Evidence for a Second Nucleotide Binding Site in Rat Elongation Factor eEF-2 Specific for Adenylic Nucleotides†

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ABSTRACT: The rat elongation factor eEF-2 catalyzes the translocation step of protein synthesis. Besides its well-characterized GTP/GDP binding properties, we have previously shown that ATP and ADP bind to eEF-2 [Sontag, B., Reboud, A. M., Divita, G., Di Pietro, A., Guillot, D., and Reboud, J. P. (1993) *Biochemistry 32*, 1976−1980]. However, whether the adenylic and guanylic nucleotide binding sites were different or not remained unclear. To further characterize these sites, eEF-2 was incubated in the presence of *N*-methylanthraniloyl (Mant) fluorescent derivatives of GTP, GDP, ATP, and ADP. This led to an increase in the probe fluorescence and to a partial quenching of eEF-2 tryptophans in each case. The Mant-derivatives and the unmodified corresponding nucleotides were shown to bind to eEF-2 with a similar affinity. Competition experiments between Mant-labeled and unmodified nucleotides suggested the presence of two different sites binding either guanylic or adenylic nucleotides. A Förster's transfer between tryptophan residues and the Mant-probe is obtained with both the adenylic and the guanylic Mant-nucleotides, and comparison of the transfer efficiencies confirmed the presence of a second binding site specific for adenylic nucleotides. A sequence alignment of EF-Gs with eEF-2s from different species suggests the presence of potential Walker A and B motifs in an insert of the G-domain of eEF-2s from higher eukaryotes. Our results raise the possibility that a site specific for adenylic nucleotides and located in this insert has appeared in the course of evolution although its physiological function is still unknown.

The eukaryotic elongation factor eEF-2[1] (94 kD) is by far not as well-studied as its prokaryotic equivalent, EF-G. Both proteins belong to the superfamily of GTP hydrolases and catalyze the translocation step in the elongation cycle of protein synthesis. This step results in a relative displacement of the ribosome along the messenger RNA by one codon and, simultaneously, a shift of the peptidyl-tRNA from the A-site to the P-site of the ribosome. GTP hydrolysis by eEF-2 and EF-G is required for achieving translation, and the function of GTP hydrolysis in translocation seems to be now understood (*1*).

The structure of EF-G free or in complex with GDP has been solved, whereas no structure of eEF-2 is available yet (*2*−*4*). A 3-dimensional comparison of EF-G with several proteins belonging to the superfamily of GTP hydrolases has led to the identification of a consensus G-core. This G-core is involved in the binding and the hydrolysis of GTP in GDP and is exemplified by the G-domain of Ras p21 (*5*, *6*). A sequence alignment of EF-G and eEF-2 suggests that the G-core is conserved in eEF-2 (*7*).

From all the available knowledge, it can be assumed that EF-G and eEF-2 work by the same overall mechanism. However, there is some evidence that the eukaryotic factor operates in a more complex and sophisticated manner than its prokaryotic equivalent. This is exemplified by the fact that eEF-2, in contrast to EF-G, is inactivated by ADP-ribosylation of the diphtamide 715 and by the phosphorylation of Thr 56 and 58 in the effector loop of the G-domain (*8*, *9*). Moreover, our group has shown that eEF-2 binds ATP and ADP with a relatively high affinity (respectively, 4.1 and 13.2 μM) and also AMP and adenosine but with a lower affinity (*10*). Up to now, there is no experimental evidence for a requirement of adenylic nucleotides in the translocation mechanism. Furthermore, the binding of an adenylic nucleotide, either to EF-G or to another GTPase, has never been reported. As it can be assumed that the G-domain carries out similar functions in all the GTPases, this observation is quite intriguing because there are structural features in the G-core that should prevent the binding of adenylic nucleotides to this site. Therefore, whether ATP/ADP and GTP/GDP share the same binding site or whether a distinct site specific for adenylic nucleotides exists in eEF-2 is an intriguing question to investigate.

