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Authors

Jameson, DM
Gratton, E
Eccleston, JF

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Intrinsic Fluorescence of Elongation Factor Tu in Its Complexes with GDP and Elongation Factor Ts[†]

David M. Jameson*

Department of Pharmacology, University of Texas Health Science Center at Dallas, Dallas, Texas 75235

Enrico Gratton

Physics Department, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

John F. Eccleston

National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

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ABSTRACT: The intrinsic fluorescence properties of elongation factor Tu (EF-Tu) in its complexes with GDP and elongation factor Ts (EF-Ts) have been investigated. The emission spectra for both complexes are dominated by the tyrosine contribution upon excitation at 280 nm whereas excitation at 300 nm leads to exclusive emission from the single tryptophan residue (Trp-184) of EF-Tu. The fluorescence lifetime of this tryptophan residue in both complexes was investigated by using a multifrequency phase fluorometer which achieves a broad range of modulation frequencies utilizing the harmonic content of a mode-locked laser. These results indicated a heterogeneous emission with major components near 4.8 ns for both complexes. Quenching experiments on both complexes indicated limited accessibility of the tryptophan residue to acrylamide and virtually no accessibility to iodide ion. The quenching patterns exhibited by EF-Tu-GDP and EF-Tu-EF-Ts were, however, different; both quenchers were more efficient at quenching the emission from the EF-Tu-EF-Ts complex. Steady-state and dynamic polarization measurements revealed limited local mobility for the tryptophan in the EF-Tu-GDP complex whereas formation of the EF-Tu-EF-Ts complex led to a dramatic increase in this local mobility.

Elongation factor Tu (EF-Tu)¹ mediates the binding of aminoacyl-tRNA to the ribosome of prokaryotic systems and is thus an essential component of the elongation cycle of protein biosynthesis [see Miller and Weissbach (1977), Weissbach (1980), and Bosch et al. (1983) for reviews]. EF-Tu interacts

with many components of the protein biosynthesis system at various stages of the cycle; these components include guanosine nucleotides, elongation factor Ts, aminoacyl-tRNA, and ribosomes. The antibiotics kirromycin and aurodox also bind tightly to EF-Tu (Parmeggiani & Swart, 1985). A body of evidence suggests that the binding of the different ligands

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* Correspondence should be addressed to this author.

¹ Abbreviations: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; ANS, anilinonaphthalenesulfonate; DTT, dithiothreitol; FWHM, full width at half-maximum; CD, circular dichroism; ORD, optical rotatory dispersion; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

FIGURE 1: Schematic of GDP binding domain of trypsin-modified EF-Tu-GDP showing location of tryptophan-184 [adapted from Jurnak (1985)].

promotes structural changes in EF-Tu. Detailed information on the nature of the structural and dynamic alterations manifested by EF-Tu in its interaction with other components of the protein biosynthesis system is essential to our understanding of the role of EF-Tu in such processes. Such information can, in principle, be derived from spectroscopic investigations of the protein in solution and will also be important when considered in light of crystallographic studies (Jurnak, 1985; LaCour et al., 1985).

Previous spectroscopic investigations of EF-Tu, using methodologies such as ANS binding, ORD, CD, and total intrinsic fluorescence signals [reviewed by Kaziro (1978)], have been directed toward detecting differences between the EF-Tu-GDP and EF-Tu-GTP complexes. These studies have not addressed questions pertaining to specific structural changes in the protein complexes. Information concerning the dynamic aspects of the protein matrix has not emerged from these investigations either. Kinetic evidence has been presented suggesting structural changes in the interaction of EF-Tu with ligands although again details of the conformational changes involved remain obscure. For example, the rate of binding of GDP and guanine nucleotide analogues to EF-Tu (Fasano et al., 1978; Eccleston, 1981a) and the rate of binding of aurodox to EF-Tu-GDP (Eccleston, 1981b) are much slower than expected for diffusion-controlled reactions and may consist of an initial binding process followed by a protein isomerization. A protein isomerization has also been implicated in the conversion of EF-Tu-GDP to EF-Tu-EF-Ts (Eccleston, 1984; Eccleston & Geeves, 1986).

In the present study, we are primarily concerned with a quantitative description of the fluorescent properties of the single tryptophan residue of EF-Tu in the EF-Tu-GDP and EF-Tu-EF-Ts complexes. This tryptophan residue is located at position 184 (Arai et al., 1980). Recent X-ray diffraction studies of crystals of a modified (protease treated) form of EF-Tu-GDP show that the tryptophan is located in the N-terminal domain, the domain containing the nucleotide binding site, at the end of α -helix F between β -strand 6 and α -helix E [see Figure 1 and Jurnak (1985)]. There are no tyrosine residues closer than 20 Å to the tryptophan residue, and the putative antibiotic binding site is located in the C-terminal

domain, at least 25 Å from the tryptophan (F. Jurnak, personal communication). EF-Ts has no tryptophan residues (An et al., 1981).

