Incorporation of Dinitrophenyl Protein L23 into Totally Reconstituted Escherichia coli 50 S Ribosomal Subunits and Its Localization at Two Sites by Immune Electron Microscopy*

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Escherichia coli ribosomal protein L23 was derivatized with [3H]2,4-dinitrofluorobenzene both at the N terminus and at internal lysines. Dinitrophenyl-L23 (DNP-L23) was taken up into 50 S subunits from a reconstitution mixture containing rRNA and total 50 S protein depleted in L23. Unmodified L23 competed with DNP-L23 for uptake, indicating that each protein form bound in an identical or similar position within the subunit. Modified L23, incorporated at a level of 0.7 or 0.4 DNP groups per 50 S, was localized by electron microscopy of subunits complexed with antibodies to dinitrophenol. Antibodies were seen at two major sites with almost equal frequency. One site is beside the central protuberance, in a region previously identified as the peptidyltransferase center. The second location is at the base of the subunit, in the area of the exit site from which the growing peptide leaves the ribosome. Models derived from image reconstruction show hollows or canyons in the subunit and a tunnel that links the transferase and exit sites. Our results indicate that L23 is at the subunit interior, with separate elements of the protein at the subunit surface at or near both ends of this tunnel.

Determination of the positions of proteins within the 30 S and 50 S ribosomal subunits has been a major goal of studies directed toward the elucidation of the structure and function of the Escherichia coli ribosome. The technique of immune electron microscopy (IEM), the visualization in electron micrographs of complexes formed between ribosomal subunits and specific antibodies, has been especially useful for this purpose (1, 2). However, in the case of protein L23, application of IEM has yielded controversial results. The object of the present work is to resolve this controversy.

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¶ The abbreviations used are: IEM, immune electron microscopy; DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; RP-HPLC, reverse phase high performance liquid chromatography; HPLC, high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; TF50, total protein from 50 S ribosomal subunits.

E. coli ribosomal protein L23 is a primary binding protein; it interacts directly with ribosomal RNA (3) and plays a significant role in the assembly of the large ribosomal subunit (4). The protein has been linked to the ribosomal peptidyltransferase center in several ways. First, photoaffinity labeling of 70 S ribosomes with either puromycin (5–7) or p-azidopuromycin (8, 9), each of which inhibits protein synthesis by acting as a peptide acceptor in the transferase reaction, led to photoincorporation into L23 as the major site of protein labeling. Second, antibodies recognizing the Nε,Nε-dimethyladenosine moiety of puromycin bound to labeled 50 S subunits in a region generally agreed to include the peptidyltransferase center, i.e. between the central protuberance and the site of protein L1, near the 30 S:50 S interface (10, 11). Third, chemical cross-linking and related studies showed L23 to neighbor other proteins (L2, L15, L16, and L27) that have been placed at or near the peptidyltransferase center (12). Finally, although peptidyltransferase activity is not altered in reconstituted 50 S subunits either lacking L23 or including puromycin-modified L23 in place of L23, the latter do show reduced aminoacyl-tRNA binding (6), which suggests proximity to the transferase region.

In contrast, the Berlin group used IEM with polyclonal antibodies to place the protein far from the transferase center, at the subunit base on its cytoplasmic surface (13) at a position similar to that at which the growing peptide emerges from the subunit (the exit site) (14). IEM of subunits that had been photoaffinity modified with puromycin and azidopuromycin also shows a small but consistent secondary puromycin localization, at the exit site (10, 11). Furthermore, some cross-linking studies placed L23 near proteins (L29, L34) located near the 50 S base (15, 16). The peptidyltransferase and exit sites are separated by more than 100 Å in common subunit models (13, 17, 18).

