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Fluorescence Polarization Studies of the Interaction of *Escherichia coli* Protein Synthesis Initiation Factor 3 with 30S Ribosomal Subunits†

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ABSTRACT: Steady-state fluorescence polarization techniques were used to study the binding of initiation factor 3 (IF3) to 30S ribosomal subunits. Covalent fluorescent derivatives of IF3 were prepared by treating the pure protein with fluorescein isothiocyanate. The fluorescein-labeled IF3 (F-IF3) contained 0.8–1.7 dye molecules per protein. Polyacrylamide gel electrophoretic analysis of the derivatized forms is consistent with the view that the probe is randomly attached, presumably to lysine ϵ-amino groups. The activity of F-IF3 is not impaired in assays for binding to 30S ribosomal subunits or in promoting formylmethionyl-tRNA binding to 70S ribosomes. Fluorescence polarization values were measured at different F-IF3 and 30S ribosomal subunit concentrations, and the association constant and number of binding sites were calculated. In buffer containing 10 mM magnesium acetate and 100 mM ammonium chloride, the association constant is (3.1 ± 1.4) × 10^7 M^{-1}, and the number of ribosomal binding sites is 1.2 ± 0.2. The value for the association constant varies inversely with the ammonium chloride and magnesium acetate concenrations by a small amount. Competition studies show that nonderivatized IF3 binds to 30S ribosomal subunits with the same affinity as F-IF3. Therefore, the association constants measured for F-IF3 are valid for IF3 as well.

Initiation of protein synthesis in bacteria involves the sequential formation of 30S and 70S initiation complexes containing mRNA and fMet-$tRNA_f$.[1] In *Escherichia coli*, three initiation factors promote this process: IF1 (M_r 8119); IF2 (M_r 115 000); and IF3 (M_r 20 668). A number of pathways have been proposed to describe the interaction of the macromolecular components [for reviews, see Grunberg-Manago et al. (1978) and Lodish (1976)], but numerous ambiguities or uncertainties exist, and no single pathway has been rigorously proven to be correct. We lack the kinetic data which could be used to distinguish reasonable alternative pathways.

In this work, we utilize the technique of steady-state fluorescence polarization to measure the equilibrium association constant for fluorescein-labeled IF3 (F-IF3) binding to the 30S ribosomal subunit. Changes in rotational mobility are reflected in changes in the polarization of fluorescence emission. Large differences in the rotational mobility of free initiation factors compared to those complexed with ribosomal particles make this technique attractive for the study of factor–ribosome interactions. Theoretical treatments of fluorescence polarization were given originally by Perrin (1926) and more recently by Weber (1952, 1971). Steady-state methods were first used in studies of dye–protein (Laurence, 1952), protein–protein, and protein–nucleic acid associations (Steiner, 1953). Further important contributions in the theoretical and experimental uses of fluorescence polarization resulted from work on the equilibria (Dandliker & Feigen, 1961; Dandliker et al., 1964, 1973; Kierszenbaum et al., 1969; Portmann et al., 1975) and rate kinetics (Dandliker & Levison, 1967; Levison et al., 1970, 1971, 1975; Levison, 1975) of antigen–antibody reactions. We demonstrate here and elsewhere[2] that the steady-state fluorescence polarization technique is suitable for studying the binding of factors to ribosomes. A preliminary account of our studies with fluorescein-labeled

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[1] Abbreviations used: fMet-tRNA, formylmethionyl transfer RNA; IF, initiation factor; F-IF3, fluorescein-labeled IF3; βME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

[2] J. Weiel and J. W. B. Hershey, unpublished experiments.

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IF3 was published previously (Weiel et al., 1978).

Experimental Procedures

Buffers. Buffer A contained 10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 7 mM 2-mercaptoethanol (βME), 5% (v/v) glycerol, and KCl as indicated in millimolar concentrations in parentheses. Buffer B contained 10 mM Tris-HCl, pH 7.4, 7 mM βME, 5 μM bovine serum albumin (325 μg/mL), and ammonium chloride and magnesium acetate as indicated sequentially in millimolar concentrations in parentheses.

Growth and Lysis of Bacteria. E. coli MRE 600 cells were grown in 12-L batches in a 14-L Microferm fermenter (New Brunswick). Per liter, the buffered (pH 6.8) medium contained 10 g of yeast extract (Difco), 34 g of KH_2PO_4, 7 g of KOH, 12 g of glucose, and 0.42 mL of Antifoam B (Sigma). A 100-mL starter culture, in the same medium, was added, and the cells were grown at 37 °C with vigorous stirring and aeration. Growth was terminated when the A_{550} value was between 3.5 and 4.0 (about 2.5–3.5 h). The cells were quickly cooled to 15 °C by the addition of crushed ice, incubated for 30 min at 15 °C, and then rapidly cooled to 0 °C by adding more crushed ice. The bacteria were harvested by centrifugation, resuspended in 400 mL of ice-cold buffer (10 mM Tris-HCl, pH 7.4, and 10 mM magnesium acetate), and centrifuged in a Sorvall GSA rotor for 20 min at 8000 rpm. This procedure yields 30–50 g (wet weight) of bacteria.