Steady-state (emission spectra, polarization, and quenching) and dynamic (time-resolved measurements) fluorescence studies of the single tryptophan residue afford us information about the local environment of this fluorophore, its motional properties and accessibility to solvent, and general hydrodynamic properties of the protein complexes. This information may be important in assessing the details of conformational changes and dynamic aspects in the EF-Tu-GDP and EF-Tu-EF-Ts complexes. These protein systems are ideally suited for such examinations since both EF-Tu-GDP and EF-Tu-EF-Ts contain the unique tryptophan residue. Although the presence of 10 tyrosine residues in EF-Tu and 3 in EF-Ts renders the tyrosine emission properties, per se, too complex for most quantitative interpretations, the possibility of tyrosine to tryptophan energy transfer can provide an additional probe of protein conformation in the complexes.

MATERIALS AND METHODS

Protein. EF-Tu was prepared by the method of Leberman et al. (1980) followed by back-extraction with ammonium sulfate (Miller & Weissbach, 1974). Since this preparation is heterogeneous with respect to the bound nucleotide, it was converted to EF-Tu-GDP as described by Eccleston (1984). The preparation and characterization of EF-Tu-EF-Ts have previously been described (Eccleston, 1984). Aurodox was a gift from Dr. P. Miller at Hoffmann-La Roche (Nutley, NJ). Protein solutions were stored in liquid nitrogen and thawed immediately before each measurement. All measurements were made in 50 mM Tris-HCl, pH 7.6, containing 10 mM $MgCl_2$ and 0.5 mM DTT. Acrylamide was electrophoresis grade from Bio-Rad (Richmond, CA); *p*-terphenyl was laser grade from Eastman Kodak (Rochester, NY). All other chemicals used were analytical grade.

Steady-State Emission Measurements. Corrected emission spectra were obtained by using the microprocessor-controlled photon-counting spectrofluorometer described by Gratton and Limkeman (1983a). Quenching and steady-state polarization studies were carried out by using an SLM Instruments (Urbana, IL) Model 8000 photon-counting spectrofluorometer equipped with calcite prism polarizers. Polarization measurements were done in the "L" configuration using 1-cm path-length square quarasil cuvettes; all other measurements were done with 3-mm square quarasil cuvettes. All measurements were made at 20 °C unless stated otherwise. The absorbance of all protein solutions in a 1-cm path-length cuvette was between 0.1 and 1.5 at 280 nm; the most concentrated solutions were used with excitation at 300 nm with corresponding decreases in absorbance.

Time-Resolved Measurements. (A) *Lifetimes.* Multifrequency phase and modulation fluorometry was utilized for lifetime and anisotropy decay determinations [see Gratton et al. (1984a) for a review]. In this approach, the intensity of the exciting light is modulated, and the phase shift and relative demodulation of the emitted light, with respect to the excitation, are determined. Lifetimes are then calculated according to the equations:

$$\tan P = \omega \tau^P$$

$$M = [1 + (\omega \tau^M)^2]^{-1/2}$$

where P is the phase shift, M the demodulation, and ω the angular modulation frequency. Two independent lifetime determinations, τ^P and τ^M , are thus obtained. These mea-

measurements have usually been carried out on instruments which utilize either acoustooptic (Debye-Sears tanks) or electrooptic (Pockel's cells) devices for sinusoidal modulation of the exciting light; both xenon arc lamps and lasers have been utilized as light sources. The recent development of techniques utilizing the harmonic content of pulsed light sources such as synchrotron radiation (Gratton et al., 1984c) or mode-locked lasers (Alcala et al., 1985) has greatly extended the UV capabilities and the frequency modulation range of phase fluorometry. Phase and modulation lifetime measurements on the intrinsic fluorescence of proteins at high modulation frequencies were first performed by using synchrotron radiation (Gratton et al., 1984b), and the preliminary time-resolved measurements on the complexes studied in this report were performed by using the synchrotron radiation facility in Frascati, Italy, on the instrumentation previously described (Gratton et al., 1984c). The final time-resolved measurements reported in this work, however, were performed with a multifrequency phase fluorometer utilizing the harmonic content of a mode-locked synchronously pumped dye laser (Alcala et al., 1985). The pulse train from the dye laser was amplitude modulated by an acoustooptic modulator and then frequency doubled, providing excitation in the region 280–310 nm. The high repetition rate pulsed source gives a set of equally spaced harmonic frequencies. Amplitude modulation of the pulse train permits variation of the modulation frequency quasi-continuously from a few hertz to the gigahertz regime. In this study, the frequency set utilized ranged from 1 to 400 MHz. An ISS1-ADC interface (I.S.S., Inc., Champaign, IL) to an IBM-PC and software from I.S.S., Inc., were used for data collection and analysis.

