FLUORESCENCE POLARIZATION STUDIES OF THE BINDING OF FLUORESCEIN-LABELED INITIATION FACTOR IF3 TO 30 S RIBOSOMAL SUBUNITS FROM *ESCHERICHIA COLI*

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1. Introduction

The mechanism of initiation of protein synthesis in bacteria has been intensively studied [1,2] but the precise pathway of assembly of initiation complexes is not yet known. In particular, the kinetic parameters of the various reactions in the pathway have not been determined. Kinetic studies have usually relied on sucrose gradient analyses of partial initiation complexes, with and without glutaraldehyde fixation, and on filtration of complexes through nitrocellulose filters [3]. These techniques suffer from obvious deficiencies if a rigorous and accurate kinetic analysis is desired. Light scattering techniques have been used to study initiation factor–ribosome interactions [4], but the method is inherently complex and does not easily lend itself to fast kinetics when small molecules are involved.

We are utilizing the technique of fluorescence polarization to study the equilibrium binding interactions of initiation factors with 30 S ribosomal subunits. Fluorescence polarization has been used to examine dye–protein binding interactions [5] and protein–protein or protein–nucleic acid associations [6]. This technique has also been used in kinetic studies of macromolecular reactions [7,8]. These approaches are based on the theoretical work initiated [9–11]. The basic principle is as follows:

An initiation factor is covalently labeled with a fluorescent probe and the polarization of fluorescence is measured in the presence and absence of 30 S ribosomal subunits. The polarization is equal to $V-H/V+H$ where V is the fluorescence intensity of vertically polarized light and H is the fluorescence intensity of horizontally polarized light when the solution is excited with vertically polarized light. Since the magnitude of the polarization is directly related to the rotational relaxation time of the fluorescent intiation factor, it becomes a measure of the proportion of factor bound to 30 S subunits to that free in solution (bound/free ratio). Due to the sensitivity of the technique, the proportion of bound and free initiation factor can be measured in the range of 10^{-6}–10^{-9} M.

IF3 is a small initiation factor (mol. wt 22 500) which binds stoichiometrically to the 30 S ribosomal subunit [12,13]. We describe here initial fluorescence polarization studies of the binding of a biologically active, fluorescein-labeled IF3 (F-IF3) to 30 S ribosomal subunits, and report the determination of the association constant for the reaction.

2. Materials and methods

Fluorescein isothiocyanate, isomer I, was obtained from Sigma and used without recrystallization. GTP was purchased from Calbiochem, dithioerythritol from Pierce Chemical Co., phosphocellulose P-11 from Whatman, and 2-mercaptoethanol from BDH Biochemicals. All other chemicals were reagent grade.

IF3 was isolated and purified from *E. coli* MRE600 as in [14]. The preparation used in this work was essentially homogeneous as determined by SDS–polyacrylamide gel electrophoresis. Ribosomal 30 S subunits were isolated by a slight modification of the procedure in [15] and heat-activated as in [16]. The subunits were of the 'A type' [17] and contained S1 and intact 16 S RNA. Formyl-[^{14}C]methionyl-tRNA (f[^{14}C]Met-tRNA) was prepared from unfractionated *E. coli* B tRNA (Schwarz-Mann) and [^{14}C]methionine (Amersham/Searle, 260 Ci/mol) as in [18]. R17 RNA was prepared by phenol extraction of R17 phage essentially as in [19].