Freshly grown bacteria were lysed immediately in 30-g lots by grinding in a chilled mortar with 60 g of alumina (Norton), 150 mg of Bentonite (National Lead Co.), and 20 μg of DNase (Worthington). The paste was suspended in 60 mL of buffer (10 mM Tris-HCl, pH 7.4, 10.1 mM magnesium acetate, 100 mM ammonium chloride, 0.1 mM EDTA, and 7.2 mM βME) and partially clarified by centrifugation at 5000 rpm for 5 min. The lysate was centrifuged in a Sorvall SS-34 rotor for 20 min at 16 000 rpm (30000*g*). The resulting supernatant was recentrifuged for 20 min at 16 000 rpm in the same rotor to yield the S30 supernatant.

Ribosomes and Ribosomal Subunits. Crude ribosomes were obtained from the S30 supernatant by centrifugation for 1 h at 58 000 rpm (100000*g*) in a Beckman Ti 60 rotor. The 70S ribosomes were separated from native subunits in two ways. Initially, a modification of the zonal sucrose gradient centrifugation method of Fakunding & Hershey (1973) was used. The Beckman Ti 15 rotor contained a 7.5–45% hyperbolic sucrose gradient (700 mL) with the magnesium acetate concentration being 6 mM; the ribosome sample (ca. 1 g) was centrifuged at 32 000 rpm for 7.5 h. Alternatively, crude ribosomes (ca. 1 g) were resuspended in 20–25 mL of buffer (10 mM Tris-HCl, pH 7.4, 6 mM magnesium acetate, 100 mM ammonium chloride, and 6 mM βME), and 2.5-mL aliquots were layered over 23 mL of 38% (w/v) sucrose (RNase free) in the same buffer and centrifuged at 50 000 rpm for 10 h in a Beckman Ti 60 rotor. The pellet fraction contains 70S ribosomes free of native subunits.

Ribosomal subunits were derived from 200-mg aliquots of the fractionated 70S ribosomes by sucrose gradient centrifugation in the Beckman Ti 14 zonal rotor essentially as described (Fakunding & Hershey, 1973). The sample in 8 mM magnesium acetate was layered over a 10–38% hyperbolic sucrose gradient (250 mL) and centrifuged at 47 500 rpm for 4 h. The resulting 30S and 50S pellets were each resuspended in 1–1.5 mL of buffer (10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 30 mM ammonium chloride, 0.1 mM EDTA, and 2 mM dithioerythritol). 30S subunits were stored at 25 mg/mL in 30-μL aliquots in liquid nitrogen until needed. 50S subunits were stored at 90 mg/mL in 50-μL aliquots. 70S ribosomes (200 mg) yielded 30–40 mg of 30S subunits and about 100 mg of 50S subunits. Once thawed, subunits were never refrozen for use again.

Fluorescein-Labeled IF3 (F-IF3). IF3 was purified from *E. coli* MRE600 as previously described (Hershey et al., 1977), and was at least 95% pure as judged by scans of stained $NaDodSO_4$–polyacrylamide gels. F-IF3 was made by adding 2.5 mg of IF3 in 700 μL of buffer A (800) to 318 μL of 0.65 M potassium carbonate buffer, pH 9.0, containing 0.65 mg of fluorescein isothiocyanate isomer I (Sigma). The conical glass reaction vial had previously been treated with Sigmacote (Sigma) to minimize adsorption of the protein to the glass. The reaction mixture was stirred at 4 °C for 24 h, diluted with 4 mL of water, and passed through a 250-μL column of tightly packed phosphocellulose (Whatman P-11) equilibrated in buffer A (100). Unreacted dye was eluted by washing with buffer A (100), and the F-IF3 was eluted with buffer A (800). In some preparations, the bulk of unreacted reagent was removed from IF3 by passing the reaction mixture through a column of Sephadex G-25 (0.6 × 12 cm) prior to the chromatography step. The molarity of fluorescein was measured spectrophotometrically by using $\epsilon^{M}_{490} = 8.5 \times 10^4$. F-IF3 or IF3 protein concentrations were determined by the method of Bradford (1976) with a standard IF3 solution whose concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Bovine serum albumin is a suitable standard for IF3 in the Lowry assay (Hershey et al., 1977) but not in the Bradford assay. The molar concentration of IF3 and its derivatives was based on a molecular weight of 20 668 (Brauer & Wittmann-Liebold, 1977).

Fluorescence Measurements. All fluorescence polarization measurements were made in 3-mL quartz fluorescence cuvettes containing 1 mL of buffer B containing ammonium chloride and magnesium acetate as indicated in the text and figure legends. The bovine serum albumin was added to minimize loss of initiation factors due to adsorption to the walls of the cuvette. The albumin stabilized the fluorescence intensity but had no effect on the polarization measurements. Even with the dilute F-IF3 solutions employed in experiments which lasted up to 2 h, the drop in fluorescence intensity was less than 5%. To the 1 mL of buffer were added freshly thawed 30S ribosomal subunits, heat activated as described by Zamir et al. (1971), and aliquots of fluorescent factor as indicated in the text. Volume changes due to all of the additions were usually less than 3% and never exceeded 5%; concentrations of components were volume adjusted whenever appropriate. Measurements were taken in a thermostated sample chamber at 25 °C.

