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Dimer Structure of the Ribosomal Protein L7/L12 Probed by Energy Transfer†

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ABSTRACT: In dimers of the ribosomal protein L7/L12 from *Escherichia coli*, we have measured the transfer of excitation energy between fluorescent probes and derived therefrom the distances between the labeling sites. *N*-[7-(Dimethylamino)-4-methylcoumarinyl]maleimide (DACM) served as the energy donor to fluorescein. The probes were coupled covalently and with great specificity either to the N-terminal serine or to Lys-51. In one set of experiments, DACM and fluorescein were located on different chains of the L7/L12 dimer. The distance between the two labels was 45 ± 5 Å at the position of the Lys-51 residues and 33 ± 5 Å at the N-termini. These long distances provide additional evidence for a staggered conformation of the L7/L12 dimer. Comparison of the distances determined in free L7/L12 with those determined in L7/L12 bound to 50S ribosomes showed that no major changes occur in the probed region of L7/L12 upon binding to the ribosome. We also measured energy transfer in L12 labeled both with DACM, at position 51, and with fluorescein, at the N-terminus. In dilute solution, this protein was monomeric; the distance between the labels was at least 75 Å. On 50S ribosomes, a distance of about 45 Å was measured, but since almost 2 equiv of the labeled protein was bound, most likely the distance between DACM and fluorescein on different polypeptides was found. A model of the dimer structure of L7/L12 is presented, which is consistent with all experimental data available.

Like their counterparts from eukaryotic and other prokaryotic ribosomes, the acidic proteins L7 and L12 from *Escherichia coli* are present in multiple copies per ribosome (Kurland, 1977; Matheson et al., 1980; Liljas, 1982). Localized in a stalklike projection of the ribosome (Lake, 1976; Strycharz et al., 1978; Möller et al., 1983), they appear to form a flexible region (Tritton, 1978; Gudkov et al., 1982). They modulate the interaction of the ribosome with several of the initiation, elongation, and termination factors, and as such, they are indispensable for speed and fidelity of protein synthesis (Möller, 1974; Pettersson & Kurland, 1980). Except for the N-terminal acetyl group of L7, the two proteins are the same (Terhorst et al., 1972). Since L7 and L12 are also identical functionally (Möller, 1974), they are normally referred to as L7/L12. In solution, L7/L12 forms dimers (Möller et al., 1972; Wong & Paradies, 1974; Gudkov et al., 1977). On the ribosome, the four copies of L7/L12 (Subramanian, 1975; Pettersson et al., 1976) are bound in the form of two dimers (Koteliansky et al., 1978; Zantema et al., 1982a).

Because of its remarkable features, L7/L12 has attracted much attention. Spectroscopic and hydrodynamic studies on the free dimers indicated an elongated structure (Wong & Paradies, 1974; Österberg et al., 1976) with a high content of α-helix (Dzionara, 1970; Möller et al., 1970; Gudkov et al., 1978; Luer & Wong, 1979). The formation of dimers was found to be due to aggregation of the N-terminal half of the molecule (Van Agthoven et al., 1975; Gudkov & Behlke, 1978; Koteliansky et al., 1978). The three-dimensional structure of the crystallizable C-terminal half has been elucidated (Leijonmarck et al., 1980). Although in most of the resulting

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models the two chains of the L7/L12 dimer are lain in the same direction and perfectly side by side (Luer & Wong, 1979; Liljas, 1982), evidence has been found that the two chains are shifted with respect to each other (Maassen et al., 1981).

The method of long-range energy transfer between fluorescent probes (Förster, 1967) has the advantage that it allows structural studies of the proteins in situ, i.e., on the ribosome (Fairclough & Cantor, 1978; Stryer, 1978). Using this method, we investigated the localization of the two L7/L12 dimers relative to L10 and to each other (Zantema et al., 1982; Thielen et al., 1984). The energy donor *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide (DACM)[1] was coupled to Cys-70 of L10, to the N-terminal serine residue of L12, or to Lys-51 of L7. Fluorescein, the acceptor group, was coupled to the N-terminus of L12 or to Lys-51 of L7. After purification, the labeled L7/L12 contained one label per polypeptide chain, i.e., two labels, either two donors or two acceptors, per dimer of L7/L12. For the interpretation of the energy transfer observed between the labeled proteins on 50S ribosomes, it was important to know where the probes are localized in the L7/L12 dimers (Thielen et al., 1984).

In order to investigate the internal structure of the L7/L12 dimer, we prepared dimers containing one DACM group and one fluorescein group on different chains. The distance separating the labels was measured both at the position of the N-termini and at the position of the Lys-51 residues. In a derivative with both chromophores attached to the same polypeptide chain, the distance between DACM at Lys-51 and fluorescein at the N-terminus was measured. All distances were measured in solution and on the 50S ribosome, which allowed us to compare the structure of free L7/L12 with that of L7/L12 in situ.