To answer this question, we have previously carried out photolabeling experiments using 8-azido derivatives of [γ-^{32}P]-labeled ATP and GTP (*11*). These experiments were not decisive since a precise identification of the residues

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[1] Abbreviations: eEF-1 or eEF-2, eukaryotic elongation factor 1 or 2; EF-G, elongation factor G; Mant-GDP or Mant-GTP, 2′(3′)-*N*-methylanthraniloyl derivatives of guanosine 5′-di- or triphosphate; Mant-ADP or Mant-ATP, 2′(3′)-*N*-methylanthraniloyl derivatives of adenosine 5′-di- or triphosphate; tRNA, transfer ribonucleic acid; AMP, adenosine 5′-monophosphate; Tris, [tris(hydroxymethyl)aminomethane]; DTT, dithiothreitol; FRET, fluorescence resonance energy transfer.

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cross-linked with each nucleotide was not possible. Moreover, the data obtained had to be interpreted with caution since the base modification brought about by the azido group could have changed possibly the binding specificity.

In the present work, we have carried out experiments using Mant-labeled derivatives of ATP, ADP, GTP, and GDP to find out whether adenylic and guanylic nucleotides bind to the same site or to different sites. The Mant-group (*N*-methylanthraniloyl) is a fluorescent probe that has been widely and successfully used in a similar context with different proteins (*12*−*14*). The Mant-probe presents several advantages as compared to other nucleotide derivatives. First, the Mant-group is covalently linked to the ribose part of the nucleotide which contributes poorly to the recognition of the guanylic nucleotides in most cases (*15*). Therefore, the affinity values and the catalytic efficiencies obtained with Mant-derivatives and unmodified nucleotides are usually very similar (*12*). Second, the binding of Mant-derivatives to proteins can be monitored easily by following either the increase of the Mant-group (extrinsic) fluorescence or the (intrinsic) fluorescence quenching of tryptophan residues which is strengthened by the large overlap between the emission spectrum of the tryptophan residue and the absorption spectrum of the Mant-group. Third, this spectral overlap gives rise to a fluorescence resonance energy transfer (FRET), the degree of which depends on the location of the Mant-nucleotide binding sites (*16*). Fourth, both the wavelength of the maximum fluorescence emission and the quantum yield of the Mant-group bound are good reporters of the polarity of the binding site. All together, these properties make the use of Mant-nucleotides a suitable strategy to characterize the adenylic nucleotide binding site of eEF-2.

MATERIAL AND METHODS

Materials. All nucleotides used were from Boehringer Mannheim, except GTP and ATP which were obtained from Pharmacia as ultrapure and stable solutions. *N*-methylanthraniloyl (Mant) derivatives of ATP, ADP, GTP, and GDP were prepared by reaction of nucleotides with *N*-methylisatoic anhydride following a method described elsewhere (*12*, *17*). The concentration of fluorescent nucleotides was determined spectrophotometrically at 255 nm for Mant-ATP and Mant-ADP ($\epsilon = 23\,300\ M^{-1}\ cm^{-1}$) and at 252 nm for Mant-GTP and Mant-GDP ($\epsilon = 22\,600\ M^{-1}\ cm^{-1}$). Rat liver eEF-2 was prepared according to Marzouki et al. (*18*).

Fluorescence Measurements. The titrations of eEF-2 with nucleotides and the subsequent fluorescence measurements were performed as previously described by Sontag et al. (*10*). Briefly, eEF-2 samples (1 μM) were preincubated for 10 min in 20 mM Tris-HCl, pH 7.6, 8 mM $MgCl_2$, 100 mM KCl, 100 mM sucrose, 1 mM DTT, and 10% glycerol (v/v) and subsequently titrated with aliquots of nucleotides added from 1 mM stock solutions. Fluorescence measurements were carried out at 25 °C. Native eEF-2 showed a maximal excitation at 280 nm and a maximal emission at 332 nm (*10*). However, for the recording of emission spectra of tryptophan fluorescence (310 to 450 nm), the excitation wavelength was set to 295 nm to minimize both the inner-filter effect of nucleotides and the contribution of tyrosines to the emission spectra. Excitation spectra of tryptophan fluorescence were recorded from 220 to 320 nm using an emission wavelength set to 332 nm. For the Mant-moiety, emission spectra (370 to 450 nm) were recorded upon excitation at 356 nm and excitation spectra (270 to 400 nm) were monitored at 440 nm. In titration experiments, the binding of Mant-labeled nucleotides to eEF-2 was monitored in two different ways: by measuring the quenching of the intrinsic fluorescence emission of eEF-2 tryptophans at 332 nm (*10*) and by following the increase of the fluorescence emission of the Mant-group around 440 nm (*14*). The raw spectra were corrected for both the contributions of Raman scattering and unbound Mant-nucleotides by subtracting the spectra of the buffer containing ligands only. All measurements were corrected for the inner-filter effect of the nucleotides and for the dilution brought about by the addition of ligands (never exceeding 5%), using the following formula (*19*):