Steady-state fluorescence polarization measurements were made with an SLM 4800S fluorometer (SLM Instruments) supplied with Glan-Thompson polarizers and interfaced to a Hewlett-Packard 9825A computer and 9862A plotter. The excitation monochromator was set at 467 nm, and either Corning 3-70 or Schott KV-500 optical filters were placed in each emission beam. Simultaneous intensity values were determined with the fluorometer arranged in a T-format. With the excitation polarizer set to pass horizontally polarized light, a grating correction factor (C) was obtained as the ratio of the channel A intensity (vertically polarized fluorescence) to that of channel B (horizontally polarized fluorescence). This ratio was determined several times for every sample and was used to correct the channel A intensity when the excitation polarizer was set to pass vertically polarized light. Great care

was taken to minimize scattered light from the sample because the polarization measurement is extremely sensitive to background scattering levels at low fluorescence intensity. The light scattered by a buffer plus 30S sample, in the presence of optical filters, was compensated for by zeroing the A and B channels with the excitation polarizer set at the horizontal position. Then, with the excitation polarizer in the vertical position, the background intensities of the A and B channels were recorded into the computer for correcting the subsequent fluorescence intensities of the A and B channels. By judicial selection of the electronic acquisition parameters, coupled with a computer program to calculate and keep track of running averages, polarization values were normally accurate to ±0.001 unit.

Polarization and intensity values were calculated by the computer as

$$P = \frac{A/(BC) - 1}{A/(BC) + 1}$$

$$I = A/C + 2B$$

where A and B represent the scatter-corrected intensities of the A and B channels (C is the grating correction factor). The fluorometer was set to "give" the computer the average of ten single measurements. The program required five of these measurements before an average was printed. This procedure, including the determination of the correction factor in a similar manner, was repeated 4 or more times until the polarization values were steady to within ±0.001 unit. Therefore, most polarization values are the result of 200 or more instrumental measurements taken over a 2–3-min time span.

Fluorescence emission spectra were recorded with the SLM 4800S fluorometer in a ratiometric mode using a glycogen solution as a scattering reference of the excitation intensity. The excitation wavelength was 467 nm; scans were taken from 490 to 550 nm at 1-nm intervals with 4-nm resolution. Fluorescence lifetimes were measured by using the phase-modulation technique inherent in the SLM 4800S fluorometer. Measurements were made predominantly at modulation frequencies of 18 and 30 MHz. Some of the initial fluorescence polarization experiments with F-IF3 were done at room temperature (22–24°) by using the homemade device described by Dandliker et al. (1978); essentially the same results were obtained.

Calculation of Free and Bound Factors. The polarization of F-IF3 free in solution (P_f) was determined by titrating aliquots of fluorescent factor into buffer B and measuring the polarization of the fluorescence. The theoretical polarization of the completely bound state (P_b) was determined by extrapolation as described (Dandliker et al., 1964).

The bound/free ratio (F_b/F_f) and the concentration of bound factor were determined by using eq 1 and 2, respectively.

$$\frac{F_b}{F_f} = \frac{Q_f}{Q_b}\left(\frac{P - P_f}{P_b - P}\right) \quad (1)$$

$$\left(\frac{F_b/F_f}{1 + F_b/F_f}\right)M = F_b \quad (2)$$

Here, Q represents the fluorescence quantum yield of the free and bound forms of F-IF3, P_f/P_b is defined above, P is the experimentally determined polarization of a given mixture of F-IF3 and 30S subunits, and M is the total concentration of fluorescent species. The values of F_b/F_f were plotted against the values of F_b according to eq 3 in the manner of Scatchard

$$\frac{F_b}{F_f} = K_a(F_{b,max} - F_b) \quad (3)$$

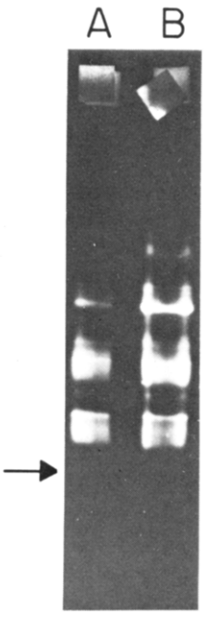

FIGURE 1: Polyacrylamide gel electrophoresis of F-IF3 preparations. Two preparations of F-IF3 containing 0.84 (lane A) and 1.2 (lane B) fluorescein groups per IF3 molecule were analyzed on horizontal polyacrylamide slab gels (12 cm long, 0.75 mm thick) essentially as described by O'Farrell et al. (1977) for their first dimension. The gels contained 7% acrylamide, 0.373% bis(acrylamide), 2% Ampholines (pH 3.5–10; LKB), 2% NP-40, and 9 M urea. Samples (10 μg) were added to 5 × 5 mm squares of Whatman 3 MM filter paper applied at the anode end of the gel lane. Electrophoresis was for 3.5 h at 400 V at 24 °C. The figure shows a photograph of the fluorescence emission obtained by irradiating the gel at 366 nm with a UV lamp and photographing through a Schott KV500 filter. The arrow indicates the position of nonderivatized IF3.