Experimental Procedures

General Procedures. Ribosomes were isolated from *E. coli* MRE600 according to Gesteland (1966) and separated into subunits by zonal centrifugation at low Mg^{2+} concentration (Möller et al., 1970). 50S subunits lacking L7/L12, i.e., P_0 cores, were prepared by addition of cold ethanol up to 50% v/v to a 1 M ammonium chloride solution of 50S ribosomes at 0 °C (Hamel et al., 1972). Ribosome concentrations were determined from the absorbance at 260 nm; 1 A_{260} unit corresponds to 25 pmol of 70S, 39 pmol of 50S, and 69 pmol of 30S ribosomes.

Proteins L7 and L12 were isolated in 6 M urea (Möller et al., 1972). Protein concentrations were measured with insulin as a standard (Lowry et al., 1951); the molecular weight of a monomer of L7 or L12 is 12 200. All preparations of proteins and ribosomes were stored at −70 °C in TMN buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM magnesium acetate, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol. The same buffer was used for the fluorescence experiments.

EF-G was isolated according to Arai et al. (1972). Measurements of EF-G-dependent GTPase activity were performed as described by Schrier et al. (1973). Whenever the term equivalents of L7/L12 is used, we refer to the number of L7 or L12 polypeptides relative to the number of ribosomal particles. Hence, if 1 equiv is added, on the average half a dimer of L7/L12 is present per 50S ribosomal subunit. Repletion of P_0 cores was accomplished by incubating the cores for 5 min at 37 °C with 12 equiv of L7 or L12. L7/L12–50S complexes were isolated by sedimentation (5 h at 55 000 rpm in a Beckman SW-60 rotor) through a 25% sucrose cushion.

Labeling Procedures. The purified proteins L7 and L12 were labeled as described before (Zantema et al., 1982a; Maassen et al., 1983). L7 was labeled with a hydrazine derivative of fluorescein or with DACM at the position of Lys-51, with a specificity of about 90%. Both labels were coupled to L7 by means of imidate compounds, (formylphenoxy)butyrimidate in the case of fluorescein and thiopropylimidate in the case of DACM. After oxidation with periodate, the N-terminal serine of L12 was reacted either directly with fluoresceinylhydrazine or via a thiopropylhydrazide spacer with DACM. The SH-containing precursors of DACM(1)L12 and DACM(51)L7 were freed from unmodified protein by affinity chromatography on thiopropyl-Sepharose 6B. We made use of the negative charge of the fluorescein moiety to remove unlabeled material from the fluorescein-labeled proteins, Fluo(1)L12 and Fluo(51)L7. The products thus obtained contained one label, either DACM or fluorescein, per polypeptide of L7 or L12. The extinction coefficients of the protein-bound probes were similar to those of the free probes, i.e., 85 000 M^{-1} cm^{-1} at 495 nm for fluorescein and 24 000 M^{-1} cm^{-1} at 395 nm for DACM.

Mixed dimers of L7, consisting of one DACM-labeled polypeptide and one fluorescein-labeled polypeptide, were prepared as follows. DACM(51)L7 and Fluo(51)L7 were monomerized in TMN buffer containing 6 M guanidinium chloride and mixed in varying ratios. The guanidinium chloride was removed on Sephadex G-25. The L7 dimers formed were freed from monomeric material by gel filtration on a 144 × 1.0 cm column of Sephacryl-200 with a flow rate of about 0.033 mL/min. The dimer fractions were combined and concentrated on a small DEAE-Sepharose 6B column. Absorption spectra were recorded on a Beckman Acta MVI and deconvoluted by hand into the composing spectra of fluorescein and DACM. Mixed dimers of DACM(1)L12 and Fluo(1)L12 were prepared in the same way.

We also prepared double-labeled L12 containing both a donor and an acceptor on the same polypeptide chain. The Lys-51 residue of Fluo(1)L12 was labeled with DACM in the same way as in the preparation of (single-labeled) DACM-(51)L7, with omission of the thiopropyl-Sepharose step.

Energy Transfer. Fluorescence was measured at 18 °C as described before (Thielen et al., 1984). The absorption by DACM at the wavelength of excitation, 395 nm, was measured very carefully. The quantum yields of DACM emission were determined by integration of the corrected emission spectra. Tetraphenylbutadiene embedded in a solid matrix (Starna Ltd., U.K.) was used as the common fluorescence standard; its fluorescence was calibrated once a month against quinine sulfate in 0.1 N sulfuric acid (Chen, 1973) and proved to be very stable.