(B) Lifetime Data Analysis. An emitting system characterized by a single exponential decay will yield identical phase and modulation lifetimes irrespective of the modulation frequency. In the case of heterogeneous emitting systems (multiple noninteracting fluorescent species), the phase lifetime will be less than the modulation lifetime, and these values will furthermore be dependent upon the modulation frequency, namely, decreasing as the modulation frequency increases (Spencer & Weber, 1969).

The measured phase and modulation values were analyzed by assuming either a sum of exponentials or a continuous distribution of lifetime values. In both cases, the goodness of the fit was judged by the value of the reduced χ^2 defined as

$$\chi^2 = \frac{\sum \{[(P_c - P_m)/\sigma^P]^2 + [(M_c - M_m)/\sigma^M]^2\}}{(2n - f - 1)} \quad (1)$$

where the sum is carried over the measured values at n modulation frequencies and f is the number of free parameters. The symbols P and M correspond to phase shift and relative demodulation values, respectively, while the indexes c and m indicate the calculated and measured values, respectively. σ^P and σ^M are the standard deviations of each phase and modulation measurement, respectively. In phase fluorometry, the standard deviations of phase and modulation values are essentially independent of the modulation frequency, i.e., constant for each measurement and hence factorize out. The χ^2 is thus independent of a common multiplicative factor, and it is convenient, vis-à-vis the speed of the calculation, to use a common fixed value for the standard deviations of the phase and modulation determinations (0.2° and 0.004 for phase angle and demodulation ratio, respectively). Consequently, the χ^2 value for a given fit includes an arbitrary factor, and the important parameter is thus the change in the χ^2 value upon using different functions for the fit rather than the absolute value of χ^2 . The calculated values of phase and modulation

were obtained by using the equations:

$$P = \tan^{-1} [S(\omega)/G(\omega)]$$

$$M^2 = S(\omega)^2 + G(\omega)^2 \quad (2)$$

where the functions $S(\omega)$ and $G(\omega)$ have different expressions depending on the fitting model utilized.

For the fit using a sum of exponentials, the functions $S(\omega)$ and $G(\omega)$ are given by

$$S(\omega) = \sum f_i \tau_i / (1 + \omega^2 \tau_i^2)$$

$$G(\omega) = \sum f_i / (1 + \omega^2 \tau_i^2) \quad (3)$$

$$\sum f_i = 1$$

where the index i depends on the number of exponentials used for the fit; f_i is the contribution to the steady-state fluorescence of the i th component; τ_i is its lifetime and ω is the angular frequency of light modulation.

For the fit using a continuous distribution of lifetime values, the $S(\omega)$ and $G(\omega)$ functions are given by

$$S(\omega) = \int_0^\infty f(\tau) \tau / (1 + \omega^2 \tau^2) d\tau$$

$$G(\omega) = \int_0^\infty f(\tau) / (1 + \omega^2 \tau^2) d\tau \quad (4)$$

$$\int_0^\infty f(\tau) d\tau = 1$$

where $f(\tau)$ is an arbitrary function. In the present work, the function $f(\tau)$ utilized was a Lorentzian, i.e.

$$f(\tau) = A / \{1 + [(\tau - \tau_0)/\frac{1}{2}W]^2\} \quad (5)$$

where A is determined by the normalization condition, τ_0 is the center of the Lorentzian, and W is its full width at half-maximum. The choice of the Lorentzian function to describe the measured decay is purely arbitrary. Other distribution functions which were tried included the uniform and Gaussian functions. In all cases investigated, the Lorentzian function gave a better fit. Also, the superposition of two Lorentzian distributions was utilized, which results in a bimodal distribution of lifetime values. In the plots presented in this study, the relative heights of the distributions correspond to the fractional contributions of each Lorentzian component to the total fluorescence intensity (Alcala et al., 1986, 1987).