(1949). A least-squares linear analysis of the resulting data points yields the equilibrium constant as the negative of the slope and the total concentration of available sites as the F_b intercept.

Results

Preparation and Characterization of F-IF3. Covalent fluorescein derivatives of IF3 (F-IF3) were prepared by treating highly purified IF3 with fluorescein isothiocyanate. Numerous pilot reactions were done to find conditions that would allow stoichiometric labeling of the IF3. Optimal conditions for covalently linking about one dye per IF3 molecule are described in detail under Experimental Procedures. Ion-exchange chromatography on phosphocellulose was used to purify the F-IF3 because significant amounts of noncovalently bound fluorescein migrated with the protein on a Sephadex G-25 column. Dialysis of crude F-IF3 to remove free dye proved unsatisfactory because it was too time consuming and was not so effective as ion-exchange chromatography. Routinely, about 75% of the IF3 was recovered as F-IF3. The degree of fluorescein labeling varied from 0.84 to 1.7 dye molecules per IF3 molecule, as determined spectrophotometrically.

On the basis of the chemistry of fluorescein isothiocyanate, it is likely that the reagent reacts primarily with the ε-amino groups of lysine and possibly with the α-amino group at the N terminus of IF3. Two preparations differing in extent of fluorescein labeling were analyzed by nonequilibrium pH gradient electrophoresis on polyacrylamide gels (O'Farrell et al., 1977), which separates IF3 forms based on the number of fluorescein groups attached. As shown in Figure 1, most of the molecules in the F-IF3 preparations contain one or two molecules of dye. Based on protein staining, approximately

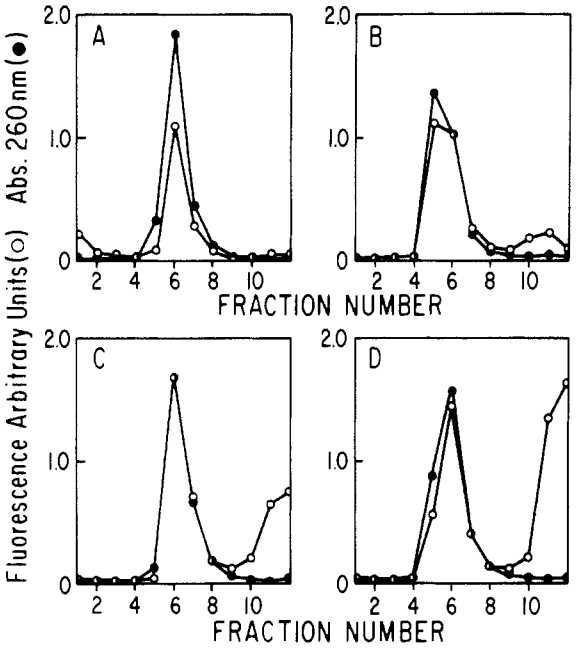

FIGURE 2: F-IF3 binding to 30S ribosomal subunits analyzed by sucrose density gradient centrifugation. Reactions in 70 μL contained 10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 100 mM NH_4Cl plus KCl, 1 mM dithioerythritol, 68 pmol of 30S ribosomal subunits, and F-IF3 as indicated below. Reaction mixtures were incubated about 10 min at room temperature and then layered onto 10–31% (w/v) hyperbolic sucrose gradients containing 10 mM Tris-HCl, pH 7.4, 10 mM magnesium acetate, 60 mM NH_4Cl, and 1 mM dithioerythritol. The gradients were centrifuged in a Beckman SW-56 rotor at 52000 rpm for 2 h at 4 °C and collected into 12 fractions which were diluted and assayed for fluorescence and absorbance at 260 nm. Sedimentation was from right to left. Reaction mixtures contained the following amounts of F-IF3 (in picomoles): panel A, 68; panel B, 136; panel C, 204; panel D, 272.

25 and 35% of the IF3 is not derivatized in the two preparations analyzed (results not shown). These patterns show that the IF3 molecules are derivatized randomly rather than extensivly on a small proportion of the molecules (i.e., denatured forms).

In order to study the binding of F-IF3 to 30S ribosomal subunits, a biologically functional derivative is required. Consequently, two independent tests for functionality were performed on the derivatized IF3. Sucrose density gradient centrifugation was used to determine the ability of F-IF3 to bind to 30S ribosomal subunits, as described in Figure 2. Since the fluorescent peak and the A_{260} peak coincide, it is clear that F-IF3 binds quite well to 30S subunits, under the conditions used. Panel A indicates that all of the recovered F-IF3 is active in 30S binding, while the other panels show that there are a finite number of 30S sites available; i.e., the binding of F-IF3 to 30S subunits is saturable. The F-IF3 was also analyzed for its ability to stimulate phage R-17 RNA-dependent f[^{14}C]Met-tRNA binding to 70S ribosomes (Hershey et al., 1977). All of the different preparations of F-IF3 retained 80–100% of the stimulating activity of nonderivatized IF3 (results not shown).

