and extracts. (Panel A) TP50 from native 50S subunits, 1.6 A_{260} equiv. (Panel B) TP50 – L23 pool used for reconstitution, 8 A_{260} equiv. (Panel C) Extract of 6 A_{260} equiv of 50S, reconstituted with TP50 – L23. (Panel D) Extract of 5.9 A_{260} equiv of 50S, reconstituted with TP50 – L23 + L23. In the reconstitution L23 was present in 3-fold excess over 50S RNA. (Panel E) Extract of 0.9 A_{260} equiv of 50S, reconstituted with TP50 – L23 + L23*. In this reconstitution L23* was present in 1.6-fold excess over 50S RNA. All analyses were performed with the analytical column. For each the column was preequilibrated at 17% solvent B, and then the following gradient was employed: 17–33% B in 25 min, curve 0.2; 33–33.8% B in 15 min, linear; 33.8–34.3% B in 20 min, linear; 34.3–44% B in 20 min, linear ; 44–60% B in 5 min, linear. The flow rate was 0.7 mL/min.

Preparation and Characterization of TP50 – L23. The TP50 – L23 pool was prepared by combining all of the fractions from a preparative run of TP50 (Figure 3A) except for those which contained L23. The advantage of this approach is its rapidity, an important feature due to the poor storage properties of 50S proteins, even at −80 °C, toward reconstitution of 50S subunits.[2] This lability convinced us that it would be futile to attempt 50S reconstitution with large numbers of purified protein pools, since the preparation time required to prepare such pools would mitigate against successful reconstitution. The drawback of our approach is that, in part because ribosomal proteins are eluted with varying yields, the relative proportions of 50S proteins in the reconstitution mix do not exactly parallel the proportions found on extraction of 50S subunits. Large deviations were handled on an ad hoc

Table I: Protein Compositions of 50S Subunits and Reconstitution Pools[a]

protein	native 50S subunits[b]	TP50 – L23 pools[c]	reconstituted 50S particles[c,d]			
			–L23	+L23	*hν*-L23[e]	+L23*
32	2.1 ± 0.1	1.1 ± 0.3	1.8[f]	1.2 ± 0.1	1.3 ± 0.0	1.7[f]
33	2.4 ± 0.2	1.2 ± 0.2	1.2 ± 0.2[e]	0.9 ± 0.1	0.8 ± 0.1	1.2[f]
27	2.8 ± 0.4	0.8 ± 0.3	1.0 ± 0.1[e]	0.6 ± 0.1	0.6 ± 0.0	0.8 ± 0.2
24	3.0 ± 0.4	0.9 ± 0.1	1.0 ± 0.3[e]	1.1 ± 0.4	1.1 ± 0.4	1.3 ± 0.3
28	3.0 ± 0.2	1.1 ± 0.1	1.3 ± 0.1[e]	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.3
25	3.7 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	1.0 ± 0.2	0.9 ± 0.2
14^+	8.6 ± 0.2	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
13^+	5.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2
2^+	20.9 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.3
18	3.4 ± 0.2	1.1 ± 0.2	1.1 ± 0.3	1.0 ± 0.1	0.9 ± 0.2	1.0 ± 0.3
22	3.5 ± 0.2	1.0 ± 0.3	1.0 ± 0.1[e]	1.2 ± 0.3	1.1 ± 0.2	1.4 ± 0.6
23	3.2 ± 0.5	<0.1	0.3 ± 0.0[e]	0.8 ± 0.0	0.8 ± 0.1	0.6 ± 0.3
29	1.2 ± 0.3	1.2 ± 0.4	1.1[f]	1.3 ± 0.4	1.5 ± 0.5	1.7 ± 0.4
6^+	15.0 ± 0.6	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.9 ± 0.1
1^+	8.3 ± 0.3	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.9 ± 0.0	0.8 ± 0.3
5	5.0 ± 0.4	1.0 ± 0.3	0.8 ± 0.0	0.9 ± 0.1	0.8 ± 0.2	0.6 ± 0.1
20	3.1 ± 0.4	1.5 ± 0.6	1.4 ± 0.3	1.4 ± 0.2	1.4 ± 0.1	1.4 ± 0.1
4^+	5.6 ± 1.1	1.4 ± 0.7	1.2 ± 0.2	1.4 ± 0.3	1.4 ± 0.1	1.4 ± 0.5