F-IF3 was made by treating 2.6 mg IF3 with 0.7 mg fluorescein isothiocyanate (a 15-fold molar excess over IF3) in total vol. 1.1 ml 0.2 M potassium carbonate, pH 9.0, containing 7.4 mM potassium phosphate, 74 mM EDTA, 5 mM 2-mercaptoethanol, 4% glycerol, and 600 mM KCl. The mixture was stirred for 24 h at 4°C, then diluted with 6 vol. doubly distilled water and slowly applied, at room temperature, to a 0.5 × 5.0 cm phosphocellulose column pre-equilibrated with 100 mM KCl in buffer A (10 mM potassium phosphate, pH 7.5; 0.1 mM EDTA; 7 mM 2-mercaptoethanol; 5% glycerol). The column was washed with copious amounts of 100 mM KCl in buffer A to remove non-covalently bound fluorescein. The F-IF3 was slowly eluted with 800 mM KCl in buffer A and stored frozen at −70°C. The molar ratio of fluorescein to protein was determined by using $E^{m}_{490} = 8.51 \times 10^4$ cm^{-1}M^{-1} for bound fluorescein and the protein concentration assay in [20] with an IF3 standard curve calibrated as in [21]. The F-IF3 used in this work had a dye/protein ratio of 0.84.

Fluorescence polarization measurements were determined by means of a device using a rotating polarizer [22], with the excitation monochromator set at 490 nm and the emission monochromator set at 520 nm. An analog computer calculated the polarization value and the total intensity, which are both read directly from the instrument. Fluorescent solutions were prepared as in fig.1 legend.

3. Results and discussion

3.1. *Biological activity of F-IF3*

The initiation factor activities of IF3 and F-IF3 were compared by measuring the stimulation of f[^{14}C]Met-tRNA binding to 70 S ribosomes with bacteriophage R17 RNA as template, essentially as in [14]. In 6 separate assays where the amounts of factor were varied, F-IF3 was 80–100% as active as the non-derivatized IF3 (data not shown). The rates of f[^{14}C]Met-tRNA binding were also identical with the two IF3 species. We conclude that treatment of IF3 with fluorescein isothiocyanate did not significantly alter the biological activity of the factor.

3.2. *Kinetic measurements*

The equilibrium binding of F-IF3 to 30 S ribosomal subunits was studied at 23°C by titrating different, fixed amounts of 30 S subunits with increasing amounts of F-IF3 and measuring the resultant equilibrium fluorescence intensities and polarization values. The polarization data was plotted and resulted in the family of curves shown in fig.1. Equation 1 relates the bound (F_b) to free (F_f) ratio of F-IF3 to the observed polarization, P. It requires that values of P_f (polarization of free F-IF3) and P_b (polarization of totally bound F-IF3) be known.

$$F_b/F_f = \frac{Q_f}{Q_b}\,\frac{P-P_f}{P_b-P} \qquad (1)$$

(Q = quantum yield of fluorescence)

Measurements of polarization values of F-IF3 in the absence of 30 S subunits gave P_f 0.190. P_b was determined to be 0.275 by extrapolation of polarization data obtained with different amounts of 30 S subunits as in [23]. The Q_f/Q_b ratio was determined to be 0.84 by measuring the fluorescence intensity of a small amount of F-IF3 in the free state and in the presence of a large excess of 30 S subunits.

By using eq. (1) and the total F-IF3 concentration at that point, F_b/F_f and F_b were calculated for every point in fig.1 and the data plotted according to

