Modification of *Escherichia coli* Ribosomes with the Fluorescent Reagent *N*-[[(Iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic Acid. Identification of Derivatized L31′ and Studies on Its Intraribosomal Properties†

Jay S. Hanas* and Melvin V. Simpson

Department of Biochemistry, State University of New York at Stony Brook, Stony Brook, New York 11794

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ABSTRACT: *N*-[[(Iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (IAEDANS) is a fluorescent reagent which reacts covalently with the free thiol groups of proteins. When the reagent is reacted with the *Escherichia coli* ribosome under mild conditions, gel electrophoresis shows modification of predominantly two proteins, S18 and L31′, which become labeled to an equal extent. When the native (i.e., untreated) ribosome is dissociated into 30S and 50S subunits, only the 30S ribosomal protein S18 reacts with IAEDANS despite the fact that L31′ is still present on the large subunit. Upon heat activation of the subunits, a procedure which alters subunit conformation, S18 plus a number of higher molecular weight proteins is modified, but not L31′; the latter reacts with IAEDANS only in the 70S ribosome or when it is free. In contrast to the relatively stable association of L31′ with native or with dissociated ribosomes, dissociation of *N*-[(acetylamino)ethyl]-5-naphthylaminesulfonic acid (AEDANS)-treated ribosomes weakens the AEDANS–L31′/ribosome interaction, resulting, upon gel filtration analysis, in ribosomes devoid of this derivatized protein.

We have been interested in the conformational changes in ribosome structure which take place upon subunit dissociation–reassociation, ribosomal translocation, and streptomycin binding, the latter with respect to the mechanism of mistranslation. Such changes have been detected by using the method of hydrogen exchange (Sherman & Simpson, 1969a,b; Chuang & Simpson, 1971). However, detailed knowledge of the nature of these alterations is lacking. Fluorescence spectroscopy is a useful tool for examining such problems. When specific ribosomal proteins (r-proteins)[1] are covalently labeled intraribosomally with a fluorescent group, they can be useful for the analysis of conformational changes of, or ligand binding to, the ribosome (Kang et al., 1979). To study these phenomena by fluorescence, it is desirable to use a probe which is environmentally sensitive, is water soluble, and, in order to limit the number of proteins derivatized, is specific for thiol groups. *N*-[[(Iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (IAEDANS) fulfills these qualifications (Hudson & Weber, 1975).

In the present study, we examine the reaction of IAEDANS with the *Escherichia coli* ribosome under mild conditions. We observe labeling of primarily two proteins in the 70S ribosome. One of these is S18, a protein implicated in codon binding (Pongs et al., 1975; Cantrell & Craven, 1977). The other, L31′, is a relatively unexplored ribosomal protein which is found at the subunit interface of the 70S ribosome in association with 5S RNA (Kenny et al., 1979; Fanning & Traut, 1981). Studies to be published (J. S. Hanas and M. V. Simpson, unpublished results) explore the effect of streptomycin and other aminoglycoside antibiotics on the fluorescence of AEDANS-derivatized ribosomes.

Experimental Procedures

Ribosome Isolation. All operations were performed at 4 °C. A frozen pellet of *E. coli* cells (strain Q13, grown to early log phase) was ground with twice its weight of alumina. The paste was suspended in 3 volumes of cold buffer A (20 mM Tris-HCl, pH 7.5, 70 mM NH_4Cl, 12 mM $MgCl_2$, and 0.5 mM DTT) containing DNase I (10 μg/mL). The suspension was centrifuged first at low speed to remove alumina and cell debris and then at 30000*g* for 30 min. The supernate was then centrifuged for 2.5 h at 38 000 rpm in a Beckman 60 Ti rotor. The ribosome pellet was then resuspended in buffer A containing 1 M NH_4Cl; the suspension was clarified by low-speed centrifugation and then respun at 38 000 rpm for 2 h. This high-salt washing was repeated, and the ribosomal suspension (in buffer A) was adjusted to 200–300 A_{260} units/mL. A ribosome concentration of 1 mg/mL is equivalent to 14.5 A_{260} units (Acjarua & Moore, 1973).

Sucrose Gradient Centrifugation of Ribosomes and Ribosomal Subunits. Ribosomes were routinely purified by centrifuging 600 A_{260} units through a 36-mL, 12.5–30% sucrose gradient (in buffer A) in an SW 27 rotor for 9 h at 21 000 rpm. Fractions around the center of the 70S peak were pooled and were recentrifuged for 7 h at 38 000 rpm in a 60 Ti rotor. The pellets were resuspended in buffer A (200–300 A_{260} units/mL), and 0.2-mL aliquots were stored at −90 °C. Ribosomal subunits were isolated by first dissociating ribosomes by overnight dialysis against buffer B (buffer A with $MgCl_2$ concentration lowered to 0.5 mM) followed by sucrose gradient centrifugation in buffer B.

Labeling of Ribosomes with IAEDANS. A 10 mM stock solution of IAEDANS (Pierce Chemical Co.) was prepared in 0.2% $NaHCO_3$ and stored at 4 °C in the dark. Ribosomes in buffer A (0.18 mL containing about 40 A_{260} units) were

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[1] Abbreviations: SDS, sodium dodecyl sulfate; IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid; IAF, 5-(iodoacetamido)fluorescein; DTT, dithiothreitol; r-protein, ribosomal protein; 1-D one dimensional; 2-D, two dimensional; PAGE, polyacrylamide gel electrophoresis; AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylaminesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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incubated at 37 °C for 10 min after which 0.02 mL of the IAEDANS stock solution was added and allowed to react in the dark for 10 min at 22 °C. Unreacted IAEDANS was removed upon chromatographing the mixture through a 3-mL column of Sephadex G-25 (medium) equilibrated with buffer A. The fluorescence of the effluent was monitored with a 360-nm lamp, and drops containing AEDANS-labeled ribosomes (found in the void volume) were pooled.

