Interaction of Guanosine Nucleotides and Their Analogs with Elongation Factor Tu from *Thermus thermophilus*†

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ABSTRACT: Transient kinetic experiments on the interaction of nucleotide-free EF-Tu from *Thermus thermophilus* with nucleotides using intrinsic protein fluorescence, extrinsic nucleotide fluoresence and fluorescence resonance energy transfer show that nucleotide binding is in general at least a two-step process. The first step is a weak initial binding, which is followed by a relatively slow isomerization of the protein−nucleotide complex in which changes of both intrinsic and extrinsic fluorescence, as well as energy transfer, occur. The values obtained for the equilibrium and kinetic constants confirm the earlier observation that EF-Tu has a higher affinity for GDP than GTP. This is mainly due to a lower dissociation rate constant for GDP, in combination with a somewhat higher effective association rate constant. Modifications of the triphosphate moiety of GTP are quite well tolerated by EF-Tu, with GTPγS displaying the same affinity as GTP and with GppNHp and GppCH$_2$p being only ca. 2−3-fold less strongly bound. Caged GTP is bound about 6-fold more weakly than GTP. These results suggest that the binding of GppNHp and GppCH$_2$p is likely to be similar to that of GTP. The photolytic protecting group of caged GTP (or the loss of one of the negative charges on the γ-phosphate group) appears to interfere to a certain extent with the interaction with the protein, but the affinity is high enough to permit generation of 1:1 complexes for dynamic structural studies. Discrimination between GDP and ADP is dramatic, with a difference of 6 orders of magnitude in affinity.

Elongation factor Tu (EF-Tu),[1] the most abundant protein in the procaryotic cell, functions as a transporter protein in bacterial protein biosynthesis by carrying the aminoacylated tRNA to the programed ribosome. This process is regulated by binding and hydrolysis of GTP, with an active conformation corresponding to the GTP-bound form and an inactive one to the GDP-bound protein (Kaziro et al., 1991). To elucidate structure−function relationships for this protein, several attempts have been made to determine the three-dimensional structure of EF-Tu·GDP (La Cour et al., 1985; Jurnak, 1985; Kjeldgaard et al., 1992). The solution of the EF-Tu structure in its active form, which was achieved recently (Berchtold et al., 1993; Kjeldgaard et al., 1993), provides the opportunity for a direct comparison of the two protein conformations. Such comparison reveals a large rigid-body movement of domains II/III relative to domain I of EF-Tu. The crystallization of the triphosphate-bound protein was made possible by the use of a slowly hydrolyzable GTP analog (Reshetnikova et al., 1991).

Such GTP analogs were also used in functional assays, for example, the study of *in vitro* polypeptide synthesis (Shorey et al., 1971; Girbes et al., 1976; Karim & Thompson, 1986) and of single steps of this process, such as the translocation reaction (Moazed et al. 1988), ternary complex formation (Thompson & Karim, 1982; Delaria et al. 1991; Nazarenko et al. 1994), and nucleotide binding relative to that of GTP (Delaria & Jurnak, 1989). All these investigations revealed that the function of the protein is affected by modification of the nucleotide structure. In particular, the rates and efficiencies of the previously mentioned reactions are altered as compared to the GTP-bound protein. We were therefore interested to know how the detailed kinetics of EF-Tu−nucleotide interactions are modulated by modification of GTP and in particular whether the EF-Tu·GppNHp structure can indeed be considered to represent the native, active state. This required establishment of methods to monitor the association and dissociation kinetics of the nucleotides, since the nucleotide affinity of the EF-Tu is too high (Arai et al. 1978) to permit the determination of dissociation constants under equilibrium conditions (Goody et al. 1991). We used the fluorescently labeled nucleotide 3′-(methylanthraniloyl)-2′-deoxyguanosine diphosphate (mantdGDP) as a reporter group, since this displays increased fluorescence upon binding to EF-Tu (Eccleston et al. 1989). By exploiting the energy transfer from the two protein tryptophans onto the mant moiety, a much improved fluorescence signal is obtained, as compared to a direct excitation of the labeled nucleotide (Remmers et al., 1994). Using this signal in stopped-flow experiments, the kinetics

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[1] Abbreviations: EF-Tu, elongation factor Tu; p21, product of the H-ras protooncogene; mantdGTP, 3′-(methylanthraniloyl)-2′-deoxyguanosine diphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GppNHp, guanosine 5′-*O*-(β,γ-imidotriphosphate); GppCH$_2$p, guanosine 5′-*O*-(β,γ-methylenetriphosphate); caged GTP, P^3-[1-(2-nitrophenyl)ethyl]guanosine 5′-*O*-triphosphate; FRET, fluorescence resonance energy transfer; DTE, dithioerythritol.

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of the association reactions could be selectively studied. In addition, dissociation of EF-Tu−nucleotide complexes could be observed in displacement reactions. The methods also allowed the determination of the kinetic constants for the interaction of ADP with EF-Tu.