[a] As determined by RP-HPLC analysis (see Figure 3). Numbers shown are the average of three to four determinations ± standard deviations, unless otherwise indicated. [b] Values shown represent the percentage of the total peak area found in the 18 protein peaks for a given protein peak. Thus, for proteins extracted from native 50S subunits, the L32 peak accounted for 2.1% of the total area. [c] Values shown are ratios of the percentages found in a given peak to the percentage found in the corresponding peak from native 50S particles. Thus, for a perfect TP50 – L23 pool or a perfectly reconstituted TP50 – L23 + L23 50S particle, all of the ratios would be 1.0. [d] –L23 refers to the composition of 50S particles reconstituted with a TP50 – L23 pool. The other columns represent compositions of 50S particles reconstituted on addition of the corresponding form of L23 (L23, *hν*-L23, or L23*) to the reconstitution mix. [e] Average of two determinations ± average deviations. [f] One determination.

basis. Thus, in some cases, removal of all of L23 necessitated removal of significant amounts of L22 and L29, and a separate pool of these two proteins was prepared and added back to the mix. Similarly, some reconstitution mixes required supplementation with proteins L4, L10, L20, and L27.

Protein pools for reconstitution were analyzed by RP-HPLC (Figure 3A,B). Such analyses typically involved determination of the areas of a total of 18 peaks, 12 corresponding to single well-resolved proteins (L proteins 5, 18, 20, 22–25, 27–29, 32, and 33) with the remaining 6 containing an additional 16 L proteins. These latter peaks were designated $L1^+$ (1, 11), $L2^+$ (2, 3, 17), $L4^+$ (4, 10), $L6^+$ (6, 9, 15, 16), $L13^+$ (13, 21), and $L14^+$ (14, 19, 30). Five L proteins were not included in reconstitution. These were L26, which is identical with S20 and which we found to be present in only very low copy number within a 50S subunit, L31, which we have not been able to identify by RP-HPLC analysis of TP50 (Kerlavage et al., 1983), and L7, L12, and L34. Neither L7 nor L12, which we recover in only limited amounts, is important for 50S reconstitution or for peptidyl transferase activity (Hampl et al., 1981).

The compositions of the TP50 – L23 pools relative to the composition of TP50 standards are presented in Table I. For most peaks, the relative percentage of total protein area found in a given peak from the TP50 – L23 pool fell within 20% of that found for the same peak from the TP50 pool, although in the worst cases ($L13^+$, $L1^+$, L20, $L4^+$) the deviation was as high as 50%. A limitation of this method of comparison is that a selective loss of one protein from a peak containing several proteins (e.g., peak $L6^+$, Table I) could go undetected.

*Reconstitution of 50S Subunits Containing L23**. Reconstitutions of 50S subunits were carried out as described (see Experimental Procedures) with RNA extracted from 50S particles and the following protein samples: (a) TP50 – L23, (b) TP50 – L23 + L23, (c) TP50 – L23 + L23*, and (d) TP50 – L23 + *hν*-L23. The following tests of structure and function were applied to the reconstituted particles: sucrose density gradient centrifugation, protein composition, uptake of L23* into the 50S subunit, peptidyl transferase activity, and tRNA binding stimulation activity. Suitable control experiments

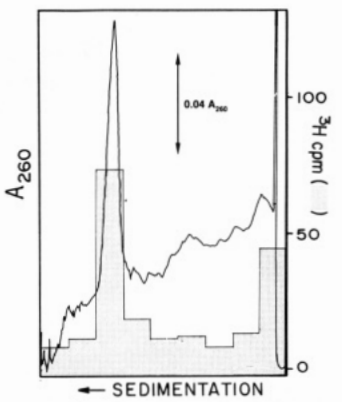

FIGURE 4: Sucrose gradient analysis of 50S subunits reconstituted with L23*. The reconstitution mixture contained (in A_{260} equiv units) TP50 – L23 (6.0 units), L23* (15.0 units), and 50S rRNA (4.0 units). Following reconstitution, the mixture was applied to a linear 15–30% sucrose gradient made up in TKM1 buffer. Centrifugation was carried out in a VTi50 rotor at 50 000 rpm for 90 min.

demonstrated no differences between 50S subunits reconstituted from TP50 prepared directly by extraction from a 50S subunit and not subjected to RP-HPLC and 50S subunits reconstituted from TP50 prepared from RP-HPLC-separated protein pools, with respect to either the yield of reconstituted 50S particles or the activity in either assay of such particles (Table IV).

