Miller, J. H. (1972a) *Experiments in Molecular Genetics*, pp 101–103, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, J. H. (1972b) *Experiments in Molecular Genetics*, p 431, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Neidhardt, F. C., Wirth, R., Smith, M. W., & Van Bogelen, R. (1980) *J. Bacteriol. 143*, 535.
Schlogl, K. (1958) *Monatsh. Chem. 89*, 377.
Silverman, R. B., & Abeles, R. H. (1976) *Biochemistry 15*, 4718.
Spudich, J. A., Horn, V., & Yanofsky, C. (1970) *J. Mol. Biol. 53*, 49.
Su, C. H., & Greene, R. C. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 367.
Uren, J. R., & Lazarus, H. (1979) *Cancer Treat. Rep. 63*, 1073.
Uren, J. R., & Ragin, R. C. (1979) *Cancer Res. 39*, 1927.
Uren, J. R., Ragin, R. C., & Chaykovsky, M. (1978) *Biochem. Pharmacol. 27*, 2807.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Wu, W. H., & Morris, D. R. (1973) *J. Biol. Chem. 248*, 1696.

Preparation and Characterization of Fluorescent 50S Ribosomes. Specific Labeling of Ribosomal Proteins L7/L12 and L10 of *Escherichia coli*†

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ABSTRACT: So that the topographic and dynamic properties of the L7/L12–L10 complex in the 50S ribosome of *Escherichia coli* could be studied, methods and reagents were developed in order to introduce fluorescent groups at specific positions of these proteins. In the case of L7/L12, this was done by attaching an aldehyde group to Lys-51 of the protein by using 4-(4-formylphenoxy)butyrimidate or by converting the amino terminus of L12 into an aldehyde group by periodate oxidation. Subsequent reaction of the aldehyde groups with newly developed hydrazine derivatives of fluorescein and coumarin resulted in specifically labeled L7/L12 derivatives. L10 was modified at the single cysteine residue with *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide. The fluorescent proteins L10 and L7/L12 could be reconstituted into 50S ribosomes. The resulting specifically labeled 50S ribosomes show 25–100% activity in elongation factor G dependent GTPase as well as in polyphenylalanine synthesis. The fluorescent properties of the labeled 50S ribosomes show that these fluorescent derivatives are suitable for energy transfer studies.

A precise understanding of the mechanism of function of the ribosome requires detailed structural information on the components and the distances to each other. For the latter, several methods are used in order to achieve this goal [for a recent review, see Chamblis et al. (1980)], for example, neutron scattering (Moore, 1980) and fluorescence energy transfer of randomly labeled proteins (Cantor et al., 1974). In both cases, a distance between the centers of mass of two specified proteins is obtained, but the conversion of these data into the actual three-dimensional picture requires the knowledge of the shape of the protein, something which is hard to obtain. In the case of the fluorescence energy transfer, the assumption of a random labeling also is questionable. Distances between specifically labeled sites lead to more insight into the topography of the ribosome. This approach has been performed initially with specifically labeled fluorescent tRNAs and erythromycin bound to the ribosome (Langlois et al., 1976; Fairclough & Cantor, 1979), while also 30S (Kang et al., 1979) and 50S ribosomes (Lee et al., 1981) have been labeled with some specificity to respectively the cysteine of S18 and Lys-120 of L7/L12.

In this paper, we report the preparation of specifically labeled L7/L12 and L10. In the case of L7/L12 (L7 is the N-acetylated form of L12), a new technique was developed which enabled us to prepare L7, specifically labeled at Lys-51, and L12, labeled at the N terminus. The properties of these modified proteins with respect to binding to ribosomal core particles and in the functional reconstitution of core particles are described.

In the following paper (Zantema et al., 1982), some of these proteins are used for distance measurements between L10 and L7/L12 on the 50S ribosomal particle.

Experimental Procedures

The following buffers were used: TMA buffer, 20 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 10 mM NH_4Cl, and 6 mM 2-mercaptoethanol; GTPase buffer, 20 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol; CM buffer, 6 M urea, 25 mM acetic acid–piperidine, pH 5.2, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] and 1 mM DTE.

NaB^3H_4, [^{14}C]formaldehyde, [γ-^{32}P]GTP, and [^{3}H]-phenylalanine were obtained from Amersham. Urea was deionized before use. Protein concentrations were determined according to Lowry et al. (1951) with insulin as standard. EF-G, EF-Tu, and EF-Ts were isolated according to Arai et al. (1972).

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[1] Abbreviations: EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; TLC, thin-layer chromatography; FPB-L7, 4-(4-formylphenoxy)butylamidine-L7; FAPB-L7, 4-(6-formyl-3-azidophenoxy)butylamidine-L7; DACM-L10, adduct of *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide with L10; EDTA, ethylenediaminetetraacetic acid; DTE, 1,4-dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

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Ribosomes were isolated from *Escherichia coli* MRE 600 according to Gesteland (1966). Separation in 50S and 30S ribosomes was performed at low Mg^{2+} concentration on a sucrose gradient in a zonal rotor (Möller et al., 1970). 50S ribosomal subunits were deprived of L7/L12 by adjusting a 1 M NH_4Cl solution of ribosomes to 50% ethanol at 0 °C (=P_0 cores) (Hamel et al., 1972), while L7/L12, L10, and part of L11 were removed by performing the same procedure either at 37 °C (=P_{37} cores) or at 0 °C by using 2 M NH_4Cl (= 2 M P_0 cores). Ribosome concentrations were determined by the absorbance at 260 nm. It is assumed that 1 A_{260} unit corresponds to 25 pmol of 70S, 39 pmol of 50S, and 69 pmol of 30S ribosomes.

Functional Assays. EF-G-dependent GTPase activity was determined as described previously (Schrier et al., 1973). Polyphenylalanine synthesis was performed essentially as described by Van Agthoven et al. (1977), using GTPase buffer and *E. coli* ribosomes and factors. Reconstitution of the 50S cores at the very low concentrations used was performed in the presence of 0.5 mg/mL bovine serum albumin to avoid loss of the protein by aspecific sticking.

Protein Isolation. The isolated proteins were routinely identified by $NaDodSO_4$–polyacrylamide electrophoresis according to Laemmli (1970) and by two-dimensional gel electrophoresis according to Kaltschmidt & Wittmann (1970).

Proteins were concentrated by binding to a small ion-exchange column (2 mL of resin), followed by elution with a buffer containing a high KCl concentration. L7, L12, and their modified derivatives were dialyzed and stored in GTPase buffer.

Protein L7/L12 was isolated by extraction of 70S ribosomes with 1 M NH_4Cl–50% ethanol at 0 °C (Hamel et al., 1972), and L7 and L12 were separately isolated as described (Möller et al., 1972). L10 was obtained from the ethanol-washed 70S core particles by extracting the remaining total protein at 0 °C with 67% acetic acid (Hardy et al., 1968). After acetic acid was removed by gel filtration on Sephadex G25, equilibrated with CM buffer, the protein was eluted from a CM-cellulose column with a linear gradient from 0 to 150 mM KCl in CM buffer. L10 elutes at 65 mM KCl. The protein was further purified on a Sephacryl S200 column in CM buffer. The protein was concentrated and stored at −70 °C in CM buffer.