$$F\mathrm{cor} = (F - B)(V/V_O)10^{0.5b(A_{\lambda ex} + A_{\lambda em})}$$

with *F*cor being the corrected value of the fluorescence intensity, *F* the measured fluorescence intensity, *B* the fluorescence of the sample without protein, *V* the volume of the sample, V_O the initial volume of the sample, *b* the pathlength of the cuvette (in cm), $A_{\lambda ex}$ and $A_{\lambda em}$ the absorbances of the sample at the excitation and emission wavelengths, respectively. A second correction procedure was performed by titrating *N*-acetyltryptophanamide instead of eEF-2 to verify the accuracy of the above-mentioned correction. Curve fitting was carried out using Grafit (Erithacus software) and the two quadratic equations described by Divita et al. (*20*).

Competition Experiments. Two types of experiments are presented. In "chase experiments", the Mant-nucleotides were first bound to eEF-2; then, successive aliquots of each unmodified nucleotide were added, and the amount of Mant-nucleotides remaining bound to eEF-2 was monitored by the residual fluorescence of the Mant-group. In the "preincubation experiments", the prevention of Mant-nucleotide binding to eEF-2 by a preincubation with several unmodified nucleotide (1 mM) was measured: Mant-nucleotide binding was specifically monitored by the enhancement of the Mant-group fluorescence as previously described. Further details about the experimental procedures are given in the legends of Figures 1 and 2.

Fluorescence Resonance Energy Transfer (FRET). There is a large overlap between the excitation spectrum of the Mant-group and the emission spectrum of eEF-2 tryptophans that gives rise to a FRET between the tryptophan(s) of eEF-2 and the Mant-probe linked to the nucleotide. The value of transfer efficiency measured with a given Mant-nucleotide bound to eEF-2 is an accurate characteristic of its binding since transfer efficiency depends directly on the distance and the orientation between the tryptophan(s) acting as the donor-(s) and the Mant-nucleotide (acceptor) (*16*, *21*). There are seven tryptophans in eEF-2 and in the absence of additional data allowing the determination of the contribution of a given tryptophan to the whole fluorescence and to the transfer process, transfer efficiency data can be hardly interpreted in terms of distance. Nevertheless, transfer efficiency was measured for each Mant-nucleotide since it is a direct reflection of the location of the Mant-nucleotide binding site in eEF-2. The calculation of the transfer efficiency can be done theoretically by measuring either the decrease in the donor (Trp) fluorescence or the increase in the acceptor

Table 1: Binding Characteristics of the Mant-Nucleotides to eEF-2

Mant-nucleotide bound to eEF-2	binding monitored by the Mant-fluorescence[a]			binding monitored by the tryptophan quenching[b]	
	λ_{Em}max (nm)[c]	increase in quantum yield[d]	K_d (μM)[e]	maximum quenching (%)	K_d (μM)[e]
Mant-GDP	440	× 1.5	2.0 ± 1.2	21.6	1.5 ± 0.2
Mant-GTP	442	× 1.2	4.4 ± 1.3	20.0	2.8 ± 0.7
Mant-ADP	437	× 2.6	4.0 ± 0.4	2.3	3.5 ± 1.5
Mant-ATP	436	× 2.7	2.1 ± 0.9	13.7	3.0 ± 1.1

[a] Fluorescence measurements were performed at the wavelength of maximum emission (λ_{Em}max) after excitation at 356 nm. [b] Fluorescence measurements were performed at 332 nm after excitation at 295 nm. [c] The maximum-emission wavelength of free Mant-nucleotides was 444 nm. [d] Related to the quantum yield of free Mant-nucleotides. [e] The dissociation constants were determined using Grafit and the two quadratic equations reported in ref *20*; indicated standard deviations represent the error of curve fitting.

