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Evidence of the Involvement of a 50S Ribosomal Protein in Several Active Sites†

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ABSTRACT: The functional role of the *Bacillus stearothermophilus* 50S ribosomal protein B-L3 (probably homologous to the *Escherichia coli* protein L2) was examined by chemical modification. The complex [B-L3·23S RNA] was photooxidized in the presence of rose bengal and the modified protein incorporated by reconstitution into 50S ribosomal subunits containing all other unmodified components. Particles containing photooxidized B-L3 are defective in several functional assays, including (1) poly(U)-directed poly(Phe) synthesis, (2) peptidyltransferase activity, (3) ability to associate with a [30S·poly(U)·Phe-tRNA] complex, and (4) binding of elongation factor G and GTP. The rates of loss of the partial functional activities during photooxidation of B-L3 indicate that at least two independent inactivating events are occurring, a faster one, involving oxidation of one or more histidine residues, affecting peptidyltransferase and subunit association activities and a slower one affecting EF-G binding. Therefore the protein B-L3 has one or more histidine residues which are essential for peptidyltransferase and subunit association, and another residue which is essential for EF-G·GTP binding. B-L3 may be the ribosomal peptidyltransferase protein, or a part of the active site, and may contribute functional groups to the other active sites as well.

The mechanism of protein biosynthesis can be represented as a series of binding interactions and partial reactions occurring on the ribosome, which can be separated operationally in vitro. Correspondingly, the ribosome is an assembly of about 50 macromolecular components, which can be separated and characterized. In order to understand the relationship between the functional complexity of protein synthesis and the structural complexity of the ribosome, it is necessary to identify the individual components which contribute to each of the functional activities.

The protein B-L3 of the large ribosomal subunit from *Bacillus stearothermophilus* has been identified as an essential ribosomal component by in vitro reconstitution studies (Fahnestock et al., 1973a). Particles reconstituted without B-L3 are defective in several functional assays, but contain all other ribosomal proteins as well as 5S and 23S RNA. It is difficult to draw any conclusions concerning direct functional roles of this protein based on such experiments, because of the high probability of relatively large scale structural perturbations caused by the omission of a component, and the demonstrable sensitivity of the functional activity of 50S subunits to structural changes (Fahnestock et al., 1973b).

Affinity labeling studies have identified a number of proteins of *Escherichia coli* ribosomes which are located close to the peptidyltransferase active center (for review, see Cantor et al., 1974). One of these, L2, is probably homologous to the *B. stearothermophilus* protein B-L3, since (1) both have direct binding sites on 23S RNA (Stöffler et al., 1971); (2) they have similar molecular weights and polyacrylamide gel electrophoretic mobilities; and (3) B-L3 cross-reacts immunologically with *E. coli* L2, but not with any of the other *E. coli* ribosomal proteins (Tischendorf et al., 1973). In view of the one-for-one functional homology between the 30S ribosomal proteins of *E. coli* and *B. stearothermophilus* which has been demonstrated by Higo et al. (1973) it is reasonable to assume that the structural homology between the 50S proteins B-L3 and L2 reflects a functional homology as well. These affinity labeling studies suggest the possibility that L2 (and therefore, presumably, B-L3) is the ribosomal peptidyltransferase, or at least may form part of the active site. In order to investigate this possibility I have subjected B-L3 to chemical modification in an effort to introduce limited structural and functional alterations into the 50S ribosomal subunit. The pH dependence of ribosomal peptidyltransferase activity suggests the involvement of a histidine residue in the active site (Fahnestock et al., 1970). Photooxidation in the presence of the dye rose bengal is the most selective method available for the modification of histidine residues in proteins (Means and Feeney, 1971), especially with regard to its total lack of reactivity toward amino groups, which are abundant in ribosomal proteins. The peptidyltransferase protein is likely to have a critical histidine residue and therefore to be sensitive to photooxidation. The experiments reported here demon-

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strate that the protein B-L3 has a histidine residue which is required for peptidyltransferase activity, and that the same protein may contribute to other active sites as well.

Materials and Methods

Ribosomes were obtained from *B. stearothermophilus* as described elsewhere (Fahnestock et al., 1974). A mixture of 50S (75%) and 30S (25%) subunits was used for all experiments for optimal reconstitution activity. Subunit separation, when necessary, was carried out after reconstitution.