Anisotropy Decay Measurements. In dynamic polarization measurements, the sample is illuminated by light polarized parallel to the vertical laboratory axis with intensity modulated at variable frequencies. The phase delay, $\Delta\Phi$, between the parallel and perpendicular polarization components of the emission can then be directly determined as well as their modulation ratios, Y . For an isotropic rotation, one obtains the expressions (Weber, 1977):

$$\Delta\Phi = \tan^{-1} \{ (3\omega r R) / [(k^2 + \omega^2) \times (1 + r - 2r^2) + R(R + 2k + kr)] \}$$

$$Y^2 = \frac{[k + 6R/(1 - r)]^2 + \omega^2}{[k + 6R/(1 + 2r)]^2 + \omega^2} \quad (6)$$

r is the limiting anisotropy, R the rotational diffusion coefficient, and k the radiative decay rate ($1/\tau$). For a residue in a protein, the expression for the differential phase and modulation ratio contains more terms. First, a protein is generally not an isotropic rotator. However, assuming that the protein can be considered as an ellipsoid and that the ratio of the long axis to the short axis is not large, the rotational relaxation times about the different axes will not differ greatly,

FIGURE 2: Emission spectra of EF-Tu-GDP and EF-Tu-EF-Ts excited at 280 and 300 nm. Excitation and emission band-passes were 2 and 4 nm, respectively.

and our values should correspond approximately to weighted averages of these relaxation times. Second, the relative weight of the rotational times along the different axes depends on the orientation of the fluorophore's absorption and emission dipoles with respect to the long and short axes of the protein [see, for example, Witholt and Brand (1970)]. Since we are interested in distinguishing internal motions from the tumbling of the entire protein molecule, we will consider only large differences in the rotational relaxation times. The following expression for time-dependent anisotropy was used for the rotational analysis:

$$r(\tau) = r_0 \sum_j g_j e^{-\tau/\theta} / \theta \quad (7)$$

where r_0 is the time zero anisotropy, θ is the rotational correlation time, g is the associated amplitude, and the index j can have a value of 1 or 2. This expression describes the rotation of the entire protein (global rotation) plus the internal motion (local rotation) of the tryptophan residue. Equation 7 was converted to the frequency domain by using the method outlined by Weber (1977), and then a fit was performed by using a nonlinear least-squares analysis (Lakowicz et al., 1985).

RESULTS

Corrected Emission Spectra. The corrected emission spectra of EF-Tu-GDP and EF-Tu-EF-Ts are shown in Figure 2. These spectra were obtained by subtracting the appropriate buffer contribution for each sample and then correcting the spectra for the detector response function. Upon excitation of the proteins at 280 nm, the emission spectra were dominated by the tyrosine contribution. For EF-Tu-GDP, an emission maximum of 307 nm and a FWHM of 47 nm were observed. For EF-Tu-EF-Ts, the emission maximum was at 305 nm, and the FWHM was 41 nm. The slight blue shift and decreased FWHM of the EF-Tu-EF-Ts complex compared to the EF-Tu-GDP complex suggest an increased tyrosine contribution in the emission relative to the tryptophan contribution due to the additional tyrosine residues in EF-Ts. Upon excitation at

300 nm (a wavelength which will excite tryptophan but not tyrosine residues), emission maxima of 336 nm for both proteins were observed.

Quenching of the Tryptophan Emission. The accessibility of the tryptophan residue in both EF-Tu-GDP and EF-Tu-EF-Ts complexes to iodide ion and acrylamide was investigated by using fluorescence quenching methodologies. Four-microliter aliquots of either 3 M sodium iodide (containing 0.5 mM sodium thiosulfate) or 6 M acrylamide were added to a solution (250 μ L in a 3 $\times$ 3 mm cuvette) of complex ($OD_{280} = 1.5$ for EF-Tu-GDP and 1.2 for EF-Tu-EF-Ts). Excitation was at 300 nm while emission was monitored at 350 nm; band-passes were 2 and 8 nm for excitation and emission, respectively. Since these quenching studies were carried out using a short path-length cuvette and since the exciting wavelength was 300 nm, corrections for absorption of the exciting light by acrylamide were insignificant. The fluorescence intensity, corrected for the very small buffer contribution and for dilution effects, was measured as a function of quencher concentration. Neither quenching agent at the concentrations used affected the GDP binding capacity of EF-Tu nor did equivalent concentrations of chloride ion affect the fluorescence intensity (data not shown). Figure 3 shows the Stern-Volmer plots for both acrylamide and iodide quenching experiments. The intensity data were analyzed according to the Stern-Volmer equation [see, for example, Eftink and Ghiron (1984)]:

$$F_0/F = 1 + K_{sv}[Q] \quad (8)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$ is the quencher concentration, and K_{sv} is the Stern-Volmer quenching constant which equals (in the case of dynamic quenching) the product of the bimolecular quenching constant (k_q) and the unquenched lifetime (τ_0).