Labeling of Isolated Individual r-Proteins with IAEDANS. For the study of the labeling by IAEDANS of the isolated individual r-proteins, L31′, L31, L30, L27, and L18, two classical 2-D gel electrophoresis systems were used. These have long been known to yield homogeneous r-proteins (Wittmann, 1974; Kenny et al., 1979), and subsequent electrophoresis of the cut-out spots in a second gel has confirmed this (data not shown).

After the 2-D electrophoresis run of 1–5 mg of acetic acid extracted r-proteins, individual Coomassie-stained spots were cut out with a 6-mm cork borer and shaken at 37 °C for 1 h in 2 mL of 100 mM Tris-HCl, pH 7.5, containing 0.5% SDS. The gel disks were then separately incubated with 1 mM IAEDANS for an additional hour in the above buffer. The reaction was stopped by addition of 100 mM DTT, and the gel slices were then transferred to slab gel wells for subsequent electrophoretic analysis either in 1-D SDS or in 1-D alkaline urea.

Quantitation of AEDANS-Labeled Ribosome Fluorescence. Fluorescence measurements were performed at 22 °C with a Perkin-Elmer MPF-44A fluorescence spectrophotometer. The excitation wavelength was 360 nm, and emission spectra were recorded between 440 and 520 nm by using a 430-nm filter. Samples were prepared separately and analyzed in duplicate. The emission intensity maxima were quantitated against a blank sample containing nonderivatized ribosomes.

Dissociation of Ribosomes by Gel Filtration in Buffer B. Ribosomes or AEDANS-labeled ribosomes (0.2 mL in buffer A) were passed through a Sephadex G-75 column (3 mL) which had been equilibrated in buffer B. AEDANS-labeled ribosome fractions were detected by their fluorescence and nonfluorescent ribosome fractions by the A_{260} of *10*-drop fractions (0.3 mL). Ribosomes chromatographed in this manner dissociate, and their reassociation is promoted by elevating the $MgCl_2$ concentration in buffer B to 12 mM. (Ribosome state was determined by sucrose gradient centrifugation.)

SDS/Polyacrylamide Gel Electrophoresis and Photography. Ribosomes or AEDANS-labeled ribosomes (at least 1 A_{260} unit) were added to an equal volume (total volume not to exceed 100 μL) of SDS sample buffer (0.2% SDS, 80 mM Tris-HCl, pH 6.8, 10% glycerol, and 20 mM DTT). The mixtures were incubated at 100 °C for 15 min. The SDS/polyacrylamide gel system utilized [18%, 100:1 acrylamide/bis(acrylamide)] was similar to the discontinuous pH, stacking gel system previously described (Laemmli, 1970). The gel was electrophoresed in the Tris/glycine/SDS running buffer for 3.5 h at 10 V/cm. It was then photographed by using a 360-nm UV light box (Transilluminator), Kodak Royal Pan sheet film, and Wratten 9 and 60 filters. The gels were then stained with Coomassie blue G-250, destained, and then photographed by using a white light box and Kodak Contrast Process Pan film.

Urea 2-D Gel Electrophoresis. Protein from ribosomes and AEDANS-labeled ribosomes were extracted by acetic acid (Hardy et al., 1969), dialyzed overnight against 10% acetic acid, and then lyophilized and stored at −20 °C. For gel electrophoresis in the alkaline urea first dimension of the Wittman gel system (Wittmann, 1974), the lyophilized proteins were dissolved in the first-dimension sample buffer (100 μL) containing 25% sucrose so that polymerization of the acrylamide solution can occur directly on top of the sample in the cylindrical tube. Both this and the second dimension (acid urea) were electrophoresed as described previously (Wittmann, 1974).

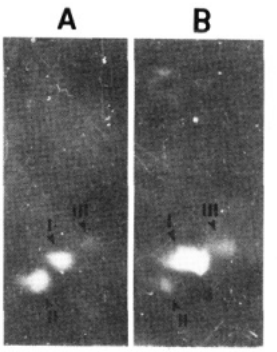

FIGURE 1: Gel electrophoresis (2-D) in alkaline urea/SDS of r-proteins from AEDANS- and AF-labeled ribosomes. All methodology is described under Experimental Procedures. Electrophoresis was carried out on 0.2 mg (by weight) of the lyophilized r-protein preparation. Basic proteins in the first dimension (alkaline urea) migrate from left to right; proteins in the second dimension (SDS) migrate from top to bottom. (A) Fluorescent proteins from IAEDANS-treated 70S ribosomes; (B) fluorescent proteins from IAF-treated 70S ribosomes.

Another 2-D gel system used was that of Traut and coworkers (Knopf et al., 1975; Kenny et al., 1979) which utilizes acid urea in both dimensions but an increased acrylamide concentration in the second. Protein sample preparation was identical with that for the Wittmann procedure except that the lyophilized protein was dissolved in 8 M urea, 10 mM DTT, and 25% sucrose. Electrophoresis of both acid urea dimensions was performed as described previously (Kenny et al., 1979).