Preparation of B-L3. The complex (B-L3·23S RNA) is obtained by dissociating ribosomes with 4 *M* urea–2 *M* LiCl at 0°C for 36 hr (Fahnestock et al., 1974). The resulting precipitate (urea–LiCl RNA fraction) contains 23S RNA, 16S RNA, approximately 30% of the 5S RNA, and 90% of the protein B-L3 (bound to 23S RNA), comprising approximately 90% of the protein in this fraction. Other proteins and 5S RNA, plus approximately 10% of the B-L3, are present in the supernatant (urea–LiCl protein fraction). The redissolved urea–LiCl RNA fraction is here referred to as "(B-L3·23S RNA) complex".

The protein B-L3 (and minor contaminating proteins) can be removed from 23S RNA by treatment of the urea–LiCl RNA fraction with 4 *M* urea–0.5 *M* $MGOAc_2$ (pH 2.0) (Fahnestock et al., 1974). The resulting precipitate contains all of the RNA and the supernatant all of the protein.

Photooxidation of the complex (B-L3·23S RNA) was carried out as follows. Urea–LiCl RNA was dissolved in and dialyzed against a buffer containing 0.01 *M* Tris-HCl (pH 7.4 at 23°C)–0.01 *M* $MgCl_2$–0.03 *M* NH_4Cl. To 2 ml of solution containing 50 A_{260} units of urea–LiCl RNA was added 25 μl of 1 mg/ml of rose bengal (Eastman; dye content 87%; used without further purification) freshly dissolved in water. The apparatus for photooxidation was a Gilson Model GP-20 respirometer equipped with a bank of 30-W reflector lamps. Samples (2 ml) in 30-ml Pyrex Warburg flasks were submerged in water at 0°C approximately 11 cm above the lamps and shaken gently. Reaction was initiated by turning on the lamps and terminated by transferring the sample to darkness in a covered ice bucket. Each experiment included a dark control which was treated identically but left in the dark. The dye was removed by precipitating the RNA with 5 ml of ethanol and the pellet was washed by redissolving in 2 ml of 0.01 *M* Tris (pH 7.4)–0.01 *M* $MgCl_2$–0.03 *M* NH_4Cl–0.006 *M* 2-mercaptoethanol (TMAI),[1] and precipitating with 5 ml of ethanol. In some experiments the complex was incorporated into reconstituted ribosomes as described in detail elsewhere (Fahnestock et al., 1974) without dissociation by incubation with 1.25 equivalent units of urea–LiCl protein fraction per A_{260} unit of urea–LiCl RNA for 2 hr at 60°C. In other experiments the complex was dissociated as described above and the RNA and B-L3 incorporated separately into reconstituted ribosomes by incubation of 1.25 equivalent units of urea–LiCl protein fraction and 2.0 equivalent units of B-L3 per A_{260} unit of RNA. (One equivalent unit is defined as the amount of material derived from 1 A_{260} unit of ribosomes.) Reconstituted particles were pelleted by centrifugation at 50000 rpm for 6 hr in the Beckman type 65 rotor and resuspended in TMAI.

Activity assays were carried out as described in detail elsewhere (Fahnestock et al., 1974). All assays were carried out in a range of ribosome concentration in which there is a linear dependence of measured activity upon the amount of 50S subunits. Activities are expressed relative to the activity of particles reconstituted from a dark control complex. For a typical set of assays 100% activity was as follows: (1) poly(phenylalanine), 70 pmol of Phe polymerized per 0.5 A_{260} unit reconstituted 50 S (compared to 114 pmol for undissociated 50 S); (2) peptidyltransferase, 0.044 pmol of fMet-puromycin per 0.1 A_{260} unit of reconstituted 50S (compared to 0.070 pmol for undissociated 50 S); (3) Phe-tRNA binding, 4.8 pmol of Phe-tRNA bound per 0.5 A_{260} unit of reconstituted 50 S (compared to 3.7 pmol for undissociated 50 S); (4) EF-G·GTP binding, 1.9 pmol of GTP bound per 0.5 A_{260} unit of reconstituted 50 S (compared to 3.4 pmol for undissociated 50 S).

Sedimentation Analysis. Linear sucrose density gradients (5.2 ml) from 5% (w/v) to 20% (w/v) ribonuclease-free sucrose (Schwartz) in TMAI or TMAII (same as TMAI but with 0.3 m*M* $MgCl_2$ instead of 10 m*M*) were subjected to centrifugation for 90 min at 50000 rpm in the Beckman SW 50.1 rotor, at 4°C, and analyzed with the ISCO Model 184 gradient fractionator.