From the decrease of DACM fluorescence induced by fluorescein, the energy transfer was calculated from

$$E_m = 1 - Q_m/Q_0 \quad (1)$$

in which E_m stands for the measured energy transfer, Q_0 is the unquenched quantum yield of DACM, and Q_m is the quantum yield measured in the presence of fluorescein. Hence, we compared the fluorescence yield of the double-labeled L7/L12 proteins with the fluorescence yield of the proteins labeled with only DACM at the same position. In practice, this was done by comparison of the integrated emission spectra

[1] Abbreviations: DACM, *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide; DACM(1)L12 and Fluo(1)L12, L12 with DACM and fluorescein N-terminally bound; DACM(51)L7 and Fluo-(51)L7, derivatives of L7 with the probes at Lys-51; Fluo(1)DACM-(51)L12, L12 with a fluorescein group at the N-terminus and a DACM group at Lys-51; EF-G, elongation factor G; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

as well as by comparison of the emission intensities at the wavelength of maximum DACM emission. Since the emission spectrum of DACM did not change upon addition of the fluorescein group, this can be done safely. For every sample, the fluorescence yield was measured 3 times by both methods. The average of the six results was used to calculate the energy transfer observed.

After mixing of DACM-labeled L7/L12 with fluorescein-labeled L7/L12, not only mixed dimers (DF) but also homogeneous dimers (DD and FF) are formed. To find the extent of energy transfer (E) within a DF-type dimer, the experimentally determined energy transfer, E_m, has to be divided by the fraction of donor molecules having an acceptor molecule in the same dimer. On the assumption of a random formation of DF- and DD-type dimers, the fraction of DACM molecules present in DF dimers is statistically given by

$$\frac{DF}{D_{total}} = \frac{1}{1+r} \tag{2}$$

in which r represents the ratio of DACM-labeled to fluorescein-labeled L7 or L12. E is then found from

$$E = (1+r)E_m \tag{3}$$

or from a plot of $1/E_m$ against the mixing ratio r:

$$\frac{1}{E_m} = \frac{1}{E} + \frac{1}{E}r \tag{4}$$

From the energy transfer results, distances were calculated according to Förster (1967):

$$R = R_0\left(\frac{1}{E} - 1\right)^{1/6} \tag{5}$$

R is the distance measured. R_0 is the distance at which 50% energy transfer would occur between the donor–acceptor couple used; it is given in angstroms by

$$R_0 = 9790(J\kappa^2 Q_0 n^{-4})^{1/6} \tag{6}$$

n is the refractive index of the medium (about 1.4). J, the overlap integral, is determined by the degree of spectral overlap of donor emission and acceptor absorbance [see, e.g., Zantema et al. (1982b)]. It is difficult to measure κ^2, the orientation factor, directly. Therefore R_0 is usually calculated with a value of $^2/_3$ for κ^2. Probability limits of κ^2, and hence also for R_0, can be derived from the anisotropies of donor and acceptor emission. We used the conversion tables of Haas et al. (1978) and found at all positions of the probes a resulting uncertainty in the determination of R_0 of ±10% [cf. Thielen et al. (1984)].

Results

During the preparation, some of the labeled L7 and L12 lost the ability to form dimers. Since the presence of monomeric material would affect the measurement of intradimer energy transfer, the monomers were removed by gel filtration on Sephacryl-200. About 10% of L7 labeled at Lys-51 and about 30% of the N-terminally labeled L12 was monomeric [cf. Thielen et al. (1984)].

In order to find out if the labeled proteins were still capable of binding functionally to 50S particles deprived of L7/L12, i.e., P_0 cores, we routinely performed measurements of EF-G-dependent GTPase activity of P_0 cores reconstituted with increasing amounts of L7/L12. The results are shown in Figure 1. The shape of the titration curve obtained with unlabeled L7 or L12 has been discussed before (Lee et al., 1981; Möller et al., 1983). The activity of the mixed dimers was about the same as that of the composing homogeneous

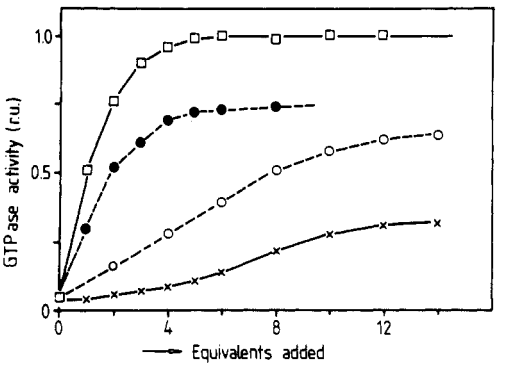

FIGURE 1: EF-G-dependent GTPase activity of P_0 cores reconstituted with 30S particles and increasing amounts of unlabeled L7/L12 (open squares) or of L7/L12 labeled with DACM and fluorescein. The GTPase activities restored by the mixed dimers were independent of the ratio of DACM(51)L7 to Fluo(51)L7 (closed circles) and of DACM(1)L12 to Fluo(1)L12 (open circles). The crosses denote the activities restored by Fluo(1)DACM(51)L12.