A sucrose density gradient profile of a TP50 – L23 + L23* reconstitution mix is shown in Figure 4 and is representative of the results obtained with all other such mixes. The single peak shown sediments as a 50S particle. As seen from Table II, the yield of reconstituted 50S particle was essentially identical for all the mixes and averaged 23% on the basis of rRNA in the reconstitution mix. As native 50S particles were recovered with a yield of 45 ± 3%, we estimate the yield in the reconstitution mix itself to be of the order of 50%. Also shown in Figure 4 is the radioactivity corresponding to L23* taken up into the reconstituted particle. A control experiment in which a mix containing 50S subunits, TP50, and L23* was incubated under reconstitution conditions and subjected to

Table II: Recoveries of 50S Subunits from Sucrose Density Gradients

sample	% recovery[a]
native 50S subunit	45 ± 1 (3)
reconstituted 50 subunits	
TP50 – L23	24 ± 3 (4)
TP50 – L23 + L23	26 ± 5 (3)
TP50 – L23 + *hν*-L23	23 ± 3 (5)
TP50 – L23 + L23*	21 ± 3 (4)

[a] For the native 50S subunit, calculated on the basis of the amount of 50S subunits applied to gradient. For the reconstituted subunits, calculated on the basis of 50S rRNA used in reconstitution. ± values are average deviations. Numbers in parentheses indicate the number of independent determinations.

sucrose density gradient centrifugation showed no radioactivity cosedimenting with the 50S particle.

Protein compositions for reconstituted particles were compared with those for authentic 50S particles by RP-HPLC analysis (Table I, Figure 3), leading to two major conclusions. First, within the limits of the reproducibility and precision of this analysis, and except for L23, no major differences in protein composition were seen for any of the reconstituted particles as compared with that found for an authentic 50S subunit. In particular, no drastic reduction in relative amount was seen for any of the resolved peaks. Second, despite intense effort, we were unable to reduce the L23 content of a 50S subunit reconstituted from a TP50 – L23 pool below about 30% of the value obtained from an authentic 50S subunit. This problem arises from our inability to completely remove unmodified L23 from TP50 – L23 and is accentuated by the nature of the reconstitution process. Thus, reconstitution is optimal with approximately 1.8 equiv of protein per RNA but proceeds in only 50% yield (Table II). As a consequence, if L23 is taken up preferentially [as would be expected, since L23 binds directly to 23S rRNA (Vester & Garrett, 1984)], a TP50 – L23 pool containing as little as 0.08 L23 equiv/pool equiv would yield a reconstituted particle containing 0.30 L23/50S. Such preferential uptake is clearly demonstrated on comparing the RP-HPLC trace of a TP50 – L23 pool (Figure 3B) with the RP-HPLC trace of a 50S subunit reconstituted with such a pool (Figure 3C). The L23 content of the pool is seen as a small shoulder of the L22 peak, whereas the extract of the subunit reconstituted with the pool shows a prominent L23 peak. It is also clear that the area of the L23 peak in Figure 3C is much reduced when compared with that obtained for an extract from an authentic 50S subunit (Figure 3A) or for a 50S subunit reconstituted with a TP50 – L23 + L23 protein pool (Figure 3D—the RP-HPLC pattern for the 50S subunit reconstituted with a TP50 – L23 + *hν*-L23 protein pool was very similar to that shown in Figure 3D).