Results

The urea–LiCl RNA fraction derived from a mixture of 50S and 30S ribosomal subunits, containing the protein B-L3 tightly bound to 23S RNA, was irradiated with visible light in the presence of the sensitizing dye rose bengal. The complex was then dissociated and protein and RNA were incorporated separately into reconstituted 50S subunits which were assayed for the following activities: (1) synthesis of poly(phenylalanine) directed by poly(U) (The 50S subunit participation is assayed specifically by adding an excess of 30S subunits. This activity requires all of the functions involved in polypeptide chain elongation including, presumably, those measured by the remaining three partial functional assays.); (2) peptidyltransferase (In this assay the ability of the 50S subunit to form peptide bonds (fMet-puromycin) is measured independent of the participation of the 30S subunit, mRNA, or elongation factors, in 33% (v/v) methanol.); (3) Phe-tRNA binding (This assay measures the ability of the 50S subunit to associate with the (30S·Phe-tRNA·poly(U)) complex as reflected by protection of bound Phe-tRNA from ribonuclease attack. This interpretation has been confirmed by the observation that loss of RNase protection activity in these experiments is accompanied by loss of ability to convert a Phe-tRNA containing complex sedimenting at about 30 S into one sedimenting at about 70 S, determined by sucrose density gradient sedimentation (data not shown).); (4) the binding of GTP to the 50S subunit, dependent on EF-G (The complex (EF-G·GTP·50S) is stabilized by fusidic acid and collected on a nitrocellulose filter. This binding activity is independent of the 30S subunit.).

Figure 1 shows the activities of particles reconstituted with RNA or protein derived from photoxidized (B-L3·23S RNA) complex as a function of time of photooxidation. All of the activities tested are affected, and both the protein and RNA fractions lose activity, the protein more rapidly than the RNA. The inactivation of the protein fraction il-

[1] Abbreviations used are: poly(U), poly(uridylic acid); TMAI, 0.01 *M* Tris (pH 7.4)–0.01 *M* $MgCl_2$–0.03 *M* NH_4Cl–0.006 *M* 2-mercaptoethanol; TMAII, same as TMAI but with 0.3 m*M* $MgCl_2$ instead of 10 m*M*.

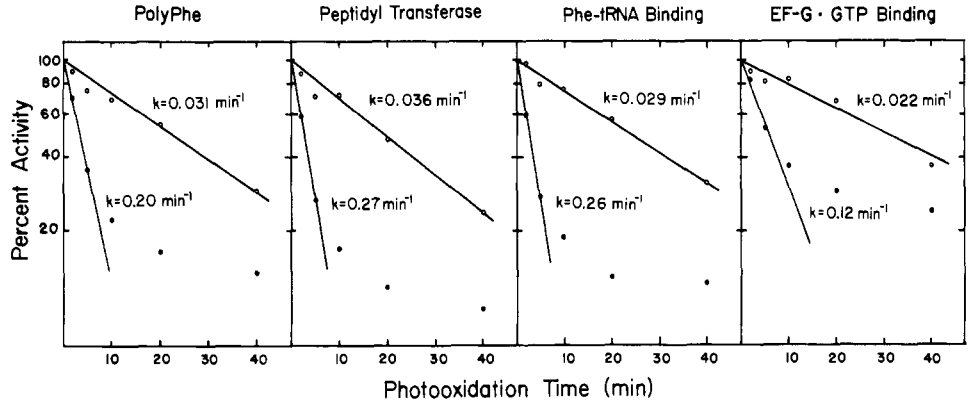

FIGURE 1: Kinetics of inactivation of 23S RNA and B-L3 on photooxidation of the complex (B-L3·23S RNA). B-L3 and 23S RNA from urea-LiCl RNA photooxidized for various times were separated as described in Materials and Methods and incorporated separately into reconstituted particles containing all other unmodified components. Aliquots of the same reconstituted particles were assayed for the four activities. (○) Reconstituted particles containing photooxidized RNA only, (●) reconstituted particles containing photooxidized B-L3 only. 100% activity is the activity of reconstituted particles containing RNA or B-L3 derived from dye-treated dark control complex.

lustrated in Figure 1 does not appear to obey simple first-order kinetics. Most of the deviation from linearity in these first-order plots can be accounted for by the known presence of B-L3 in small amounts in the urea-LiCl protein fraction used for reconstitution, which is not exposed to photooxidation. However, these data cannot exclude the possibility that particles containing extensively oxidized complex retain partial activity. This is likely to be true of the EF-G·GTP binding activity, since particles deficient in B-L3 have significantly greater activity in this assay than in the others (Fahnestock et al., 1973a).

The rate of loss of EF-G·GTP binding activity is significantly lower than the rates of loss of the other activities. This difference in rate is interpreted as reflecting at least two different inactivation events one (or possibly two) affecting peptidyltransferase and Phe-tRNA binding and another affecting EF-G·GTP binding. For convenience, these rates are characterized by first-order rate constants illustrated by the straight line drawn in Figure 1. These constants apply to the bulk of (B-L3·23S RNA) complex, though their precise interpretation depends on the nature of the deviation from first-order kinetics.