MATERIALS AND METHODS

GDP, GTP, GppNHp, and GTPγS were purchased from Boehringer (Mannheim, Germany). GppCH$_2$p and dGDP were from Sigma (Deisenhofen, Germany), and caged GTP was from Calbiochem (San Diego, CA). Since EF-Tu binds nucleoside diphosphates more strongly than triphosphates, all nucleoside triphosphates were purified before use by column chromatography on QAE-Sephadex A25 at 4 °C. Elution was with a gradient of NaCl (50−250 mM) in 50 mM Tris-HCl, pH 7.0, and desalting was achieved by gel permeation chromatography on a Sephadex G10 column. Methylanthraniloyl nucleotides of dGDP, dGTP, and dGppNHp were prepared according to Hiratsuka (1983) with the modifications described by John et al. (1990). The purities of the nucleotides were analyzed by HPLC on a C18 reversed-phase column (Supelco, Bellefonte, PA) in the presence of tetrabutylammonium hydrogen sulfate under isocratic conditions (Tucker et al., 1986) for nonfluorescent nucleotides or with a gradient of 0−50% acetonitrile for nucleotide derivatives, respectively (John et al., 1990).

Nucleotide-free EF-Tu from *Thermus thermophilus* (EF-Tu$_f$) was purified as described by Limmer et al. (1992). For the removal of tightly bound GDP, EF-Tu was denatured by 5 M urea and passed through a CM-Sephadex CL6B column. Then the protein was slowly refolded at 4 °C by dialysis. This procedure provided the EF-Tu$_f$ which was stable and did not change its properties with respect to interaction with nucleotides even after prolonged storage at −20 °C (over 6 months). The concentration of EF-Tu$_f$ was determined both spectrophotometrically (Whitaker & Granum, 1980) and by its [^{3}H]GDP binding activity in the nitrocellulose filter assay according to Arai et al. (1972). In the case of EF-Tu•GDP the samples were incubated for 10 min at 37 °C. In the case of EF-Tu$_f$, the incubation was performed for 3 min at 0 °C.

Rapid kinetics were measured with a stopped-flow apparatus (High Tech Scientific, Salisbury, U.K.) with EF-Tu$_f$ concentrations of 0.5−1.0 μM. Excitation of tryptophan fluorescence was at 297.5 nm, with detection through a 320 nm cutoff filter. Fluorescence of mantdGDP was excited either directly at 370 nm or via FRET at 290 nm, with emission through a 389 nm cutoff filter. Data collection and primary analysis for determination of the rate constants were with the PC package from High Tech Scientific, while secondary analysis was with the program Grafit 3.0 (Erithacus software). Analysis of the ADP kinetic data was with the program NIODE (R. Umathum, Heidelberg). Fluorescence spectra and long-time fluorescence measurements were performed with an SLM 8000 spectrophotometer (Aminco, Silver Spring, MD).

All fluorescence measurements were carried out in a buffer containing 50 mM Tris-HCl, pH. 7.6, 10 mM $MgCl_2$, 0.5 mM DTE, and 1 mM NaN_3.

RESULTS AND DISCUSSION

Fluorescence Changes Occurring on Nucleotide Binding to EF-Tu from T. thermophilus. Elongation factor Tu (EF-Tu) from *T. thermophilus* contains tryptophan residues at positions 195 and 200. On excitation at 280−290 nm, binding of GTP or GDP to the nucleotide-free protein in the presence (or absence) of magnesium ions leads to a 12% reduction in fluorescence intensity at 334 nm (Figure 1a). This quenching of fluorescence on nucleotide binding can be used in transient kinetic experiments, as will be described below.

