Interaction of a Fluorescent Analogue of GDP with Elongation Factor Tu: Steady-State and Time-Resolved Fluorescence Studies†

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Received October 8, 1986; Revised Manuscript Received February 11, 1987

ABSTRACT: A fluorescent derivative of GDP was prepared by the reaction of 2′-amino-2′-deoxy-GDP with fluorescamine. This derivative binds tightly ($K_D \sim 4.5 \times 10^{-8}$ M) to elongation factor Tu (EF-Tu) from *Escherichia coli*. The emission properties, including spectra, polarizations, and lifetimes, for fluorescamine–GDP free in solution and bound to EF-Tu are presented. Emission data on the fluorescamine-ethylamine conjugate are also given. A multifrequency phase and modulation lifetime study (using nine modulation frequencies over the range of 2–80 MHz) indicated that the emissions of these three systems were well characterized by single exponential decays corresponding to 1.45 ns for the fluorescamine–ethylamine derivative in buffer and to 7.74 and 11.03 ns for the fluorescamine–GDP derivative free in buffer and bound to EF-Tu, respectively. Multifrequency differential polarized phase fluorometry results indicated a rotational relaxation time of the protein–probe complex of approximately 88 ns; these data also indicated the lack of significant local motion for the probe. Addition of excess GDP to the EF-Tu–probe complex led to displacement of the fluorescamine–GDP derivative as evidenced by the change in both the steady-state and dynamic polarization values. The observed increase in fluorescence intensity upon displacement allowed us to follow the kinetics of the dissociation reaction; a dissociation rate constant of 5.0×10^{-3} s^{-1} was determined. These results demonstrate the utility of this 2′-amino-2′-deoxy-GDP analogue as a probe of guanosine nucleotide dependent systems.

The importance of guanosine nucleotide binding proteins in the regulation of many biological processes has become apparent in recent years. For example, we now know that guanosine nucleotide binding proteins are intimately connected with the hormone-sensitive adenylate cyclase system (Gilman, 1984), the light-activated cGMP-specific phosphodiesterase in the retina (Stryer et al., 1981), and the stability of microtubules (Purich & Kristofferson, 1984). The protein products of ras oncogenes are also guanosine nucleotide binding proteins (Gibbs et al., 1985). The involvement of guanosine nucleotides in the initiation, elongation, and termination steps of protein biosynthesis has been recognized for a longer time (Weissbach, 1980; Bosch et al., 1983).

In order to investigate hydrodynamic properties of these proteins and to make equilibrium and kinetic measurements of their interaction with nucleotides and other components of the relevant system, it is advantageous to use fluorescent analogues of guanosine nucleotides. 2-Azido-6-oxypurine riboside and 2-aminopurine riboside both exhibit fluorescent properties (Weigand & Kaleja, 1976; Ward et al., 1969). However, guanosine nucleotide binding proteins often show high specificity for the purine ring. For example, elongation factor Tu (EF-Tu)[1] binds 2-azido-6-oxypurine riboside 5′-diphosphate 90-fold weaker than GDP and exhibits no detectable binding of 2-aminopurine riboside 5′-diphosphate (Eccleston, 1981).

Modification of the ribose moiety of guanosine presents a second class of fluorescent analogues. The three-dimensional structure obtained by X-ray diffraction techniques on crystals of EF-Tu·GDP shows that the 2′, 3′-*cis*-diol of GDP projects out of the protein (Jurnak, 1985). This finding suggests that modification of the nucleotide at these ribose positions, e.g., by incorporation of a fluorescent moiety, may not adversely affect the binding properties of such guanine nucleotide analogues.

2′-Amino-2′-deoxyguanosine is a suitable precursor to such analogues since the presence of the reactive 2′-amino group allows the introduction of fluorophores with a wide range of fluorescent properties such as differing lifetimes or environmental sensitivities. We report here the synthesis of a fluorescamine (4-phenylspiro[furan-2(3*H*),1′(3′*H*)-isobenzofuran]-3,3′-dione) derivative of 2′-amino-2′-deoxyguanosine 5′-diphosphate and a characterization of its binding properties to EF-Tu as well as its absorption and fluorescence properties free in solution and bound to protein.

Materials and Methods

The synthetic approach to the synthesis of the fluorescamine derivative of 2′-amino-2′-deoxyguanosine 5′-diphosphate is shown in Figure 1.

Preparation of 2′-Amino-2′-deoxyguanosine 5′-Monophosphate. 2′-Amino-2′-deoxyguanosine (Nakanishi et al.,

† This work was supported by the Medical Research Council, U.K. (J.F.E.), by U.S. Public Health Service Grant GM 29603 (J.F.E.), and by National Science Foundation Grants PCM-8403107 (E.G.) and PCM-8402663 (D.M.J.). A preliminary report on this work has been presented at the Feb 1984 meeting of the Biophysical Society.
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[1] Abbreviations: EF-Tu, elongation factor Tu; fluorescamine–GDP, 2′-amino-2′-deoxyguanosine 5′-diphosphate derivatized on the 2′-amino group with fluorescamine; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography; DTE, dithioerythritol.