(Mant-) fluorescence (*19*, *22*). Transfer efficiency determined by the decrease of the emission of the donor assumes that the binding of the acceptor to the protein does not modify the quantum yield of the donor except by the energy transfer process itself. On the contrary, the increase in the excitation of the acceptor can be used even if the local environment of the donor becomes different upon acceptor binding. In our experiments, the latter method was used only since we did observe a quenching of eEF-2 fluorescence when unmodified nucleotides were bound to the factor (*10*). Therefore, to monitor only the part of the process that is due to the transfer between the Mant-group and the tryptophan residue, transfer efficiency (*E*) was calculated in the excitation spectrum of the acceptor using the following formula (*22*):

$$E = [F_{295}/F_{350} - \epsilon_{A295}/\epsilon_{A350}](\epsilon_{A350}/\epsilon_{D295})$$

with F_{295} and F_{350} being the corrected intensities of the excitation spectrum, ϵ_{A295} (2.85×10^3 M^{-1} cm^{-1}) and ϵ_{A350} (6.66×10^3 M^{-1} cm^{-1}) being the extinction coefficients of Mant-nucleotides at 295 and 350 nm, respectively, and ϵ_{D295} (70.5×10^3 M^{-1} cm^{-1}) being the extinction coefficient of the protein at 295 nm.

RESULTS

Characteristics of the Binding of the Mant-Labeled Nucleotides to eEF-2. Binding of Mant-adenylic and Mant-guanylic nucleotides to eEF-2 was monitored either by following the increase in fluorescence emission of the Mant-moiety or the decrease in tryptophan intrinsic fluorescence. Binding parameters are summed up in Table 1.

Concerning the emission properties of the Mant-group, binding to eEF-2 resulted in both a blue-shift and an increase of the Mant-probe quantum yield for all the Mant-nucleotides. In contrast to Mant-guanylic nucleotides, the fluorescence properties of adenylic analogues were markedly changed upon eEF-2 binding: fluorescence increase was at least 2.6-fold for Mant-adenylic nucleotides, whereas the enhancement was 1.5 at most for Mant-guanylic nucleotides. In addition, the blue-shift in the emission spectra of bound Mant-nucleotides was more pronounced for adenylic than for guanylic derivatives (7−8 nm for adenylic versus 2−4 nm for guanylic Mant-nucleotides). Both parameters reflected a polarity lower for the adenylic Mant-nucleotides binding site as compared to that of the guanylic Mant-nucleotides site.

Binding of Mant-labeled nucleotides to eEF-2 resulted also in a partial quenching of the intrinsic fluorescence of eEF-2 due to the presence of seven tryptophan residues. Maximum quenching values were significantly higher for guanylic than for adenylic Mant-nucleotides. This suggested that the binding of the two classes of nucleotides affected differently the conformation of the protein.

Both the decrease of the intrinsic fluorescence and the increase of the extrinsic fluorescence of the Mant-probe showed hyperbolic saturations that enabled the calculation of apparent dissociation constants. Values obtained by each method were in relatively good agreement with each other and were close to the values obtained for the corresponding unmodified nucleotides when their binding was monitored by tryptophan fluorescence quenching (*10*). These values were, respectively, 2.5, 3.0, 13.2, and 4.1 μM for GDP, GTP, ADP, and ATP. Hence, Mant-derivatives of GTP, GDP, and ATP bound to eEF-2 with a similar affinity and Mant-ADP with an affinity around 4 times higher than its unmodified counterpart. Therefore, modifications of the nucleotides by the Mant-probe have not modified significantly their binding properties toward eEF-2.