As described previously (12), these conflicting results could be explained by one of three possibilities. First, the IEM placement of puromycin may not reflect placement of L23. We considered this unlikely because 70% of photoincorporation of puromycin into 50 S subunits was into L23. Second, the IEM placement using anti-L23 may be in error, either because the anti-L23 preparation is contaminated with antibodies to other ribosomal proteins or because an incorrect site has been identified. However, the authors argue persuasively for the validity of their results. Third, the IEM results cited above may all be correct; the puromycin binding site on L23 and epitopes recognized by anti-L23 IgG could differ and involve separate areas of an asymmetric protein. It is also possible that the two sites are not as far apart as is suggested by the visually derived subunit models. Reconstructed images of negatively stained subunits (19, 20) and of frozen unstained ribosomes (21–23) and electron
microscopy of crystalline ribosome arrays (24) each show a more porous particle than the "classical" models of Lake (18), Vasiliev (17), or Sto¨ffler (13) would suggest.

We here report the definitive localization of L23 within the 50 S subunit by reconstitution of 50 S subunits with dinitrophenyl (DNP) derivatives of L23 in place of L23 and visualization of the complexes that such subunits make with anti-DNP. This approach, applied by us earlier to the localization of proteins within the 30 S subunit (25–28), avoids the possible ambiguities associated with the earlier L23 studies. In particular, derivatizing highly purified L23 with dinitrofluorobenzene ensures that anti-DNP will recognize only L23 in the reconstituted 50 S subunit. Our results clearly support the validity of both of the earlier IEM studies (i.e. of the third possibility) with interesting implications for 50 S structure.

EXPERIMENTAL PROCEDURES

Preparation and Purification of 50 S Proteins for Reconstitution—

Preparation of L23—This procedure was the same as described previously (6, 26, 28). RP-HPLC was performed on both analytical (250 mm × 4.6 mm) and preparative (250 mm × 10 mm) Synchropak RP C18 and C8 columns (Synchrom Inc.) as described previously (6, 26, 28) and in the figure legends.

Preparation and Purification of 50 S Proteins for Reconstitution—

Preparation of 50 S subunits—Reconstitutions were carried out by combining RNA, [3H]DNFB-L23, TP50-L23, and placentale RNase inhibitor protein (4 units/A260 equivalent) as described for the reconstitution of 50 S subunits containing puromycin-L23 in place of L23 (6). The molar ratio of protein to RNA was 1.8. Proteins dissolved in 300 mM NaCl were dialyzed versus HEPES (lacking urea) before being used in reconstitution. Reconstituted subunits were stored at −70 °C in 50 mM Tris-HCl, pH 7.6, 10 mM MgCl2, 50 mM KCl.

Acid Hydrolysis of [3H]DNFB-L23—In a typical hydrolysis, 25 µg of [3H]DNFB-L23 in 0.1% trifluoroacetic acid was added to an ampoule containing 40 µg each of N2-DNP-Lys and N2-DNP-Met and lyophilized. The sample was dissolved in 400 µl of 6 N HCl, purged with nitrogen gas, sealed, and heated for 10–16 h at 105 °C. Followings transfer and lyophilization, the sample was dissolved in 10 ml methanol followed by 1 ml of 0.1% trifluoroacetic acid for analysis by RP-HPLC. [3H]DNFB-L23 derived from reconstituted 50 S subunits was partially purified by RP-HPLC on a C18 column prior to acid hydrolysis. This procedure also removed contaminating sucrose and nucleic acid that interfered with the analysis of hydrolysis products.

Preparation of Complexes for Electron Microscopy—Two samples of reconstituted subunits were used. One sample was prepared using protein that contained 1.3 DNP/L23, and the resulting 50 S particles obtained 0.65 DNP/50 S. A second sample was prepared with protein that contained 0.9 DNP/L23, yielding subunits that included 0.4 DNP/L23, with one of the complexes that such subunits make with anti-DNP L23, was used in reconstitution. The L23 sample was further purified and TP50 was prepared from 50 S subunits as described (6). RP-HPLC was performed on both analytical (250 mm × 20 mm) and C8 columns (SynChrom Inc.) as described previously (6, 26, 28) and in the figure legends.

Buffers and reagents were prepared and obtained essentially as described previously, as were E. coli Q13 70 S ribosomes and 50 S subunits (6, 26, 28). RP-HPLC was performed on both analytical (250 mm × 4.6 mm) and preparative (250 mm × 10 mm) Synchropak RP C18 and C8 columns (Synchrom Inc.) as described previously (6, 26, 28) and in the figure legends.