Fluorescence emission spectra of the F-IF 3 derivatives were taken in the presence and absence of 30S ribosomal subunits. As shown in Figure 3, there is no difference in the wavelength maxima of emission between the F-IF3 in the free and ribosome-bound states. Within experimental error, there also is no difference in fluorescence intensity (quantum yield) between the two states. Comparison of the fluorescent lifetimes of F-IF3 in the free and bound states indicates that there is no preceptable change in the excited-state fluorescent lifetime

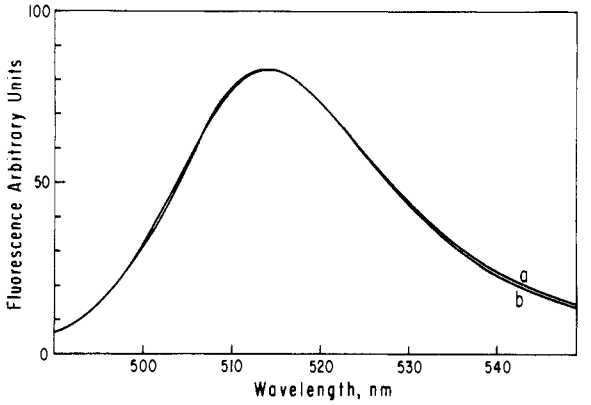

FIGURE 3: Fluorescence emission spectra of F-IF3. Spectra were recorded as described under Experimental Procedures. Spectrum a represents an emission scan of free F-IF3 at 1×10^{-7} M in buffer B (100, 5). Spectrum b is a scan of the same solution with 30S ribosomal subunits added to a concentration of 1.6×10^{-7} M, where about 80% of the F-IF3 is bound.

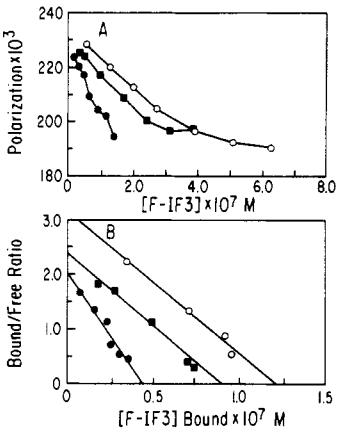

FIGURE 4: F-IF3 binding to 30S ribosomal subunits. (A) Polarization values. Aliquots of F-IF3 were added to 30S ribosomal subunits in buffer B (100, 10) at 0.4×10^{-7} (●), 0.8×10^{-7} (■), and 1.2×10^{-7} M (○), and polarization values were determined as described under Experimental Procedures. (B) Scatchard plots of data from (A). Bound and free F-IF3 were calculated and plotted as described under Experimenal Procedures. Values for P_f and P_b are 0.180 and 0.250, respectively. The calculated values for association constants and the number of binding sites per ribosomal subunit (given in parentheses) are the following: (●) 4.6×10^7 M^{-1} (1.1); (■) 2.7×10^7 M^{-1} (1.1); (○) 2.6×10^7 M^{-1} (1.0).

upon binding. Both lifetimes were 3.7 ± 0.2 ns as measured in the SLM 4800S instrument by phase-modulation techniques. The steady-state polarization value (P_f) for free F-IF3 in buffer B (100, 10) is 0.180,indicating that the fluorescein probes are somewhat restricted in movement.

F-IF3 Binding to 30S Ribosomal Subunits. The binding of F-IF3 to 30S ribosomal subunits was studied by measuring the steady-state fluorescence polarization of F-IF3 in the presence of 30S subunits. Fixed amounts of 30S ribosomal subunits (range 2×10^{-8} to 2×10^{-7} M) were titrated with aliquots of F-IF3 (range 5×10^{-9} to 5×10^{-7} M), and polarization values were determined as described under Experimental Procedures. Typical polarization vs. F-IF3 concentration curves obtained in buffer B (100, 10) are shown in Figure 4, panel A. At a given 30S subunit concentration, the addition of increasing amounts of F-IF3 resulted in a decrease in the polarization. Alternatively, at a fixed F-IF3 concentration, the polarization rose with increasing ribosome concentration. Such behavior is consistent with the expectation that F-IF3 bound to 30S ribosomal subunits exhibits a higher polarization value than that in the free state.