The ionic conditions during the EF-G·GTP binding assay (TMAI) are different from the conditions during the peptidyltransferase assay (0.04 *M* Tris-HCl (pH 7.4 at 23°C); 0.013 *M* $MgCl_2$; 0.27 *M* KCl; 0.004 *M* 2-mercaptoethanol; 33% (v/v) methanol). The observed difference in rate of inactivation of particles containing photooxidized B-L3 in these two assays is not due to this difference in assay conditions, however, since the kinetics of inactivation of EF-G·GTP binding are the same as shown in Figure 1 when assayed under the conditions of the peptidyltransferase assay (data not shown).

The protein derived from the urea-LiCl RNA fraction is approximately 90% pure B-L3. Other proteins in this fraction are present in minor amounts, being found mainly (>90%) in the urea-LiCl protein fraction. Therefore the inactivation of the urea-LiCl RNA fraction by photooxidation can be attributed mainly to the single protein B-L3. This interpretation is confirmed in Figure 2. In the experiment shown by the broken line, pure (electrophoretically homogeneous) unmodified B-L3 was added in increasing amounts to a reconstitution mixture containing the protein derived from photooxidized urea-LiCl RNA. The addition of pure B-L3 restored most of the poly(phenylalanine) synthesizing activity to the reconstituted particles, confirming that B-L3 is the effective target of photooxidation.

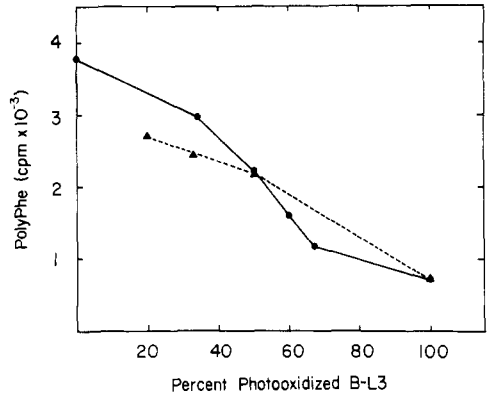

FIGURE 2: Competition between photooxidized and untreated B-L3. Ribosomes were reconstituted in the presence of mixtures of B-L3 derived from photooxidized (15 min) and untreated (B-L3·23S RNA) complexes and assayed for poly(phenylalanine) synthesizing activity directly, without purification. In one experiment (●) each reconstitution mixture contained (in 0.3 ml) 2.0 A_{260} units of protein-free RNA (derived from a mixture of 75% 50S subunits and 25% 30S), 2.5 A_{260} equivalent units of urea-LiCl proteins, 4.0 A_{260} equivalent units of untreated "crude" B-L3 (the total protein derived from the urea-LiCl RNA fraction—approximately 90% B-L3), and varying amounts of crude photooxidized B-L3. In another experiment (▲) each reconstitution mixture contained 4.0 A_{260} equivalent units of "crude" photooxidized B-L3 and varying amounts of untreated pure B-L3, prepared as described previously (Fahnestock et al., 1973), in addition to RNA and proteins as above.

Earlier experiments have shown that reconstituted 50S subunits lacking B-L3 are deficient in these four functional activities, retaining somewhat more activity in the binding of EF-G and GTP than in the other assays. Thus, one possible explanation of the loss of all activities on photooxidation of B-L3 might be that the photooxidized protein is not incorporated into reconstituted ribosomes. In the competition experiment illustrated by the solid line in Figure 2 photooxidized B-L3 was added in increasing amounts to a reconstitution system containing saturating amounts of unmodified B-L3. If the modified protein has lost its binding affinity it should have no effect, assuming that it does not interact with unmodified B-L3. The results show that photooxidized B-L3 is able to compete with unmodified B-L3 for incorporation into reconstituted ribosomes and that modified B-L3