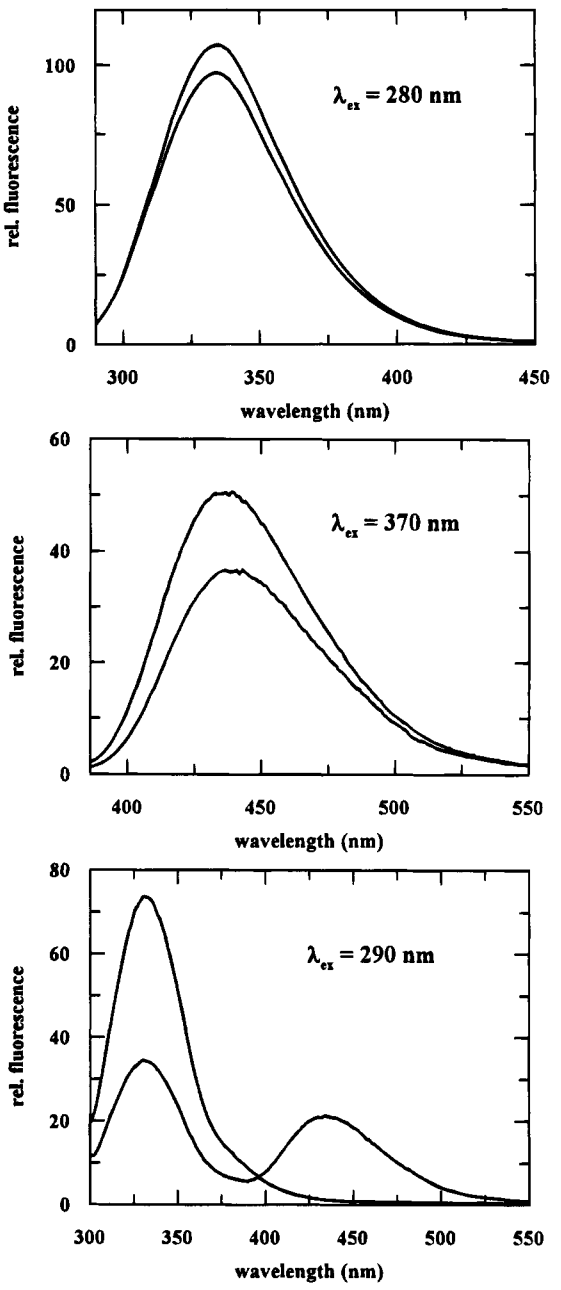

FIGURE 1: Emission spectra of (a, top) 0.45 μM EF-Tu$_f$ (upper curve) and after addition of 0.5 μM GDP (lower curve) with excitation of tryptophan fluorescence at 280 nm, (b, middle) 2 μM mantdGDP (lower curve) and the same solution after addition of 2.5 μM EF-Tu$_f$ (upper curve) with excitation at 370 nm, and (c, bottom) 2 μM EF-Tu$_f$ (upper curve) and after addition of 10 μM mantdGDP (lower curve) (energy transfer; excitation at 290 nm). All measurements were at 15 °C.

Methylanthraniloyl derivatives of nucleotides have been used in investigations of a number of enzymes, including myosin (Woodward et al., 1991), the ras oncogene product p21 (John et al., 1989, 1990; Neal et al., 1990; Rensland et

al., 1995; Moore et al., 1993), F_1 ATPase (Divita et al., 1993), and adenylate kinase (Reinstein et al., 1990). In all cases, interaction of the fluorescent derivatives with the protein leads to a substantial increase in fluorescence quantum yield. In keeping with these observations, binding of mantdGDP or mantdGTP to EF-Tu also leads to a fluorescence enhancement, but this is uncharacteristically small (ca. 30%; see Figure 1b). A more typical case is that of H-ras p21, which shows an enhancement which is about 10-fold higher than this. The reasons for this difference are not clear at present. However, the structures of different GTPases [α-subunit of transducin (Noel et al., 1993), $G_{i\alpha 1}$ (Coleman et al., 1994), Ha-ras p21 (Pai et al., 1990), and EF-Tu as referenced above] are not uniform with respect to the interaction of the protein with the *cis*-diol group of the bound nucleotide. In the structure of H-ras p21 with a (methylanthraniloyl)guanine nucleotide at the active site, the fluorescent group is oriented away from the nucleotide in a direction opposite to that of the nucleobase (Goody et al., 1992). There are no obvious hydrogen-bonding interactions of the group with the protein, the only detectable interaction being an apparently weak aromatic interaction with Tyr-32 (in the GTP conformation) of H-ras p21. It seems likely that the large fluorescent enhancement seen with most enyzmes to which the methylanthraniloyl nucleotides bind is due to alleviation of quenching interactions of the fluorescent group with the nucleobase when free in solution. This interpretation is supported by the observation that the 2′- and 3′-isomers of (methylanthraniloyl)-GTP have significantly different fluorescence yields, which is probably due to the different degree of quenching interactions of the fluorescent group with the base (Rensland et al., 1991). Thus, for EF-Tu, we conclude tentatively that either these interactions are not fully alleviated when the nucleotides are at the active site or that other quenching interactions with the protein occur, so that the observed enhancement is significantly reduced.

Despite the small magnitude of the fluorescent enhancement seen when the fluorescent nucleotides bind to EF-Tu, this can still be used as a signal of binding in transient kinetic experiments. However, a much more sensitive signal is provided by energy transfer from one (or both) of the tryptophan residues at positions 195 and 200 to the methylanthraniloyl group (Figure 1c). As shown in Figure 2, both of these residues are in the G-domain of EF-Tu and are at less than 25 Å distance to the nucleotide binding site. It is therefore not surprising that efficient energy transfer occurs.

Association Kinetics of Nucleotides and EF-Tu. Using intrinsic tryptophan fluorescence as a signal of binding, the association kinetics of GDP, GTP, and GppNHp could be registered by the stopped-flow method. Typical traces obtained are shown in Figure 3a, and they can be well described by a single exponential function as long as the nucleotide was in large excess over enzyme. At an enzyme concentration of 1 μM, data could be collected at nucleotide concentrations of up to ca. 500 μM at 15 °C and over 1 mM at 5 °C. At this point, pseudo-first-order reaction rates were of the order of 200 s^{-1}, and at these rates signal amplitudes began to decrease due to the dead time of the instrument. In addition to this, the overall intensity was decreased due to absorption of light by the high concentrations of nucleotide, leading to increased noise. Despite these limitations, there is indication of deviation from linearity as the nucleotide