In both Tu complexes, acrylamide quenched the emission substantially better than did iodide ion. In the case of

FIGURE 3: Stern-Volmer plots of intensity quenching ($\lambda_{ex} = 300$ nm; $\lambda_{em} = 350$ nm) of EF-Tu-GDP and EF-Tu-EF-Ts by acrylamide or iodide ion. EF-Tu-GDP: acrylamide (O), iodide (●). EF-Tu-EF-Ts: acrylamide (□), iodide (Δ).

Table I: Phase and Modulation Lifetime Values

Frequency (MHz)	EF-Tu-GDP		EF-Tu-EF-Ts	
	τ^P (ns)	τ^M (ns)	τ^P (ns)	τ^M (ns)
7.09	3.881	5.366	4.315	5.019
21.10	3.572	5.341	3.947	4.876
29.10	3.225	4.822	3.660	4.725
39.10	2.861	4.559	3.366	4.453
73.20	1.953	3.950	2.525	4.154
83.21	1.737	3.909	2.445	4.000
112.31	1.321	3.510	1.754	3.933
141.40	1.088	3.234	1.587	3.832
203.52	0.733	2.610	1.038	3.652
336.92	0.533	1.960	0.734	2.885

acrylamide quenching, the Stern-Volmer quenching constants were $K_{sv} = 1.34$ and 1.68 M^{-1} for EF-Tu-GDP and EF-Tu-EF-Ts, respectively. The effect of acrylamide upon the fluorescence lifetime, i.e., verification of the dynamic nature of the quenching, was also investigated (see Fluorescence Lifetime Measurements and Discussion). Iodide was completely ineffective at quenching the tryptophan emission of EF-Tu-GDP and only slightly effective in the EF-Tu-EF-Ts system.

Fluorescence Lifetime Measurements. The phase and modulation lifetime values for EF-Tu-GDP and EF-Tu-EF-Ts at a number of modulation frequencies are shown in Table I. These data were obtained by using 300-nm exciting light and cutoff filters on the emission side (either Corning 0-52 or two Corning 0-54 filters) chosen to pass wavelengths greater than 340 nm. The exciting light was polarized at 35° relative to the vertical laboratory axis and observed without a polarizer to eliminate possible polarization effects upon the lifetime determination (Spencer & Weber, 1970). A solution of *p*-terphenyl in cyclohexane was used as the phase reference solution, instead of the usual glycogen scatterer, to eliminate small systematic errors which were previously observed at very high modulation frequencies (>200 MHz) (Alcala et al., 1985, 1986). The differences between the phase and modulation lifetime values for EF-Tu-GDP and EF-Tu-EF-Ts and their frequency dependence clearly indicate that the fluorescence decay of the tryptophanyl residue in these proteins is not well described by a single exponential.

Two-component analysis of these data yielded lifetimes of 4.9 and 0.34 ns with fractional intensities of 0.77 and 0.23, respectively, for EF-Tu-GDP and 4.7 and 0.36 ns with frac-

FIGURE 4: Multifrequency phase (closed symbols) and modulation (open symbols) data for EF-Tu-GDP (squares) and EF-Tu-EF-Ts (circles) excited at 300 nm; emission viewed through Corning 0-52 cutoff filters ($\lambda_{em} > 340$ nm). Solid lines correspond to 2-component fits: for EF-Tu-GDP, $\tau_1 = 4.9$ ns, $\tau_2 = 0.34$ ns, and $f_1 = 0.77$; for EF-Tu-EF-Ts, $\tau_1 = 4.7$ ns, $\tau_2 = 0.36$ ns, and $f_1 = 0.86$.

FIGURE 5: Continuous distribution lifetime plots for EF-Tu-GDP (A, C) and EF-Tu-EF-Ts (B, D) in the absence of (A, B) and presence (C, D) of acrylamide ([acrylamide] = 0.52 M).

tional intensities of 0.86 and 0.14, respectively, for EF-Tu-EF-Ts (Figure 4). The χ^2 values were 13 and 22, respectively, for EF-Tu-GDP and EF-Tu-EF-Ts while a three-exponential component fit gave lower χ^2 values (4 and 20, respectively), demonstrating that the decay is more complex than two discrete exponentials.

A new and alternative approach to lifetime heterogeneity analysis involves the use of a distributional model (Gratton et al., 1986; Alcala et al., 1986, 1987). In this approach, a distribution of lifetime values is assumed, and the results are displayed as shown in Figure 5. The results shown for EF-Tu-GDP and EF-Tu-EF-Ts give lower or comparable χ^2 values (4 and 15, respectively) than were obtained with the two- and three-exponential component analyses and indicate major components near 4.8 and 4.6 ns for EF-Tu-GDP and EF-Tu-EF-Ts, respectively (Figure 5A,B). These results were obtained by using a bimodal Lorentzian distribution (see Discussion).