Preparation and Purification of 50 S Proteins for Reconstitution—

Preparation of RNA for Reconstitution—RNA for reconstitution was dissolved in TM4 buffer (10 mM Tris HCl, pH 7.6, 150 mM NaCl, 10 mM MgCl2). Samples were incubated at 37 °C for 5–15 min and then on ice for 12–16 h. They were then fractionated by size exclusion HPLC using either a 7.5 × 300-mm Beckman SpheroSpher TSK 3000 SW column or a 7.8 × 300-mm Supelco Progel TSK 3000 SW XL column that had been equilibrated with 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM MgCl2, (10/150/10 buffer) as described (33).

In a second set of experiments, 15–16 pmol (0.37–0.40 A260 units) of reconstituted 50 S subunits were incubated first with 60–120 pmol (4–8 IgG equivalents) of anti-DNP (26, 28). Each IgG-purified protein was incubated with 0.5–0.5 ml of 25/150/10 buffer, pH 7.5, 50 mM KCl, at 37 °C for 15 min. Then 2–4 binding equivalents of anti-DNP IgG were added, and the buffer was adjusted with NH4Cl to raise the monovalent cation level to 150 mM. The samples were incubated at 37 °C for an additional 5–15 min followed by 12–16 h on ice, and fractionated as described above.

Reconstituted 50 S subunits were also studied in 70 S ribosomes. About 8 pmol (0.2 A260 unit) of reconstituted 50 S proteins containing DNP-modified L23 were mixed with 16 pmol (0.24 A260 unit) of 30 S subunits in 10 ml of 10/150/10 buffer and incubated at 37°C for 15 min. Then 4 binding equivalents of anti-DNP IgG in 2 µl of 25/150/10 buffer were added, and the mixtures were incubated for 15 min at 37 °C followed by 16 h on ice. Immune complexes were fractionated at 0 °C as above using 10/150/10 buffer. Alternatively, 8 pmol (0.2 A260 unit) of reconstituted 50 S particles containing DNP-modified L23 were incubated with 4 binding equivalents of anti-DNP IgG in 10 µl of 10/150/10 buffer for 15 min at 37 °C and then on ice for 12 h. Then 16 pmol (0.24 A260 unit) of 30S subunits in 5 µl of 25/150/10 buffer were added, and samples were incubated for 10 min at 37 °C, followed by HPLC fractionation as described above.

Electron Microscopy—Size-exclusion HPLC fractions containing ribosomes and immune complexes were immediately adsorbed to thin carbon films and negatively contrasted with 0.7% (w/v) uranyl acetate using the double carbon technique as described previously (18, 25). Electron micrographs were obtained and evaluated as described (25). Nomenclature is from Lake (18).

RESULTS

Purification of L23—Confidence in our interpretation of the immuno electron microscopy depends on the DNP derivatization of highly purified L23, i.e. sites of antibody attachment must reflect binding to derivatized L23 only, rather than to L23 and other proteins. We purified L23 through the use of multiple steps of HPLC, using both C18 and C8 columns, as described in Fig. 1. The final material was homogeneous on both RP-HPLC and SDS-PAGE analysis (Fig. 2) and had the A260/A280 ratio expected for a protein containing one Trp and one Tyr (0.047). Protein L22, containing only one Tyr and no Trp, has a much lower value: 0.007, and L29, lacking both Tyr and Trp has a value of <0.001 (see Ref. 29). Simultaneously satisfying all of these criteria is important since two proteins of the 70 S ribosome (S14 and L24) have molecular weights similar to L23 and are not well resolved from it by SDS-PAGE, but elute from L23 on RP-HPLC (34). Finally, quantitative amino acid sequence analysis gave results consistent with L23 and ruled out more than trace contamination with the two proteins, L22 and L29, that elute close to L23 on RP-HPLC (Table I). We estimate that the L23 preparation derivatized with DNFB was ≥98% pure.