Reductive methylation was performed by incubation of the protein for 0.5 h with 1–4 mM formaldehyde, followed by reduction with $NaBH_4$. For ^{14}C or ^{3}H labeling, [^{14}C]formaldehyde or NaB^3H_4 was used respectively. In the case of L7/L12, a buffer of 50 mM sodium tetraborate, pH 9.2, was used, while for L10 a buffer of 100 mM $NaHCO_3$, pH 8.0, 3 mM 2-mercaptoethanol, and 25% diethylene glycol or 100 mM $NaHCO_3$, pH 8.0, 3 mM 2-mercaptoethanol, and 6 M urea was used.

Affinity Chromatography of L10. Protein L10, prepared as described above, contains approximately 0.6 SH group/polypeptide chain as determined by DTNB titration (Ellman, 1959), possibly due to irreversible oxidation or modification during the isolation procedure. In order to get L10 containing one SH group per polypeptide chain, we introduced an SH-affinity chromatography step. A solution of 10 mg of [^{3}H]-methyl-L10 (230 cpm/pmol) in 50 mM sodium acetate, pH 5.0, 6 M urea, and 1 mM EDTA (this buffer was preflushed with nitrogen) was shaken with 6 mL of thiopropyl-Sepharose 6B (Pharmacia) for 6 h at room temperature. Subsequently, a small column was poured, and nonbound L10 was washed off with 16 mL of 20 mM Tris-HCl, pH 7.4, 10 mM NH_4Cl, 6 M urea, and 1 mM EDTA. The SH-containing protein was released by using the buffer, mentioned above, containing in addition 60 mM 2-mercaptoethanol. Then L10 was passed over Sephadex G25, equilibrated with a degassed buffer of 6 M urea, 50 mM sodium citrate–HCl, pH 6.0, 10 mM NH_4Cl, and 1 mM EDTA, after which modification with DACM took place immediately.

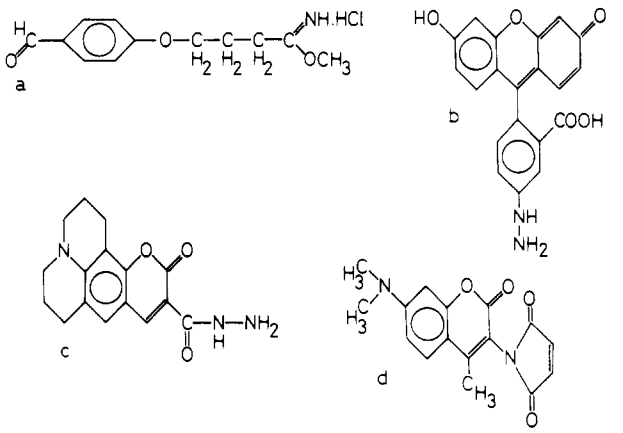

FIGURE 1: Structures of the used reagents. (a) 4-(4-Formylphenoxy)butyrimidate (FPB-imidate); (b) fluorescein hydrazine isomer I; (c) coumarin-314 hydrazide; (d) *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM).

Synthetic Procedures. Thin-layer chromatography was performed on SiO_2-coated aluminum plates (F254, Merck, West Germany). The chemicals were obtained from Aldrich or Merck; coumarin-314 was from Eastman.

(*A*) *4-(4-Formylphenoxy)butyrimidate* (*FPB-imidate*). This compound (Figure 1a) was synthesized via an analogous procedure as described for FAPB-imidate (Maassen, 1979); *p*-hydroxybenzaldehyde was converted into 4-(4-formylphenoxy)butyronitrile (yield 53%): TLC (ether) R_f 0.56; NMR ($CDCl_3$) δ 9.94 (singlet, CHO), 7.88 and 7.03 (two doublets, aromatic H's), 4.15 (triplet, OCH_2–), 2.61 (triplet, $-CH_2CN$), 2.17 (quintet, $-CCH_2C$).

Subsequently the nitrile was converted into FPB-imidate by treatment with methanol–HCl: yield 50%; NMR (CD_3-OCD_3) δ 9.76 (singlet, CHO), 7.94 and 7.17 (two doublets, aromatic H's), 4.16 (triplet, OCH_2–), 4.06 (singlet, $-OCH_3$), 3.40 (broad singlet, C=N·HCl), 2.82 (triplet, $-CH_2C$=N–), 2.12 (quintet, $-CCH_2C$–); UV_{max} (H_2O) 280 nm (ϵ_{max} 16 200 M^{-1} cm^{-1}).

(*B*) *Fluorescein Hydrazine Isomer I* (*Figure 1b*). Fluorescein amine isomer I (1 mmol) in 10 mL of concentrated hydrochloric acid was diazotized at 0 °C with 1.2 mmol of $NaNO_2$ in 5 mL of H_2O. Then, 5 g of $SnCl_2$, dissolved in 5 mL of concentrated hydrochloric acid, was added at once. The mixture was stirred for 2 h at 0 °C and for 20 h at room temperature. The reaction mixture was made pH 12 by addition of 10 M NaOH and heated for 5 min at 60 °C. Subsequently, by addition of concentrated hydrochloric acid, the pH of the reaction mixture was carefully adjusted to 3.5. The precipitate, consisting of fluorescein hydrazine in its zwitterion form and tin salts, was isolated by centrifugation and extracted 3 times with 5 mL of boiling ethanol. Ethanol was removed by evaporation, the residue was reextracted with ethanol, and the resulting impure fluorescein hydrazine was converted into a hydrazone by addition of 2 mL of acetone and 100 μL of 1 M sodium acetate, pH 4.8, to the alcoholic extract. After the solution was stirred for 30 min at room temperature and cooled to 0 °C, it was made pH 1.0 by addition of about 50 mL of ice cold 1% hydrochloric acid. The uncharged hydrazone was extracted twice with ether (2 times 25 mL), and

the combined extracts together with 20 mL of 10% hydrochloric acid were heated for 45 min on a boiling water bath which results in back-conversion of the hydrazone into the hydrazine. Insoluble material was filtered off, and the hydrazine was precipitated by raising the pH to 3.5 with NaOH. The precipitate was washed twice with ice-cold water and dried in vacuo: yield of fluorescein hydrazine, 190 mg (55%) as dark brown powder; TLC (ethanol) R_f 0.86, R_f (fluorescein amine) 0.75; UV_{max} (GTPase buffer) 489 nm; fluorescence emission maximum at 513 nm with a quantum yield of 0.14. In contrast, fluorescein amine has a much lower fluorescence.