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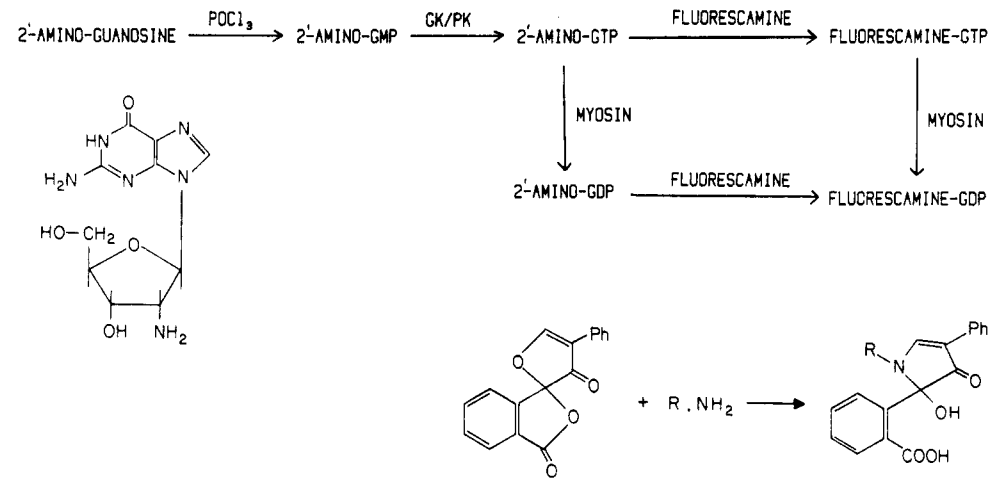

FIGURE 1: Synthetic route for the preparation of 2′-amino-2′-deoxyguanosine 5′-mono-, 5′-di-, and 5′-triphosphates and their derivatives with fluorescamine. 2′-Amino-2′-deoxyguanosine is abbreviated to 2′-aminoguanosine. The nucleotides are similarly abbreviated. The lower scheme shows the reaction of fluorescamine with primary amines (DeBernardo et al., 1974) where RNH_2 is 2′-amino-2′-deoxyguanosine nucleotide.

1974) was a generous gift from Dr. F. Tomita (Kyowa Hakko Kogyo Co. Ltd., Tokyo); 67 μmol of this nucleoside was dissolved in 1.5 mL of triethyl phosphate with gentle heating. The solution was then cooled on ice after which 133 μmol of water and 200 μmol of $POCl_3$ were added. Additional 200-μmol aliquots of $POCl_3$ were added after 30, 60, and 150 min. Samples of the reaction mixture were taken at intervals, diluted 100-fold with water, and analyzed by HPLC. The Waters μBondapak C_{18} column (3.9 mm × 30 cm) was eluted under isocratic conditions at 2 mL min^{-1} with 10 mM KH_2PO_4, pH 5.5, buffer containing 4% methanol. The absorbance of the eluant was monitored at 254 nm. Under these conditions, 2′-amino-2′-deoxyguanosine elutes at 4.2 min. During the reaction, the amplitude of the 2′-amino-2′-deoxyguanosine peak decreased while a new peak formed eluting at 1.8 min. This peak contained a shoulder on the trailing edge and was presumed to be mainly the 5′-monophosphate together with some 3′-monophosphate. After 180 min, more than 80% of the nucleoside had been phosphorylated. The reaction mixture was then diluted with 50 mL of cold water, adjusted to pH 7.6, and applied to a DE-52 column (Whatman) (30 cm × 2.5 cm) in the bicarbonate form. This column was eluted with a linear gradient of 0.01–0.40 M triethylammonium bicarbonate, pH 7.6 (total volume 3000 mL). A major peak of absorbance was eluted at approximately 0.25 M triethylammonium bicarbonate. Its absorption spectrum was typical of that of guanosine nucleotides with an absorption maximum at 252 nm and a shoulder at 270 nm; it formed a fluorescent product with fluorescamine (DeBernardo et al., 1974; see below). The ^{31}P NMR spectrum in 50 mM Tris, pH 8.0, showed a triplet at 2.47 ppm downfield from 85% H_3PO_4 which collapsed to a single peak upon proton decoupling. The triplet had a splitting of 4.4 Hz. This result is expected if the phosphate group is at the 5′-position since the two C(5′) protons have near-magnetic equivalence in guanine nucleotides with an average coupling constant, $^1/_2(J_{5'P} + J_{5''P})$, of 4.5 Hz in GMP and 4.7 Hz in 2′dGMP (Davies & Danyluk, 1974). A total of 33 μmol of product was obtained.