Competition Experiments between Unmodified and Mant-Labeled Nucleotides. The fluorescence of the Mant-probe can be used in competition experiments as a reliable reporter of the binding of the Mant-derivatives to the protein since the signal of the bound derivatives should not be modified by the presence of the unmodified nucleotides. Figure 1 represents the released of Mant-nucleotides bound to eEF-2 by the addition of successive amounts of ATP, ADP, GTP, and GDP. Concentrations of Mant-nucleotides used were 15 μM for Mant-ATP (Figure 1, panel A) and 24 μM for Mant-GTP (Figure 1, panel B), which corresponded to a theoretical site occupancy of about 90 and 85% respectively, before the addition of the competitors (values calculated from the dissociation constants in Table 1). Results indicated that Mant-ATP was released very efficiently from eEF-2 by ATP and ADP but hardly by GTP and GDP (extrapolated maximal values given by a reciprocal representation were 100 and 100% versus 30 and 40%, respectively). Similar results were obtained for Mant-ADP (results not shown). In the case of bound Mant-GTP, GTP and GDP behaved as very efficient competitors in contrast to ATP and ADP (extrapolated maximal values of chase were 100 and 95% versus 43% and less than 10%, respectively). This result is in agreement with a result of the Kaziro group reporting that [^{3}H]-GDP was efficiently released from pig liver eEF-2 by GDP and GTP but not by ATP (*23*). The overall results suggested that Mant-nucleotides bound to eEF-2 at the same site as the corresponding unmodified nucleotides. Furthermore, since the maximal efficiency of the Mant-nucleotide chase by the same class of unmodified nucleotides was 100%, these results

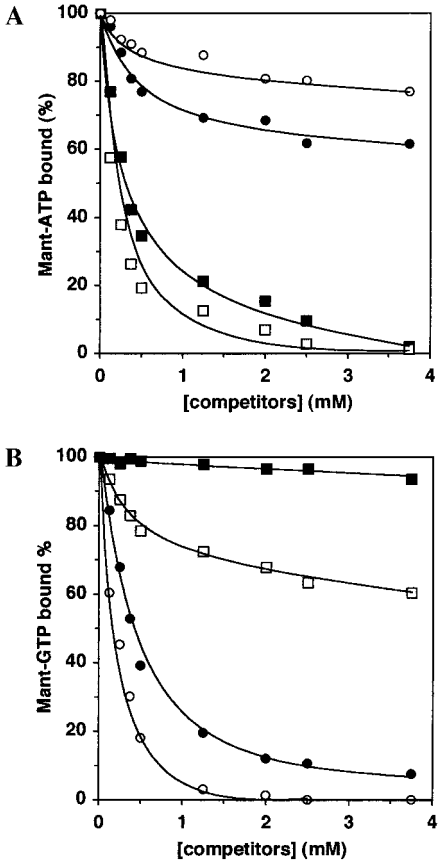

FIGURE 1: Chase of Mant-nucleotides bound to eEF-2 by unmodified nucleotides. Upon excitation at 356 nm, the Mant-nucleotide binding to eEF-2 was specifically monitored by the increase in the Mant-group fluorescence at 436 nm for Mant-ATP (A) and at 442 nm for Mant-GTP (B). Unmodified nucleotides used as competitors were GDP (●), GTP (○), ADP (■) or ATP (□).

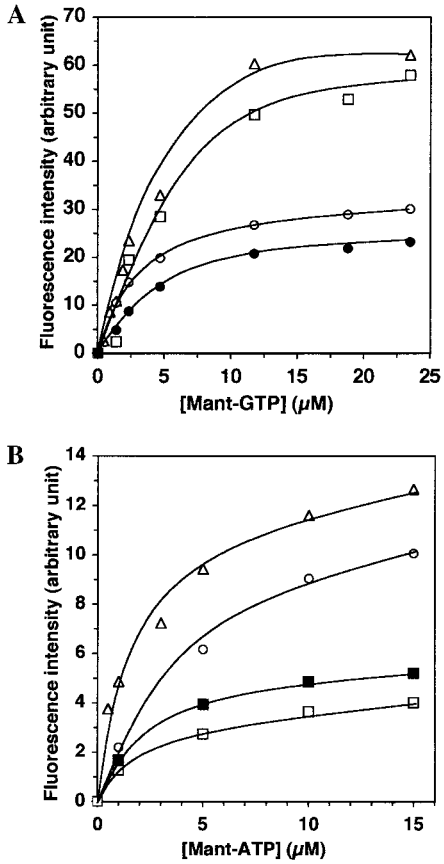

FIGURE 2: Prevention of Mant-nucleotide binding to eEF-2 by a preincubation with unmodified nucleotides. Mant-GTP (A) and Mant-ATP (B) binding to eEF-2 was monitored under the conditions described in Figure 1 after a 15-min preincubation with 1 mM GTP (○), GDP (●), ATP (□), ADP (■), or without any competitor (△).

indicated also that there was no additional binding of the Mant-derivative outside the nucleotide binding sites and, therefore, that the specificity of the binding was not brought by the Mant-moiety.