dimers (Thielen et al., 1984) and showed little, if any, variation with the ratio of DACM to fluorescein in the dimer preparations. Like the unlabeled proteins, L7 labeled at Lys-51 showed about maximal restoration of GTPase activity, when 4 equiv was added to the cores. However, maximal restoration of activity by N-terminally labeled L12 required about 3-fold higher excess. The maximal EF-G-dependent GTPase activity induced by the labeled proteins was about 70% of the control value, found with unlabeled L7 or L12, and was independent of r and of whether Lys-51 or the N-terminus was labeled.

The preparation of double-labeled L12 with both DACM and fluorescein attached to the same polypeptide chain started from 1:1 labeled Fluo(1)L12. Modification of its Lys-51 was done as for the preparation of DACM(51)L7. To avoid losses of L12, the SH-containing intermediate (Maassen et al., 1983) was not purified on thiopropyl-Sepharose. The final product was labeled to 100% at its N-terminus with fluorescein, and to about 75% at Lys-51 with DACM. The incomplete labeling with DACM has no consequences for the interpretation of the fluorescence experiments, because energy transfer was determined from the quenching of donor emission. In this kind of experiment, only the degree of labeling with fluorescein, our energy acceptor, is important [cf. Epe et al. (1983)]. On passage through Sephacryl-200, the double-labeled L12 proved to be monomeric, even after treatment with dithioerythritol to ascertain reduction of the methionines (Gudkov & Behlke, 1978). The maximum EF-G-dependent GTPase activity restored to P_0 cores by this protein was about 35% (Figure 1).

The emission yield of DACM was determined both from the intensity in the emission maximum, where interference by the fluorescein emission is null, and from the fluorescence yield integrated over the whole spectrum. For the preparations containing both DACM and fluorescein, the absorption by DACM was found by analysis of the absorption spectra; an example is shown in Figure 2. Energy transfer was calculated with the aid of eq 1; the emission yield of DACM in the preparations containing DACM and fluorescein was compared with the emission yield of DACM at the same labeling site in the absence of fluorescein. As a control, we incorporated unlabeled L7 or L12 together with DACM-labeled protein into mixed dimers. The emission yield of DACM appeared to be independent of whether a second DACM group was present in the same dimer or not. So it seems safe to conclude that the quenching induced by the introduction of a fluorescein group in a dimer is solely due to the occurrence of energy transfer from DACM to fluorescein. The measured distances

Table I: Energy Transfer and Distances in Double-Labeled L7/L12 Dimers

prepn	distance	Q_0(DACM)	E^a	R_0 (Å)^b	R (Å)^c
mixed L7	Lys-51 → Lys-51	0.27	0.57 ± 0.03^d	47	45 ± 5
mixed L12	N-terminus → N-terminus	0.17	0.64 ± 0.07^d	36	33 ± 5
Fluo(1)DACM(51)L12	Lys-51 → N-terminus				
	in solution	0.27	<0.03	47	>75
	on 50 S	0.27	0.35 ± 0.02	47	52 ± 5
			(0.70 ± 0.04)^e		(41 ± 4)^e

^{a}Energy transfer, if necessary corrected for the 90% specificity of Lys-51 labeling. ^{b}Assuming a value of $^2/_3$ for κ^2. ^{c}Uncertainty in orientation factor included. ^{d}From Figure 3. ^{e}Assuming dimer with shifted chains.

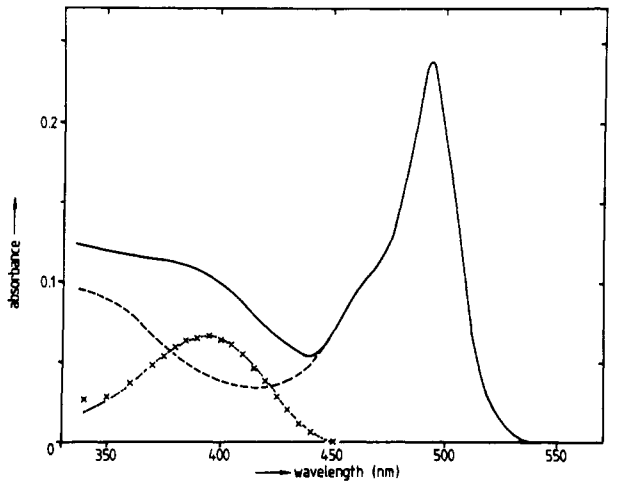

FIGURE 2: Absorption spectra of the dimer preparation obtained by mixing DACM(1)L12 and Fluo(1)L12 in a ratio of 1.0 (solid line) and of Fluo(1)L12 (dashed line, normalized at 495 nm). The absorption remaining after subtraction of the latter from the former spectrum is indicated by the crosses and matches the absorption spectrum of DACM(1)L12 (dotted line).