Preparation of 2′-Amino-2′-deoxyguanosine 5′-Triphosphate. The 5′-monophosphate was converted to the 5′-triphosphate by guanosine 5′-monophosphate kinase and pyruvate kinase in the presence of a catalytic amount of ATP. The initial reaction mixture (12 mL) contained 2 mM 2′-amino-2′-deoxyguanosine 5′-monophosphate, 67 mM phosphoenolpyruvate, 50 mM Tris, 5 mM $MgCl_2$, and 17 μM ATP adjusted to pH 7.6; 2500 units of pyruvate kinase (from rabbit muscle, Boehringer) and 10 units of guanosine 5′-monophosphate kinase (from hog brain, Boehringer) were added, and the mixture was incubated at 30 °C. Further additions of the same amounts of both enzymes were made at 6 and 24 h. The progress of the reaction was followed by HPLC analysis using a Whatman Partisil 10 SAX column (4.6 mm × 25 cm) eluted under isocratic conditions with 0.6 M ammonium hydrogen phosphate, pH 4.0, at 2 mL min^{-1}. The 5′-monophosphate eluted at 2.0 min, and the formation of 5′-triphosphate was monitored by the formation of a peak eluting at 4.4 min. After 48 h, approximately 80% of the 5′-monophosphate had been converted to the 5′-triphosphate. The reaction was terminated by the addition of Dowex 50W resin in the H^+ form to adjust the solution to pH 2; the solution was then filtered and the filtrate adjusted to pH 7.6. The 5′-triphosphate was purified on a DE-52 column as described for the 5′-monophosphate except that the gradient was from 0.01 to 0.60 M triethylammonium bicarbonate.

Preparation of 2′-Amino-2′-deoxyguanosine 5′-Diphosphate. The 5′-triphosphate was hydrolyzed to the 5′-diphosphate with myosin subfragment 1. The reaction solution contained 2 mM 2′-amino-2′-deoxyguanosine 5′-triphosphate, 10 mM Tris, 1 mM $MgCl_2$, and 2 mg of myosin subfragment 1 (Weeds & Taylor, 1975) at pH 7.5 and 23 °C. The reaction was followed by HPLC analysis using the conditions described for the 5′-triphosphate, the 5′-diphosphate eluting after 2.6 min. The hydrolysis proceeded smoothly with 100% conversion after 2 h. The reaction was terminated and the product purified as described for the 5′-triphosphate.

Preparation of the Fluorescamine Derivative of 2′-Amino-2′-deoxyguanosine 5′-Diphosphate. One milliliter of 5 mM fluorescamine (Sigma) in acetone was added to an equal volume of 1 mM 2′-amino-2′-deoxyguanosine 5′-diphosphate in 25 mM sodium bicarbonate, pH 8.2. After 5 min at room temperature, the solution was diluted to 50 mL with water and purified on a DE-52 column (30 cm × 2.5 cm) in the bicarbonate form using a gradient of 0.01–0.67 M triethylammonium bicarbonate (total volume 3000 mL). The eluant was monitored by measuring the absorption at 254 nm and the fluorescence emission at 480 nm (excitation 390 nm). A major fluorescent peak was eluted at approximately 0.4 M triethylammonium bicarbonate. It was evaporated to dryness and the remaining triethylamine removed.

The fluorescamine derivative of 2′-amino-2′-deoxyguanosine 5′-triphosphate was synthesized by a similar procedure. This compound could be hydrolyzed to the 5′-diphosphate derivative

Table I: TLC Properties of 2′-Amino-2′-deoxyguanosine Nucleotides and Their Fluorescamine Derivatives

nucleotide	R_f(system 1)[a]	R_f(system 2)[b]
GMP	0.61	0.78
GDP	0.47	0.53
GTP	0.33	0.64
2′-amino-2′-deoxy-GMP	0.58	0.46
2′-amino-2′-deoxy-GDP	0.51	0.28
2′-amino-2′-deoxy-GTP	0.43	0.30
fluorescamine–GDP	0.15	0.91
fluorescamine–GTP	0.07	0.93

[a] System 1: Cellulose PEI-F TLC plates (J. T. Baker Chemical Co., Phillipsburg, NJ) developed in 2 M LiCl. [b] System 2: Silica gel 60 F254 TLC plates (E. Merck, Darmstadt, W. Germany) developed with 75% 1 M ammonium acetate–25% ethanol.

by myosin subfragment 1 as described for the synthesis of 2′-amino-2′-deoxyguanosine 5′-diphosphate.

The position of the labeling by fluorescamine at the 2′-amino group was confirmed by the fact that no reaction occurred between fluorescamine and GDP or GTP.

The TLC properties of these derivatives are shown in Table I.