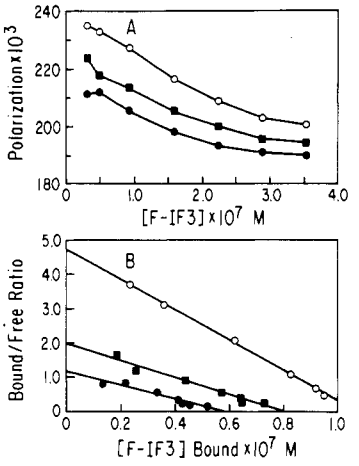

FIGURE 5: Effects of NH_4Cl concentration on F-IF3 binding. (A) Polarization values. F-IF3 was added to 30S ribosomal subunits at 0.76×10^{-7} M in buffer B containing 10 mM magnesium acetate and varying amounts of NH_4Cl as follows: 50 mM (○); 100 mM (■); 150 mM (●). Measurements were made as described under Experimental Procedures. (B) Scatchard plots of data from (A). The data were treated as described in the legend to Figure 4; P_f and P_b values were unchanged. Calculated association constants and number of binding sites (in parentheses) are the following: (○) 4.3×10^7 M^{-1} (1.4); (■) 2.5×10^7 M^{-1} (1.0); (●) 2.0×10^7 M^{-1} (0.8).

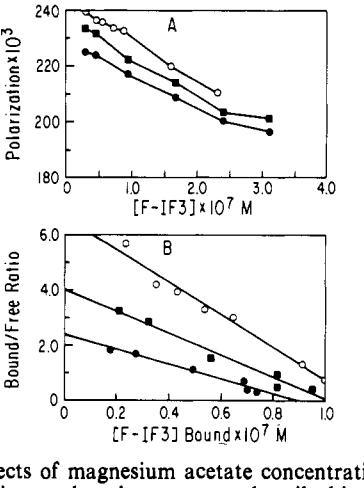

FIGURE 6: Effects of magnesium acetate concentration on F-IF3 binding. Titrations and analyses were as described in the legend to Figure 5, except that 0.79×10^{-7} M 30S ribosomal subunits were used, and buffer B contained 100 mM NH_4Cl and the following concentrations of magnesium acetate: 10 mM (●); 5 mM (■); and 2.5 mM (○). (A) Polarization values. (B) Scatchard plots. Calculated association constants and number of binding sites (in parentheses) (same P_f and P_b values) are as follows: (●) 2.6×10^7 M^{-1} (1.1); (■) 3.9×10^7 M^{-1} (1.3); (○) 5.8×10^7 M^{-1} (1.4).

The concentrations of bound and free F-IF3 were calculated from the polarization data and were analyzed by the method of Scatchard, as described in detail under Experimental Procedures and shown in Figure 4, panel B. The Scatchard lines are linear and indicate that all of the F-IF3 molecules bind to the ribosomal site(s) with equal affinity. Similar measurements were made in buffer B (100, 10) with a number of different F-IF3 and 30S ribosomal subunit preparations. The association constant for the F-IF3–30S interaction was determined to be $(3.1 \pm 1.4) \times 10^7$ M^{-1} (the standard deviation is $N - 1$ weighted, $N = 27$); the apparent number of binding sites on 30S subunits is 1.2 ± 0.2.

Effect of Salt Concentration. In order to determine whether salt concentration influences the association constant, values were measured in buffer B with varying amounts of ammonium chloride or magnesium acetate. In Figure 5 are shown the results of measurements in buffer B with 10 mM magnesium acetate and 50, 100, and 150 mM ammonium chloride. Equilibrium constants of 4.3, 2.5, and 2.0×10^7 M^{-1} were calculated from these data for the three salt concentrations. The small decrease in the association constant with increasing ammonium chloride concentration indicates that the binding of IF3 is not very sensitive to the ionic strength of the medium. The effect of magnesium acetate concentration in buffer B with 100 mM ammonium chloride is shown in Figure 6. Association constants of 5.8, 3.9, and 2.6×10^7 M^{-1} were obtained at 2.5, 5, and 10 mM magnesium acetate, showing a small decrease with increasing Mg concentration. Changes in the ammonium chloride or magnesium acetate concentration had no effect on the polarization of free F-IF3 (P_f) or on the completely bound polarization (P_b).

While differences in the equilibrium constant due to changes in the ammonium or magnesium ion concentration could easily be detected in individual experiments, the averaged value for all independent experiments (using different preparations of F-IF3 and 30S subunits) showed smaller significant differences, due to relatively large standard deviations. Since the equilibrium constants consistently decreased with increasing salt concentration in any given experiment, the changes can be expressed as changes in ratio. Averaged ratios of 1.0:0.65:0.50 were obtained for the equilibrium constants in buffer B and 10 mM magnesium acetate at 50, 100, and 150 mM ammonium chloride, respectively. The averaged ratios are 1.0:0.72:0.42 at 2.5, 5.0, and 10.0 mM magnesium acetate in buffer B and 100 mM ammonium chloride.

Competitive Binding of IF3 against F-IF3. Although F-IF3 has been shown to be biologically active and capable of binding to 30S ribosomal subunits (see above), it was not known how the binding of the fluorescent factor compares quantitatively with the binding of nonderivatized IF3. Several types of competitive experiments were done to directly compare the binding of IF3 with that of F-IF3. An initial study consisted of two titrations of 30S subunits with F-IF3; one was a control titration, and the other was done in the presence of a 50-fold excess of IF3 over 30S subunits. The control was normal, showing initially high polarization values decreasing with added F-IF3; but in the presence of excess IF3, virtually no F-IF3 was bound (constant very low polarization) to 30S subunits (data not shown). This indicates that F-IF3 and IF3 compete for the same site on the 30S subunit.