A total of four reconstitutions were carried out with TP50 – L23 + L23*, in which the ratio of L23*/50S rRNA in the reconstitution mix varied from 1.1 to 3.6. As summarized in Table III, the value of L23* taken up never exceeded 0.5/50S subunit. Our failure to increase beyond this value is probably due to contamination of L23* with unmodified L23 and a preferential uptake of unmodified L23 as compared with L23*. An RP-HPLC analysis of such a reconstituted particle (Figure 3E) shows L23* eluting between the elution position of unmodified L23 and L29.

Activities of Reconstituted Particles. The four kinds of reconstituted 50S subunits discussed above (Table II) were tested for two activities, stimulation of tRNA binding and peptidyl transferase. The first assay measures the stimulation by added 50S subunits of poly(U)-dependent Phe-tRNA^{Phe} binding to 30S subunits. Such stimulation presumably occurs

Table III: L23* Uptake into Reconstituted 50S Subunits

L23*/50S rRNA[a]	L23*/50S[b]
1.1	0.54
1.6	0.42
3.0	0.51
3.6	0.36

[a] In reconstitution mix. L23* was measured by radioactivity, assuming photoincorporation of 1.0 puromycin per L23. [b] In reconstituted particle, 50S was measured by the sum of the integrated protein areas on RP-HPLC analysis compared with that obtained for an authentic 50S subunit.

Table IV: Relative Activities of Reconstituted 50S Subunits[a]

protein pool	stimulation of tRNA binding	peptidyl transferase
TP50 – L23	1.00	1.00
TP50 – L23 + L23[b]	1.27 ± 0.04 (3)	1.10 ± 0.03 (2)
TP50 – L23 + *hν*-L23	1.24 ± 0.09 (3)	1.07 ± 0.13 (3)
TP50 – L23 + L23*[c]	0.64 ± 0.07 (3)	0.97 ± 0.23 (4)
native 50S subunits	2.9 ± 1.1 (6)[d]	14.3 ± 5.0 (5)[e]

[a] Ranges given are average deviations. Numbers in parentheses indicate the number of independent determinations. [b] Relative activities of 50S subunits reconstituted with unfractionated TP50, not subjected to RP-HPLC, gave essentially the same results. [c] Average L23* uptake: 0.46/50S. [d] Typically, 10 pmol of native 50S subunits added to 13 pmol of 30S subunits increased tRNA binding from 0.3 to 1.9 pmol out of a total of 7 pmol in the reaction mixture. [e] In the typical assay with a 1-h incubation, 20 pmol of native 50S subunits transferred 1.2–1.4 pmol of *N*-AcPhe to puromycin, out of a total of 2.5 pmol of *N*-AcPhe-tRNA added to the reaction mixture.

via formation of 70S ribosomes (Kerlavage & Cooperman, 1986). The second assay measures the formation of *N*-AcPhe-puromycin from *N*-AcPhe-tRNA^{Phe} and puromycin. In the presence of 33% ethanol this reaction is catalyzed by 50S subunits in the absence of both 30S subunits and mRNA. Both assays were carried out under conditions in which activity was proportional to added 50S particles. Although the limited quantities of reconstituted 50S subunits available resulted in data having considerable scatter, the results obtained (Table IV) permit four principal conclusions to be drawn:

(1) The omission of L23 from a TP50 pool leads to a small loss (~20%) of tRNA binding stimulation and slight if any loss of peptidyl transferase activity. The latter result is in accord with previous results of Hampl et al. (1981) and Schulze and Nierhaus (1982).

(2) *hν*-L23 is indistinguishable from L23, at least as measured in these assays.

(3) The inclusion of L23* in a 50S particle has no apparent effect on peptidyl transferase but results in a clear decrease in tRNA binding stimulation activity. Indeed, within the limits of precision of the experiment, the most straightforward interpretation of our results is that 50S subunits containing an L23* are completely unable to stimulate tRNA binding activity.

(4) Reconstituted 50S subunits have considerably lower activity in both assays than native 50S subunits. Toward stimulation of poly(U)-dependent Phe-tRNA^{Phe} binding, the specific activity obtained with a TP50 – L23 + L23 reconstitution mix averaged 35% of the value found for 50S subunits, with a range of 31–45%. The relative activity of the reconstituted particle was much lower in the peptidyl transferase assay, averaging 17% of the value found for 50S subunits, with a range of 4–26%.