(*C*) *Coumarin-314 Hydrazide* (*Figure 1c*). The ethyl ester moiety of coumarin-314 was converted into its hydrazide (see Figure 1c) by reacting 0.1 mmol of coumarin-314 and 0.2 mL of hydrazine hydrate (100%) in 3 mL of ethanol for 6 h at room temperature. The reaction mixture was kept overnight at −20 °C, and the formed crystals were isolated by centrifugation. After the coumarin-314 hydrazide was washed with some ice cold ethanol, it was dried in vacuo: yield 80%; TLC (CH_3OH–ether, 1:1 v/v) R_f 0.36; UV_{max} (GTPase buffer) 448 nm; fluorescence emission maximum at 497 nm with a quantum yield of 0.36. For coumarin-314, these values are respectively 0.80, 448 nm, 492 nm, and 0.72.

Modification of Ribosomal Protein L7/L12. (*A*) *Preparation of FPB–L7.* This was done as described for FAPB–L7 (Maassen, 1979). On the basis of a molar extinction coefficient for the formylphenoxy chromophore at 280 nm of 16 200 M^{-1} cm^{-1}, the degree of modification of the protein was 0.7–1.0 mol of FPB-imidate/mol of protein.

(*B*) *Preparation of Coumarin-314–FPB–L7.* A solution of 12 mg of FPB–L7 (degree of modification 0.9) in 8 mL of 6 M guanidinium chloride and 50 mM sodium acetate–acetic acid (pH 4.3) was made 2 mM in coumarin-314 hydrazide by addition of a 20 mM solution of coumarin-314 hydrazide in aldehyde-free ethanol. The mixture was incubated for 90 min at room temperature, low molecular weight material was removed by gel filtration on Sephadex G25 (fine) in 6 M guanidinium chloride, and the intensely yellow colored protein fraction was dialyzed against TMA buffer and concentrated; yield, 4 mg of coumarin-314–FPB–L7. On the basis of a molar extinction coefficient of the coumarin-314 chromophore at 448 nm of 42 000 M^{-1} cm^{-1} (based on Eastman catalog data), 0.86 mol of coumarin-314 hydrazide is present per mol of protein.

For determination of the modification site, 3.2 mg of coumarin-314–FPB–L7, dissolved in 1% ammonium carbonate solution, was digested by 65 μg of elastase for 4.5 h at 37 °C. The elastase peptides were separated by high-performance liquid chromatography on a μBondapak C18 column, developed by a linear gradient of 20 mM triethylammonium phosphate, pH 5.8, to 20 mM triethylammonium phosphate, pH 5.8, and 50% 2-propanol (v/v).

(*C*) *Preparation of Fluorescein–FPB–L7.* By use of the same procedure as for coumarin-314–FPB–L7, we modified 15 mg of FPB–L7 with fluorescein hydrazine. Because attachment of fluorescein moieties to the protein introduces at neutral pH a negative charge in the protein, fluorescein–FPB–L7 was purified by ion-exchange chromatography on DEAE-Sepharose CL 6B by using a linear 0–250 mM KCl gradient in 6 M urea, 20 mM Tris-HCl, pH 8.0, 10 mM NH_4Cl, and 6 mM 2-mercaptoethanol. First protein without fluorescein is eluted, followed by a protein peak with fluorescein absorption; yield, 2 mg of fluorescein–FPB–L7 containing one fluorescein group. On the basis of this one to one modification and the protein concentration according to Lowry et al. (1951), the molar extinction coefficient at the absorbance maximum, 495 nm, is 89 000 M^{-1} cm^{-1}.

(*D*) *Preparation of N-Terminally Labeled L12.* The N-terminal serine residue in L12 was converted into an aldehyde group by oxidation with 5 mM $NaIO_4$ for 1 h at 0 °C in 100 mM sodium acetate, pH 5.0 (Clamp & Hough, 1965; Knowles, 1965). The oxidized protein was modified with fluorescein hydrazine or coumarin-314 hydrazide as described for FPB–L7. After modification, the solution was made 5 mM in DTE and kept for 10 min at 37 °C. The fluorescein–L12 was purified in the same way as described for fluorescein–FPB–L7 (yield 30%). The absorbance maximum is at 491 nm, and the extinction coefficient is 86 000 M^{-1} cm^{-1}, based on the protein concentration and a one to one modification.

For determination of the modification site, 1 mg of N-terminal fluorescein–L12, dissolved in 1 mL of 1% ammonium carbonate, was digested by 30 μg of trypsin for 2 h at 37 °C. Either tryptic peptides were separated on paper as described (Maassen et al., 1981), or the core peptides were precipitated at pH 4.3 (Terhorst et al., 1973), and the resulting supernatant (containing more than 80% of the fluorescein absorption) was subjected to high-performance liquid chromatography on a μBondapak C18 column developed with a linear gradient of 20 mM tertiary butylammonium acetate, pH 6.0, to 20 mM tertiary butylammonium acetate, pH 6.0, and 50% acetonitrile (v/v).

Modification of Ribosomal Protein L10. L10 (3 mg) dissolved in 3 mL of 50 mM sodium citrate–HCl, pH 6.0, 10 mM NH_4Cl, 6 M urea, and 1 mM EDTA was modified by addition of a 40 mM solution of *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (Machida et al., 1975) (Wake Pure Chemical Industries, Japan) (Figure 1d) in acetone to a final 3-fold excess with respect to the sulfhydryl groups present. After reaction for 20 min at 0 °C, the protein was separated from the excess of reagent on Sephadex G25 in 6 M urea, 20 mM Tris-HCl, pH 7.4, 10 mM NH_4Cl, and 6 mM 2-mercaptoethanol. The yellow protein solution had an absorption maximum at 402 nm. The degree of modification was calculated by using an extinction coefficient of 24 200 M^{-1} cm^{-1} (Yamamoto et al., 1977). Prior to the use of DACM–L10 in reconstitution experiments, the urea concentration was made below 0.6 M by dilution with the appropriate buffer containing 0.5 mg/mL bovine serum albumin.

Reconstitution. Cores (50 S) and proteins were mixed in GTPase buffer and incubated for 10 min at 37 °C. The reconstituted 50S particles were isolated by centrifugation through a cushion of 15% sucrose in GTPase buffer at 4 °C in a SW 50.1 swing-out rotor.

The degree of reconstitution of fluorescein- and coumarin-314–L7/L12's into 50S particles was estimated from the optical densities of the fluorescent ribosomes by using the molecular extinction coefficients of the fluorescent chromophores as given in Table IV. Due to the low value of the molar extinction coefficient of the DACM chromophore with respect to the high background of ribosome absorption at the absorption maximum of DACM, ^3H-labeled DACM–L10 was used in order to determine the degree of reconstitution.

Fluorescence Measurements. Fluorescence measurements were performed on a Perkin-Elmer MPF-2A apparatus. Cuvettes were thermostated at 20 °C. Excitation and emission slits of 10 nm were used.