The effect of acrylamide on the tryptophan lifetime for both protein complexes was evaluated by using a multifrequency study at several different acrylamide concentrations (specif-

FIGURE 6: Excitation polarization spectra for EF-Tu-GDP (squares) and EF-Tu-EF-Ts (circles) for emission viewed through paired Schott filters WG 340 and WG 360 ($\lambda_{em} > 345$ nm).

ically, 0, 0.275, 0.522, and 0.745 M) and analyzing the lifetime data using the distributional approach. Although the decay is complex, a clear decrease in the lifetime and relative weight of the major component in the presence of the quencher was observed as shown in Figure 5C,D, indicating that the quenching is largely dynamic. At the 0.522 M acrylamide level, τ_0/τ values for both EF-Tu-GDP and EF-Tu-EF-Ts systems were in the range of 1.6–1.8, in reasonably good agreement with the intensity quenching results.

Excitation Polarization Studies. Excitation polarization spectra for EF-Tu-GDP and EF-Tu-EF-Ts are shown in Figure 6. These data correspond to the polarization of the emission at the wavelengths greater than 345 nm, i.e., emission corresponding to tryptophan regardless of the exciting wavelength (data were corrected for the small buffer contribution). The striking aspect of these spectra is the lower polarization values for EF-Tu-EF-Ts compared to EF-Tu-GDP at exciting wavelengths greater than 295 nm. This decrease in polarization, in conjunction with the similarities of the lifetime data for the two complexes, indicates increased motional properties for the tryptophan residue upon formation of EF-Tu-EF-Ts despite the increase in the size of the complex. At 300 nm, the polarization values were 0.270 and 0.216 for EF-Tu-GDP and EF-Tu-EF-Ts, respectively. A starting point for analysis of steady-state polarization values is the Perrin equation:

$$1/P - 1/3 = (1/P_0 - 1/3)(1 + 3\tau/\rho_h) \quad (9)$$

where P is the observed polarization, P_0 the limiting or intrinsic polarization, τ the fluorescence lifetime, and ρ_h the harmonic mean of the rotational relaxation times. For tryptophan, the P_0 value upon excitation at 300 nm is 0.405 (Valeur & Weber, 1978). With this value (which we also obtained on our instrumentation for tryptophan in glycerol at 0 °C) and average lifetimes of 4.8 and 4.6 ns for EF-Tu-GDP and EF-Tu-EF-Ts, respectively, the Perrin equation yields ρ_h values of 25 and 14 ns for these complexes. These values are lower than what one would expect for globular proteins of molecular weights 43 000 (EF-Tu-GDP) and 74 000 (EF-Tu-EF-Ts) and may indicate the existence of local mobility of the tryptophan residue in addition to the global rotation of the protein system. The dynamic polarization results presented in the next section reveal the extent of this local mobility.

Another interesting feature in the polarization spectra is the increased ratio of the polarization values at exciting wavelengths of 302 and 270 nm (i.e., the 302/270-nm ratio). These ratios were 1.91 and 1.61 for EF-Tu-GDP and EF-Tu-EF-Ts, respectively, compared to a ratio of 1.45 for tryptophan in glycerol at 0 °C. As Weber has pointed out (Weber, 1960), this ratio is an indication of tyrosine to tryptophan energy transfer in proteins. We will discuss this point further under

FIGURE 7: Multifrequency differential phase (closed symbols) and modulation ratio (open symbols) data for EF-Tu-GDP (circles) and EF-Tu-EF-Ts (squares). Solid lines were calculated for the following: EF-Tu-GDP, $\tau_1 = 4.8$ ns, $\tau_2 = 0.31$ ns, $f_1 = 0.79$, $\rho_1 = 63$ ns, $r_1 = 0.23$, $\rho_2 = 1.5$ ns, and $r_2 = 0.05$; EF-Tu-EF-Ts, $\tau_1 = 4.60$ ns, $\tau_2 = 0.31$ ns, $f_1 = 0.82$, $\rho_1 = 84$ ns, $r_1 = 0.19$, $\rho_2 = 0.3$ ns, and $r_2 = 0.12$.

Discussion. In the presence of saturating concentrations of aurodox, the excitation polarization spectrum for EF-Tu-GDP was essentially unchanged (data not shown).