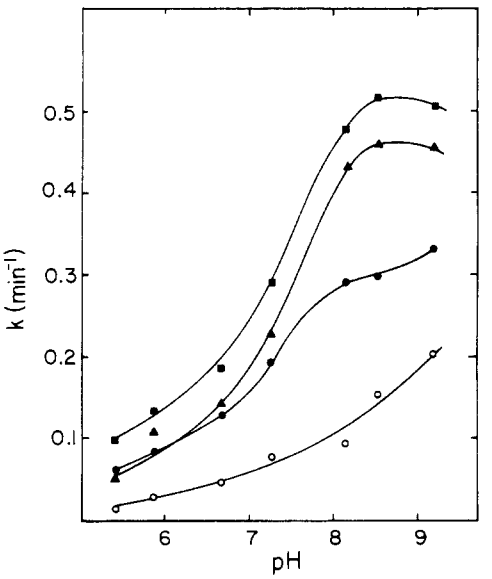

FIGURE 3: Dependence of rate of inactivation of the complex (B-L3·23S RNA) on pH of photooxidation. Aliquots of the complex (B-L3·23S RNA) were photooxidized for 0 (dark controls), 2 min (above pH 8), or 4 min (below pH 8) in buffers at various pH values (measured at 0°). Buffers below pH 7 contained 0.05 *M* potassium cacodylate, 0.01 *M* $MgCl_2$, and 0.03 *M* NH_4Cl. Buffers above pH 7 were the same, except 0.05 *M* Tris-HCl was substituted for cacodylate. First-order rate constants (k) for loss of activity were calculated by comparing the activity of reconstituted particles containing photooxidized (B-L3·23S RNA) and those containing the complex treated with dye in the dark in the same buffer, assuming first-order kinetics. (●) Poly(phenylalanine) synthesis; (▲) peptidyltransferase; (■) Phe-tRNA binding; (○) EF-G·GTP binding.

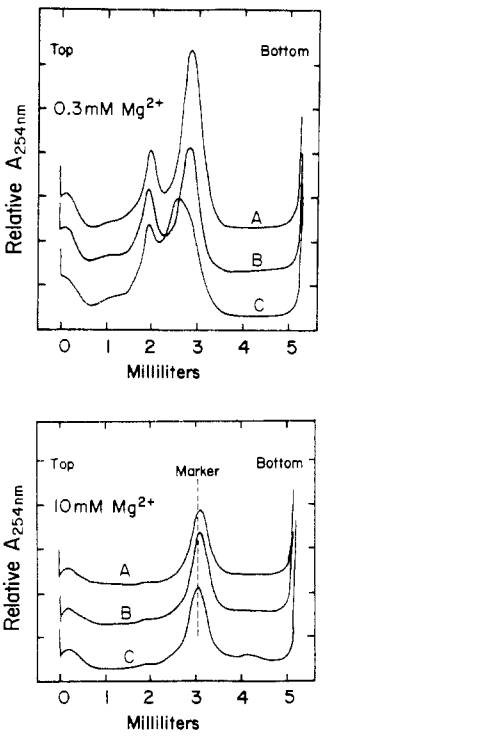

FIGURE 4: Sedimentation analysis of reconstituted ribosomes. The same reconstituted particles described in Figure 1 were analyzed by sucrose density gradient sedimentation in buffer containing 0.3 m*M* $MgCl_2$ (upper panel) and 10 m*M* $MgCl_2$ (lower panel). For low Mg^{2+} analysis the entire mixture of reconstituted particles containing 30S and 50S subunits was applied. For analysis at higher Mg^{2+}, 50S subunits were first purified by sedimentation at low Mg^{2+}, in order to eliminate subunit association. The high Mg^{2+} gradient samples contained added ^3H-labeled *E. coli* 50S subunits as marker, and the patterns are aligned with marker peaks coincident. (A) Ribosomes reconstituted with untreated RNA and dark control B-L3. (B) Ribosomes reconstituted with photooxidized (10 min) RNA and untreated B-L3. (C) Ribosomes reconstituted with untreated RNA and photooxidized (10 min) B-L3.

is essentially unimpaired in its ability to bind to the reconstituted particle. The presence of photooxidized B-L3 in the finished reconstituted particle is demonstrated directly in Figure 5.

Figure 3 shows the dependence of the rate of loss of each of the functional activities of reconstituted 50S subunits containing the undissociated (B-L3·23S RNA) complex upon the pH at which the urea–LiCl RNA fraction was photooxidized. The pH dependence of loss of peptidyltransferase and Phe-tRNA binding activities is indicative of oxidation of a required histidine residue, resembling the characteristics of photooxidation of enzymes in which an active site histidine residue is affected (Freude, 1968; Martinez-Carrion et al., 1967; Westhead, 1965), and the pH dependence of histidine oxidation in model systems (Weil, 1965). Such pH dependence reflects the resistance of the protonated imidazole residue to oxidation. The rate of loss of EF-G·GTP binding is consistently lower than the others, and the shape of its curve is somewhat different at the high pH end. This suggests the possibility that the loss of EF-G·GTP binding is due to oxidation of a different amino acid, perhaps a tyrosine or tryptophan. In preliminary experiments tetranitromethane treatment of the urea–LiCl RNA, expected to modify preferentially tyrosine residues, had little effect on any of the activities of reconstituted particles, offering no evidence of the involvement of an available tyrosine residue in any function (data not shown).