Modification of L23 with [3H]DNFB—Modification condi-
tions were chosen to afford a stoichiometry of approximately 1 DNP/L23, based on the earlier observation of Olah et al. (28) that, at least in 30 S reconstitution, less modified proteins are selected over more heavily modified proteins. Prior to use in reconstitution, residual unreacted L23 was removed by RP-HPLC fractionation (Fig. 3), in order that it not compete with [3H]DNP-L23 for reconstitution into 50 S subunits.

Sites of Modification in [3H]DNP-L23—DNFB was originally introduced by Sanger (35, 36) as a reagent to specifically modify the α-amino group of a protein, Met in the case of L23. The major competing reaction is with the ε-amino group of internal lysines. Nα-DNP-Met and Nε-DNP-Lys are readily separable by RP-HPLC, allowing quantification of modification sites following acid hydrolysis of DNP-L23. The Nα-DNP-Met/Nε-DNP-Lys ratio (Fig. 4) was determined both for DNP-L23 prepared by reaction of DNFB with L23 and for DNP-L23 partially purified from reconstituted 50 S subunits (see below). In the modification of L23 we find a 1:1 ratio, i.e. the 13 Lys residues in L23 have a cumulative reactivity equal to that of the single N-terminal Met. This ratio shifts to about 3:2 in the L23 incorporated by 50 S subunits.

Reconstitution of 50 S Subunits with [3H]DNP-L23—50 S reconstitutions were performed by combining [3H]DNP-L23, TP50-L23, and rRNA. When a sample containing 1.3 DNP/L23 was employed the resulting particles contained 0.65 ± 0.02 DNP/50 S, as measured following sucrose density gradient purification of reconstituted 50 S. This figure did not change following purification on a second sucrose density gradient and on a size-exclusion column, thus demonstrating that DNP-L23 is stably incorporated into the 50 S subunit. Unmodified L23 competes efficiently with DNP-L23 in reconstitution; when each was present at an equal concentration, sucrose gradient analysis showed that incorporation of [3H]DNP-L23 was reduced by at least half.

Electron Microscopy of Reconstituted 50 S Subunits—Ribosomal subunits containing DNP-L23 were reconstituted with a preparation of TP50 that was depleted for protein L7/L12. Electron micrographs of this preparation showed very few particles that displayed the stalk that is characteristic of protein L7/L12 (33), but the subunit images were otherwise typical of 50 S ribosomal subunits. These subunits were incubated with purified protein L7/L12 and examined by electron microscopy. Roughly half of the particles showed a stalk; a similar proportion of stalks was seen in preparations of native 50 S subunits and in subunits that had been treated with NH₄Cl and ethanol to remove the stalk and then incubated with purified L7/L12 to restore it. We conclude that by the criterion of electron microscopy these reconstituted subunits have the characteristics of native particles.

Localization of DNP Ribosomal Protein L23 at Two Sites

FIG. 1. Purification of L23 from TP50 by RP-HPLC. Samples loaded were as follows. A, TP50. Approximately 2 mg of TP50 dissolved in 0.1% trifluoroacetic acid were applied per run. B, L22/L23/L29 pool from A. C, L23/L29 pool (fractions eluting from 45–50 min in B). D, Partially purified L23 (fractions eluting from 75–80 min in C). Column A, preparative C18; columns B and D, analytical C18; column C, analytical C8. Gradients (all in 0.1% trifluoroacetic acid) are as follows. A, all linear: 15–35% ACN (10 min), 35–40% ACN (45 min), 40–75% ACN (15 min). B, linear: 36–40% ACN (40 min). C, concave: 15–38% ACN (90 min). D, linear: 35–40% ACN (40 min). All flow rates were 0.7 ml/min. Solid traces show A₂₁₅. Abscissas give elution time in minutes.

FIG. 2. Characterization of purified L23. Upper panel, RP-HPLC on an analytical C8 column; concave gradient, 15–45% ACN (60 min). Flow rate, 0.7 ml/min. Solid trace shows A₂₁₅. Lower panel, SDS-PAGE (20% polyacrylamide). Lane 1, control sample showing migrations of L22, L23, and L29; lane 2, purified L23; lane 3, highly purified L23 (peak from upper panel).
in these experiments. In the first case, a total of 125 micrographs showing 7.8\(\times 10^3\) subunits were analyzed, and 200 immune complexes were identified and interpreted. In the second case we analyzed 43 micrographs showing about 4.3\(\times 10^3\) particles and identified and interpreted 154 immune complexes. The location of the DNP hapten, and thus the placement of the modified protein in the three-dimensional structure of the ribosomal subunit, was identified from the apparent point of contact of the antibody molecule with the subunit as seen in each of its characteristic two-dimensional projections, each of which can be seen in two orientations that are mirror images of each other (18). The results of these observations are summarized in Table II.