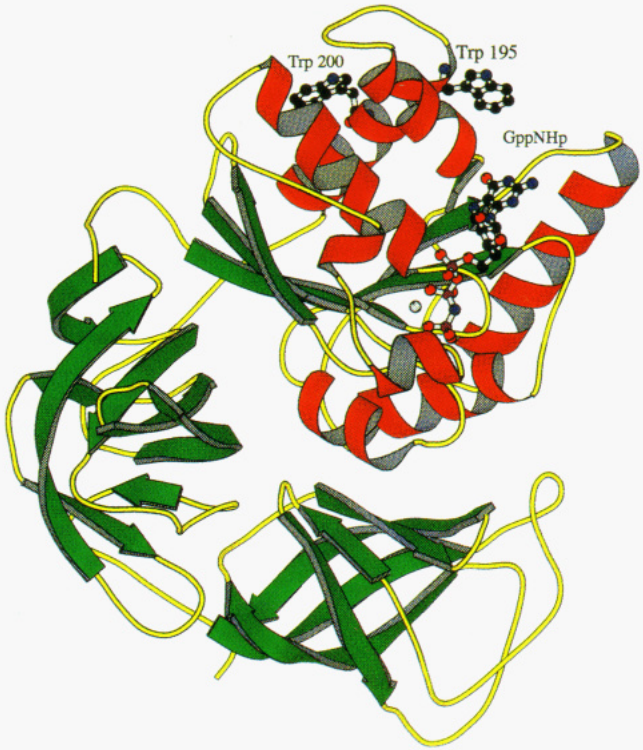

FIGURE 2: Structural model of EF-Tu•GppNHp. Secondary structure elements were as defined by the program DSSP (Kabsch et al., 1983) and were drawn using MOLSCRIPT (Kraulis, 1991). The nucleotide (GppNHp), the magnesium ion, and the two tryptophan residues in domain I are highlighted (top). Domain II is at the left and domain III at the bottom of the illustration.

concentration increases at 15 °C and obvious departure at 5 °C (Figure 3b). Fitting a hyperbolic curve to the data leads to a value of the maximum rate constant at 15 °C of 450 s^{-1}. This value cannot be regarded as well determined, due to the limited concentration range, but the obvious curvature at 5 °C adds confidence to the basic interpretation of a hyperbolic dependence of the rate constant on concentration at both temperatures. At 5 °C, the maximum rate is 284 s^{-1}. The concentration for the half-maximal pseudo-first-order rate constant is similar at both temperatures (0.22 and 0.2 M^{-1} at 15 and 5 °C, respectively).

The kinetic behavior described above (i.e., strictly monoexponential transients and a hyperbolic dependence of the observed pseudo-first-order rate constant on the nucleotide concentration) is not unusual for nucleotide-binding enzymes and is usually attributed to a two-step binding mechanism in which an initial complex is formed in a reaction which is in rapid equilbrium followed by a relatively slow quasi-irreversible isomerization reaction in which the fluorescence change occurs. Well-documented examples are myosin ATPase (Bagshaw et al., 1974) and H-ras p21 (John et al., 1990). Although other mechanisms can be envisaged to explain the kinetic pattern seen, this remains the most likely and most direct interpretation [see Bagshaw et al. (1974), for a discussion of this point]. When EF-Tu changes its conformation from the active GTP form to the inactive GDP form or vice versa, the pattern of interactions between the domains becomes different (Berchtold et al., 1993); i.e., this process requires a dissociation and reassociation of the domains. Such a process is expected to be slow. The relatively slow kinetic step after initial binding of the nucleotide to $EF\text{-}Tu_f$ may reflect this reaction.

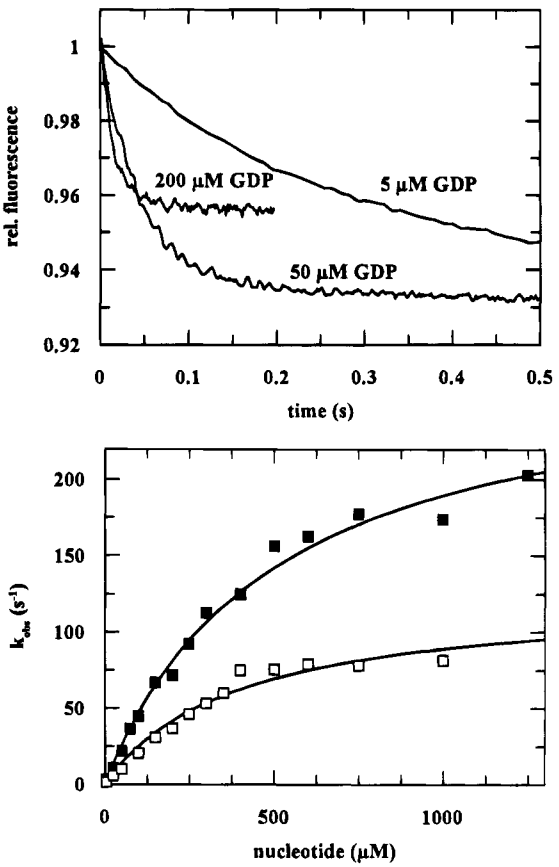

FIGURE 3: Kinetics of association of GDP−GTP to EF-Tu$_f$. (a, top) Original curves obtained by binding of 5, 50, and 200 μM GDP to 1 μM EF-Tu$_f$ at 15 °C. Excitation was at 297.5 nm detection via a cutoff filter (320 nm). (b, bottom) Concentration dependence of the pseudo-first-order rate constant for the association of GDP (■) and GTP (□) at 5 °C.