Preparation of the EF-Tu·Fluorescamine–GDP Complex. EF-Tu·GDP was prepared as described by Leberman et al. (1980). The GDP was replaced by fluorescamine–GDP by the method described previously for the preparation of the EF-Tu·thio-GDP complex (Eccleston, 1981). The fluorescamine derivative of ethylamine was prepared and characterized as described by DeBernardo et al. (1974).

Instrumentation. Emission spectra of fluorescamine–GDP bound to EF-Tu and displaced by excess GDP and the time course of the dissociation were measured on a Farrand Mark 1 spectrofluorometer. Absorption spectra were obtained by using a Beckman DU8B spectrophotometer. Corrected excitation spectra and emission spectra were obtained on a SPEX fluorometer.

Excitation polarization spectra were obtained by using an SLM 8000 spectrofluorometer (SLM Instruments, Urbana, IL). Time-resolved measurements were carried out by using the multifrequency phase fluorometer described by Gratton and Limkeman (1983).

Results

Spectroscopic Properties of Fluorescamine–GDP. The absorption spectrum of fluorescamine–GDP in buffer (Figure 2A) shows maxima at 259 and 380 nm. The 259-nm peak derives from both the guanine and fluorescamine moieties (since fluorescamine–ethylamine also absorbs at 260 nm). Concentrations of fluorescamine–GDP were therefore measured by absorption at 380 nm where only the fluorescamine absorbs. The molar extinction coefficient (ϵ) of the fluorescamine–GDP was determined by measuring the labile phosphate content (Mahoney & Yount, 1984) of a fluorescamine–GDP solution and comparing this value to the absorbance at 380 nm; ϵ was determined to be 2.4×10^3 M^{-1} cm^{-1}.

Figure 2B shows the corrected excitation spectrum with a maximum at 385 nm and the emission spectrum (uncorrected) with a maximum at 484 nm. These spectroscopic properties are qualitatively similar to those of other fluorescamine–primary amine adducts described by DeBernardo et al. (1974).

Binding of Fluorescamine–GDP to EF-Tu. The ability of 2′-amino-2′-deoxy-GDP and fluorescamine–GDP to bind to EF-Tu was determined by competition experiments with [^{3}H]GDP. Experimental details and analysis of the data were as previously reported (Eccleston, 1981).

Under these conditions (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 0.5 mM dithiothreitol, 37 °C), we found that

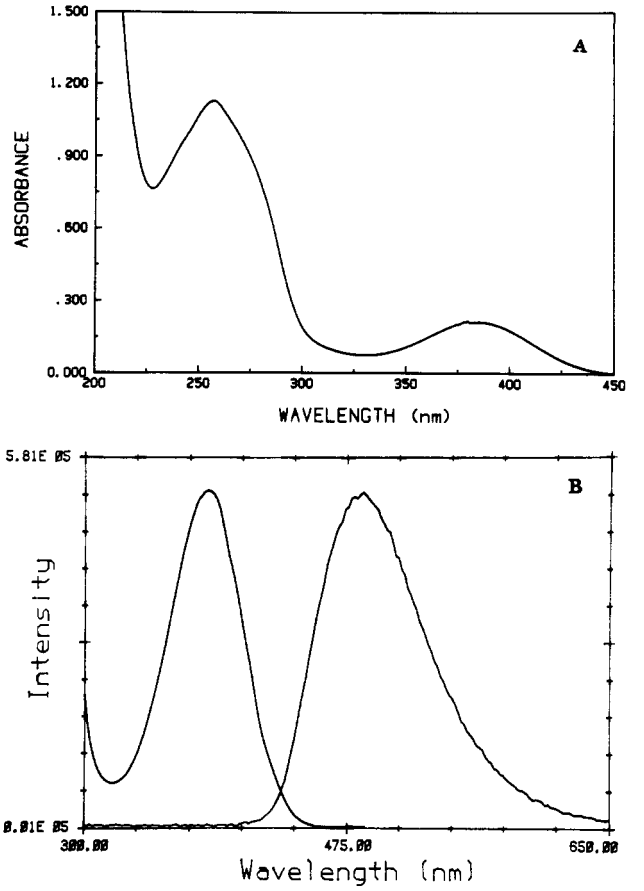

FIGURE 2: Spectroscopic properties of fluorescamine–GDP. (A) Absorption spectrum. The 1-cm path-length cuvette contained 36 μM fluorescamine–GDP. (B) Corrected excitation spectrum (emission 480 nm) and uncorrected emission spectrum (excitation 390 nm) of a 12 μM solution of fluorescamine–GDP. Excitation and emission band-passes were 2 nm. The solutions also contained 50 mM Tris-HCl–10 mM $MgCl_2$, pH 7.6.

Table II: Equilibrium and Kinetic Constants for the Interaction of EF-Tu with GDP and GDP Analogues

nucleotide	K_D (M)	k_{diss} (s^{-1})
GDP	3.0×10^{-9} [a]	4.4×10^{-3} [b]
2′-amino-2′-deoxy-GDP	1.5×10^{-8} [c]	
fluorescamine–GDP	4.5×10^{-8} [c]	5.0×10^{-3} [c]

[a] Miller & Weissbach (1970). [b] Fasano et al. (1978). [c] This work.