Extraction of L31′ at pH 3.8. The $MgCl_2$ concentration of ribosomes or AEDANS-labeled ribosomes in buffer A was increased to 100 mM by addition of 1 M $MgCl_2$ prior to the lowering of the pH to 3.8 by dropwise addition of 1 M acetic acid (Schwabe, 1972). The precipitated ribosomes were pelleted by low-speed centrifugation, and the supernate was dialyzed overnight against 10% acetic acid. After lyophilization, the r-proteins were stored at −20 °C.

RESULTS

Identification of AEDANS-Derivatized r-Proteins

Selection of Gel Electrophoresis Conditions. We had observed that the acid urea dimension of either of the "standard" gels usually employed to identify r-proteins (Wittman, 1974; Knopf et al., 1975) strongly quenches the fluorescence of AEDANS-labeled proteins. Attempts to reverse this effect by diffusing in various solutions after electrophoresis were not successful. Consequently, we used alkaline urea and/or SDS gels to detect fluorescent proteins and, later, employed the Wittmann and the Traut 2-D gel systems in concert with large amounts of AEDANS-labeled proteins to overcome the quenching problem.

Identification of AEDANS–Protein I as AEDANS–S18. r-Proteins extracted from AEDANS-labeled ribosomes were electrophoresed in a 2-D alkaline urea/SDS gel. Two fluorescent spots of approximately equal intensity were observed (I and II, Figure 1A). Also, a faint spot (III) was observed slightly to the "northeast" of spot I; it will be discussed briefly later. The identification of these proteins is difficult from this

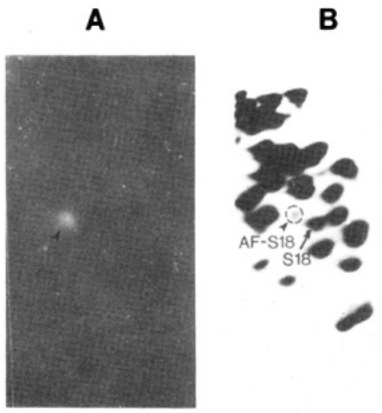

FIGURE 2: Gel electrophoresis (2-D) in alkaline urea/acid urea (Wittmann, 1974) of r-proteins from AF-labeled ribosomes. Basic r-proteins migrate in the first dimension (alkaline urea) from left to right and in the second dimension (acid urea) from top to bottom. (A) AF-derivatized r-proteins visualized by fluorescence; (B) gel in panel A stained with Coomassie Blue G-250. For a marker, S18 in this panel is identified from the map of Wittmann (1974). The dotted circle marks the position of AF-derivatized protein I as determined by a pinhole punctured in the center of the fluorescent spot in panel A.

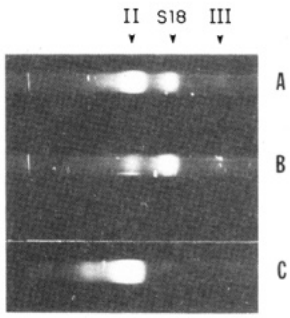

FIGURE 3: Gel electrophoresis (1-D) in alkaline urea showing the results of pH 3.8 extraction of AEDANS-labeled ribosomes. The pH 3.8 extraction procedure is described in the text. Electrophoresis of this first dimension, shown in a left to right direction, and fluorescence photography were performed as described in the text and the Figure 1 legend. Lane A, 0.2 mg (20%) of total proteins from unextracted AEDANS-labeled 70S ribosomes loaded; lane B, 0.2 mg (20%) of residual proteins from pH 3.8 extracted AEDANS-labeled ribosomes loaded; lane C, 100% of pH 3.8 extracted proteins.

electrophoretogram alone since no r-protein map exists for this gel system. Thus, in order to identify the two predominant AEDANS-labeled proteins, we used the standard 2-D gel system of Wittmann (1974). In an attempt to circumvent the acid urea quenching problem, we turned to a surrogate thiol-specific fluorescent label, IAF (Molecular Probes Co.); we had observed that AF-labeled proteins are less quenched than AEDANS-labeled proteins in acid urea, and we could expect that the reactivity of this iodoacetyl probe toward intraribosomal r-proteins would be at least qualitatively similar to that of IAEDANS. Moreover, the mobilities of the two AEDANS-labeled proteins differ from one another in an alkaline urea gel (Figure 1A), which is also the first dimension of the Wittmann gel, and we could anticipate that this would probably hold in the acid urea second dimension of the Wittmann gel system. Thus, the identification of surrogate AF–proteins I and II might be accomplished in this gel system, and, therefore, the identity of AEDANS–proteins I and II might thereby be inferred. [It should be pointed out here that however useful for this identification, IAF itself was not suitable for our later conformational change studies (J. S. Hanas and M. V. Simpson, unpublished results).]

In an initial experiment examining the reaction of IAF with ribosomes, we employed alkaline urea/SDS 2-D gel electrophoresis on r-proteins from AF-derivatized ribosomes. Under these conditions, we observed three AF-labeled spots (I, II, and III; Figure 1B) in positions identical with those of the AEDANS derivatives in Figure 1A. Thus, qualitatively, the reactivity of the 70S r-proteins toward I-AF appears to be the same as that toward IAEDANS. Although protein II was labeled considerably less by IAF than by IAEDANS, this in no way interfered with the identification of the proteins. Indeed, as will be seen, the higher intensity of AF–protein I aided in its identification. Upon electrophoresis of the proteins of the AF-labeled ribosomes in the alkaline urea/acid urea gel (Figure 2A), only the predominant fluorescent spot, AF–protein I, can be observed. After the position of this spot had been fixed with a pinhole, the acid urea gel was stained with Coomassie blue (Figure 2B). The position of AF–protein I corresponded to a lightly stained spot slightly to the "northwest" of S18 (Figure 2B) in a position one would expect an AF derivative of S18 to appear, that is, close to S18 but slightly retarded in both dimensions because of the presence of an additional negatively charged group (COO^-) on the fluorescein adduct. In an experiment to be described later, we demonstrate that IAEDANS derivatization also alters the mobilities of proteins which it modifies (Figure 7B). In the present experiment (Figure 2B), most of S18 remains underivatized because of the mild reaction conditions (10 min at 22 °C). Hence, these observations support the view that AF–protein I and, by inference, AEDANS–protein I are derivatives of S18.