Discussion

This work constitutes the second part of our ongoing effort to determine the functional effects of photoaffinity labeling

by puromycin of specific ribosomal proteins. The present study with L23* proved technically more difficult than our previous study with puromycin–S14 (Kerlavage & Cooperman, 1986) in three important respects. First, we were unable to exceed an uptake of L23* of 0.5 per reconstituted 50S subunit, whereas we achieved a level of 0.9 puromycin–S14 per reconstituted 30S subunit. This is a direct consequence both of the preferential uptake of L23 vs L23* into reconstituting 50S subunits (Figure 3B,C) and of our inability, because L23 falls in a rather crowded region of the chromatogram, to prepare either a homogeneous sample of L23* [our preparation contained significant amounts of unmodified L29 although only limited amounts of L23 (Figure 2B)] or a TP50 – L23 protein pool that was completely devoid of L23. By contrast, S14 is well resolved from other ribosomal proteins, making it straightforward to prepare homogeneous samples of both S14 and puromycin–S14, as well as to prepare a TP30 – S14 pool that completely lacked S14. Second, in our hands 50S reconstitution proceeded in yields (based on 50S rRNA added) of less than 50% and yielded subunits having substantially lower activities than native subunits (Table IV), whereas 30S reconstitution proceeded in high yield and routinely yielded subunits with activities quite comparable to that of authentic 30S subunits. Third, since L23 is not essential for either of the two ribosome functions we tested, our measurement of functional effects depended on being able to demonstrate an inhibition of ribosomal function as a consequence of L23* uptake. With 0.5 mol of L23* taken up per 50S subunit, a full inhibitory effect would be expected to decrease the observed activity to 50% of the control value. We were able to detect such a full effect, but our data were insufficiently reproducible to allow for reliable estimation of partial inhibitory effects. Again in contrast, 30S subunits lacking S14 are almost completely devoid of poly(U)-dependent Phe-tRNA^{Phe} binding activity. Thus, in our earlier study we could compare the measured activity on uptake of puromycin–S14 with the full activity on uptake of unmodified S14, allowing easy detection of partial activity.

Despite the limitations imposed by these technical difficulties, the major conclusions that can be drawn from our work are that replacement of L23 by L23* in a reconstituted 50S subunit interferes with the ability of a 50S subunit to stimulate poly(U)-dependent Phe-tRNA^{Phe} binding but has no major inhibitory effect on 50S-dependent peptidyl transferase activity. Given the low activity of our reconstituted 50S subunits in the peptidyl transferase assay, the validity of this latter conclusion depends on the assumption that L23* is not taken up preferentially by inactive subunits. Although this possibility cannot be definitively excluded, we consider it unlikely, especially since the rate-limiting conformational changes in the assembly of the 50S subunit occur subsequent to the binding of L23 to 23S rRNA (Dohme & Nierhaus, 1976).

The peptidyl transferase assay used in this study, which is carried out in 33% ethanol, looks directly at the peptidyl transferase center and has no requirement for tRNA binding aside from the 3′-terminal portion of the peptidyl donor. Thus, although we have used *N*-AcPhe-tRNA^{Phe} as a peptidyl donor for this assay, *N*-AcPhe linked to the 3′-OH of CACCA, the 3′-terminal pentanucleotide common to tRNAs, is a perfectly good donor (Monro, 1971). This being the case, the failure of L23* to markedly inhibit peptidyl transferase shows only that photoincorporation into L23 is not taking place precisely at either the pentanucleotide donor site or the puromycin acceptor site. This is consistent with earlier results showing that neither puromycin nor *p*-azidopuromycin is active as a peptidyl transferase acceptor when covalently incorporated into ribosomes (Jaynes et al., 1978; Nicholson et al., 1982b). On the other hand, the marked inhibitory effect of L23* on the ability of 50S subunits to stimulate poly(U)-dependent Phe-tRNA^{Phe} binding provides strong evidence that the site occupied by photoincorporated puromycin is critical for a region of tRNA binding to the 50S subunit and/or for 50S association with 30S subunits and is consistent with previous results showing a loss of mRNA-dependent peptidyl transferase activity at high stoichiometries of puromycin or *p*-azidopuromycin photoincorporation (Jaynes et al., 1978; Nicholson et al., 1982b).