Prevention of Mant-nucleotide binding to eEF-2 by a previous incubation with unmodified nucleotides were also performed (Figure 2). Mant-GTP binding was very similar in the presence of ATP and in the absence of any competitor, whereas it was significantly inhibited by GTP or GDP: extrapolated maximal values given by the curve fitting were 100% versus 45 and 40%, respectively (Figure 2, panel A). These results confirmed that there was no Mant-GTP bound to the ATP binding site. Mant-ATP binding was prevented efficiently by a preincubation with ATP or ADP but not with GTP (Figure 2, panel B). Results similar to those obtained with Mant-ATP were obtained with Mant-ADP (data not shown). These observations showed that no Mant-adenylic nucleotide bound to the GTP/GDP binding site.

Put together, these competition experiments demonstrated first that the binding of the Mant-nucleotides was strictly restricted to the site of their corresponding unlabeled nucleotides. Second, these results suggested that two different sites existed on eEF-2, one specific for the adenylic nucleotides, the other one specific for the guanylic nucleotides.

Measurement of Fluorescence Energy Transfer Efficiencies. A clear evidence of a Förster's transfer between tryptophan residue(s) and the Mant-group was provided in the emission spectrum of eEF-2 tryptophans by the appearance of a peak specific of the Mant-group (peak centered around 430 nm). The increase of this peak was concomitant with a quenching of the tryptophan fluorescence (Figure 3, panel A). In the excitation spectrum of the Mant-group, a peak due to the excitation of tryptophan residue(s) and centered around 280 nm was also observed in the presence of eEF-2 (Figure 3, panel B).

The maximal values of quenching of intrinsic fluorescence mentioned in Table 1 for the Mant-labeled nucleotides were higher than those found with the corresponding unmodified nucleotides in very similar conditions, except for Mant-ADP (*10*): binding to eEF-2 resulted in quenching values of 21.6, 20, 2.3, and 13.7% with the Mant-derivatives of GDP, GTP, ADP, and ATP, respectively, versus 5.3, 14, 6.4, and 11.1% for the corresponding unabeled nucleotides. This confirmed that the quenching of tryptophan fluorescence due to the binding of the nucleotide itself was strengthened by the Förster's transfer due to the Mant-moiety. Consequently, only the method emphasized in Materials and Methods was appropriate to calculate the FRET efficiency. Transfer efficiencies measured for the two classes of nucleotides were significantly different [about 65% versus about 15% for guanylic and adenylic Mant-nucleotides, respectively (see Table 2)]. As mentioned in Materials and Methods, owing to the presence of several tryptophan residues in eEF-2, no interpretration of transfer efficiencies in terms of distances is made.

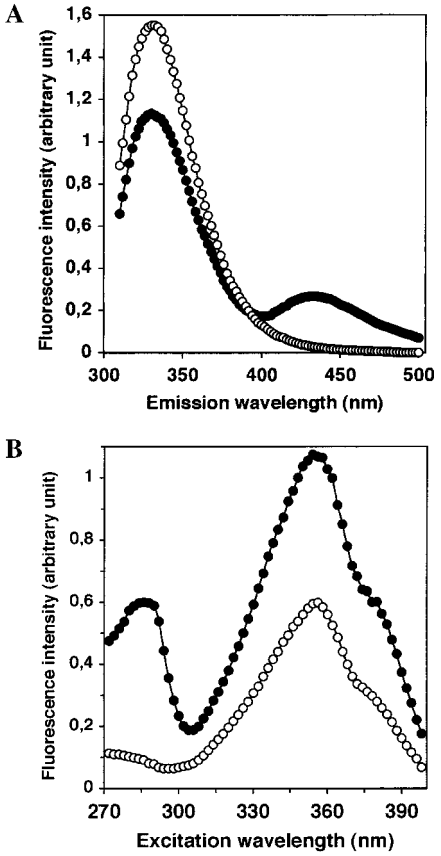

FIGURE 3: Evidence for a FRET between eEF-2 Trp and Mant-GTP. (A) The emission spectrum of eEF-2 tryptophans was recorded upon excitation at 295 nm in the absence (○) and in the presence of 24 μM Mant-GTP (●). (B) The excitation spectrum of 24 μM Mant-GTP was recorded at 440 nm in the absence (○) and in the presence of eEF-2 (●).