The excitation intensity was calibrated by the rhodamine B method (Chen, 1967). The detector system was calibrated by a standard tungsten filament lamp (Chen, 1967; Melhuish, 1973). The corrected spectra of the fluorescence standards 2-aminopyridine, quinine sulfate, and 3-aminophthalimide

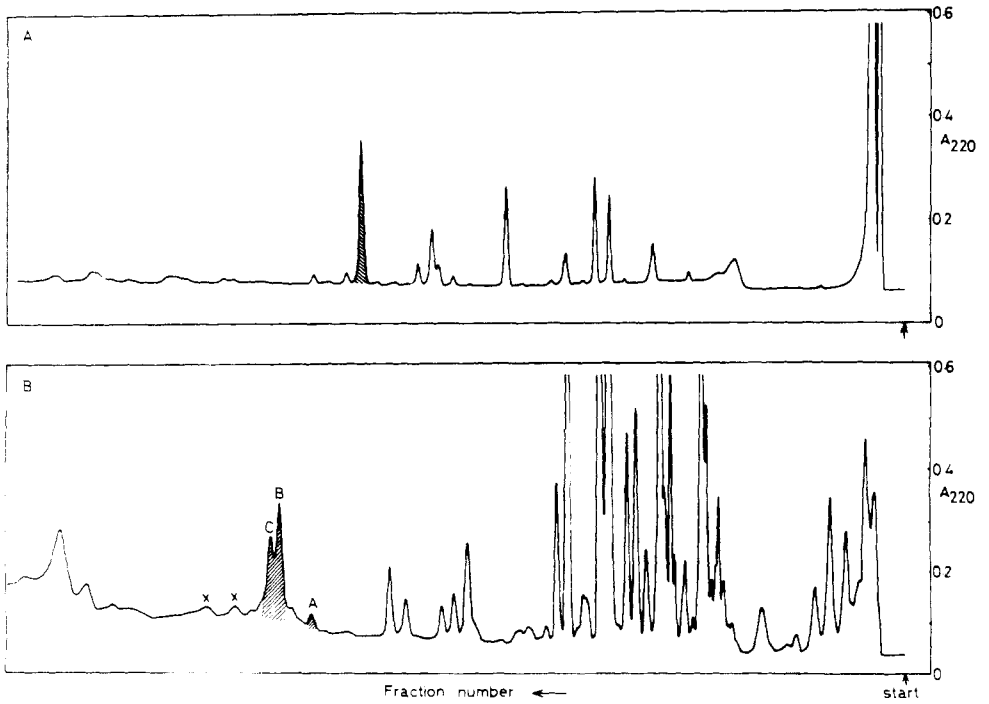

FIGURE 2: High-performance liquid chromatography of (A) noncore tryptic peptides of N-terminal fluorescein–L12 and (B) elastase peptides of coumarin-314–FPB–L7. The hatched peaks contain fluorescein (A) or coumarin-314 (B). The peaks denoted by a × in (B) contain some coumarin-314. For details, see text.

agreed with the spectra published by Melhuish (1973). The fluorescence spectra presented in this paper are corrected for the fluorescence and scattering of a solution without a fluorophore (at the fluorescence maximum always less than 10%) and are presented as arbitrary units per unit wavelength interval.

Quantum yields are determined relative to quinine sulfate in 0.05 M sulfuric acid by using a quantum yield of 0.51 for quinine sulfate (Chen, 1973).

Emission anisotropy $[(I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})]$ measurements were made with a polarizer–analyzer set of Aminco-Bowman. If necessary, the fluorescence spectra were corrected for the inner filter effect.

Analytical Ultracentrifuge. The molecular weights were determined by sedimentation equilibrium by using a Spinco Model E analytical ultracentrifuge equipped with interference optics. The high-speed, short-column procedure of Yphantis (1964) has been used at a rotor speed of 24 000 rpm.

Results and Discussion

Modification of L7/L12. In order to introduce fluorescent labels at specific positions in L7/L12, we have developed a two-step procedure. First, we introduce in the protein an aldehyde group, which is subsequently used for a specific reaction with a fluorescent hydrazine derivative. The finding to attach specifically an aldehyde to Lys-51 originated from a cross-linking study by Maassen et al. (1981) using 4-(6-formyl-3-azidophenoxy)butyrimidate. In analogy, we used the similar, photochemically more stable, compound 4-(4-formylphenoxy)butyrimidate (FPB-imidate), which reacts at pH 8.5 also predominantly with Lys-51 (see below). The introduction of an aldehyde at the N terminus of L12 is possible because of the presence of an unblocked N-terminal serine in L12 which can be oxidized easily by periodate (Clamp & Hough, 1965). Both types of aldehyde residues were modified with fluorescein hydrazine as well as with coumarin-314 hydrazide (see Figure 1). We found fluorescein hydrazine superior to the commercially available fluorescein thiosemicarbazide, which showed a slightly nonspecific, sluggish reaction. Because oxidation of the N-terminal serine by periodate will also oxidize part of the methionines (Knowles, 1965), the protein was reduced afterward with DTE.

Modification with fluorescein introduces an extra negative charge in the protein. Therefore, it is possible to separate the fluorescein–L7/L12 from unmodified and multiply modified protein by ion-exchange chromatography as described under Experimental Procedures, resulting in products with one fluorescein residue per protein monomer. The reaction of FPB–L7 with coumarin-314 hydrazide resulted in 0.86 coumarin-314 residue per L7. In the case of periodate-oxidized L12, 0.26 coumarin-314 residue per L12 was incorporated. Increased reaction times (up to 15 h) for coumarin-314 hydrazide with oxidized L12 resulted in a higher modification degree up to 0.49. Because of this low degree of modification, this product was not studied in detail.

Determination of the Modification Site in Coumarin-314–FPB–L7 and Fluorescein–FPB–L7. The following approaches were used in order to test whether the fluorescein and coumarin-314 moieties are attached to Lys-51, via the aldehyde group in FPB–L7.

The tryptic peptide map of fluorescein–FPB–L7 by the procedure described by Maassen et al. (1981) is similar to the peptide map of L7, except for fluorescein at the origin. This indicates a labeling at one of the two lysines (29 and 51) of the core peptides (Terhorst et al., 1973).

Coumarin-314–FPB–L7 was digested with elastase, and the peptides were separated by high-performance liquid chromatography. The resulting chromatogram of the elastase peptides of coumarin-314–FPB–L7 is shown in Figure 2B. The two prominent peaks have amino acid analyses corresponding with the sequence from residues 41 to 58 for peak B and with the sequence of residues 47–58 for peak C (Terhorst et al., 1973). Both peptides contain only one lysine, which is located at position 51. From the coumarin-314 extinction, we find that peaks B and C in Figure 2B represent about 75% of the total coumarin-314 extinction. About 15% of the extinction is found

in peak A, which has an amino acid analysis corresponding to residues 87–106. Minor labeling has thus taken place at Lys-95 and/or Lys-100. The remaining 10% of the coumarin-314 extinction is found in two small peaks (denoted by × in Figure 2B). These fractions do not give conclusive amino acid analysis. The labeling pattern of the coumarin-314 results from the reaction specificity of FPB-imidate with L7. This implies that also the fluorescein in fluorescein–FPB–L7 will have the same labeling pattern as coumarin-314 in coumarin-314–FPB–L7.