Time-Dependent Anisotropy Studies. The plot of the differential phase as a function of the log of the frequency should give a bell-shaped curve for each distinct rotational motion. The position of the maximum on the frequency axis depends upon the rotational rate and the lifetime (see eq 6). The modulation ratio should instead give a sigmoidal curve for each rotational motion (see eq 6). The value of the modulation ratio at low frequency depends upon the steady-state anisotropy whereas the value at very high frequency depends only upon the time zero anisotropy. The results for EF-Tu-EF-Ts and EF-Tu-GDP complexes are shown in Figure 7. The solid lines in the figure correspond to the best fit using two rotational motions. The corresponding rotational relaxation times for EF-Tu-GDP were 63 and 1.5 ns with relative amplitudes (anisotropies) of 0.23 and 0.05, respectively. For EF-Tu-EF-Ts, the best fit was obtained with values of 84 and 0.3 ns and relative amplitudes of 0.19 and 0.12, respectively.

DISCUSSION

As the spectra in Figure 2 indicate, the emissions of both EF-Tu-GDP and EF-Tu-EF-Ts, upon 280-nm excitation, are dominated by the tyrosine contribution—this observation is consistent with the earlier results of Arai et al. (1977). Such dominance by tyrosine in a tryptophan-containing protein is unusual since proteins with a single tryptophan typically exhibit an emission characteristic of tryptophan with at best a shoulder on the blue side of the spectrum due to the tyrosines, although some exceptions to this generality such as subtilisin, Carlsberg and subtilisin inhibitor have been reported [for a review, see Longworth (1983)]. In the case of the EF-Tu complexes, then, it may be concluded either that the quantum yield of the tryptophan residue is unusually low or that at least some of the tyrosine residues have higher quantum yields than is usual. The relatively long major lifetime component observed for the tryptophan residue, discussed later, argues against the first position, but clearly a detailed examination of the various quantum yields is required before conclusions can be drawn on this point. The observation that the tryptophan emission maxima in both EF-Tu-GDP and EF-Tu-EF-Ts are at 336 nm does not permit us to draw firm conclusions regarding the degree of solvent exposure of the tryptophan residue. Although a general tendency toward “bluer” emissions from buried tryptophans has been noted in the literature, there exist sufficient exceptions (Longworth, 1983) to warrant caution in drawing conclusions about the degree of solvent exposure or polarity of the environment simply on the basis of the position

emission maxima. The microscopic details of the environment, such as the extent of polarizability, the presence of water molecules, and the specific interactions between the indole ring and polar groups in the protein, will all influence the maximum emission wavelength.

The observation that acrylamide quenches the tryptophan emission of both protein complexes substantially better than does iodide suggests that, in both systems, the local charge environment of the tryptophan residue may be somewhat negative, thus inhibiting access of the negatively charged iodide ion. The primary sequence of EF-Tu supports this interpretation; in particular, the residues on either side of the tryptophan are glutamic acids (Arai et al., 1980). The K_{sv} values for acrylamide quenching, namely, 1.34 and 1.68 M⁻¹ for EF-Tu-GDP and EF-Tu-EF-Ts, respectively, fall within the lower range of the acrylamide quenching constants measured by Eftink and Ghiron (1983) for a number of single tryptophan-containing proteins. The bimolecular quenching constants, calculated by using the major lifetime components of 4.8 and 4.6 ns for EF-Tu-GDP and EF-Tu-EF-Ts, respectively, are 2.8×10^8 and 3.7×10^8 M⁻¹ s⁻¹. These values indicate that the tryptophan, in both complexes, is marginally accessible to the solvent but that formation of the EF-Tu-EF-Ts complex enhances the accessibility. The X-ray structure of the EF-Tu-EF-Ts complex is not yet available, but our quenching data suggest that the tryptophan residue in this protein dimer does not become buried in the interface region upon formation of the complex. Furthermore, the increase in the efficiency of iodide quenching upon formation of the EF-Tu-EF-Ts complex suggests that the overall charge environment around the tryptophan may be altered in the dimer complex relative to EF-Tu-GDP.

The analysis of the lifetime data reveals that in both EF-Tu complexes the decay law associated with the single tryptophan is quite complex. Such complex decays for proteins containing single tryptophan residues are, in fact, generally observed (Beechem & Brand, 1984). In both EF-Tu complexes, the χ^2 values obtained by using single exponentials were about 2000 and dropped to about 20 when two exponentials were used. Using three exponentials gave still lower χ^2 values in both cases. The distributional analysis, which assumes that a quasi-continuum of lifetime values exists, gave comparable or lower χ^2 values relative to the three discrete exponential cases. The observation that very large decreases in the χ^2 values occur upon passing from the single to the dual exponential component analysis indicates that there are possibly at least two gross conformations for the tryptophan surroundings. However, these two potential conformations are not unique as indicated by the large spread of the lifetime values obtained from the distributional analysis. The FWHM's of about 1 ns are among the widest distributions that have been observed for single tryptophan proteins at room temperature (Gratton et al., 1986). In the EF-Tu-EF-Ts complex, the magnitude of the main lifetime component is slightly less than in EF-Tu-GDP, but the width associated with this component is decreased relative to EF-Tu-GDP.