Antibodies were observed to be bound at either of two major sites: beside the central protuberance and, with slightly higher frequency, at the base of the particle. The gallery of complexes shown in Fig. 5 illustrates the predominant observations upon which this conclusion is based. Subunits in rows A and B are in the quasisymmetric projection. In row A antibody contact is near the central protuberance with the contacting Fab arm at least partially obscured by the subunit body. In row B antibody contact is seen at the base of the subunit, slightly removed from the vertical axis of near symmetry and with the antibody Fab arm partially obscured by the subunit body.

Subunits in row C show subunits in the asymmetric projection. In row C antibody contact is in the region of the central protuberance, while in row D contact is on the surface opposite the central protuberance. Subunits in row E are shown in the quasisymmetric projection; two antibodies are bound to a single subunit, one antibody at each site identified above.

The subunits shown in Fig. 5 were reconstituted from protein mixtures that lacked L7/L12, the protein that generates the stalk of 50 S subunits, and no stalks were seen on subunits in these micrographs. The absence of the stalk makes antibody identification much less ambiguous, but the stalk is a major asymmetric element in the quasisymmetric projection of the 50 S subunit. To establish the side of the line of near symmetry at which antibody is bound, it was necessary to restore the stalk

Table I

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Figure 3. RP-HPLC analysis of [3H]DNP-modified L23. A sample derived from reaction of [3H]DNFB with L23 was dissolved in 0.1% trifluoroacetic acid, loaded on an analytical C18 column, and eluted with a concave gradient of 15–55% ACN (60 min) at a flow rate of 0.7 ml/min. Panel A shows [H] measured in the eluent, and panel C shows the A215 trace. Positions of unmodified L23 and DNP-L23 are indicated, and pooled fractions, indicated with a bracket, were the source of DNP-L23 in the reconstitution experiments. Approximate DNP/L23 stoichiometries are indicated in panel B, showing A360. These values were calculated from the ratio of [H]/A360 (26, 31).

Figure 4. RP-HPLC analysis of an acid hydrolysate of [3H]DNP-L23. Non-radioactive standards N\(^{\text{a}}\)-DNP-Lys and N\(^{\text{e}}\)-DNP-Met were added to the samples. Lower panel, A215. Middle and upper panels, radioactivity in the acid hydrolysates of [3H]DNP-L23 (middle) and of [3H]DNP-L23 purified from 50 S subunits containing [3H]DNP-L23 (upper). C18 Synchropak, linear gradient 0–35% ACN in 60 min, flow rate 0.7 ml/min.
to these subunits. Reconstituted subunits containing DNP-L23 were first incubated with a 2-fold excess of protein L7/L12 and then with anti-DNP IgG. Unincorporated L7/L12 and uncomplexed antibodies were removed by size exclusion HPLC, and the fraction containing 50 S subunits and immune complexes was prepared for electron microscopy. The field of Fig. 6A shows that in at least half of the subunits the characteristic stalk is present. From these micrographs we have identified and analyzed 94 antibody-subunit complexes in which a stalk is also clearly seen on subunits in the quasisymmetric projection; panels B–D of Fig. 6 illustrate these results. In panel B antibody contact is near or beside the central protuberance and on the side opposite the stalk. In panel C antibody contact is at the base of the subunit and also on the side opposite the L7/L12 stalk, while in panel D two antibodies are bound, one at each of the sites identified above.