The sedimentation behavior of ribosomes containing photooxidized B-L3, at two different concentrations of Mg^{2+} ion, is shown in Figure 4. At 10 m*M* Mg^{2+} there is a very slight (about 1S) difference in sedimentation velocity between reconstituted 50S subunits containing photooxidized B-L3 and those containing either dark control B-L3, or unmodified B-L3 and photooxidized 23S RNA. The difference in sedimentation is much more pronounced at 0.3 m*M* Mg^{2+}, where 50S subunits containing photooxidized B-L3 sediment approximately 4S units more slowly than those containing dark control B-L3 or photooxidized 23S RNA. The change in sedimentation of 50S subunits containing photooxidized B-L3 is reversible, since the particles analyzed in Figure 4b were purified by sedimentation at low Mg^{2+} as in Figure 4a, from a profile identical with that in Figure 4a. Such behavior must be due mainly to a reversible structural alteration which takes place at low Mg^{2+} and not to the loss of any protein or RNA. This indicates a greater flexibility or reduced stability of particles containing photooxidized B-L3. But at higher Mg^{2+} concentration, closer to the conditions of the functional assays, there is very little structural difference detectable by sedimentation.

Figure 5 shows the protein composition of reconstituted 50S subunits (purified by sedimentation at 0.3 m*M* Mg^{2+}) containing photooxidized and dark control B-L3. There are no reproducible differences between the patterns. However, there are apparent differences in this set of gels, in the lower right portion, where the smaller basic proteins migrate. One spot (B-L32 according to the nomenclature of Horne and Erdmann, 1972) is reduced in intensity in the pattern derived from photooxidized B-L3-containing particles, and two spots (B-L36-38) traveling at the ion front

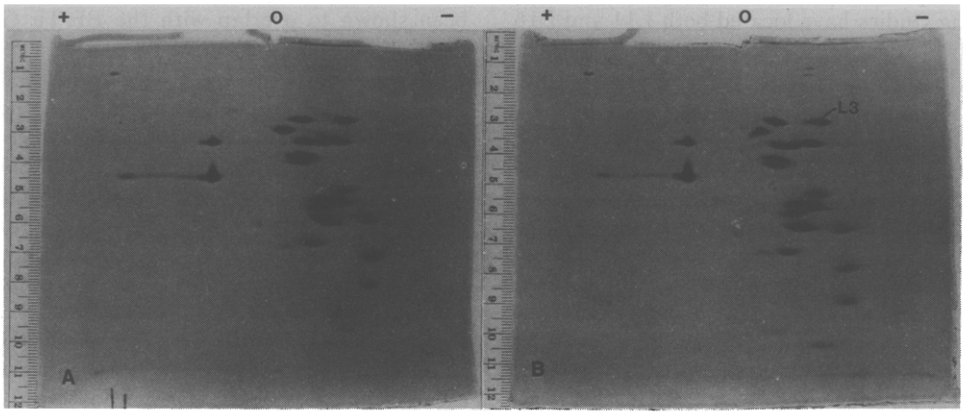

FIGURE 5: Protein composition of 50S subunits reconstituted with photooxidized (15 min) and dark control (B-L3·23S RNA) complexes. The complex was incorporated without dissociation into reconstituted particles then 50S subunits were purified by sucrose density gradient sedimentation, and analyzed by two-dimensional polyacrylamide gel electrophoresis by the method of Kaltschmidt and Wittmann (1970) as modified by Howard and Traut (1973). For the first dimension two separate gels were run at 2.7 mA/3-mm diameter gel for 6 hr with opposite polarity. Both were at pH 8.7, the gel for the acidic proteins containing 8% acrylamide, 0.3% *N,N'*-methylenebisacrylamide (bis), and the gel for the basic proteins containing 4% acrylamide and 0.13% bis. First dimension gels were polymerized onto a slab with tops adjacent (at "0") for the second dimension (18% acrylamide, pH 4.6). (A) 50S subunits reconstituted from photooxidized (B-L3·23S RNA); (B) 50S subunits reconstituted from dark control (B-L3·23S RNA).

(normally somewhat variable) are missing. These differences have not been observed in other similar preparations. Furthermore the mixed reconstituted 30S and 50S subunits from which the 50S particles analyzed in Figure 5 were purified gave identical two-dimensional gel patterns, including all of the small basic proteins. Thus, under assay conditions, where the subunits are not separated, all of the proteins are present. The differences observed in Figure 5 can be attributed to (variable) loss of the smallest proteins from the 50S subunit containing photooxidized B-L3, as it is sedimented in a somewhat unfolded state at 0.3 m*M* Mg^{2+} during the 50S purification prior to gel analysis.[2] Polyacrylamide gel electrophoretic analysis (not shown) also revealed the presence of equal amounts of 5S RNA in each of the 50S preparations analyzed in Figure 5.