Because there are other lysines modified next to Lys-51, a small amount of L7 with more than one fluorescent moiety will also be present. For fluorescein–FPB–L7, we separated the protein with one fluorescein moiety per polypeptide chain from unmodified L7 and from protein with more than one fluorescein moiety. Using FPB–L7 with 0.9 FPB/L7, it is estimated that the isolated fluorescein–FPB–L7, having one fluorescein per polypeptide chain, will have its fluorescein for 89% at the Lys-51, 7% at the Lys-95 and/or Lys-100, and 4% at any other lysine.

Isoelectric focusing (see Figure 3a) for coumarin-314–FPB–L7 shows one band comigrating with unmodified L7. For fluorescein–FPB–L7, an extra negative charge is present, resulting in a lower isoelectric point. As can be seen from Figure 3a, lane 2, the low amount of fluorescein attached to another position as Lys-51 shows up as a minor band with a slightly different isoelectric point.

Determination of the Modification Site in N-Terminal Fluorescein–L12 and N-Terminal Coumarin-314–L12. The tryptic peptide map of N-terminal fluorescein–L12 (see Experimental Procedures) shows one peptide-containing fluorescence near the position of peptide T4 (Terhorst et al., 1972). In addition, the N-terminal peptide T_{12} is absent, in agreement with a modification at the N terminus. In addition, we separated the noncore tryptic peptides (see Experimental Procedures) also by high-performance liquid chromatography. The chromatogram is shown in Figure 2A. Only one peak contains fluorescein absorption, indicating a high specificity of the reaction. The amino acid analysis of this peak yields Thr, Ile, and Lys, in accordance with the N-terminal peptide Ser-Ile-Thr-Lys (Terhorst et al., 1973), showing specific labeling of the N-terminal serine.

As expected, the N-terminally acetylated L12, i.e., L7, did not show a detectable labeling (<0.04 label/L7) after periodate oxidation.

Isoelectric focusing (see Figure 3a) of N-terminal fluorescein–L12 and N-terminal coumarin-314–L12 shows in both cases one band; in the case of N-terminal fluorescein–L12, it is shifted to a lower isoelectric point because of the negative charge of the fluorescein. The result for the N-terminal fluorescein–L12 shows that no unmodified L12 or L12 with an oxidized N terminus (expected at the isoelectric point of L7) is present.

Modification of L10. Under nondenaturing conditions, ribosomal protein L10 is hard to handle (Dijk & Littlechild, 1979). For this reason, we kept L10 throughout the preparation in 6 M urea until the final experiments, e.g., reconstitution (see Experimental Procedures). L10 has one cysteine at position 70 (Heiland et al., 1976; Dovgas et al., 1976). In accordance with the fact that maleimides will react at pH 6.0 primarily with sulfhydryl groups (Means & Feeney, 1971), we found a labeling degree equal to the SH content determined by DTNB titration (Ellman, 1959). Because our L10 preparation contained only about 0.6 SH group per polypeptide chain, we isolated the reactive SH-containing fraction by

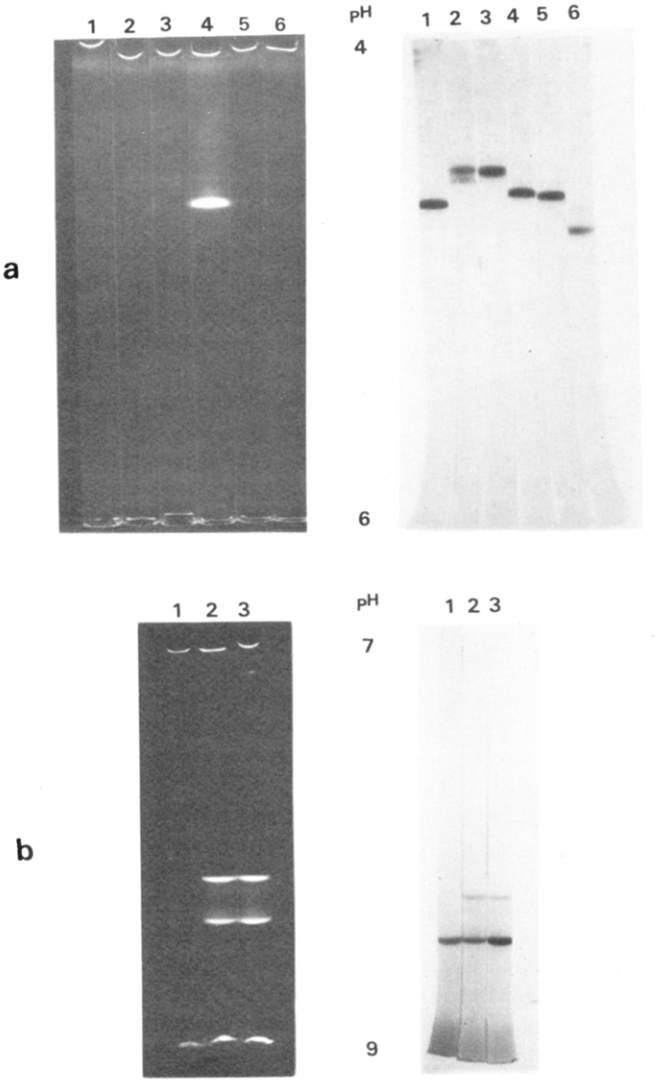

FIGURE 3: Isoelectric focusing of unmodified and modified L7/L12 (a) and L10 (b). The left panel shows the fluorescence under illumination with light of 366 nm and the right panel the Coomassie staining. (a) L7/L12 proteins are focused on a pH gradient from 4 to 6; the samples are 12 μg of (1) L7, (2) fluorescein–FPB–L7, (3) N-terminal fluorescein–L12, (4) coumarin-314–FPB–L7, (5) N-terminal coumarin-314–L12, and (6) L12. (b) L10 is focused on a pH gradient from 7 to 9; the samples are 12 μg of (1) L10, (2) DACM–L10, and (3) L10 + DACM–L10.

affinity chromatography on thiopropyl-Sepharose 6B. Next, the SH-containing L10 was modified with DACM (Figure 1d), resulting in 1.1 DACM/L10. This degree of modification is based on an ϵ_{max} of 24 200 M^{-1} cm^{-1}, which is the value of the open form of the DACM–2-mercaptoethanol adduct (Yamamoto et al., 1977). Under the same reaction conditions, the non-SH-containing fraction of L10 showed no reaction with DACM. Thus, we modified L10 specifically at Cys-70.

In isoelectric focusing (Figure 3b), the DACM–L10 shows two bands, one with the same isoelectric point as L10 and the other with a lower isoelectric point. This is due to the open and closed forms of the maleimide adducts (Yamamoto et al., 1977). In the open form, there is an extra carboxylate group, resulting in a lower isoelectric point.