Scheme 1: General Scheme for the Binding of Nucleotides to EF-Tu[a]

$$\text{EF-Tu} + \text{N} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{EF-Tu•N} \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} \text{EF-Tu}^{*}\text{•N}$$

[a] N is any nucleotide which has affinity for the EF-Tu active site. The experimental data can be explained if the first step is in rapid equilibrium compared with the second step at all concentrations of nucleotide. Formally, this condition is fulfilled if k_{-1} is much larger than k_{+2}.

The dependence of the pseudo-first-order rate constant for the proposed mechanism of nucleotide binding (Scheme 1) on the concentration of nucleotide is given in eq 1. This

$$\text{EF-Tu} + \text{N} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} \text{EF-Tu•N} \underset{k_{-2}}{\overset{k_{+2}}{\leftrightarrow}} \text{EF-Tu*•N} \qquad (1)$$

equation describes a hyperbola starting from a value equal to k_{-2} and reaching a maximum determined by $k_{+2} + k_{-2}$. Within the limits of experimental error, the hyperbola starts at the origin, suggesting that k_{-2} is very small compared with k_{+2}, in accordance with the previously documented slow rates of dissociation of nucleotides (see also the section on dissociation kinetics). Thus, the maximum gives the value of k_{+2}, and the concentration required to reach half of this value gives the value of K_1, the equilibrium constant for the first step. The results provide only indirect information on the individual rate constants for the first step. The kinetic characteristics indicate that k_{-1} is much larger than k_{+2}. In the case of GDP at 15 °C, this would mean that it must be at least 10-fold larger than 450 s^{-1}. The first step, represented by K_1, reflects a very weak affinity (K_d or $1/K_1$ = ca. 0.2 mM). If we assume that the initial reaction is a diffusion-controlled association, the second-order rate constant is expected to be of the order of 10^8 M^{-1} s^{-1}. Together with the measured value of K_1, this would lead to a calculated value of 5×10^4 s^{-1} for k_{-1}, which is very much larger than k_{+2} and demonstrates the consistency of the results and the interpretation.

The results for several nucleotides are collated in Table 1. In all cases, there was a nonlinear relationship between the observed rate constant and the nucleotide concentration, and the data could be well fitted by a hyperbolic curve.

Association kinetics could also be examined by using the fluorescence of methylanthraniloyl nucleotide derivatives. An example is given in Figure 4, where fluorescent energy transfer from tryptophan to mantdGDP was used as a signal of binding. By exciting the tryptophan fluorescence at 290 nm, where the fluorescence of the methylanthraniloyl group is hardly excited, and measuring the emission at wavelengths greater than 385 nm, it was possible to work at large excess of the nucleotide over EF-Tu, which is not possible if the nucleotide fluorescence is excited directly. As in the experiments with intrinsic fluorescence, there was a nonlinear dependence of the rate constant of the fluorescence change on the nucleotide concentration. Fitting these data to a hyperbolic function and making the same assumptions as for the experiments with intrinsic fluorescence, values for K_1 and k_{+2} are obtained which are shown in Table 1. It can be seen that the initial binding is somewhat stronger for mantdGDP (possibly due to an additional hydrophobic interaction between the fluorescent group and the protein) but the second step is slower. The effective second-order rate constant for association (K_1k_{+2}) remains approximately equal to that for GDP, since these two opposing effects almost cancel. Thus, mantdGDP is a good analog of GDP in terms of its association kinetics with EF-Tu. As can be seen in Table 1, this also applies to mantdGTP and mantdGppNHp.

The association kinetics of unlabeled nucleotides could also be examined by using them as competitors for the association of mantdGDP as described for H-ras p21 by John et al. (1990). Some values shown in Table 1 were determined using this method. The kinetic characteristics of ADP were determined by this method, but this nucleotide represents a special case in terms of its interaction with EF-Tu and will be considered below in a separate section.

Dissociation Kinetics of Nucleotides from Their Complexes with EF-Tu. Dissociation reactions were studied using mantdGDP as a displacing reagent in the fluorescence energy transfer mode. This allowed the use of a large excess of fluorescent nucleotide over the respective EF-Tu-nucleotide complex, so that the displacement reaction was efficient and the rate was limited only by the rate constant for dissociation of the complex. Example results are shown in Figure 5, where mantdGDP was used to displace GDP from the EF-Tu•GDP complex at two different temperatures (5 and 15 °C). Dissociation rate constants for the different nucleotides tested are given in Table 1. As already mentioned, ADP represents a special case and will be discussed below.