In order to quantitate the competitiveness of IF3, further experiments were performed. To 30S subunits (either 5.7×10^{-8} or 11.3×10^{-8} M), in buffer B (100, 5), was added a constant amount of total factor (either 4×10^{-8} or 10×10^{-8} M, respectively), that differed in the mole fraction of IF3 to F-IF3 (Figure 7). If IF3 and F-IF3 bind to 30S subunits with equal affinity, the amount of F-IF3 bound should decrease linearly to zero, as the mole fraction of IF3 changes from 0 to 1. This is precisely what is observed in Figure 7. Therefore, we can conclude that F-IF3 and nonderivatized IF3 bind to 30S ribosomal subunits with the same affinity. The competition experiments also indicate that a true equilibrium is observed by the fluorescence polarization technique. The order of addition of IF3 and F-IF3 had no effect on the resulting polarization (results not shown). After the addition of an aliquot of IF3 or F-IF3, a new equilibrium is attained within a minute.

Discussion

Steady-state fluorescence polarization is an ideal technique for studying the interaction of a macromolecule with a relatively large particle. Its feasibility for determining equilibrium association constants and number of binding sites is established

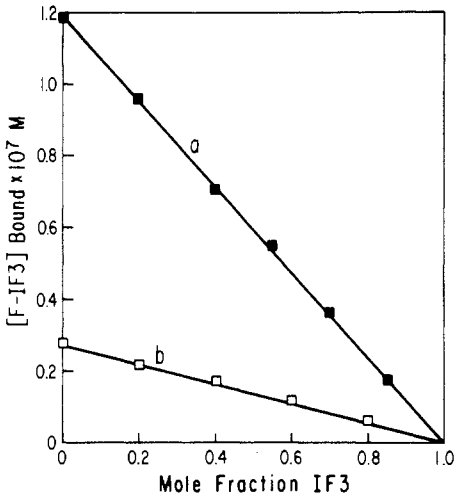

FIGURE 7: Competitive binding of F-IF3 and IF3. Mixtures of F-IF3 and nonderivatized IF3 were prepared which differed in the mole fraction of IF3. Polarization values were determined with two concentrations of 30S ribosomal subunits and total factor: 1.1×10^{-7} M subunits and 1.0×10^{-7} M total factor (■); 0.57×10^{-7} M subunits and 0.40×10^{-7} M total factor (□). Bound F-IF3 was calculated from the polarization values and P_f and P_b values of 0.180 and 0.250, as describd under Experimental Procedures. The experimental values are shown by the symbols; lines a and b represent theoretical concentrations of bound F-IF3, assuming equal affinities for F-IF3 and IF3.

here in the case of the binding of initiation factors to ribosomal subunits. In this work, we have employed a covalent fluorescent derivative of IF3 prepared by treating the factor with fluorescein isothiocyanate. The resulting F-IF3 preparations contained on the average about one dye molecule attached (apparently randomly) to the protein. The fluorescence properties of the F-IF3 were examined in the presence and absence of 30S ribosomal subunits. There is no change in the fluorescence emission spectrum or fluorescence lifetime upon binding to 30S ribosomal subunits. The observed change in polarization upon addition of 30S ribosomal subunits to the F-IF3 must therefore be due to the difference in rotational mobilities of the factor and its complex. Given the precision of our polarization measurements (±0.001), a difference of 0.070 between free (P_f) and ribosome-bound (P_f) F-IF3 is sufficient for quantitative work.

It is imperative that the covalently labeled factor mimicks the activity of the native, nonderivatized protein. Different F-IF3 preparations, containing 0.8–1.6 fluorescein molecules per factor, were capable of binding to 30S ribosomal subunits when analyzed by sucrose density gradient centrifugation and were fully active in assays for initiation of protein synthesis. Scatchard plots of the polarization data are linear, i.e., monophasic, which implies homogeneous or identical binding interactions by all of the IF3 derivatives. This would not be the case if the fluorescein group strongly suppressed or enhanced normal binding because F-IF3 preparations would then show heterogeneous binding, dependent on the degree of labeling. The Scatchard plots and gradient analyses show that the binding is saturable and within experimental error stoichiometric in a one to one ratio. Finally, polarization studies involving competition between IF3 and F-IF3 for binding to 30S subunits indicate that the affinities are quantitatively indistinguishable. The equilibrium association constant determined for F-IF3 is therefore valid for IF3 also. The competition experiments also indicate that true equilibria are established in less than a minute following mixing of the reagents, since the same polarization values are reached irrespective of the order of addition of F-IF3 and IF3.