Although L23* does not inhibit peptidyl transferase, in previous work we have presented quite strong evidence that puromycin photoincorporation into L23 takes place from the peptidyl transferase center. One part of this evidence was the strong parallelism between the abilities of various puromycin analogues and derivatives to act as substrates or competitive inhibitors of peptidyl transferase and their abilities to block puromycin (or *p*-azidopuromycin) photoincorporation into L23 or to themselves photoincorporate into L23 (Jaynes et al., 1978; Nicholson et al., 1982b; Weitzmann & Cooperman, 1985). In addition, *p*-azidopuromycin, which, as noted above, photoincorporates into L23 to a greater extent than into any other ribosomal proteins, also labels other ribosomal components [L15 and L18/22 (Nicholson et al., 1982a); 23S rRNA bases U-2504 and G-2502 (Hall et al., 1988)] that have been directly implicated in peptidyl transferase activity in other studies (Teroaka & Nierhaus, 1978; Hampl et al., 1981; Schulze & Nierhaus, 1982; Cooperman, 1987; Arêvalo et al., 1988; Steiner et al., 1988; Moazed & Noller, 1989). These prior results and our current work lead us to conclude that although L23 is not at the peptidyl transferase center, and indeed is not required for peptidyl transferase activity, it is located sufficiently close by that a puromycin photoincorporated into it can prevent proper tRNA binding to the 70S ribosome.

This conclusion supports one side in the controversy currently surrounding the placement of L23 within the 50S subunit. In addition to the photoaffinity labeling results with puromycin and *p*-azidopuromycin summarized above, four additional sets of results are relevant, three of which place L23 close to the peptidyl transferase center, in agreement with the current work, while the fourth places L23 far from the peptidyl transferase center but is somewhat ambiguous. Thus, L23 has been found to be cross-linked to a set of ribosomal proteins (L15, L16, L18, and L27) that are strongly linked to peptidyl transferase and may be considered as forming a distinct domain at or close to the peptidyl transferase center (Traut et al., 1986). In addition, as shown by immunoelectron microscopy (Olson et al., 1982, 1985), antibody to N^6,N^6-dimethyladenosine (which recognizes puromycin) binds to 50S subunits that have been photoaffinity labeled with either puromycin or *p*-azidopuromycin. Such binding takes place in the region of the 50S subunit that has been identified as containing the peptidyl transferase center, i.e., at or near the notch between the central protuberance and the protuberance containing protein L1 and on the side of the 50S subunit facing the 30S subunit (Stöffler & Stöffler-Meilicke, 1984, 1986). This result is of particular significance for the puromycin-labeled 50S subunit, in which 70% of photoincorporation was into L23. Further, tetracycline, which specifically stimulates puromycin photoincorporation into L23 [Grant et al. (1979) and this work], has recently been shown to specifically photomodify 23S rRNA bases A-2503 and U-2504 (Steiner et al., 1988). These bases fall within the central loop of domain

V that has been identified as being at the peptidyl transferase center, at essentially the same location as the sites of *p*-azidopuromycin photoincorporation (Hall et al., 1988).

On the other hand, the most recent immunoelectron microscopy studies of 50S subunits using antibody to L23 as a probe (Stöffler-Meilicke et al., 1983; Hackl & Stöffler-Meilicke, 1988) place L23 near the base of the 50S subunit and on the side facing away from the 30S subunit, quite far from the peptidyl transferase center, although earlier results from the same laboratory had placed L23 at a site reasonably consistent with the location of the peptidyl transferase center (Tischendorf et al., 1974). With present models of 50S structure and function it is difficult to reconcile the current positioning of L23 with the effect of puromycin–L23 on tRNA binding. It remains to be seen whether the more detailed model of the 50S subunit that is emerging from computerized image processing of both ice-embedded subunits (Wagenknecht et al., 1988) and quasi-crystalline subunit arrays (Yonath et al., 1988), which appears to have channels through the subunit, will permit a satisfactory resolution to this controversy.

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