Discussion

These results indicate that the protein B-L3 has one or more histidine residues which are essential for peptidyltransferase and subunit association activity of the 50S ribosomal subunit, and another functional group which is necessary for optimal binding of EF-G and GTP. It is not possible without detailed structural information to determine conclusively whether the functional groups in question participate directly in the affected active sites, or whether they are required for the maintenance of a proper structure in the active site area of the ribosome. The observed effects of modification of these functional groups are not due to a gross derangement of the ribosome structure or reconstitution process since (1) reconstituted particles containing photooxidized B-L3 have all 50S ribosomal proteins and RNAs; (2) difference in sedimentation velocity is slight under ionic conditions resembling activity assay conditions; and (3) at least two independent inactivation events are observed during photooxidation, evidenced by the difference in rate of loss of EF-G·GTP binding compared to the rate of loss of peptidyltransferase and subunit association activities. This last observation serves to localize the effects of the more rapid inactivation events to some extent, and argues for the directness of the effects.

This evidence, taken together with the evidence from affinity labeling studies, makes the protein B-L3 and its *E. coli* counterpart L2 the best candidates at present for identification as the peptidyltransferase proteins in their respective ribosomes. The peptidyltransferase active site need not be confined to a single protein, however, and affinity labeling experiments have identified an abundance of proteins which are close enough to the active site to participate in it. These include in addition to L2 (in *E. coli*), L11, L14, L15, L16, L18, and L27 (Czernilovsky et al., 1974; Eilat et al., 1974a,b; Hsiung et al., 1974; Pellegrini et al., 1974; Sopori et al., 1974). By means of partial reconstitution studies Nierhaus and Montejo (1973) demonstrated that fractions containing L11 could convert inactive LiCl core particles into particles with peptidyltransferase activity and concluded that L11 may participate in the peptidyltransferase active site. However, other core particles lacking L11 have been shown to have peptidyltransferase activity (Ballesta and Vazquez, 1974; Howard and Gordon, 1974). More recently Moore et al. (1975), in experiments similar to those of Nierhaus and Montejo, found that L16 is required and sufficient to restore peptidyltransferase activity to LiCl core particles which have very little L11. Dietrich et al. (1974) have demonstrated a functional interdependence between L11 and L16 in such reconstitution experiments. The functional importance of L2 could not be examined in those experiments since L2 is present in the core particles. The possibility of severe structural effects makes interpretation of such partial reconstitution experiments difficult. Neverthe-

[2] Cohlberg and Nomura (personal communication) have examined the functional requirement for each of the *B. stearothermophilus* 50S ribosomal proteins by means of reconstitution using purified proteins. With regard to the proteins which are occasionally lost from reconstituted particles containing photooxidized B-L3 (Figure 5) they have observed the following. (1) B-L33, which is present in Figure 5, is probably a derivative of the protein present in reduced amount, B-L32. Reconstituted particles lacking B-L32 (and B-L33) are fully active (97 ± 5% activity compared to complete particles) in poly(Phe) synthesis. (2) B-L36 is only occasionally present in 50S gel patterns, and may be a derivative of B-L38. It is not required for activity. (3) Omission of B-L37 has no effect on the activity of reconstituted particles. (4) Omission of B-L38 has only a weak effect on activity, particles lacking B-L38 having 65 ± 3% of the activity of complete particles. The lability of these proteins on particles containing photooxidized B-L3 is clearly not responsible for the effects described in this paper.

less affinity labeling studies have located both L11 and L16 near the peptidyltransferase site, and one or both of them may well participate in the active site, but further confirmation is required.

The present results also indicate the importance of B-L3 in the binding site for EF-G and GTP, a site which plays a critical role in the process of translocation. This suggests an intimate connection between peptide bond formation and translocation, and the possibility of direct coordination of the two processes. The proximity of *E. coli* L2 to the EF-G binding site is supported by the results of experiments in which proteins have been cross-linked in situ by bifunctional reagents. Traut et al. (1974) observed cross-linking of L2 to both L7 and L12, proteins which have been shown to be required for GTPase activities on the ribosome, including that involving EF-G (Brot et al., 1972; Hamel et al., 1972; Sander et al. 1972; Fakunding et al., 1973). Also, P. B. Moore (personal communication) has obtained preliminary evidence that L2 can be cross-linked to ribosome-bound EF-G. On the other hand, Highland et al. (1973, 1974) have examined the effect on EF-G·GTP binding of reaction of either 70S ribosomes or 50S subunits with antibodies directed against each of 50 ribosomal proteins and found evidence of the involvement only of L7 and L12 in the EF-G binding site. Anti-L2 was not inhibitory. It is possible, however, that a weak binding antibody could be displaced by a stronger binding ligand under equilibrium conditions, in which case no effect on ligand binding would be observed even though the binding sites may overlap. Anti-L2 was identified by Morrison et al. (1973) (who suggested this possibility with reference to subunit association—see below) as one of the weaker binding antibodies.