Identification of Protein III as S21. We established first that protein III is derived from the 30S subunit by isolating these subunits from AEDANS-labeled ribosomes and electrophoresing the proteins in an alkaline urea/SDS 2-D gel. Two fluorescent spots were observed, one of which is AEDANS–S18 (data not shown). The position of AEDANS–protein III can be seen in an alkaline urea 1-D gel (Figure 3, lanes A and B), which is identical with the first dimension of Figure 1A and the first dimension of the Wittman 2-D gel. The only 30S proteins with corresponding mobility are S20 and S21. S20 does not contain thiol groups (Wittmann-Liebold et al., 1976), and we found the isolated protein not to react with IAEDANS (data not shown). We thus conclude that AEDANS–protein III is S21, in accord with the results of Kang et al. (1979).

Identification of Protein II as L31′. The position of AEDANS–protein II in an alkaline urea first dimension (Figure 3, lane A, or Figure 1A) is slightly to the west of AEDANS–S18. Thus, since the IAEDANS derivatization of both these proteins would be expected to retard them equally, native protein II would also be expected to be found slightly to the west of native S18. While a number of r-proteins occupy such a position in this first dimension, the second (SDS) dimension (Figure 1A), in which mobility is a function of size, shows that protein II possesses a molecular weight smaller than that of S18. Perusal of catalogs of r-proteins (Wittmann, 1974; Kenny et al., 1979; Fanning & Traut, 1981) shows that only a few 50S subunit r-proteins are likely candidates, most notably L30 and L31′, the latter protein a prominent variant of L31 (Kenny et al., 1979; Fanning & Traut, 1981). We have isolated L30 and have found that it does not react with IAEDANS (data not shown), in accord with the absence of cysteine in this protein (Ritter & Wittmann-Liebold, 1975) and the SH-group specificity of IAEDANS. In contrast, isolated L31′ readily reacts with IAEDANS (Figure 7), consonant with the presence of thiol groups in this protein (Brosius, 1978).

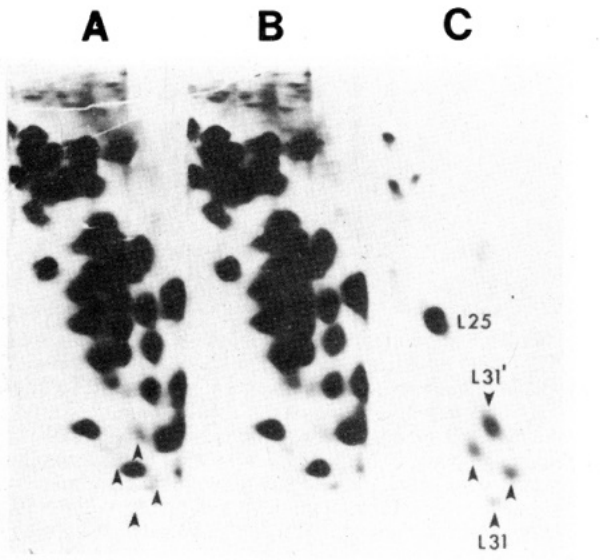

FIGURE 4: Gel electrophoresis (2-D) in acid urea/acid urea showing the results of pH 3.8 extraction of native ribosomes. Extraction conditions and r-protein quantities electrophoresed were identical with those in Figure 3. Proteins were visualized by Coomassie blue staining. r-Proteins in this gel system migrate from left to right in the first dimension and from top to bottom in the second dimension. The identity of L31′ was established by reference to the acid urea/acid urea gel map (Kenny et al., 1979; Fanning & Traut, 1981). (A) Total proteins from unextracted underivatized ribosomes; (B) residual proteins from pH 3.8 extracted underivatized ribosomes; (C) pH 3.8 extracted proteins.

To further examine the possibility that protein II is L31′, we took advantage of the possibility that it might be selectively extractable as the nearly homologous protein L31. Extraction of *E. coli* ribosomes at pH 3.8 releases only a few proteins including L31 (Schwabe, 1972). Thus, we extracted AEDANS-labeled ribosomes at pH 3.8 and electrophoresed both the extract and the residual pellet in an alkaline urea 1-D gel (Figure 3). The control, lane A, containing all the r-proteins, clearly shows the presence of AEDANS-labeled proteins I (S18) and II, as well as protein III (S21). Lane B, containing the residual proteins, reveals no decrease in S18 and S21, but most of protein II has disappeared. The extracted proteins are shown in lane C; the intense fluorescence of protein II is clearly visible.

Total r-proteins, pH 3.8 residual proteins, and extracted r-proteins were electrophoresed in the acid urea/acid urea 2-D gel system of Traut and co-workers (the gel system originally employed to identify L31′). Coomassie staining (Figure 4) showed the presence in the total r-protein extract (panel A) of L31′ of a cluster of closely related proteins (arrows). This cluster disappears from the extracted pellet (panel B) and reappears in the pH 3.8 extract (panel C). The proteins in the cluster have all been shown to be genetically related (Dabbs, 1981). We routinely observe, as does Traut's group, that L31′ is the predominant species in this cluster. The extractability property of identifiable L31′ is thus in accord with the extractability of AEDANS–protein II.