In our micrographs we also observed several dimeric complexes in which a pair of subunits is linked by a single bivalent IgG molecule. Subunits were seen in each of the orientations described above, and antibody contact sites were consistent with the observations made on monomeric IgG complexes. Examples are shown in Fig. 7. Antibody contact to each subunit is seen near the central protuberance (frames 1 and 2) or at the subunit base (frame 3), or one subunit is contacted near the central protuberance while the second subunit is bound at the base (frames 4–6). Those subunits in the quasisymmetric projection that show a stalk (in frames 1 and 5 the subunit on the right; in frame 6 the upper subunit) are contacted by antibody on the side opposite the stalk. The lower subunit in frame 6 also shows a stalk, but it is in the asymmetric projection so it is not possible to determine on which side of the central protuberance antibody contact occurs.

The 50 S particles that had been reconstituted with DNP-L23 could associate with 30 S subunits to form 70 S ribosomes that bound anti-DNP IgG. Examples of such complexes are shown in frames 7–9 of Fig. 7; the ribosomes are oriented with the 30 S subunit lying atop the 50 S particle. In frames 7 and 8 antibody contact is seen at the base of the larger subunit, well removed from the smaller (head) segment of the small subunit. Such complexes were seen with a frequency similar to that observed with 50 S subunits. Frame 9 shows an example in which the site of antibody contact is at or very near the plane of subunit interaction and on the same end as the 30 S subunit head, indicating contact in the peptidyltransferase region. This type of complex was very rare. The result was expected; the 30 S subunit should block contact at the transferase region, and in addition any bound antibody could easily be obscured by the ribosome. Nevertheless, the sites we observe with 70 S ribo-
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The validity of the conclusions in Fig. 8 depends entirely on the successful replacement of native L23 by DNP-L23 in the reconstituted 50 S subunits. Previously, we demonstrated the validity of this approach using DNP-modified proteins of the 30 S subunit (25–28). In accord with the earlier results, incorporated DNP-L23 appears to bind within the 50 S subunit in the same or similar manner as native L23, as evidenced by the ability of unmodified L23 to efficiently compete with [3H]DNP-L23 uptake. This result provides strong evidence that the localization of DNP-L23 within reconstituted 50 S subunits by immune electron microscopy faithfully reflects the location of unmodified L23, at the resolution achievable by this technique.

The results we present here indicate that, as shown in Fig. 8, ribosomal protein L23 spans the 50 S subunit, from a site at the subunit interface and at or near the peptidyltransferase center (37) to a second site, at the base of the 50 S particle and indistinguishable from the exit site (14) at which the growing peptide chain emerges from the ribosome. There are several potential explanations for this unexpected observation, including: (i) more than one physical site in the ribosome for protein L23, (ii) a very elongated, asymmetric configuration for L23, and (iii) inaccuracy or inappropriateness of the 50 S models commonly used. We believe that the combination of a somewhat asymmetric configuration of L23 and a porous and indented 50 S subunit surface adequately explains both our results and a large body of apparently contradictory data that place protein L23 in two distant parts of the subunit.

Early work on the placement of ribosomal proteins by IEM often resulted in the identification of multiple sites for a single protein, many of which were incorrect (38). These errors were due to the use of impure ribosomal proteins as immunogens. The resulting antisera interacted with several proteins, and, unfortunately, the results were interpreted as if the antibodies were monospecific. The approach used here excludes this kind of error. Protein L23 was HPLC-purified prior to modification, and any possible contamination (<2%) is far less than that necessary to constitute one of the two binding sites we observe. It is equally improbable that a compact, globular protein can occupy two different sites at two separated positions in the 50 S particle. Protein L23 exists in one copy per 50 S particle and it enters the large subunit early in its assembly (4). Subunit biogenesis is an ordered process that involves specific interactions of the proteins with both the RNA and other ribosomal proteins. L23 is a primary binding protein that binds and protects from RNase action a terminal loop and part of an internal loop in 23 S RNA domain III (3); this binding motif is phylogenetically conserved in large subunit rRNA and is required for L23 binding to eubacterial and chloroplast RNA and for the binding of its eukaryotic homologue, L25 (39, 40). Inactivation of the yeast genes for either the mitochondrial or cytoplasmic ribosome homologues of L23 is lethal (41, 42), suggesting a central role in the ribosome. It is difficult to see how the protein could participate in an alternative set of highly specific but mutually exclusive interactions with the RNA, and we are unaware of any other ribosomal protein with such properties. We conclude that each of the two sites we identify represents a valid localization of a part of a single molecule of L23.