Properties of the Modified Proteins. (A) Molecular Weight of Modified L7/L12. The L7/L12 protein forms under nondenaturing conditions a stable dimer (Möller et al., 1972). In order to test whether modification at Lys-51 or at the N terminus alters the dimeric structure, we performed sedimentation equilibrium experiments of L7, N-terminal fluor-

Table I: Molecular Weights Determined by Sedimentation Equilibrium at 24 000 rpm at 10 °C in GTPase Buffer

protein	mol wt
L7	23 000
N-terminal fluorescein-L12	20 000
coumarin-314-FPB-L7	24 000

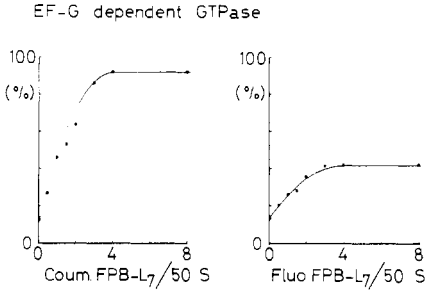

FIGURE 4: EF-G-dependent GTPase of coumarin-314-FPB-L7 (left) and fluorescein-FPB-L7 (right) added to P_0 cores. Activity of 100% is obtained by the addition of 6 equiv of L7 to P_0 cores. The curves are calculated based on the model that two dimers can bind to each 50S P_0 core; the binding to both sites is equally strong and independent of each other, while one dimer is sufficient for the final activity.

escein–L12, and coumarin-314–FPB–L7. The ln c vs. r^2 plots were linear and resulted in the molecular weights shown in Table I. It is clear that the modified proteins are also dimers. In the case of N-terminal fluorescein–L12, this means that after periodate oxidation, modification, and DTE reduction, the major fraction of the methionine residues apparently is not oxidized, since L7/L12 in which the methionine residues are oxidized behaves as a monomer (Gudkov & Behlke, 1978).

(*B*) *EF-G-Dependent GTPase.* Reconstitution of P_0 cores with modified L7/L12 gives a stimulation of the EF-G-dependent GTPase. In the case of coumarin-314–FPB–L7, fluorescein–FPB–L7 (Figure 4), and unmodified L7, the titration curves are the same; only the final level of activity is changed. Coumarin-314–FPB–L7 gives almost the same level of EF-G-dependent GTPase as the unmodified L7, while fluorescein–FPB–L7 gives about 40% of the activity. Thus, the negative charge and more bulky fluorescein group seem to disturb the EF-G-dependent GTPase activity while the coumarin-314–FPB group causes no significant interference. The shape of the titration curves (Figure 4) is consistent with a model of two equal binding sites, each for an L7/L12 dimer, and ribosomes with one dimer bound already show full EF-G-dependent GTPase activity. Thus, the GTPase data suggest that coumarin-314–FPB–L7 and fluorescein–FPB–L7 bind to 50S ribosomes in the same way as L7, i.e., in four copies (Subramanian, 1975). Besides, full EF-G-dependent GTPase is obtained if one modified L7/L12 dimer is bound, a situation similar to unmodified L7/L12 (Lee et al., 1981).

EF-G-dependent GTPase activity of P_0 cores as a function of increasing concentrations of N-terminal fluorescein–L12 gave a gradual rise in GTPase activity. In order to determine the relation between the EF-G-dependent GTPase and the amount of modified L12 bound, we incubated P_0 cores with increasing amounts of N-terminal fluorescein–L12. The reconstituted 50S particles were isolated by centrifugation through a sucrose cushion, and the EF-G-dependent GTPase and the amount of N-terminal fluorescein–L12 bound to the 50S particles were determined. These results are shown in Figure 5. It is seen that about one-third of the N-terminal fluorescein–L12 is able to bind to P_0 cores. The bound N-terminal fluorescein–L12 yields also maximal GTPase activity after binding of one dimer, the maximum activity being 70%

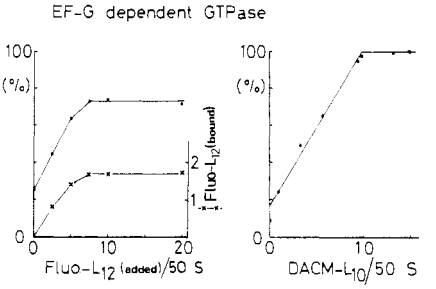

FIGURE 5: (Left) P_0 cores were incubated with the indicated amount of N-terminal fluorescein–L12, the 50S ribosomes were isolated by centrifugation through a sucrose cushion, and the amount of bound N-terminal fluorescein–L12 and the EF-G-dependent GTPase were determined. The EF-G-dependent GTPase is expressed relative to the addition of 6 equiv of L7 to P_0 cores. (Right) EF-G-dependent GTPase of P_{37} cores + L7 + L11 + the indicated amount of DACM-L10 (relative to the addition of 1.5 equiv of L10).

Table II: Polyphenylalanine Synthesis[a]

	% activity
P_0 cores	0
P_0 cores + L7	100
P_0 cores + fluorescein-FPB-L7	26
P_0 cores + coumarin-314-FPB-L7	63
P_0 cores + N-terminal fluorescein-L12	34
DACM-L10-P_0 cores	0
DACM-L10-P_0 cores + L7	93

[a] Effect of 8 equiv of L7 and modified L7/L12 on the polyphenylalanine synthesis of P_0 cores and 2 M P_0 cores, prereconstituted with 0.2 equiv of L11 and 1.0 equiv of DACM-L10. One hundred percent activity corresponds to 3.7 pmol of polyphenylalanine incorporated, and the core background (0%) corresponds to 0.37 pmol of polyphenylalanine incorporated.

of the value obtained with unmodified L7/L12. The fact that only one-third of the N-terminal fluorescein–L12 is able to reconstitute is probably caused by irreversible periodate oxidation of some methionine residues which are not involved in the formation of L7/L12 dimers. By isolating the N-terminal fluorescein–L12–50S ribosome, we select the fraction of the protein molecules which is able to bind. From the fact that this modified 50S ribosome is 70% active in EF-G-dependent GTPase, we conclude that the fraction of N-terminal fluorescein–L12 molecules, which still can bind to core particles, is bound in a functionally active way.

EF-G-dependent GTPase of P_{37} cores + L7 + L11 together with increasing concentrations of DACM–L10 is shown in Figure 5. Clearly DACM–L10 binds well and is fully active in this assay. This shows that DACM–L10, reconstituted in 50S ribosomes, is able to bind the L7 required for GTPase activity.

(*C*) *Polyphenylalanine Synthesis.* The effect of saturating concentrations of L7/L12 on the polyphenylalanine synthesis is shown in Table II. All modified proteins show a significant stimulation of the polyphenylalanine synthesis. The degree of stimulation correlates very well with the EF-G-dependent GTPase results (see above). Only the stimulation in the polyphenylalanine synthesis of the N-terminal fluorescein–L12 is somewhat lower than its stimulation of the EF-G-dependent GTPase. However, it is possible that the excess (8 equiv) of N-terminal fluorescein–L12 added in polyphenylalanine synthesis was not sufficient to saturate all binding sites in view of the partial binding of the N-terminal fluorescein–L12.