2′-amino-2′-deoxy-GDP binds to EF-Tu 5-fold weaker than GDP while fluorescamine–GDP binds 15-fold weaker. These values correspond to equilibrium dissociation constants of 1.5×10^{-8} and 4.5×10^{-8} M, respectively.

The change in the fluorescence emission and absorption properties of fluorescamine–GDP upon binding to EF-Tu was investigated. Since EF-Tu containing no bound nucleotide is extremely unstable, the measurements were made by preparing EF-Tu·fluorescamine–GDP and determining the emission and absorption spectra before and after displacement with an excess of GDP. Figure 3A shows the absorption spectra for fluorescamine–GDP bound to EF-Tu and displaced by GDP. Figure 3B shows that upon displacement from EF-Tu and with excitation at 390 nm the emission intensity of fluorescamine–GDP is increased by a factor of about 2. This process is accompanied by an increase in the absorbance at 390 nm of 27% (Figure 3A). This change in fluorescence enabled us to measure the dissociation rate constant of fluorescamine–GDP from EF-Tu by recording emission intensity with time after addition of excess GDP. As shown in Figure 4, this rate constant was determined to be 5.0×10^{-3} s^{-1}. Table II sum-

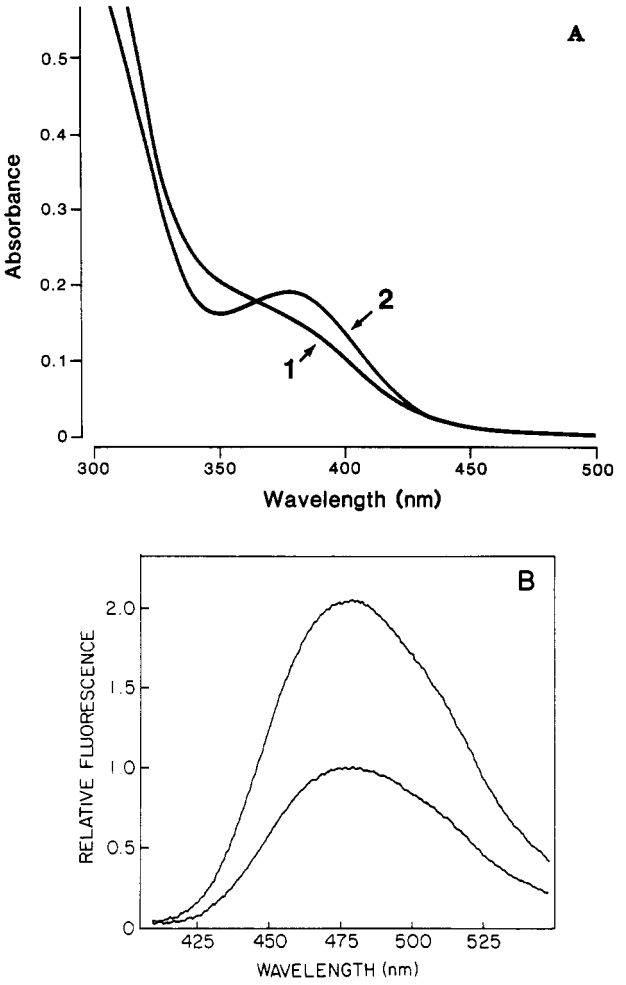

FIGURE 3: Change in absorption and emission spectra of fluorescamine–GDP after displacement from EF-Tu by GDP. (A) Absorption spectra. The 1-cm path-length cuvette contained 80 μM EF-Tu·fluorescamine–GDP. After spectrum 1 was recorded, the solution was made 1 mM GDP, and spectrum 2 was measured after leaving the solution at 23 °C for 20 min. The solution also contained 50 mM Tris-HCl–10 mM $MgCl_2$, pH 7.6. (B) Emission spectra. These were recorded with excitation at 390 nm following the conditions described in (A) except that the initial concentration of EF-Tu·fluorescamine–GDP was 20 μM. The lower trace is before addition of GDP, and the upper trace is 20 min after the addition of GDP.

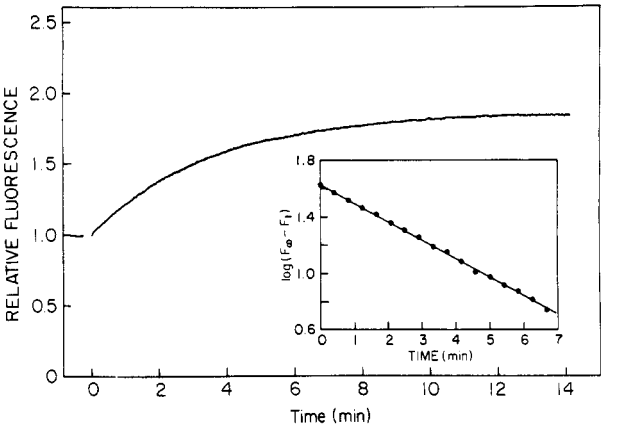

FIGURE 4: Rate of dissociation of fluorescamine–GDP from EF-Tu·fluorescamine–GDP. The increase in fluorescence intensity (excitation 390 nm) on addition of GDP to EF-Tu·fluorescamine–GDP was recorded with time. Experimental details are as in Figure 3B.