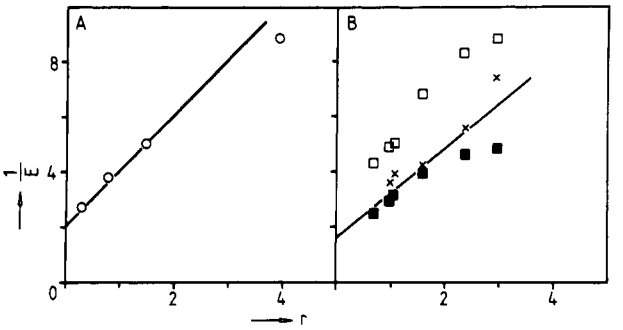

FIGURE 3: Reciprocal of the measured energy transfer plotted against the mixing ratio of DACM(51)L7 to Fluo(51)L7 (A) and of DACM(1)L12 to Fluo(1)L12 (B). In (A), the results obtained in solution and on 50S ribosomes were averaged. In (B), the results are shown separately for the dimers in solution (crosses) and for the dimers bound to 50S ribosomes (open squares); the closed squares were calculated from the open squares to account for differences in R_0 (see the text). The precision of the measurements of $1/E$ was highest for values of r around 1 and 2 (about ±0.2) and decreased on both sides (about ±0.5 at r = 0.3, and about ±1 at r = 4). The straight lines were drawn such that the slopes equalled the intercepts (cf. eq 4).

(see below) also exclude a possible interaction of the labeled sites with each other.

It has been shown before (Zantema et al., 1982a,b) that after reconstitution of P_0 cores with L7 or L12 and isolation of the ribosomes by sedimentation through a sucrose cushion, only one L7 or L12 dimer remains bound. The second binding site, called the weak site, is emptied in the absence of free dimers. We measured the energy transfer in the L7 and L12 proteins in solution as well as bound to the strong site of 50S ribosomes. Similar results were obtained when the proteins were added to the weak binding site of 50S subunits pretreated with unlabeled L7 to occupy the strong binding site.

The results obtained with the mixed dimers composed of DACM(51)L7 and Fluo(51)L7 are shown in Figure 3A. Since the yield and the spectrum of DACM emission and the spectrum of fluorescein absorption are left unchanged by binding of the proteins to 50S particles (Thielen et al., 1984), the R_0 value of this donor–acceptor couple is the same in solution and on the ribosome. Therefore, differences between the energy transfer values measured in solution and on the ribosome would reflect conformational changes in the dimers induced by binding to the ribosomes. However, no significant differences were observed between the two sets of data. Hence, the two labels bound to the Lys-51 residues apparently do not rearrange upon binding. In Figure 3A, for each value of r the average of the two results was plotted. The actual energy transfer between DACM and fluorescein attached to the two lysine-51 molecules of an L7 dimer was determined from the intercept of the straight line and is shown in Table I.

When energy transfer was measured between the labels attached to the N-termini of L12, the results found for the 50S-bound proteins were different from those obtained in solution (Figure 3B). This finding does not immediately imply different structures. In accordance with an earlier report (Thielen et al., 1984), the emission yield of N-terminally bound DACM was about halved, when the labeled L12 was bound to the 50S subunit. In solution, DACM(1)L12 had a quantum yield of 0.17; bound to the ribosome, it had a quantum yield of 0.084. As a result, the R_0 for energy transfer between DACM and fluorescein was 36 Å in solution and 32 Å on the 50S ribosome. With the aid of eq 5, we calculated the fraction of the excitation energy, which would be transferred in the ribosome-bound dimers, in case the emission yield of DACM was as high on the ribosome as it was in solution. The values thus found (Figure 3B, closed squares) match much better with the values determined for the L12 dimers in solution. The interchain distances, which can be calculated separately from the two sets of data, only differ by about 2 Å. Therefore, we took all data together and calculated the straight line shown in Figure 3B. The energy transfer values between the N-termini derived therefrom and the distances found are given in Table I.