There is evidence to support the location of parts of a possibly asymmetric protein L23 at each site we identify here. Photoaffinity labeling of the 50 S subunit by puromycin (7) or azidopuromycin (8) results in their predominant incorporation into L23. Puromycin is an acceptor in the peptidyltransferase reaction and functionally defines the A site of the transferase center. Puromycin-modified L23 has been incorporated into reconstituted 50 S subunits, with a proportional decrease in mRNA-dependent aminoacyl-tRNA binding activity but no specific effect on the peptidyltransferase reaction (6). Tetracycline interacts directly with the central loop of domain V, the region of 23 S RNA most strongly implicated in peptidyltransferase activity (43, 44), and it also specifically stimulates incorporation of puromycin into protein L23 (7). Streptomycin affects the elongation step of protein synthesis primarily by interfering in ternary complex binding. Streptomycin derivatives that photoaffinity-label large subunits in 70 S ribosomes are incorpo-
L23 by photoaffinity labeling, have been localized by IEM at a site beside the central protuberance (10, 11), helping to define the peptidyltransferase region on the 50 S subunit and placing L23 near the transferase center. Affinity labeling with a bro- moacetyl derivative of puromycin also places the antibiotic at the transferase center, on the subunit shoulder, and results in the modification of proteins L1, L2, L23, and L27 (46). Cross-linking of L23 to 30 S proteins (47) puts it at the subunit interface, and cross-linking to 50 S proteins L5, L15, L16, L18, and L27, all of which have been localized at or near the central protuberance by IEM and linked to transferase activity by functional studies (37), further indicates a site at or near the transferase center.

Early IEM from the Sto¨ffler laboratory (48) used polyclonal antibodies directed against L23 and placed it in the transferase region; additionally, anti-L23 Fab fragments were found to inhibit subunit association, suggesting an interface site (49). However, uncertainty about antibody specificity led the Berlin workers to question their results. Well controlled IEM placed both E. coli (13) and Bacillus subtilis (50) protein L23 at the subunit base, at the position of the exit site (as marked by an antibody initially thought to be specific for L23 but later des- ignated LY; Ref. 51). Placement of L23 at the exit site in the model of Walleczek et al. (52) was based on IEM; it is supported by cross-linking that places the N terminus of L23 near L29 (15) and by cross-linking of a segment of 23 S rRNA to both L23 (53) and the N terminus of a 30–33 residue long emerging peptide (54). However, identification of an epitope at the exit site does not rule out the possibility that other elements of the protein, not recognized by the antibody preparation, exist at a separate position in the particle.

Several results support the existence of two separate sites for distinct elements of L23 on different surfaces of the subunit. Cross-linking of the protein to nucleotides near the 5’ end of the 23 S rRNA (53) in conjunction with identification of the primary L23 binding site in domain III (3) indicates that the protein interacts simultaneously with separate elements of RNA tertiary structure. Mueller et al. (55) consider “circum- stantial evidence” that L23 lies along the path of the nascent peptide: a 25-residue-long peptide also cross-links to RNA do- main III while a longer peptide cross-links at nucleotide 91 (54), both near the L23 sites. A C-terminal region of the yeast homologue of L23 interacts with rRNA in a crucial assembly step that follows RNA binding (56), indicating a role for the protein in forming or maintaining RNA tertiary structure. In work from our laboratories, IEM of 50 S particles that were photoaffinity-labeled with both puromycin and azido-puromycin showed a second site at the base of the particle that ac- counted for up to 25% of the complexes seen (10, 11). It was unclear if the second site was a result of nonspecific binding, a genuine placement of puromycin-L23, or unrelated to L23.