The third functional activity affected by photooxidation of B-L3 is the ability of the 50S subunit to join the [30S·poly(U)·Phe-tRNA] complex. B-L3 is therefore implicated in this site as well. However, the interpretation of this assay is somewhat ambiguous because the interactions involved may be between the 50S subunit and either Phe-tRNA or the 30S subunit, or most likely both. Indeed, it may be that interaction with Phe-tRNA at the peptidyltransferase active site is important in stabilizing the complex, in which case it could be that inactivation of peptidyltransferase and subunit association activities are the same event. On the other hand, B-L3 may interact directly with 30S components to stabilize the complex. This latter interpretation is more likely because photooxidation of B-L3 also affects subunit association which takes place in the absence of added tRNA at 10 m*M* Mg^{2+} (preliminary data not shown). Furthermore, Litman and Cantor (1974), and Michalski and Sells (1975) have demonstrated that L2 can be protected from iodination in the ribosome by the presence of the 30S subunit, which is evidence that L2 is located at the subunit interface. Chang (1973) similarly observed protection of L2 from reaction with *N*-ethylmaleimide by the 30S subunit, but attributed the difference in reactivity between 70S ribosomes and 50S subunits to a conformational change in the 50S subunit on association with the 30S subunit. This interpretation has been disputed by Glick and Brubacher (1975). On the other hand, antibodies directed against L2 do not inhibit subunit association (Morrison et al., 1973), though this observation does not necessarily preclude the presence of L2 at the interface.

Taken together, many of these observations and the present work suggest that the peptidyltransferase site is located close to the EF-G-dependent GTPase site, which has been shown to overlap with the EF-Tu GTPase site (and therefore the tRNA binding site) (for a review see Möller, 1974) and that all of these sites are close to the subunit interface and the protein B-L3 (L2). Another site in this region is suggested by the work of Tate et al. (1975), who found that reaction of 50S subunits (but not 70S ribosomes) with Fab derived from antibody directed against L2 (in addition to L11, L16, L7/L12, and others) inhibits the binding to ribosomes of release factors, proteins required for polypeptide chain termination. This suggests that L2 is close to, or contributes to, the release factor binding site. Clearly confirmation from different experimental approaches is necessary before the direct functional involvement of any of the components in this area, including L2 (B-L3), can be deduced.

Poly(phenylalanine) synthesizing activity is lost more slowly than peptidyltransferase and subunit association activities during photooxidation of B-L3. Since both of these partial functional activities are presumably required for polypeptide synthesis one might expect that poly(Phe) synthesis would be inactivated at least as rapidly as the most rapid inactivation of partial function. However, if neither of these partial functions is rate limiting in poly(Phe) synthesis, then the assays in which they are measured directly may be much more sensitive than the overall functional assay. For example, a preparation of ribosomes which is able to make peptide bonds at a small fraction of the rate of normal ribosomes will appear inactive in the peptidyltransferase assay, but may make poly(Phe) at a normal rate if peptide bond formation is not rate limiting in the overall process. Apparently neither peptidyltransferase nor subunit association activities limit poly(Phe) formation in the (uninhibited) assay system used here.

The pH dependence of the rate of loss of peptidyltransferase and subunit association activities indicates that for these functions the effective target of photooxidation of B-L3 is one or more histidine residues. The nature of the functional group responsible for loss of EF-G and GTP binding is less clear. Photooxidation under these conditions can affect, in addition to histidine, tyrosine, tryptophan, methionine, and cysteine. Oxidation of methionine and cysteine may be reversed during reconstitution when the photooxidized protein is incubated for 2 hr at 60°C in the presence of 6 m*M* 2-mercaptoethanol. Therefore the target is probably either a histidine residue which is less exposed than those responsible for loss of peptidyltransferase and subunit association activity, or a tyrosine or tryptophan. Further studies are underway aimed at elucidating this point and attempting to separate the inactivation events identified here.

Some of these results were presented at the 59th FASEB meeting, April 1975 (Fahnestock, 1975).

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