The lower half of Table I shows the energy transfer in the preparation of Fluo(1)DACM(51)L12. The discrepancy between the values determined in solution and on the ribosome, for the distance between the N-terminal fluorescein and DACM linked to Lys-51, will be discussed in the following section.

Discussion

As the measured EF-G-dependent GTPase activities showed, the mixed L7 and L12 dimers had retained their ability to bind to P_0 cores and to form active 50S subunits. Compared to the L7/L12 dimers labeled with only DACM or fluorescein (Thielen et al., 1984), they were just as active in restoring the

GTPase activity. The lower affinity of the N-terminally labeled proteins for binding to 50S core particles has been noted before (Zantema et al., 1982a,b; Thielen et al., 1984) and most likely is due to the presence of the probes in a region involved in the binding of L7/L12 to L10 and the rest of the 50S subunit.

The preparation of the L7 and L12 dimers containing a fluorescence donor and a fluorescence acceptor, DACM and fluorescein, respectively, on different polypeptides, involves an uncontrolled step, the random formation of dimers upon removal of guanidinium chloride. After chromatography on Sephacryl-200, the composition of the dimer fraction might be different from the composition of the monomer fraction. Analysis of the absorption spectra of the mixed dimers always yielded a ratio of DACM to fluorescein which closely resembled the mixing ratio. On dimerization, not only the wanted DACM/fluorescein dimers (DF dimers) but also homogeneous dimers with two DACM or two fluorescein groups (DD and FF dimers) were formed. In the fluorescence experiments, the emission of all DACM molecules, both in DF- and in DD-type dimers, is detected. Therefore, the measured energy transfer has to be extrapolated to 100% DF dimers. We assumed that the DACM-labeled L7 or L12 had no preference for association either with itself or with fluorescein-labeled protein. In general, the relationship between the fraction of DF-type dimers formed and the mixing ratio r is very complex. When plotted as in Figure 3, this relationship deviates significantly from linearity only if a broad range of r values is taken into consideration. The energy transfer measurements can be done with sufficient precision only in a narrow range around $r = 1$. So our experimental system does not allow the results to be fitted with a theoretical curve and the equilibrium between homogeneous (DD + FF) and heterogeneous (DF) dimers to be determined. The absorption and emission spectra gave no evidence for an interaction of the probes; also, there was no evidence from the GTPase measurements for different tertiary structures of the DACM-labeled and the fluorescein-labeled L7/L12. Hence, the actual distribution of DACM over DD- and DF-type dimers will not be far off from the random distribution.

In the dimers of L7 and L12, we determined the distances between the two N-termini and between the two Lys-51 residues, or more precisely between, in either case, two covalently bound probes. Both distances were measured in the dimers free in solution, and in the dimers bound to the strong binding site of the 50S subunit. The results showed that the structure of the dimers, at least as far as the N-terminal half is concerned, is essentially the same in solution as on the ribosome. At the N-termini, a small change may take place upon binding, but the slightly different energy transfer observed (Figure 3B) could also reflect a change in the orientation of the transition moments of the probes with respect to each other. This factor determines the actual value of the orientation factor κ^2, and thereby affects R_0.

The composing protein chains of an L7/L12 dimer are arranged in a parallel fashion [for a summary of the evidence available, see Liljas (1982)]. Even if the size of the probes and the length of the spacers, used to connect the probes to the proteins, are taken into account, the distances listed in the top half of Table I are remarkably long. The N-terminal distance, 33 ± 5 Å, is especially meaningful in this respect. Since at this position fluorescein is coupled directly to the protein chain, and DACM is coupled via a small thiopropylhydrazide group, the N-termini must be at least about 25 Å apart. It may be that the two chains of a dimer are far apart at the positions where the distances were measured, i.e., residues 1 and 51, and only interact at a position somewhere in between. But attempts to ascribe the dimerization behavior of L7/L12 to a certain region of the protein only led to the conclusion that apparently the whole stretch between residues 1 and 73 is involved (Gudkov & Behlke, 1978; Koteliansky et al., 1978; Schop & Maassen, 1982). On the other hand, our data could also reflect a staggered alignment of the two interacting L7/L12 chains. The idea of two parallel, shifted chains was put forward by Maassen et al. (1981) on the basis of the preferential cross-linking of lysines-29 and -51 on different chains of the L7/L12 dimer. The symmetric dimer structure found in crystals of the C-terminal half of L7/L12 (Liljas, 1982) would argue against this model, but in solution, this part of L7/L12 does not form dimers (Gudkov & Behlke, 1978).

The distance which we found between the probes at the lysine-51 residues is consistent with a shift of about 30 Å. In addition, it shows that the DACM and fluorescein moieties, coupled to the protein via thiopropylimidate and (formylphenoxy)butyrimidate, respectively, apparently are sticking out on opposite sites of the L7/L12 dimer, so that the distance separating them is increased to about 45 Å.