It remained to finally identify the derivatized protein as L31′ by identifying AEDANS–L31′ on the Traut 2-D gel map, the only published map on which this protein has been identified (Kenny et al., 1979; Fanning & Traut, 1981). We attempted to overcome the strong fluorescence quenching of this gel system by overloading the gel with the pH 3.8 AEDANS–protein II extract, a device which leads to extremely poor gels if attempted on total r-proteins. From a knowledge of the position of underivatized L31′ on the Traut gel (Figure 4C),

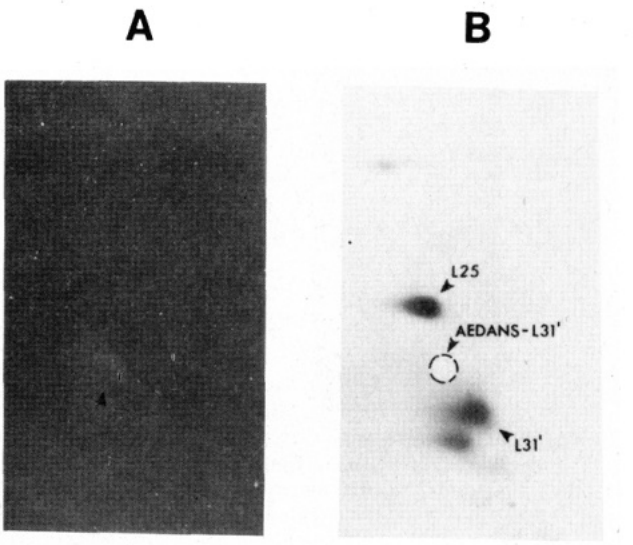

FIGURE 5: Gel electrophoresis (2-D) in acid urea/acid urea of the pH 3.8 extract of AEDANS-labeled ribosomes. Electrophoresis conditions were identical with those described for Figure 4C. (A) Fluorescence of the AEDANS-labeled pH 3.8 extract (arrow). (B) Coomassie stained panel A gel; the position, obtained from panel A, of AEDANS–protein II (dotted circle) can be seen relative to that of underivatized L31′.

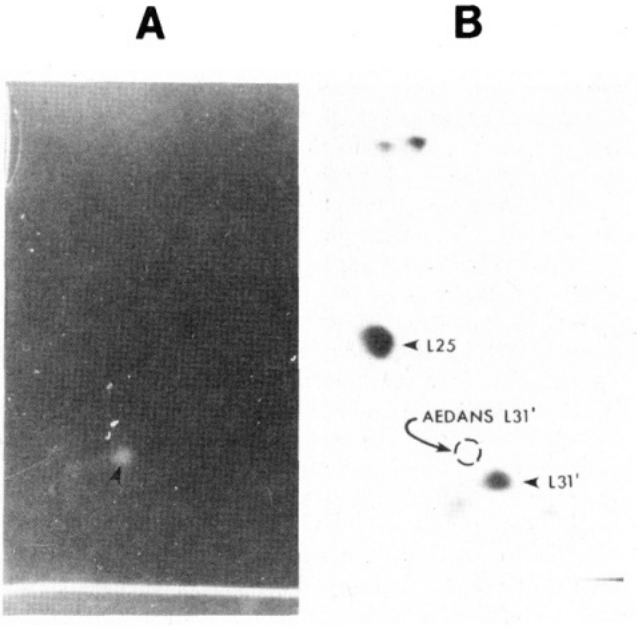

FIGURE 6: Gel electrophoresis (2-D) in alkaline urea/acid urea of the pH 3.8 extract of AEDANS-labeled ribosomes. Electrophoresis conditions are identical with those in the Figure 2 legend. (A) Fluorescence of the pH 3.8 extract (arrow); (B) Coomassie stained gel from panel A.

we anticipated observing a fluorescent spot slightly to the northwest of this position (because of the slightly lower positive charge, as discussed earlier). As expected, only one fluorescent spot (arrow, Figure 5A) is visible in this area under consideration, and that spot is in the anticipated position, slightly to the northwest of native L31′. The position of the latter was obtained by staining (Figure 5B).

We could also employ the Wittmann 2-D gel system (alkaline urea/acid urea) for identification of AEDANS–protein II. Our experiments with the pH 3.8 concentrate had indicated that quenching of AEDANS-labeled proteins in this system is less than that in the Traut system. All r-proteins (except the acidic ones) run virtually identically in the two systems (Knopf et al., 1975), and the position of native L31′ on the

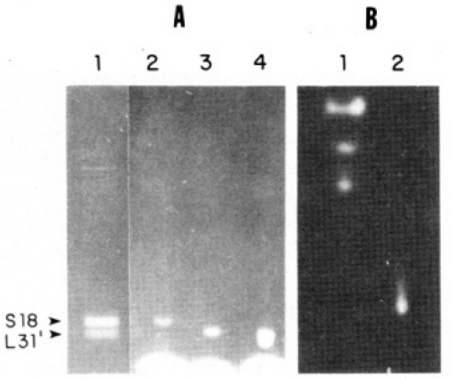

FIGURE 7: Gel electrophoresis (1-D) of isolated r-proteins reacted with IAEDANS. r-Protein isolation and reaction conditions are described under Experimental Procedures. Protein migration is from top to bottom. Panel A (SDS gel): lane 1, AEDANS-labeled r-proteins from intact ribosomes; lane 2, AEDANS–S18; lane 3, AEDANS–L31′; lane 4, AEDANS–L31. Panel B (alkaline urea gel): lane 1, AEDANS–L31′; lane 2, AEDANS–L27.