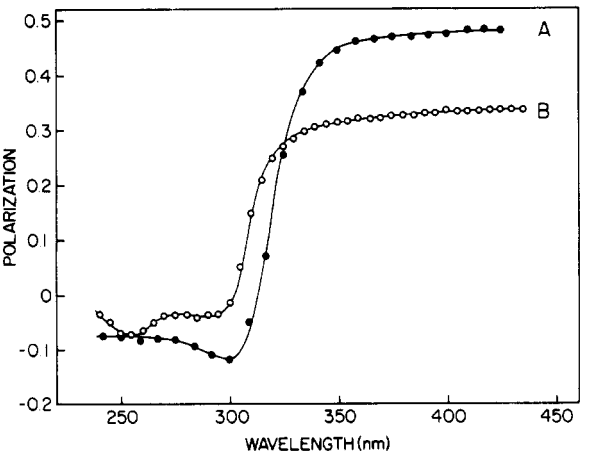

FIGURE 5: Excitation polarization spectra. The solution contained fluorescamine–GDP in glycerol at 0 °C or 10 μM EF-Tu·fluorescamine–GDP in 50 mM Tris-HCl, 10 mM $MgCl_2$, and 0.5 mM DTE, pH 7.6 at 20 °C. Excitation band-pass was 2 nm; emission was observed at 470 nm with an 8-nm band-pass.

marizes some equilibrium and kinetic constants for the interaction of EF-Tu with GDP and GDP analogues; this table is intended only to convey the general range of these constants since precise experimental conditions differ slightly, and the original references should be consulted for these details.

Excitation Polarization Spectra. The excitation polarization spectra of EF-Tu·fluorescamine–GDP in aqueous conditions at 20 °C and of fluorescamine–GDP in glycerol at 0 °C are shown in Figure 5.

At 400-nm excitation, the limiting polarization (approximated by the polarization value in glycerol at low temperature) of fluorescamine–GDP was 0.475. The corresponding value for EF·Tu·fluorescamine–GDP was 0.338. Standard deviations for steady-state polarization measurements were less than ±0.005. These steady-state polarization values may be related to the rotational parameters of the EF-Tu system according to the Perrin equation:

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

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 about the principal axes of rotation. The calculated value of ρ_h, for the EF-Tu·fluorescamine–GDP complex using a fluorescence lifetime of 11.03 ns (see Lifetime Measurements section), is 69 ± 6 ns. This calculation assumes, however, that the observed fluorescence corresponds to 100% bound fluorescamine–GDP. As we shall discuss later, the differential phase measurements indicated the presence of a small amount of free material under the conditions utilized for the polarization determinations.

Lifetime Measurements. The fluorescence lifetimes of EF-Tu·fluorescamine–GDP, fluorescamine–GDP, and fluorescamine–ethylamine were determined by using the multifrequency phase and modulation fluorometry techniques. In this approach, the phase shift and relative demodulation of the emitted light relative to a scattering solution are determined, and the phase (τ^P) and modulation (τ^M) derived lifetimes are given by

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

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

where P is the derived phase shift in degrees, M the relative demodulation, and ω the angular modulation frequency (Spencer & Weber, 1969).

The results for the three samples are shown in Figure 6. In all cases, the data were well fit by a single exponential decay [χ^2 values were near 1; see Jameson et al. (1984) for a review