Kinetics of Interaction of ADP with EF-Tu. It is a common property of the GTP-binding proteins that ATP and

Table 1: Kinetic and Thermodynamic Data for Nucleotide Binding to *T. thermophilus* EF-Tu

nucleotide	association K_1 (10^4 M^{-1})	k_{+2} (s^{-1})	k_{on} (10^6 M^{-1} s^{-1})	dissociation k^{-2} (10^{-2} s^{-1})	K_D (10^{-9} M)	affinity K_A (10^9 M^{-1})
GDP, 15 °C[a]	0.22	450	1	0.18	1.8	0.55
GDP, 15 °C[b]	(1)	(156)	(1.66)		(1.08)	(0.92)
GDP, 5 °C[a]	0.2	284	0.57	0.085	1.49	0.67
GTP, 15 °C[a]	0.13	264	0.35	1	29	0.035
GTP, 15 °C[b]	(0.47)	(82)	(0.39)		(0.26)	(0.039)
GTP, 5 °C	0.25	124	0.31	0.5	16	0.062
mantdGDP, 15 °C[c]	0.9	134	1.2	0.37	3.1	0.32
mantdGTP, 15 °C[c]	nd[d]	nd	0.29	1.54	53	0.18
mantdGppNHp[c]	nd	nd	0.113	1.45	128	0.078
GppNHp, 15 °C[b]	0.36	49	0.18	0.9	50	0.02
GppNHp, 5 °C[a]	0.2	84.1	0.17	0.47	28	0.036
$GppCH_2p$, 15 °C[b]	0.43	48.6	0.21	1.44	69	0.015
GTPγS, 15 °C[b]	0.5	79.6		0.4	27	0.037
caged GTP (*R,S*)[b]	0.116	62.88	0.073	nd	nd	nd
caged GTP (*R*), 5 °C[b]	0.32	37.8	0.12	1.06	88	0.011
ADP 15 °C[b]	nd	nd	0.000165	175	10606060	0.000000094

[a] From tryptophan fluorescence. [b] From competition of nucleotide with mantdGDP (energy transfer). [c] From energy transfer. [d] Not determined.

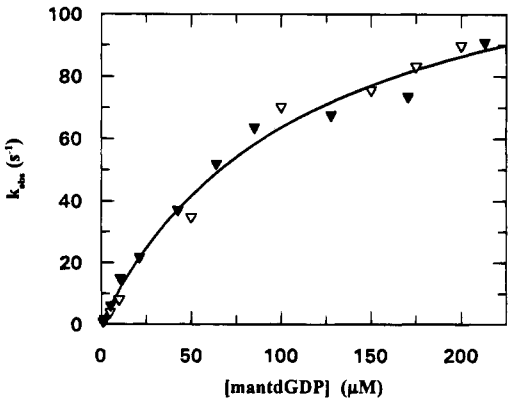

FIGURE 4: Association of mantdGDP with EF-Tu_f at 15 °C. The observed pseudo-first-order rate constants are not linearly related to the nucleotide concentration. Two sets of data are shown to illustrate the reproducibility of the experiments: (▼) obtained with freshly prepared EF-Tu_f ; (▽) obtained with EF-Tu_f which was stored for 6 month at −20 °C. The excitation wavelength was at 290 nm, with detection through a filter with a cutoff at 389 nm. The continuous line shows the hyperbolic fit according to the model shown in Scheme 1.

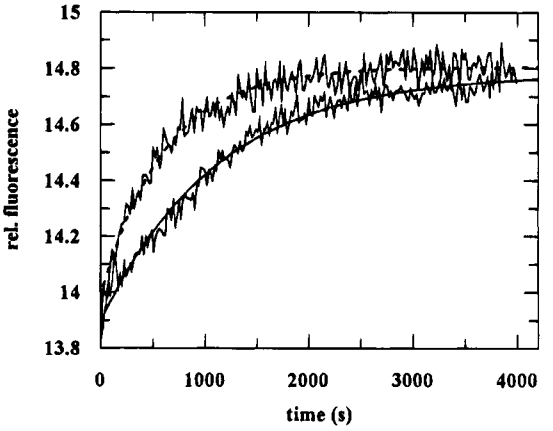

FIGURE 5: Dissociation of GDP from the EF-Tu·GDP complex. The monoexponential time trace of the displacement of 1 μM GDP by 50 μM mantdGDP is shown at 15 °C (broken line) and 5 °C (continuous line). Excitation was at 290 nm and emission was measured at 440 nm.

ADP are bound many orders of magnitude less strongly than the corresponding guanosine nucleotides. Standard methods of measuring nucleotide affinities (e.g., the filter-binding method) are not suitable for quantitating this weak interaction, since high concentrations of an adenosine nucleotide would be needed to displace a tightly bound guanosine nucleotide. We have recently determined the affinity of ATP to the GTP-binding protein H-ras p21 using a transient kinetic method and have shown that the discrimination between GTP and ATP is of the order of 10^6-fold (Rensland et al., 1995). Since this was much greater than the discrimination against other purine nucleotides which differ structurally in the base from GTP, it is of interest to determine whether other GTP-binding proteins discriminate equally strongly and specifically against adenine as the nucleobase.