Wittmann gel can be inferred from its position on the Traut gel. Figure 6A clearly shows a fluorescent spot (arrow) which corresponds to AEDANS–protein II. When the Wittmann gel was stained with Coomassie blue (Figure 6B), the position of the fluorescent spot (dotted circle) is observed to the northwest of L31′. This is the expected position of the slightly more negatively charged L31′ derivative.

Properties of L31′

AEDANS Derivatization Alters the Electrophoretic Mobility of r-Proteins. We have shown that intraribosomal derivatization of S18 with IAF (Figure 2) or L31′ with IAEDANS (Figures 5 and 6) leads to a slight decrease in its mobility on charge-sensitive gels. However, inasmuch as some of the evidence for the identity of AEDANS–L31′ rests on these observations, we wished to obtain confirmation by demonstrating this charge-induced retardation directly on isolated L31′. When isolated L31′ was reacted with IAEDANS, a single fluorescent band on a 1-D SDS gel was observed, as expected (Figure 7A, lane 3). When a charge-sensitive gel such as alkaline urea was employed, however, four bands were observed (three prominent and one faint, top to bottom), each with a different degree of retardation (Figure 7B, lane 1). This observation is consistent with the known presence of four SH groups in the homologous protein L31 (Brosius, 1978) and with the formation of tetra-, tri-, di-, and mono-AEDANS derivatives, conferring added charges of 4–, 3–, 2–, and 1–, respectively. As a control, a protein with only one SH group, L27 (Chen et al., 1975), was treated with IAEDANS, and only one fluroescent spot was observed (lane 2, Figure 7B). Thus, only one of the four thiol groups of L31′ is accessible to IAEDANS in the intact ribosome, and the extent of retardation of the AEDANS derivative corresponds to that of the mono-AEDANS derivative formed on direct action of IAEDANS on isolated L31′.

Reactivity of L31′ to IAEDANS Is Dependent upon the Conformational State of the Ribosome. Ribosomes were dissociated by lowering the Mg^{2+} concentration to 0.5 mM by gel filtration in buffer B. Treatment of the subunit mixture with IAEDANS under conditions which label both S18 and L31′ in intact ribosomes (Figure 1A) and subsequent SDS/PAGE of the subunit proteins showed that L31′ no longer becomes labeled (Figure 8, lane 1) whereas the usual S18 fluorescent band appeared. Heating the subunit mixture at 37 °C for 15 min prior to reaction with IAEDANS resulted in the appearance of four additional fluorescent bands, all of

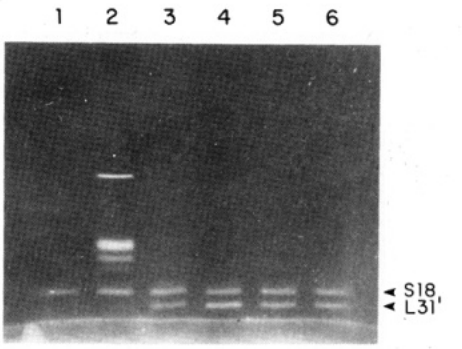

FIGURE 8: Effect of dissociation and reassociation of underivatized ribosomes on subsequent IAEDANS labeling of S18 and L31′, as shown by 1-D SDS gel electrophoresis. Ribosomes were treated as described below and then exposed to IAEDANS, and the r-proteins were subsequently electrophoresed as described in the text. Electrophoresis of r-proteins is from top to bottom. Lane 1, ribosomes dissociated to subunits by gel chromatography in buffer B (see text); lane 2, same as lane 1 except that the subunit mixture was incubated at 37 °C for 15 min; lane 3, same as lane 1 except that the subunit mixture was incubated at 22 °C for 15 min in buffer B containing 12 mM $MgCl_2$ (i.e., buffer A); lane 4, same as lane 3 except that incubation was at 37 °C; lanes 5 and 6, controls: ribosomes incubated in buffer A for 15 min at either 22 °C (lane 5) or 37 °C (lane 6).

higher molecular weight than S18 (Figure 8, lane 2). However, again no fluorescence was visible in the position corresponding to L31′. The absence of L31′ reactivity to IAEDANS does not result from the possibility that it is lost during the gel filtration step. This possibility was tested by exposure of the subunits to 12 mM Mg^{2+} which resulted in reassociation of 70–80% of the subunits (as evidenced by sucrose gradient centrifugation, data not shown) and also restoration of the reactivity of L31′ to IAEDANS (lanes 3 and 4). Thus, the most likely explanation of the resistance of L31′ to attack by IAEDANS when ribosomes are in the dissociated state is that an altered conformational state in or around this protein has rendered the normally reactive thiol group inaccessible to this reagent. That the reactivity of L31′ is environmentally sensitive was also observed with the reagent IAF (Figure 1B) and by the failure of this protein to become labeled by *N*-ethylmaleimide (data not shown).

AEDANS Derivatization Weakens the Association of L31′ with the Ribosome. A sample of AEDANS-labeled ribosomes was dissociated into subunits by lowering the Mg^{2+} concentration to 0.5 mM by means of gel chromatography through Sephadex G-75. Upon SDS/PAGE of the isolated subunit proteins, AEDANS–L31′ was found to be missing (Figure 9A, lane 2). In a parallel control experiment, the Mg^{2+} concentration (and therefore the 70S state) was maintained at 12 mM during the gel filtration; here, AEDANS–L31′ was not lost (lane 1).