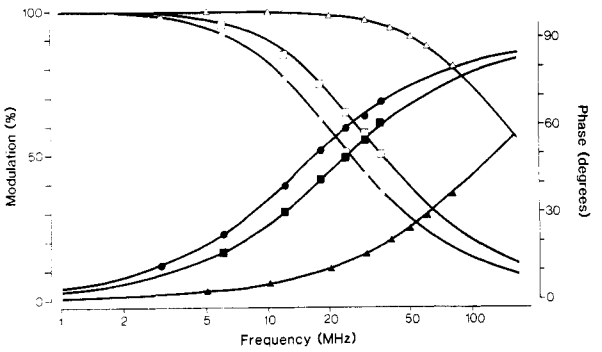

FIGURE 6: Lifetime measurements of fluorescamine–GDP, EF-Tu·fluorescamine–GDP, and fluorescamine–ethylamine. Lifetime mesurements were performed by using a multifrequency phase fluorometer at the indicated modulation frequencies. Excitation wavelength was 351 nm; emission was measured through a Corning 3-74 sharp cutoff filter ($\lambda_{em} > 400$ nm). The starting protein solution contained ~20 μM EF-Tu·fluorescamine–GDP in 50 mM Tris-HCl, 10 mM $MgCl_2$, and 0.5 mM DTE, pH 7.6 at 20 °C. After the measurements were completed, 100 μM GDP was added to displace the fluorescamine–GDP, and measurements were made after 15 min. A second solution contained 10 μM fluorescamine–ethylamine in the same buffer as the protein case. Fluorescamine–GDP, (■) phase; fluorescamine–GDP, (□) modulation; EF-Tu·fluorescamine–GDP, (●) phase; EF-Tu·fluorescamine–GDP, (○) modulation; fluorescamine–ethylamine, (▲) phase; fluorescamine–ethylamine, (△) modulation. The solid lines represent a single component fit to the phase and modulation data for each sample; the corresponding lifetimes were 7.74 ± 0.04 ns for fluorescamine–GDP, 11.03 ± 0.05 ns for EF-Tu·fluorescamine–GDP, and 1.45 ± 0.01 ns for fluorescamine–ethylamine.

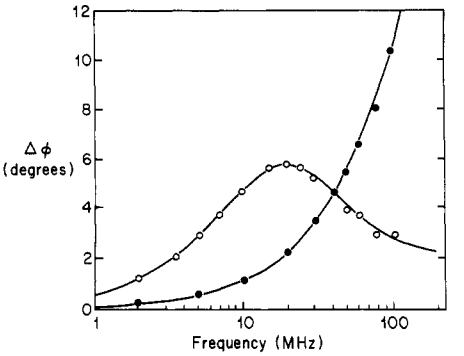

FIGURE 7: Differential phase measurements of fluorescamine–GDP and EF-Tu·fluorescamine–GDP. Experimental details as in Figure 5. Data correspond to EF-Tu·fluorescamine-GDP (○) and displaced (by 100 μM GDP) fluorescamine·GDP (●). The solid lines represent the best-fit differential phase ($\Delta\phi$) values for ρ = 1.1 ns, τ = 7.74 ns, and r_0 = 0.31 (free fluorescamine·GDP) and ρ_1 = 88 ns, τ_1 = 11.03 ns, ρ_2 = 1.1 ns, τ_2 = 7.74 ns, and r_0 = 0.31 for EF-Tu·fluorescamine–GDP.

of analysis of multifrequency data]; lifetime values of 7.74, 11.03, and 1.45 ns were obtained for fluorescamine–GDP, EF-Tu·fluorescamine–GDP, and fluorescamine–ethylamine, respectively. The lifetime observed for the displaced fluorescamine–GDP was the same as that observed for fluorescamine–GDP placed directly in buffer.

Differential Phase Measurements. Dynamic polarization measurements on the EF-Tu·fluorescamine–GDP complex were also performed. This measurement is the frequency domain equivalent of the decay of anisotropy measurement performed in the time domain. A description of the dynamic polarization methodology including the relevant equations and the details of the data analysis is given in Jameson et al. (1987). The data shown (Figure 7) represent measured differential phase values for fluorescamine–GDP bound to elongation factor Tu and then displaced by excess GDP. The solid lines represent the best fit of the nonlinear least-squares data analysis. The best fit for the bound case was obtained by using a two-component analysis which corresponds to fractional contributions of 0.95 for bound fluorescamine–GDP (τ = 11.03 ns) and 0.05 for free fluorescamine–GDP (τ = 7.74 ns), respectively. Rotational relaxation times of approximately 88 and 1.1 ns for bound and free fluorescamine–GDP, respectively, were obtained. The displaced (free) fluorescamine–GDP gave an excellent fit for a single rotational component with a rotational relaxation time of 1.1 ns and a lifetime of 7.74 ns.

Discussion

We have demonstrated that a fluorescent derivative of GDP can be prepared by the reaction of fluorescamine with 2′-amino-2′-deoxyguanosine 5′-diphosphate. The fluorescent properties of this derivative, i.e., its excitation and emission spectra, intrinsic polarization properties, and lifetime characteristics, suggest its utility as a probe with guanosine nucleotide binding proteins. The similarities of the binding kinetics of GDP and fluorescamine–GDP to the EF-Tu protein, inferred from the displacement reaction, further support the utility of this particular probe.

The differences in the lifetimes observed for the free and bound fluorescamine–GDP analogues and the previously reported sensitivity of the lifetimes of fluorescamine–primary amine adducts to solvent polarity (DeBernardo et al., 1974) suggest that detailed lifetime and quantum yield investigations may convey information concerning the local environment around the nucleotide binding site. Such studies, however, will first require a more detailed examination of the lifetime and quantum yield of fluorescamine–GDP in a wide range of solvents of varying dielectric constants and viscosities. The observed increase in the relative yield of free over bound fluorescamine–GDP despite the decreased lifetime of the free probe (we note that this large increase in yield is not compensated by the small absorption change) further indicates the complex photophysics of such probe–protein interactions. We may note that the alterations in the excitation polarization spectrum of the protein-bound probe relative to the free probe (in glycerol) also suggest changes in the relative orientations of the absorption and emission dipoles of the fluorophore in different environments.