In the preparation of Fluo(1)DACM(51)L12, the equilibrium between monomers and dimers was shifted to the monomeric form. The EF-G-dependent GTPase activity restored by it to P_0 cores was low (Figure 1). So any conclusion regarding the structure of the native L7/L12, if solely based on the distances measured in this protein, would be doubtful. Our reason to include in the present report the results obtained with this preparation is that they were in agreement with the other distances measured and completed our set of data. In solution, the distance between the probes at positions 1 and 51 of the same protein chain was too long for energy transfer to be observed, i.e., at least 75 Å. On the 50S ribosome, however, energy transfer did occur. This finding might indicate that the structure of the monomers was different after binding. On the other hand, under conditions which normally lead to the exclusive occupation of the so-called strong L7/L12 binding site on the 50S cores (Zantema et al., 1982a,b; Thielen et al., 1984), about 1.4 polypeptides of this double-labeled L12 could be bound per 50S subunit. This value is to be compared with the 1.7 equiv of labeled L7/L12 which is normally bound (Zantema et al., 1982a; Thielen et al., 1984). Therefore, the energy transfer observed most likely was due to transfer between DACM and fluorescein attached to different polypeptides. The labeled L12 may have bound to the two L7/L12 binding sites on the 50S ribosomes in the form of monomers, as was observed for the monomeric fragment 1–59 (Schop & Maassen, 1982). The distance of about 52 Å between DACM and fluorescein on separately bound monomers would be compatible with the distances determined between labeled dimers on the ribosome (Thielen et al., 1984). However, the somewhat sigmoidal rise in the GTPase activity, which we found when titrating the P_0 cores with Fluo(1)DACM(51)L12 (Figure 1), might indicate that this protein was bound to the cores in the form of dimers. Assuming a staggered structure, one of the DACM groups at Lys-51 is relatively close to an N-terminal fluorescein group (see Figure 4). In such a system, the actual energy transfer between this specific donor–acceptor pair is twice the one measured (Thielen et al., 1984). The distance between the two labels then is about 41 Å.

In Figure 4, the measured distances are visualized in a model of the L7/L12 dimer. The distances measured in the monomeric, double-labeled L12 is not shown, although the ex-

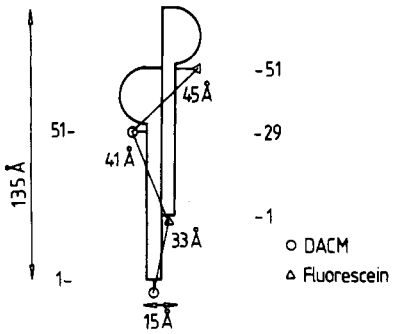

FIGURE 4: Model of a dimer of L7/L12 showing the labeled sites and the distances measured. The numbers to the left and right of the model refer to the sequence of amino acids.

perimental result would nicely fit the distance of about 80 Å in the model. The sizes of the probes and the cross-linkers used were estimated, with the flexibility of the aliphatic parts taken into consideration, and were drawn approximately on scale. The model presented here has the quality of being simple and is consistent with the experimental data (see below), but maybe other models can be thought of which do as well.

In solution and, since we observed no structural changes upon binding to 50S subunits, probably also on the ribosome, the L7/L12 dimers are very elongated (Möller et al., 1972; Gudkov et al., 1977). A length of 135 Å or more was found for the dimers (Wong & Paradies, 1974; Österberg et al., 1976; Liljas, 1982). The C-terminal half of the L7/L12 chains is globular and contributes only about 30 Å (Leijonmarck et al., 1980). The N-terminal half, about 53 amino acids (Leijonmarck et al., 1980), then must span 100 Å or more. CD measurements on L7/L12 indicated a high content of α-helix (Dzionara, 1970; Möller et al., 1970; Gudkov et al., 1978; Boublik et al., 1979; Luer & Wong, 1979). Relatively, the highest amount of helicity was found in the N-terminal fragment 1–73 (Gudkov et al., 1978). Therefore, in our model, the N-terminal part is assumed to be predominantly helical [cf. Gudkov et al. (1977) and Luer & Wong (1979)]. If it consists of several helical fragments, these fragments must all extend in the same direction. With a translation of about 1.5 Å per amino acid in an α-helix, the length of the N-terminal stick still is only about 80 Å. Hence, a shift of about 30 Å of the two L7/L12 chains with respect to each other is essential, not only to explain the cross-linking data of Maassen et al. (1981) and the distances measured at present but also to understand how a distance of about 135 Å can be spanned by the dimers of this small and partly globular ribosomal protein. In the same time, it may provide an explanation for the apparent sensitivity of the dimerization properties of L7/L12 toward the intactness of the whole stretch of residues 1–73.