The method used to obtain the binding parameters for ADP is based on the fact that any ligand which interacts with EF-Tu at reachable concentrations of the ligand will have an effect on the binding kinetics of a guanosine nucleotide if present at a concentration which is not vanishingly small compared with its K_d value. Thus, if the K_d value is in the region of 1 mM, an effect on the binding kinetics of a guanosine nucleotide which is present at micromolar concentrations will occur if the competing (weakly bound) nucleotide is present at around 1 mM, even though there will be no effect on the equilibrium amount of the guanosine nucleotide bound because of the difference in affinities of about 10^6-fold. In the present work, the effect of the presence of ADP on the association of 5 μM mantdGDP with 1 μM EF-Tu was examined. Three different possible kinetic situations can be imagined. If ADP had similar binding characteristics to those of GDP or GTP (i.e., the overall affinity is high and the rate of release is slow compared to the effective rate constants of binding), increasing the ADP concentration would lead to an increase in the effective rate constant seen for the association with EF-Tu with a corresponding decrease of amplitude (John et al., 1990). If the nucleotide displayed two-step binding, this increase in rate

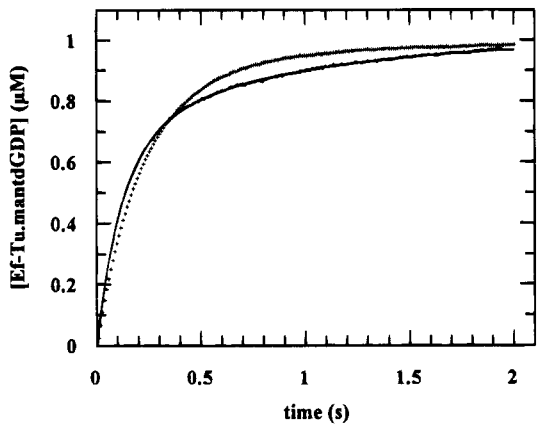

FIGURE 6: Competition between ADP and mantdGDP in the association reaction with EF-Tu_f. The reference data (+) showing the association of 1 μM EF-Tu_f with 5 μM mantdGTP could be fitted with an equation containing a single exponential component (fit not shown to allow data to be distiunguished from the second curve). In the presence of 10 mM ADP and 5 μM mantdGDP (continuous line), the time dependence could not be described by a single exponential equation. The fitted curve (hardly distinguishable from the data) was obtained using a program which allows numerical integration of the rate equations for a particular kinetic model and which fits values of the rate constants to the data. The simplified model used includes single-step association of EF-Tu with mantdGDP with the known (fixed) constants given in Table 1 and the analogous process for ADP association, for which the constants were allowed to float. The curve shown is for rate constants of 1.65×10^2 M^{-1} s^{-1} for association and 1.75 s^{-1} for dissociation.

constant would be hyperbolic. Such behavior is seen with, for example, GDP or GTP as competitors for mantdGDP (data not shown). The other extreme type of behavior would be shown by a competing ligand which is bound weakly and in rapid equilibrium. This leads to a hyperbolic decrease of the pseudo-first order-rate constant for mantdGDP association toward zero at high (saturating) concentrations of competitor. This was observed for H-ras p21 with guanosine and GMP as competitors for mantdGDP (John et al., 1990). For intermediate kinetic behavior, i.e., when the competing nucleotide is weakly bound but its rate of dissociation from EF-Tu is neither very slow nor very fast compared with the time scale of the binding event monitored, more complex behavior is seen. The individual kinetic curves are not monoexponential, and an interpretation cannot easily be made in terms of sums of exponentials. This has been observed for the system containing H-ras p21, mantdGDP, and ATP and has been interpreted in terms of a two-step binding of ATP, with an easily reversible second step, in contrast to the quasi-irreversible second step with guanosine nucleotides.

When ADP was used as a competitor for mantdGDP in the binding transient to EF-Tu, no effect was seen until millimolar concentrations of ADP were reached, at which point the curves began to depart from monexponential behavior. Figure 6 shows an example of such a curve at 10 mM ADP, and it can be seen that it can be qualitatively divided into two parts. The beginning is steeper than in the absence of ADP, and this arises from the fact the the effective rate constant in the intial binding reaction is dependent on the sum of the products of each nucleotide concentration multiplied by its respective effective second-order rate constant. The slower second part of the transient, which is not seen in the absence of ADP, is due to the release of initially bound ADP, which, because of its high dissociation rate, is only able to compete with mantdGDP transiently but not at equilibrium. Since there is not enough information in these results to quantitatively describe a two-step binding reaction for ADP (although this must pertain), the data were analyzed in terms of two competing reversible second-order reactions, with the effective second-order rate constants representing the product of K_1 and k_{+2} in Scheme 1. Using a computer program which simulates the data by numerical integration based on the differential equations describing the temporal behavior of each species, and fitting the rate constants to the data, the values of K_1k_{+2} and k_{-2} shown in Table 1 were obtained. It can be seen that the effective second-order rate constant for association is reduced from 1.2×10^6 M^{-1} s^{-1} for mantdGDP to 1.7×10^2 M^{-1} s^{-1}, while the rate constant for ADP release (k_{-2}) is increased from 3.7×10^{-3} s^{-1} to 1.75 s^{-1}. Thus, the overall affinity is now ca. 100 M^{-1} (K_d = 10 mM), compared with 5.5×10^8 M^{-1} ($K_d = 1.8 \times 10^{-9}$ M) for GDP. Thus, EF-Tu discriminates by a factor of ca. 5×10^6 between GDP and ADP. An almost identical factor was found for the discrimination between guanosine and adenosine nucleotides for H-ras p21 (Rensland et al., 1995). In addition to the loss of important hydrogen-bonding interactions between H-ras p21 and the guanine base (in particular between Asp-119 and N1 and N2; the equivalent residue and interactions in EF-Tu is Asp-139), this dramatic difference is attributed to a sterically unfavorable interaction between the 6-amino group of the adenine base and the backbone carbonyl group of Ala-146 (Ala-175 in EF-Tu) (Rensland et al., 1995).