The weakening of the AEDANS–L31′/ribosome interaction is brought about by ribosomal dissociation itself and is not dependent upon the particular procedure by which Mg^{2+} concentration is lowered. When dialysis was substituted for gel filtration, not only were the 50S subunits devoid of fluorescence (Figure 9B) but also, upon centrifugation of the subunits, gel electrophoresis showed the presence of AEDANS–L31′ in the supernate and AEDANS–S18 in the pellet (data not shown).

DISCUSSION

When the *E. coli* ribosome is incubated with IAEDANS, primarily two proteins become labeled. One of these, S18, is well studied and has previously been shown to become so labeled (Kang et al., 1979). The other, L31′, is a relatively

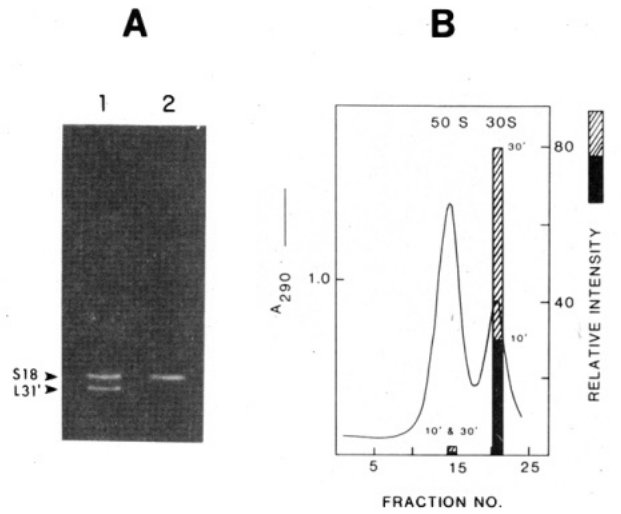

FIGURE 9: Effect of dissociation of AEDANS-labeled ribosomes to subunits on the release of AEDANS–L31′, as shown by 1-D SDS gel electrophoresis. AEDANS-labeled ribosomes (approximately 40 A_{260} units) were dissociated either by G-75 gel chromatography or by overnight dialysis in buffer B. Sucrose gradient analysis of ribosomal subunits was performed as described in the text. Panel A: lane 1, SDS gel electrophoresis of AEDANS-labeled r-proteins after gel chromatography in buffer A; lane 2, same as lane 1 except that chromatography was performed in buffer A containing 0.5 mM $MgCl_2$ (i.e., buffer B). Panel B: sucrose gradient analysis of subunits of AEDANS-labeled ribosomes reacted with the reagent for 10 or 30 min. Fluorescence intensity is expressed in relative intensity units.

recently discovered r-protein first identified by Traut and his group (Kenny et al., 1979; Fanning & Traut, 1981). Dabbs (1981) also (unknowingly) observed L31′ in his study of a cluster of r-proteins genetically related to L31.

Our identification of L31′ as one of the proteins labeled by IAEDANS was hampered by the severe fluorescence quenching of AEDANS-labeled proteins by acid urea gels, in particular the gel system which played a major role in its discovery by Traut's group. The difficulty was surmounted by the use of several different gel systems, including Traut's, and by taking advantage of its selective extractability from the ribosome, enabling us to concentrate it, thereby overwhelming the quenching. It addition, employment of an analogous fluorescent probe less sensitive to quenching enabeled us to identify AEDANS–S18, an important step in the identification of AEDANS–L31′.

Our findings are not in accord with Kang et al. (1979), who showed that only S18 becomes labeled by IAEDANS. While it is conceivable that their ribosome preparations did not contain L31′, this is unlikely since the ribosomes in both studies were identically prepared. However, the dissociability of AEDANS–L31′from the ribosome and its subsequent loss from the ribosome during subunit purification lead to a more plausible explanation. The procedure used by Kang et al. to identify the r-proteins labeled by IAEDANS involved isolation of the ribosomal subunits prior to analysis for fluorescent proteins. Thus, any AEDANS–L31′ present in the intact ribosome would have been lost and therefore have remained undetected by their analytical procedures.

The reactivity of L31′ to IAEDANS is influenced by the conformational state of the ribosome. In the intact ribosome, IAEDANS labels L31′ at least to an equal extent as S18; in dissociated ribosomes, only S18 reacts. Moreover, when the subunits are activated by brief heating, a treatment which induces a change in subunit conformation (Zamir et al., 1974; Ghosh & Moore, 1979), at least four additional proteins are labeled by IAEDANS. The identification of these proteins should provide added information about the subunit activation process.

Cross-linking experiments show the proximity of L5 and L31′ (Fanning & Traut, 1981). L5 is located in the subunit interface region of the 70S ribosome (Kenny et al., 1979). Thus, the cross-linking results also place L31′ in this region. Our finding that the reactivity of L31′ with IAEDANS is dependent upon the subunits being associated offers further support for the interface location of this r-protein.

The cross-linking results also suggest close association between L31′ and L18 (Fanning & Traut, 1981). L5 and L18 bind specifically to 5S rRNA (Horne & Erdmann, 1972) and more recently, L31′ has also been found associated with 5S RNA (Fanning & Traut, 1981). The availability of a fluorescent derivative at the ribosomal subunit interface provides an experimental handle for the study of this unique and most interesting region of the *E. coli* ribosome. For example, experiments on intraribosomal AEDANS–L31′ fluorescence indicate that streptomycin may induce a conformational change in this region (J. S. Hanas and M. V. Simpson, unpublished results).