Stimulation of polyphenylalanine synthesis by L7 occurs only after binding of two L7 dimers (i.e., 4 equiv of L7) (W. Möller, P. I. Schrier, J. A. Maassen, A. Zantema, E. Schop, H. Reinalda, A. F. M. Cremers, and J. Mellema, unpublished

Table III: Amount of Modified L7/L12 or L10 Bound to 50S Cores As Determined by Isolation of 50 S by Pelleting through a Sucrose Cushion[a]

50 S	protein added	equiv added	equiv bound
P_0	coumarin-314-FPB-L7	2.0	1.41
P_0	coumarin-314-FPB-L7	6.0	1.31
P_0	fluorescein-FPB-L7	2.0	1.50
P_0	fluorescein-FPB-L7	5.0	1.76
P_0	N-terminal fluorescein-L12	20.0	1.73
P_0	[^{3}H]methyl-L7	5.0	2.09
P_0	[^{14}C]methyl-L7	6.0	1.76
DACM-L10-P_0	fluorescein-FPB-L7	5.0	1.66
DACM-L10-P_0	N-terminal fluorescein-L12	15.0	1.66
P_{37} + L11	DACM-L10	1.2	0.76

[a] The level of reconstitution is determined as described under Experimental Procedures.

results). Thus, the polyphenylalanine synthesis results from Table II suggest that the modified L7/L12's can bind in four copies to P_0 cores and that P_0 cores containing DACM-L10 can bind four copies of L7. The functional behavior of these specifically modified ribosomes shows their suitability for further studies.

(D) Binding. Using the appropriate cores, we found reconstitution of the L7/L12 derivatives as shown in Table III. Controls with intact 50S ribosomes showed at the most 10% binding with respect to values obtained with cores. Isolation of the reconstituted 50S ribosomes by centrifugation through a sucrose cushion results in a binding of somewhat less than two copies of these proteins. Because cores in general still contain about 10% residual L7/L12 and our modified L7/L12's are dimers, we will have close to one dimer of modified L7/L12 bound to our cores. This holds also for [^{3}H]methyl-L7 and [^{14}C]methyl-L7 which normally behave as unmodified L7. Only coumarin-314-FPB-L7 binds somewhat less to the P_0 cores. This may be caused by the presence of some unmodified L7 in this preparation, because unmodified L7 may bind somewhat faster than coumarin-314-FPB-L7.

Table III shows that addition of low concentrations of fluorescein-FPB-L7 and coumarin-314-FPB-L7 results in almost quantitative binding of the added protein, in agreement with the GTPase results. This means that these preparations are homogeneous, in contrast to N-terminal fluorescein-L12, of which only about one-third binds (see Figure 5).

The binding of one L7/L12 dimer, as determined by centrifugation through a sucrose cushion, is in apparent contrast to the normal binding of two dimers to the 50S ribosome (Subramanian, 1975). By analyzing our results of reconstitution, the picture arises of a strong and a weak dimer binding site as reported for the binding of L7/L12 to a complex of L11, L10, and 23S rRNA by Dijk et al. (1979). Our modified preparations will be slightly altered, resulting in a somewhat lower affinity. For the weak binding site, the binding of an L7/L12 dimer is not strong enough to allow isolation by centrifugation through a sucrose cushion. Therefore, the apparent binding of one dimer via the centrifugation assay is not in disagreement with the actual binding of two dimers. The binding of four copies of L7/L12 has indeed been demonstrated from the stimulation of polyphenylalanine synthesis (Table II). Also our energy transfer measurements suggest a binding of two dimers [see Zantema et al. (1982)].

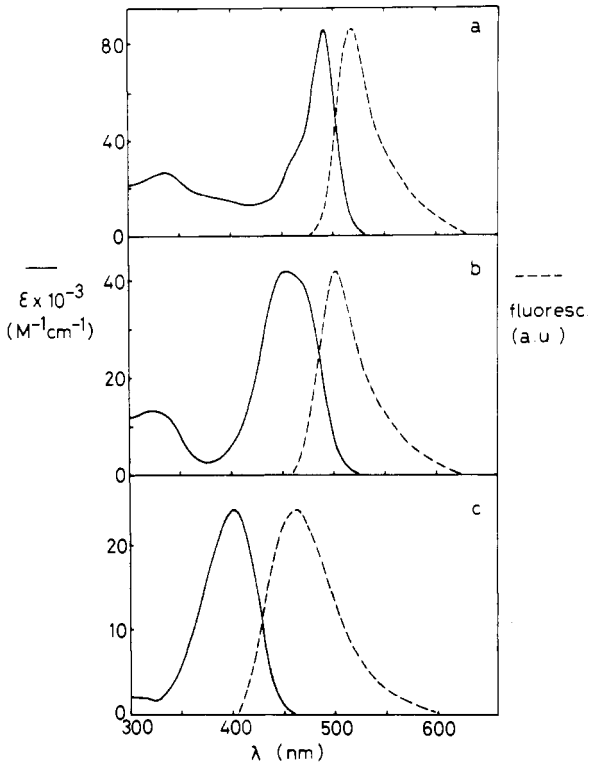

FIGURE 6: Absorbance (solid line) and fluorescence (dashed line) spectra of N-terminal fluorescein-L12 (a), coumarin-314-FPB-L7 (b), and DACM-L10 (c). The fluorescence spectra were scaled to the same height as the absorbance spectra. For the quantitative comparison of the fluorescence, see the quantum yields in Table IV.

Spectroscopic Properties. The fluorescence pattern of isoelectric focusing gels shows that only coumarin-314-FPB-L7 and DACM-L10 are strongly fluorescent (Figure 3). More detailed information is given in Table IV. The absorbance maximum of fluorescein hydrazine (489 nm) shifts to longer wavelength upon formation of a hydrazone. As expected, this effect is more pronounced in the case of FPB-L7 in which a hydrazone of an aromatic aldehyde is formed. Both products are only weakly fluorescent. Reconstitution does not shift the maxima of absorbance or fluorescence; only the quantum yield alters. For fluorescein-FPB-L7, the quantum yield increases, whereas for the N-terminal fluorescein-L12 the low fluorescence decreases even further as a result of reconstitution (Table IV). The absorbance and fluorescence spectra of N-terminal fluorescein-L12 are shown in Figure 6a. The coumarin-314-FPB-L7 is very well fluorescent (Table IV). Upon re-

Table IV: Spectroscopic Properties of the Modified Proteins

	λ_{max} (nm)		ϵ ($\times 10^3$ M^{-1} cm^{-1})		emission max (nm)		quantum yield	
	free protein	in 50 S	free protein	in 50 S	free protein	in 50 S	free protein	in 50 S
fluorescein-FPB-L7	495	495	89	89[c]	515	515	0.008	0.011
N-terminal fluorescein-L12	491	491	86	86[c]	518	518	0.012	0.006
coumarin-314-FPB-L7	453	453	42[a]	42[a,c]	503	503	0.23	0.10
N-terminal coumarin-314-L12	458	nd[d]	42[a]	nd	498	nd	0.035	nd
DACM-L10	402	*b*	24.2[a]	24.2[a,c]	463	472	0.31	0.25

[a] Literature, see Experimental Procedures. [b] Not possible to measure. [c] The same value as for the free protein is assumed. [d] nd = not determined.