We should emphasize that at this time insufficient information exists on the precise influences of protein structure and dynamics on the excited-state lifetime of tryptophanyl residues to warrant a detailed modeling of results such as those on the Tu systems in terms of distinct conformational states. The width of the lifetime distributions has been observed to be related to the dynamics of the tryptophan residues (Gratton et al., 1986) which leads us to conclude that formation of the EF-Tu-EF-Ts complex results in increased mobility for the

tryptophan residue compared to the EF-Tu-GDP case. The analysis of the rotational motions of the tryptophan residue in both protein complexes also demonstrates an increased mobility for the tryptophan in the EF-Tu-EF-Ts complex compared to EF-Tu-GDP. In the case of both proteins, the decay of the emission anisotropy must be analyzed by using at least two rotational components. The Debye rotational relaxation times (note that rotational relaxation time is 3 times the rotational correlation time) obtained for EF-Tu-GDP were 63 and 1.5 ns; the longer relaxation time is consistent with the rotational relaxation time expected for a globular protein with a molecular weight of 43 000 [assuming a spherical conformation, the calculated ρ_0 would be ~ 50 ns; see Eccleston et al. (1987)]. A dynamic polarization study using a fluorescent nucleotide, described in Eccleston et al. (1987), determined a rotational relaxation time of 88 ns for the EF-Tu-GDP complex, a value which is more consistent with the ellipsoidal model for EF-Tu proposed by Sjöberg and Elias (1978) and Morikawa et al. (1978) than the 63-ns value determined by using the tryptophan emission. The 63-ns value, however, is not likely to reflect the overall hydrodynamic parameters of the protein system as well as the 88-ns value since the associated calculations assume a unique lifetime value but, as is noted, the actual tryptophan lifetime data are better described by a distribution of lifetime values. The relative orientation of the absorption and emission dipoles of the tryptophan moiety with respect to the rotational axis of the protein will also affect the calculated rotational relaxation time. The corresponding relaxation times for EF-Tu-EF-Ts were 84 and 0.3 ns. In this case, however, the contribution of the faster rotating component is much larger than that observed for EF-Tu-GDP. The relaxation time of 0.3 ns is exceptionally rapid considering that free tryptophan in buffer at 20 °C has a rotational relaxation time of about 0.25 ns (Alcala et al., 1985). This rapid rotational motion of tryptophan has also been observed in lysozyme (Marriott et al., 1986) using the same technique.

Although the tyrosine emission per se, originating as it does from a number of tyrosine residues, is too complex to be useful for most quantitative conclusions, the possibility of tyrosine to tryptophan energy transfer may provide information on protein conformational changes. As Weber has demonstrated (Weber, 1960), the ratio of the tryptophan polarization upon excitation at wavelengths (>300 nm) where only tryptophan is excited to the polarization upon excitation at wavelengths (270 nm) where both tyrosine and tryptophan are excited can give an indication of the extent of tyrosine to tryptophan energy transfer. For tryptophan or indole in viscous solvents at low temperature, this ratio is around 1.4–1.5, whereas in most of the proteins Weber studied ratios of around 2 were observed. The change in this ratio from 1.9 to 1.6 upon formation of the EF-Tu-EF-Ts complex suggests that the efficiency of tyrosine to tryptophan energy transfer is decreased in the protein dimer and is an additional indication of a conformational change upon formation of this complex.

Our results demonstrate that the intrinsic fluorescence of EF-Tu in its complexes with GDP and EF-Ts, in particular the emission properties of tryptophan-184, is a useful probe of dynamic aspects of these protein complexes. The time-resolved studies, both lifetime and rotational analysis, indicate that the mobility of the tryptophan residue increases markedly upon formation of the EF-Tu-EF-Ts complex. These results and the quenching data suggest that, upon formation of the EF-Tu-EF-Ts complex, the tryptophan residue is not buried in an interface; the data in fact suggest that formation of the EF-Tu-EF-Ts complex leads to increased mobility of the

protein matrix in the GDP binding domain. These results may have general implications concerning the mechanism of GDP release from other guanosine nucleotide binding proteins.

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