Sjöberg and Elias (1978) have reported small-angle X-ray scattering data on EF-Tu which are consistent with an oblate ellipsoid having a 4:1 axial ratio. However, preliminary low-resolution X-ray diffraction studies of a modified form of EF-Tu crystals indicated that the overall dimensions of the protein are 75 Å × 50 Å × 45 Å (Morikawa et al., 1978). The rotational relaxation time of a rigid, spherical protein may be approximated by

$$\rho_0 = \frac{3\eta V}{RT} = \frac{3\eta M(\bar{v} + h)}{RT}$$

where V is the protein's molar volume, η the solvent viscosity, R the gas constant, T the absolute temperature, M the molecular weight, $\bar{v}$ the partial specific volume, and h the degree of hydration. For EF-Tu with a molecular weight of 43 000 in aqueous buffer (η = 1 cP) and assuming a partial specific volume of 0.73 mL/g and a hydration of 0.2 mL/g, one calculates a rotational relaxation time of 50 ns. Actual rotational relaxation times are, however, determined by the detailed hydrodynamic aspects, such as axial ratios, and by the relative orientations of the absorption and emission oscillators of the fluorophore to the principal rotational axes of the protein. Our value for ρ_h of 88 ns, determined by the

dynamic polarization method, is consistent with an asymmetric protein, but the data at present do not allow us to distinguish unambiguously between the published models; more detailed studies which include modulation ratio data should permit us to evaluate these models. The slight discrepancy between the steady-state and dynamic rotational relaxation values indicates the contribution to the steady-state polarization of a small amount of free material. For example, using the values of 0.95 and 0.05 for fractional intensities due to the bound and free nucleotide, obtained from the dynamic polarization study and the additivity of polarization principle (Weber, 1952), one can calculate from the steady-state polarization value a rotational relaxation time of ~80 ns for the fluorescamine–GDP–protein complex, in reasonable agreement with the dynamic result. We interpreted the steady-state and dynamic polarization results in terms of the presence of a small amount of unbound fluorophore since, in the sample used for these polarization measurements, the lifetime was slightly heterogeneous and consistent with the presence of a few percent of a 7.7-ns component; also, the fit of the differential phase data to the dual lifetime, bound and free model was superior to that for a model of a single component exhibiting both global and local motion. The rotational relaxation time of 88 ns obtained by using the fluorescamine–GDP analogue may be compared to the 63-ns value obtained for EF-Tu·GDP on the basis of the intrinsic tryptophan polarization as described in the preceding paper (Jameson et al., 1987). As suggested in that paper, the 88-ns value is more likely to reflect the overall hydrodynamics of the EF-Tu·GDP system since the 63-ns value is based on the intrinsic tryptophan emission which exhibits a heterogeneous lifetime complicating the rotational analysis. Also, the relative orientations of the absorption and emission oscillators in both cases (intrinsic or extrinsic probe) with respect to the various rotational axes will influence the results. In any case, the 88-ns figure represents a weighted average of rotational rates about the principle axes of rotations, and more detailed analyses are required to determine the extent of anisotropic rotation.

In summary, we have shown that analogues of guanosine nucleotides with modification at the 2′,3′-*cis*-diol of the ribose moiety may be prepared which maintain strong binding to a GDP binding protein. The particular fluorescent analogue described, fluorescamine–GDP, has a homogeneous and relatively long (~11 ns) lifetime when bound to EF-Tu; additionally, the dynamic polarization data indicate the absence of significant local mobility of the probe; i.e., the probe monitors the global protein motion. This long lifetime and the high intrinsic polarization of the fluorophore excited into the last absorption band suggest its general utility in hydrodynamic and equilibrium and kinetic binding studies of guanosine nucleotide binding proteins.

ACKNOWLEDGMENTS

We thank Dr. F. Tomita (Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan) for the gift of 2-amino-2′-deoxyguanosine and Dr. J. Hobbs (City University, London, U.K.) for helpful advice on synthetic methods. We are also grateful to Jo Hicks for preparing the manuscript.

Registry No. 2′-Amino-2′-deoxy-GDP, 108269-12-1; fluorescamine-GDP, 108269-11-0; 2′-amino-2′-deoxy-GMP, 72189-87-8; 2′-amino-2′-deoxy-GTP, 108269-13-2; 2′-amino-2′-deoxyguanosine, 60966-26-9; triethyl phosphate, 78-40-0; fluorescamine, 38183-12-9.

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