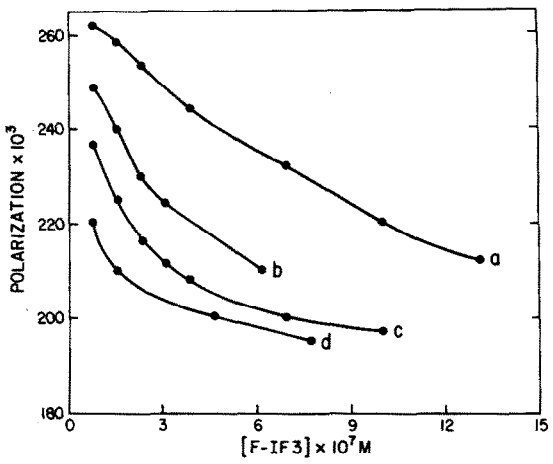

Fig.1. Equilibrium fluorescence polarization values. Curves were generated by titrating fixed amounts of 30 S subunits with increasing amounts of F-IF3. Buffer, 3 ml (10 mM Tris–HCl, pH 7.5; 10 mM magnesium acetate; 100 mM ammonium chloride; 7 mM 2-mercaptoethanol) were added to a 3 ml fluorescence cuvette followed by an aliquot of heat-activated 30 S subunits. Increasing amounts of F-IF3 were added to the subunits to give the concentrations indicated in the figure and the polarization value determined after each addition. Concentrations of 30 S subunits were: curve (a) 2.5×10^{-7} M; curve (b) 1.43×10^{-7} M; curve (c) 0.71×10^{-7} M; and curve (d) 0.36×10^{-7} M. Volume changes due to additions were less than 3%, but were taken into account in subsequent calculations of the concentrations.

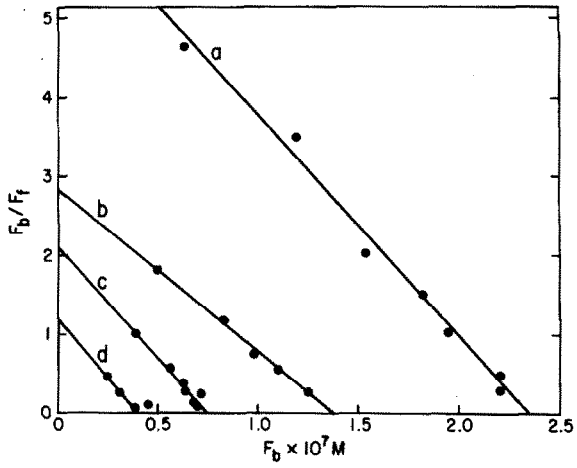

Fig.2. Scatchard plots based on the polarization data. The data reported in fig.1 was converted to F_b/F_f ratios as described in the text and plotted according to Scatchard [24] by using the equation: $F_b/F_f = K_{ass}(F_{bmax} - F_b)$. Lines were drawn according to a least squares analysis. The slope is $-K_{ass}$ and the F_b intercept is F_{bmax}. Lines (a–d) were obtained from data of the corresponding lettered curves of fig.1.

Scatchard [24] as shown in fig.2. The association constants and stoichiometric ratios were calculated by least squares analysis of the data and are reported in table 1. The results of these studies clearly indicate a 1:1 stoichiometry between F-IF3 and the 30 S ribosomal subunit and give an association constant of about 2.8×10^7 M^{-1}. The association constant for F-IF3 is in good agreement with that for unmodified IF3 determined [4], 2.5–4.0 $\times 10^7$ M^{-1}, as determined by light scattering measurements. Our value is somewhat larger than those determined by using zonal centrifugation [25], 1×10^7 M^{-1}, and [26], 1–2 $\times 10^7$ M^{-1} at 4°C and 5×10^6 M^{-1} at 24°C, but zonal centrifugation tends to underestimate association constants due to pressure-induced dissociation [26].

The results presented here establish the feasibility of using fluorescence polarization techniques to study initiation factor–ribosomal interactions. We are

Table 1
Stoichiometric ratios and association constants for the binding of F-IF3 to 30 S ribosomal subunits

[30 S]	F_{bmax}	F_{bmax}/[30 S]	K_{ass}
2.50×10^{-7} M	2.35×10^{-7} M	0.94	2.8×10^7 M^{-1}
1.43×10^{-7}	1.38×10^{-7}	0.97	2.1×10^7
0.71×10^{-7}	0.74×10^{-7}	1.05	2.7×10^7
0.36×10^{-7}	0.40×10^{-7}	1.12	2.9×10^7

currently preparing active fluorescent derivatives of IF1 and IF2 in order to study the binding equilibria and kinetics of all three initiation factors with the 30 S ribosomal subunit.

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