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In Vitro Conversion of a Methionine to a Glutamine-Acceptor tRNA†

LaDonne H. Schulman* and Heike Pelka

Department of Developmental Biology and Cancer, Division of Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: A derivative of *Escherichia coli* $tRNA^{fMet}$ containing an altered anticodon sequence, CUA, has been enzymatically synthesized in vitro. The variant tRNA was prepared by excision of the normal anticodon, CAU, in a limited digestion of intact $tRNA^{fMet}$ with RNase A, followed by insertion of the CUA sequence into the anticodon loop with T_4 RNA ligase and polynucleotide kinase. The altered methionine tRNA showed a large enhancement in the rate of aminoacylation by glutaminyl-tRNA synthetase and a large decrease in the rate of aminoacylation by methionyl-tRNA synthetase. Measurement of kinetic parameters for the charging reaction by the cognate and noncognate enzymes revealed that the modified tRNA is a better acceptor for glutamine than for methionine. The rate of mischarging is similar to that previously reported for a tryptophan amber suppressor tRNA containing the anticodon CUA, su^+7 $tRNA^{Trp}$, which is aminoacylated with glutamine both in vivo and in vitro [Yaniv, M., Folk, W. R., Berg, P., & Soll, L. (1974) *J. Mol. Biol. 86*, 245–260; Yarus, M., Knowlton, R. E., & Soll, L. (1977) in *Nucleic Acid-Protein Recognition* (Vogel, H., Ed.) pp 391–408, Academic Press, New York]. The present results provide additional evidence that the specificity of aminoacylation by glutaminyl-tRNA synthetase is sensitive to small changes in the nucleotide sequence of noncognate tRNAs and that uridine in the middle position of the anticodon is involved in the recognition of tRNA substrates by this enzyme.

A number of aminoacyl-tRNA synthetases are known to require specific anticodon nucleotides for recognition of cognate tRNAs (Kisselev, 1983). In addition, the anticodon sequence appears to play a critical role in determining the specificity of aminoacylation by at least one of these enzymes. *Escherichia coli* glutaminyl-tRNA synthetase (GlnRS)[1] loses the ability to discriminate against *E. coli* $tRNA^{Trp}$ following a single base change in the tRNA, converting the anticodon sequence from CCA to CUA. The resulting amber suppressor su^+7 $tRNA^{Trp}$ is mischarged with glutamine both in vivo and in vitro (Yaniv et al., 1974; Yarus et al., 1977). A mutant tyrosine tRNA, su^+3 $tRNA^{Tyr}$, containing the anticodon CUA, accepts only tyrosine but can also be mischarged with glutamine when additional mutations are introduced in the acceptor stem region (Hooper et al., 1972; Shimura et al., 1972; Smith & Celis, 1973; Celis et al., 1973; Ghysen & Celis, 1974). *E. coli* $tRNA_1^{Gln}$ and $tRNA_2^{Gln}$ contain the anticodon sequences U*UG and CUG, respectively (Yaniv & Folk, 1975). The uridine in the middle position of the anticodon is therefore common to the wild-type glutamine tRNAs and the mutant suppressor tRNAs mischarged by GlnRS. In order to further investigate the role of the anticodon sequence in recognition of tRNAs by GlnRS, we have examined the ability of the enzyme to aminoacylate a methionine tRNA in which the normal anticodon sequence CAU has been permuted to yield the anticodon CUA. This tRNA has been found to be a better substrate for GlnRS than the single mutant tyrosine suppressor tRNA and to be aminoacylated with glutamine in vitro at a rate comparable to that measured by others for su^+7 $tRNA^{Trp}$.

Materials and Methods

Materials. The trinucleotide GpCpU was purchased from Sigma Chemical Co. Adenosine 3′,5′-diphosphate and ribonucleases PhyM and *Bacillus cereus* were obtained from P-L Biochemicals. RNases T_1, T_2, and U_2 were purchased from Calbiochem, and RNase A was from Worthington Biochemicals. Nuclease P_1, calf intestinal alkaline phosphatase, and nuclease-free BSA were obtained from Boehringer-Mannheim. [^{14}C]Glutamine and *Pse*TI T_4 polynucleotide kinase (Cameron et al., 1978) were purchased from New England Nuclear. [γ-^{32}P]ATP, [α-^{32}P]ATP, and [^{35}S]methionine were obtained from Amersham. *E. coli* methionyl-tRNA synthetase was purified from *E. coli* K-12 strain EM20031 (Schulman & Pelka, 1977), and T_4 RNA ligase was purified from *E. coli*

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[1] Abbreviations: $tRNA^{fMet}$, the *E. coli* initiator methionine tRNA; $tRNA^{fMet}_{CUA}$, tRNA containing the sequence CUA in the anticodon position that has been enzymatically synthesized in vitro from half-molecule-sized fragments of *E. coli* $tRNA^{fMet}$; $tRNA_2^{Gln}$, the *E. coli* glutamine tRNA containing the anticodon CUG; su^+7 $tRNA^{Trp}$, the *E. coli* amber suppressor $tRNA^{Trp}$ containing the anticodon CUA; MetRS, *E. coli* methionyl-tRNA synthetase; GlnRS, *E. coli* glutaminyl-tRNA synthetase; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTT, dithiothreitol; p*, ^{32}P-labeled phosphate; U*, a derivative of 2-thiouridine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; BSA, bovine serum albumin.

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