At 100 mM ammonium chloride and 10 mM magnesium acetate, i.e., buffer B (100, 10), the equilibrium association constant for F-IF3 binding to 30S subunits is $(3.1 \pm 1.4) \times 10^7$ M^{-1}. This equilibrium constant is similar to that measured by light scattering techniques, $(1.5–4.0) \times 10^7$ M^{-1} (Godefroy-Colburn et al., 1975), but higher than those measured with radioactively labeled IF3 by sucrose density gradient centrifugation, 1×10^7 M^{-1} (Subramanian & Davis, 1970) and $(0.5–0.7) \times 10^7$ M^{-1} (Sabol et al., 1973).

The association constant for F-IF3 binding to 30S subunits is somewhat sensitive to the ionic strength of the buffer, decreasing about 2-fold when the NH_4Cl concentration is raised from 50 to 150 mM. The sensitivity to NH_4Cl concentration is considerably less than that observed[2] for F-IF2, which decreases more than 15-fold over the same concentration range. The binding of radiolabeled IF3 to 30S subunits, as analyzed by sucrose density gradient centrifugation (Pon & Gualerzi, 1976), also is sensitive to salt concentration, but to a somewhat lesser extent than reported here for F-IF3. When the Mg concentration is varied from 2.5 to 10 mM, the association constant for F-IF3 decreases about 2.5-fold, whereas that for F-IF2 remains constant.[2] This result contradicts those obtained by light scattering or gradient centrifugation (Godefroy-Colburn et al., 1975; Vermeer et al., 1973; Sabol & Ochoa, 1971) but is consistent with the notion that the 16S RNA is an important contributor to the stability of IF3 binding (Gualerzi & Pon, 1973; Pon & Gualerzi, 1976).

We have not yet attempted to measure the rate constants for the formation and dissociation of the IF3–30S complex. In a recent report, Goss et al. (1980) describe the use of stopped-flow fluorometric techniques to determine the rate of binding of dansylated IF3 to 30S subunits in a buffer containing 100 mM KCl, 1 mM $MgCl_2$, and 50S subunits. Their value of 1.7×10^4 M^{-1} s^{-1} is unexpectedly low and is inconsistent with our results. Assuming by extrapolation from our data that the association constant in their buffer would be about 1×10^8 M^{-1}, we calculated the rate of complex dissociation to be of the order of 10^{-4} s^{-1}, a value much too slow to enable equilibrium to be attained within a minute. The much higher rate constant (4×10^8 M^{-1} s^{-1}), determined by stopped-flow fluorometry in 100 mM KCl and 10 mM $MgCl_2$ by Woolley & Box (1980), is entirely compatible with the results reported here. The discrepancies in the results from different laboratories may be due to differences in the IF3 and ribosome preparations. Nevertheless, these studies demonstrate the power of fluorometric techniques for measuring the reaction rates of protein synthesis and constitute a begining for the quantitative characterization of the kinetic parameters of the initiation pathway.

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Identification of 25,26-Dihydroxyvitamin D_3 as a Rat Renal 25-Hydroxyvitamin D_3 Metabolite†

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ABSTRACT: 25,26-Dihydroxyvitamin D_3 [25,26-$(OH)_2D_3$] was unequivocally identified as a major renal microsomal metabolite of 25-hydroxyvitamin D_3 in rats fed a vitamin D sufficient diet. The structural assignment was based on a comparison of the high-performance liquid chromatograms of synthetic and in vitro generated 25,26-$(OH)_2D_3$ through four different systems, the ultraviolet absorbance and mass spectral characteristics of biological 25,26-$(OH)_2D_3$, and the chromatographic and mass spectral characteristics of the sodium metaperiodate cleavage product of the metabolite. The enzymic synthesis of 25,26-$(OH)_2D_3$ was inhibited 60–80% by a semipurified goat anti-rat NADPH–cytochrome P-450 reductase. This implicates cytochrome P-450 as the probable terminal oxidase of the 25-hydroxyvitamin D_3-26-hydroxylase system. The methodology used to assay rat renal 25-OH-D_3-hydroxylases is also discussed.

25-Hydroxyvitamin D_3 (25-OH-D_3)[1] is the major circulating metabolite of vitamin D_3 (Haddad et al., 1977; Horst et al., 1977, 1979a). This metabolite is converted in several tissues to numerous more polar metabolites (Bikle & Rasmussen, 1974; Takasaki et al., 1978; Ribovitch & DeLuca, 1978; Norman, 1979). However, only the biological role of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is certain (Omdahl & DeLuca, 1973; Napoli & DeLuca, 1979; Stern, 1980). 1,25-$(OH)_2D_3$ is a hormone that mediates the traditionally measured actions of vitamin D. The function of the other metabolites, many of their structures, and, for the most part, their site(s) of synthesis are unknown. Yet evidence is accumulating to support the notion that 25-OH-D_3, and/or its metabolites other than 1,25-$(OH)_2D_3$, may be important in regulating calcium metabolism (Bordier et al., 1978; Rasmussen & Bordier, 1980). Consequently, investigation of metabolic pathways which bypass 1-hydroxylation, occurring

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[1] Abbreviations used: 25-OH-D_3, 25-hydroxyvitamin D_3; lactone, 25-hydroxyvitamin D_3 26,23-lactone; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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