Table V: Emission Anisotropy of Modified 50S Ribosomes

	emission anisotropy
fluorescein-FPB-L7-50 S	0.16
N-terminal fluorescein-L12-50 S	0.24
coumarin-314-FPB-L7-50 S	0.31
DACM-L10-50 S	0.32

constitution, the fluorescence decreases, without a shift in the absorbance and fluorescence maxima. The spectra for coumarin-314-FPB-L7 are shown in Figure 6b.

The DACM-L10 absorbance and fluorescence spectra are shown in Figure 6c. As a result of reconstitution, the fluorescence maximum shifts 9 nm to a longer wavelength while the quantum yield decreases somewhat (see Table IV).

In order to get information on the mobility of the fluorescent probes, we measured the static emission anisotropy shown in Table V. Actually one has to measure the limiting emission anisotropy, but because of the large size of the 50S ribosome, these values are reasonable representive for the limiting anisotropy (Fairclough & Cantor, 1979). Similar values have been measured by other groups for the fluorescent 50S particles (Odom et al., 1980; Fairclough & Cantor, 1979).

Concluding Remarks. In this paper, we have described a new method for preparing ribosomal proteins containing fluorescent probes at unique locations. These modified proteins reconstitute ribosomal activities, indicating a native situation which makes these compounds useful for the study of structure and dynamics of the ribosome. The coumarin derivatives are well fluorescent and suitable as energy donors for the fluorescein absorption, as can be seen from Figure 6. The reconstituted donor–acceptor couples yield R_0 values (the distance at which 50% energy transfer occurs) of about 45 Å for the DACM-L10 to fluorescein-L7/L12 couple and about 38 Å for the coumarin-314-FPB-L7 to fluorescein-L7/L12 couple. A first series of experiments in order to determine the distances between the different sites is presented in the following paper (Zantema et al., 1982) in this issue.

Acknowledgments

We thank Ing. W. J. M. Pluijms and Dr. R. Amons for assisting in peptide and amino acid analyses.

References

Arai, K. I., Kawakita, M., & Kaziro, Y. (1972) *J. Biol. Chem. 247*, 7029–7037.

Cantor, C. R., Huang, K.-H., & Fairclough, R. (1974) in *Ribosomes* (Nomura, M., Tissières, A., & Lengyel, P., Eds.) pp 587–599, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Chambliss, G., Craven, G. R., Davies, J., Davis, K., Kahan, L., & Nomura, M., Eds. (1980) *Ribosomes, Structure, Function and Genetics*, University Park Press, Baltimore.

Chen, R. F. (1967) *Anal. Biochem. 20*, 339–357.

Chen, R. F. (1973) *NBS Spec. Publ. (U.S.) No. 378*, 183–196.

Clamp, J. R., & Hough, L. (1965) *Biochem. J. 94*, 17–24.

Dijk, J., & Littlechild, J. (1979) *Methods Enzymol. 59*, 481–502.

Dijk, J., Garrett, R. A., & Müller, R. (1979) *Nucleic Acids Res. 6*, 2717–2729.

Dovgas, N. V., Vinokurov, L. M., Velmoga, I. S., Alakhov, Yu. B., & Ovchinnikov, Yu. A. (1976) *FEBS Lett. 67*, 58–61.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Fairclough, R. H., & Cantor, C. R. (1979) *J. Mol. Biol. 132*, 575–586.

Gesteland, R. F. (1966) *J. Mol. Biol. 18*, 356–371.

Gudkov, A. T., & Behlke, J. (1978) *Eur. J. Biochem. 90*, 309–312.

Hamel, E., Koka, T., & Nakamoto, T. (1972) *J. Biol. Chem. 247*, 805–814.

Hardy, S. J. S., Kurland, C. G., Voynow, P., & Mora, G. (1968) *Biochemistry 8*, 2897–2905.

Heiland, I., Brauer, D., & Wittmann-Liebold, B. (1976) *Hoppe-Seyler's Z. Physiol. Chem. 357*, 1751–1770.

Kaltschmidt, E., & Wittmann, H. G. (1970) *Anal. Biochem. 36*, 401–412.

Kang, C., Wells, B., & Cantor, C. R. (1979) *J. Biol. Chem. 254*, 6667–6672.

Knowles, J. R. (1965) *Biochem. J. 95*, 180–190.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Langlois, R., Lee, C. C., Cantor, C. R., Vince, R., & Pestka, S. (1976) *J. Mol. Biol. 106*, 297–313.

Lee, C. C., Cantor, C. R., & Wittmann-Liebold, B. (1981) *J. Biol. Chem. 256*, 41–48.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Maassen, J. A. (1979) *Biochemistry 18*, 1288–1292.

Maassen, J. A., Schop, E. N., & Möller, W. (1981) *Biochemistry 20*, 1020–1025.

Machida, M., Ushijima, N., Machida, M. I., & Kanaoka, Y. (1975) *Chem. Pharm. Bull. 23*, 1385–1386.

Means, G. E., & Feeney, R. E. (1971) *Chemical Modification of Proteins*, Holden-Day, San Francisco.

Melhuish, W. H. (1973) *NBS Spec. Publ. (U.S.) No. 378*, 137–150.

Möller, W., Castleman, H., & Terhorst, C. P. (1970) *FEBS Lett. 8*, 192–196.

Möller, W., Groene, A., Terhorst, C., & Amons, R. (1972) *Eur. J. Biochem. 25*, 5–12.

Moore, P. B. (1980) in *Ribosomes, Structure, Function and Genetics* (Chambliss, G., et al., Eds.) pp 111–133, University Park Press, Baltimore.

Odom, O. W., Robbins, D. J., Lynch, J., Dottavio-Marin, D., Kramer, G., & Hardesty, B. (1980) *Biochemistry 19*, 5947–5954.

Schrier, P. I., Maassen, J. A., & Möller, W. (1973) *Biochem. Biophys. Res. Commun. 53*, 90–98.

Subramanian, A. R. (1975) *J. Mol. Biol. 95*, 1–8.

Terhorst, C., Wittmann-Liebold, B., & Möller, W. (1972) *Eur. J. Biochem. 25*, 13–19.

Terhorst, C., Möller, W., Laursen, R., & Wittmann-Liebold, B. (1973) *Eur. J. Biochem. 34*, 138–152.

Van Agthoven, A. J., Maassen, J. A., & Möller, W. (1977) *Biochem. Biophys. Res. Commun. 77*, 989–998.

Yamamoto, K., Sekine, T., & Kanaoka, Y. (1977) *Anal. Biochem. 79*, 83–94.

Yphantis, D. A. (1964) *Biochemistry 3*, 297–317.

Zantema, A., Maassen, J. A., Kriek, J., & Möller, W. (1982) *Biochemistry* (following paper in this issue).