Comparison of Affinities and Kinetics. All of the nucleotides investigated, with the exception of ADP, bind with relatively high affinity to EF-Tu. In most cases, it was possible to show directly that the binding mechanism is at least a two-step process, with a rapid equilibrium step being followed by a relatively slow isomerization step. The signal change monitored using both intrinsic fluorescence and fluorescence energy transfer from tryptophan to the fluorescent methylanthraniloyl group on a nucleotide was that occurring in the second step. However, it cannot be excluded with certainty that a change also occurs in the first step within the dead time of the stopped-flow apparatus. This would be of small amplitude for tryptophan fluorescence, according to the amplitude of the signal seen, but it seems likely that some energy transfer would occur in the initial step using the fluorescent nucleotides because of the proximity of the tryptophans to the active site (see Figure 2). Exact quantitation of amplitudes is difficult in this type of stopped-flow application, but the saturation behavior of the signal observed together with the lack of obvious deviation from monoexponential behavior suggests that the second step is being observed.

A comparison of the kinetic characteristics of GTP and GDP confirms the already reported difference in affinities of a factor of approximately 10 (GDP greater than GTP). This is the result of both a higher effective rate of association (mainly because k_{+2} is larger) of GDP than GTP, coupled with a lower rate of dissociation (k_{-2}). Thus, the difference in affinities arises mainly because of a change in the value of K_2, with K_1 being similar for both nucleotides. In similar work on H-ras p21 (Rensland et al., 1995), it has been shown that the most important interactions occurring in step 1 of a similar mechanism involve mainly base−protein interactions,

and this is likely to be the case for EF-Tu as well, and in accordance with this idea, there is smaller difference between the values of K_1 than of K_2 for the two nucleotides.

As with other GTP-binding proteins, nucleotide analogs have been extensively used in structural and mechanistic studies. We report here the kinetic constants for several of the most frequently used derivatives (Table 1). GppNHp was of particular interest, since the X-ray structure of EF-Tu from *T. themophilus* has been determined with this analog at the active site (Berchtold et al., 1993), and the structure of the resulting complex is regarded as a model of the GTP state of the protein. At 15 °C, only the apparent second-order rate constant for the association was measured (in competition with mantdGDP binding). It can be seen that this is slightly slower than in the case of GTP. Direct measurement of K_1 and k_{+2} at 5 °C confirmed this finding and showed that k_{+2} is somewhat slower than for GTP. The rate constant for dissociation of the analog from EF-Tu was almost identical to that of GTP, so that the overall affinity was within a factor of 2 of that for GTP. This confirms that GppNHp behaves as a good analog of GTP in this system and justifies the use of the EF-Tu·GppNHp structure as a model for the complex with the natural triphosphate. GTPγS showed almost identical overall affinity to GTP, and GppCH$_2$p was a factor of ca. 2−3 less tightly bound.

Caged GTP was found to bind 6-fold weaker than GTP to EF-Tu. This is similar to its relative affinity (compared with GTP) to H-ras p21 (Schlichting et al., 1990). Thus, the affinity should still be high enough to allow crystallization of a 1:1 complex with EF-Tu, which would be a requirement for performing time-resolved structural studies of the type reported by Schlichting et al. (1990) for H-ras p21. In the present case, the large conformational difference seen between the GTP and GDP states of the protein gives reason to doubt whether experiments intending to follow the transition between these two states in the crystal will be feasible. However, if caged GTP induces a GTP-like state in terms of the overall conformation of the protein (which seems likely from the present results) and the GTP state can be generated by photolysis, this might still allow the determination of the structure of the EF-Tu·GTP complex. This could be of interest with respect to details of the catalytic mechanism, and in the case of H-ras p21 there are indications from more recent work of a possible difference in the structure of the GppNHp and GTP complexes with respect to the exact orientations of groups thought to be involved in catalysis (Scheidig et al., 1992, 1994).

Although the differences in the binding affinities between GTP and its analogs to EF-Tu were not large, they may reflect some slight differences in the structure of the formed complexes. Even if such small deviations are not detectable in the X-ray structure analysis (e.g., EF-Tu·GppNHp and EF-Tu·GppCH$_2$p structures are identical within the limits of present refinement at 1.74 Å; R. Hilgenfeld, personal communication), they could be significant for the function of the protein. For example, several investigators observed altered properties of EF-Tu with respect to the interaction with tRNA (Förster et al., 1994; Nazarenko et al., 1994) or ribosomes (Girbes et al., 1976) in the presence of GppNHp.

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