In part to accommodate the apparently conflicting data on L23 localization, Nagano et al. (57) suggested that L23 is a dumbbell-shaped protein in which the N terminus and C ter- minus form independent domains that are separated by 90 ± 30 Å. Protein L9 provides an example of such asymmetry in the ribosome. The crystal structure (58) shows two RNA-binding globular domains linked by an exposed α-helix, yielding a protein that is 82 Å long. IEM places an epitope of L9 near the L1 shoulder, but antibody binding most strongly affects activities at the translocational domain, between the central protuber- ance and the base of the L7/L12 stalk (59).

FIG. 7. Electron micrographs of complexes of ribosomes containing DNP-L23 with anti-DNP IgG. Frames 1–6, dimeric complexes of 50 S subunits linked by a single bivalent IgG molecule. Frames 7–9, DNP-L23–50S reconstituted subunits associated with 30 S subunits to form 70 S ribosomes that bound anti-DNP IgG. An interpretive drawing is below each frame. Bar length, 50 nm.
Localization of DNP Ribosomal Protein L23 at Two Sites

Asymmetry of protein L23 may partially explain our results, but we also believe that the common (visually derived) models of the 50 S subunit (38) do not accurately describe the subunit and the channel (60) or tunnel (22, 23, 61) that is traversed by the growing peptide chain. Approximately 25–40 amino acids of the nascent peptide are protected by the ribosome from proteinase K, and slightly greater lengths are protected from access to IgG or Fab fragments (62). Hardesty et al. (62) estimate that this peptide length (considered to be α-helical) indicates a transerase-exit site separation of 65–105 Å, significantly less than the 150 Å estimate (14) which was based on the separation of these sites in the common 50 S models. Protein L23 has a length of 100 amino acids (63), and the isolated protein has an axial ratio of 2.8 ± 0.9 (64). It is unlikely that it could span the full distance in the usual models, but the two sites appear to be much closer together (about 80 Å) in structures reconstructed from images of unstained frozen ribosomes (22, 23, 65). Reconstructions show several pockets or cavities in the 50 S subunit, as well as the tunnel that traverses the particle and links the transerase and exit sites (see Fig. 8).

Images reconstructed from micrographs of two dimensional arrays of subunits also show a tunnel (66). As shown in Fig. 8, one of our L23 sites is in the “interface canyon” (65) and at the peptidyltransferase end of the tunnel, while the second site includes indentations on the back of the particle and an exit from the tunnel. The relatively porous structure of the subunit at each site could provide at least partial access to a Fab arm and thus allow antibody to approach different elements of L23 from two separate directions. From a different perspective, our results represent an independent confirmation of the indented and porous structure of the ribosome, as it is seen in cryo-EM reconstructions. It may be necessary to re-interpret much of the protein localization literature in this light.

L23 can thus be described as a protein of the subunit interior with segments surfaceing near the tunnel entrance and its exit. Analysis of DNP-L23 in reconstituted subunits indicates that the Met derivative is slightly preferred in reconstitution to the average Lys derivative and that reconstitution may select against L23 molecules in which some specific Lys residues have been dinitrophenylated. In the present work, no attempt was made to quantify the extent of modification at specific lysine residues. We emphasize that whereas N-terminal derivatization occurs at a well defined position within a protein, derivatization of e-amino groups can occur at several of the 13 lysines within L23. This point is important for interpretation of the immune electron microscopy results. Since about 60% of the DNP incorporated by L23 is at the N-terminal methionine, which has been cross-linked to protein L29 (15) and thus placed at the subunit base (52), and a similar percentage of antibody complexes are seen at the subunit base, it is likely that DNP-Met generates these complexes. We see no evidence for DNFB modification at positions other than the N terminus or the e-amino group of internal lysines for any of the proteins we have studied. This may be due to the instability of other DNP derivatives toward nucleophilic displacement by thiolis (8-mercaptoethanol is a component of buffer REC20U), as discussed earlier (26). Modification of one or more internal lysine residues would then generate the complexes we see near the peptidyltransferase center. L23 would thus lie parallel to or along the 50 S tunnel with its N terminus at the exit site and the more flexible C terminus near the transferase center.

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Incorporation of Dinitrophenyl Protein L23 into Totally Reconstituted *Escherichia coli* 50 S Ribosomal Subunits and Its Localization at Two Sites by Immune Electron Microscopy

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