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Photoaffinity Labeling of the Hemolymph Juvenile Hormone Binding Protein of *Manduca sexta*†

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ABSTRACT: A synthetic analogue of the insect juvenile hormone (JH) III, 10,11-epoxy[10-^{3}H]farnesyl diazoacetate ([^{3}H]-EFDA), binds to several proteins in a partially purified preparation of hemolymph protein from fourth instar larvae of *Manduca sexta* when irradiated with UV light. Approximately 80% of this binding could be inhibited by the addition of excess unlabeled JH I. To compare the relative affinity of EFDA for the juvenile hormone binding protein (JHBP) with that of the various JH homologues, the ability of unlabeled EFDA and JH homologues to displace [^{3}H]JH I from binding sites was measured. The relative affinities were EFDA > JH I > JH II > JH III. When Scatchard analysis of the binding of [^{3}H]EFDA or [^{3}H]JH I to the larval JHBP was performed, an estimated apparent K_D of 4.5×10^{-8} M was found for EFDA, whereas for JH I a slightly higher K_D of 8.8×10^{-8} M was calculated. To determine if [^{3}H]EFDA bound at the JH I binding site, displacement of [^{3}H]JH I from the JHBP complex with unlabeled JH I, JH II, and JH III was compared to the displacement of [^{3}H]EFDA with the same homologues. The results demonstrated that the photoaffinity label bound covalently at the JH I binding site on the hemolymph binding protein of *Manduca sexta*. Fluorescence autoradiography of [^{3}H]EFDA photoaffinity labeled proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that [^{3}H]EFDA bound covalently to two major proteins in the absence of JH I. The presence of JH I prevented the binding of [^{3}H]EFDA to one protein having a molecular weight of approximately 32 000, a molecular weight similar to that of the larval JHBP of *Manduca* hemolymph.

The juvenile hormones (JH) are one of the principal regulators of insect development and differentiation. Therefore, precise control of JH hemolymph titers is essential for normal growth and development (Gilbert & Goodman, 1981), and in the tobacco hornworm *Manduca sexta* a specific hemolymph JH binding protein (JHBP) plays an important role in regulating the JH titer [see Gilbert & Goodman (1981)]. This protein consists of a single 28 000-dalton polypeptide chain (Kramer et al., 1976a; Goodman et al., 1978a), is synthesized in the fat body (Nowock et al., 1975; Nowock & Gilbert, 1976), and functions both to transport the hormone in the hemolymph (Gilbert et al., 1976) and to protect JH from degradation by nonspecific esterases (Hammock et al., 1975; Sanburg et al., 1975).

The molecular interaction of the JHBP with JH has been studied extensively. Each JHBP molecule possess a single high-affinity, JH binding site (Kramer et al., 1976a), which binds JH I with an apparent K_D of approximately 9×10^{-8} M (Kramer et al., 1976a). The more nonpolar homologues JH 0 and JH I are bound preferentially by the JHBP, whereas it exhibits decreasing affinities for the increasingly polar homologues JH II and JH III (Goodman et al., 1976; Kramer et al., 1976b). In addition, the JHBP has little or no affinity for either of the breakdown products of JH, the JH acid and the diol (Kramer et al., 1974), indicating that the epoxide and ester functions are essential for hormone recognition and binding. On the basis of these and other findings, the JH binding site of the JHBP is purported to be a sterically defined hydrophobic region with polar sites for the recognition of the epoxide and ester groups (Goodman et al., 1978b).

Details of the molecular interaction between hormones and their binding components can be studied by the use of photoreactive analogues of these hormones. In insects, photoaffinity labeling has been used to study the sex pheromone of the moth *Antheraea polyphemus* (Gonjian et al., 1978) and 20-hydroxyecdysone in *Drosophila* (Schaltmann & Pongs, 1982). In addition, a JH III analogue, 10,11-epoxyfarnesyl diazoacetate (EFDA) has been synthesized as a photoaffinity label for juvenile hormone binding proteins (Reich, 1978). More recently, carbon-labeled (Kraft et al., 1982) and tritium-labeled EFDA have been synthesized (Prestwich et al., 1984b). The tritium-labeled analogue, [^{3}H]EFDA, has been used successfully to label the JH binding site on the hemolymph and ovarian proteins in the cockroach *Leucophaea maderae* (Koeppe et al., 1984). This paper demonstrates that EFDA attaches covalently to the JH binding site of the *Manduca* JHBP with relatively high affinity and specificity. Thus, EFDA may now be used as a photoaffinity label for

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