Table 2: Transfer Efficiencies between eEF-2 Tryptophans and the Mant-Nucleotides

acceptor	transfer efficiency (*E*) (%)
Mant-GDP	58
Mant-GTP	79
Mant-ADP	11
Mant-ATP	19

DISCUSSION

These experiments using Mant-derivatives of nucleotides were designed to elucidate whether there were two different binding sites for adenylic and guanylic nucleotides in eEF-2. As the compounds used in this work are not native nucleotides but fluorescent derivatives with a probe linked to the ribose part, we have to examine first if the different Mant-nucleotides bind to the same sites as the corresponding unmodified nucleotides.

Evidence that the Mant-Nucleotides Bind Specifically to the Sites of their Unlabeled Counterparts and that these Sites are Distinct for the Two Classes of Nucleotides. Several data clearly indicate that the binding of Mant-GTP, Mant-GDP, Mant-ATP, or Mant-ADP to eEF-2 results from a specific process. Binding can be monitored following different experimental procedures that arise from different physical mechanisms. The use of multiple reporters of the binding lowers the chance that the binding is aspecific: (i) all the Mant-nucleotides induce a quenching of the intrinsic fluorescence of eEF-2; (ii) there is an increase in the extrinsic fluorescence when eEF-2 is present in the incubation buffer; (iii) a Förster's transfer between eEF-2 tryptophan(s) and the Mant-group can be observed in both the emission spectra of tryptophan residues and the excitation spectra of Mant-nucleotides. Moreover, whatever the signal monitored is, it always follows a curve with a saturable shape. The dissociation constants obtained by the different methods are similar and low enough not to be related to a nonspecific binding. These characteristics indicate that the Mant-nucleotides bind to eEF-2 in a specific fashion.

The extrinsic fluorescence properties of bound adenylic and guanylic Mant-nucleotides are shown to be different: the Mant-adenylic binding site exhibits more hydrophobic characteristics than the Mant-guanylic one concerning the way the Mant-moiety is bound. This observation indicates that the specificity of the Mant-nucleotide recognition is not supported by the Mant-moiety but by the nucleotide part of the derivatives. This conclusion is also supported by both the similarity between the affinity values found for the unlabeled and the corresponding Mant-nucleotides and the results of the competition experiments. Therefore, all these results prove that the Mant-derivatives of one class (either guanylic or adenylic) bind to the same sites as do their corresponding unmodified nucleotides. Furthermore, they indicate that there are two different sites in eEF-2, one for each class of nucleotide.

Calculations of transfer efficiency support, ab absurdo, this assumption. If the sites were identical for adenylic and guanylic Mant-nucleotides, then the transfer efficiency would be identical for both classes of Mant-nucleotides, but this is not the case. The efficiency was approximately 70% for Mant-guanylic nucleotide whereas that of Mant-adenylic nucleotides was approximately 15%.

Are the Two Nucleotide Binding Sites Independent? The above conclusions might be slightly modulated considering that Mant-ATP seems to be partly released from eEF-2 by GTP or GDP used at very high concentrations as compared to Mant-derivatives (several mM versus few μM) (Figure 1, panel A). Conversely, Mant-GTP seems to be partly released by high concentrations of ATP (Figure 1, panel B). These results can be interpreted according to different hypotheses. First, adenylic and guanylic nucleotides might bind with a very low relative affinity to the guanylic and adenylic nucleotides binding sites, respectively. Second, the affinity of one nucleotide binding site might be decreased by the binding of a second nucleotide to the other site. Thus, the observation that Mant-GTP is partly released from eEF-2 by ATP addition suggests that ATP might lower the affinity for Mant-GTP. However, it is noteworthy that a preincubation with ATP does not prevent efficiently Mant-GTP binding to eEF-2 (Figure 2, panel A). Third, it is possible that the quantum yield of the bound probe might change upon the binding of a second nucleotide, reflecting a modification in the environment of the probe. Therefore, the partial release of one class of Mant-nucleotides by the other class of unmodified nucleotides in the chase experiments might be interpreted as a change in the quantum yield of the probe. It is likely that the last two hypotheses contribute to the observed results. Indeed, that eEF-2 could be submitted to structural adaptations that would modify only the quantum

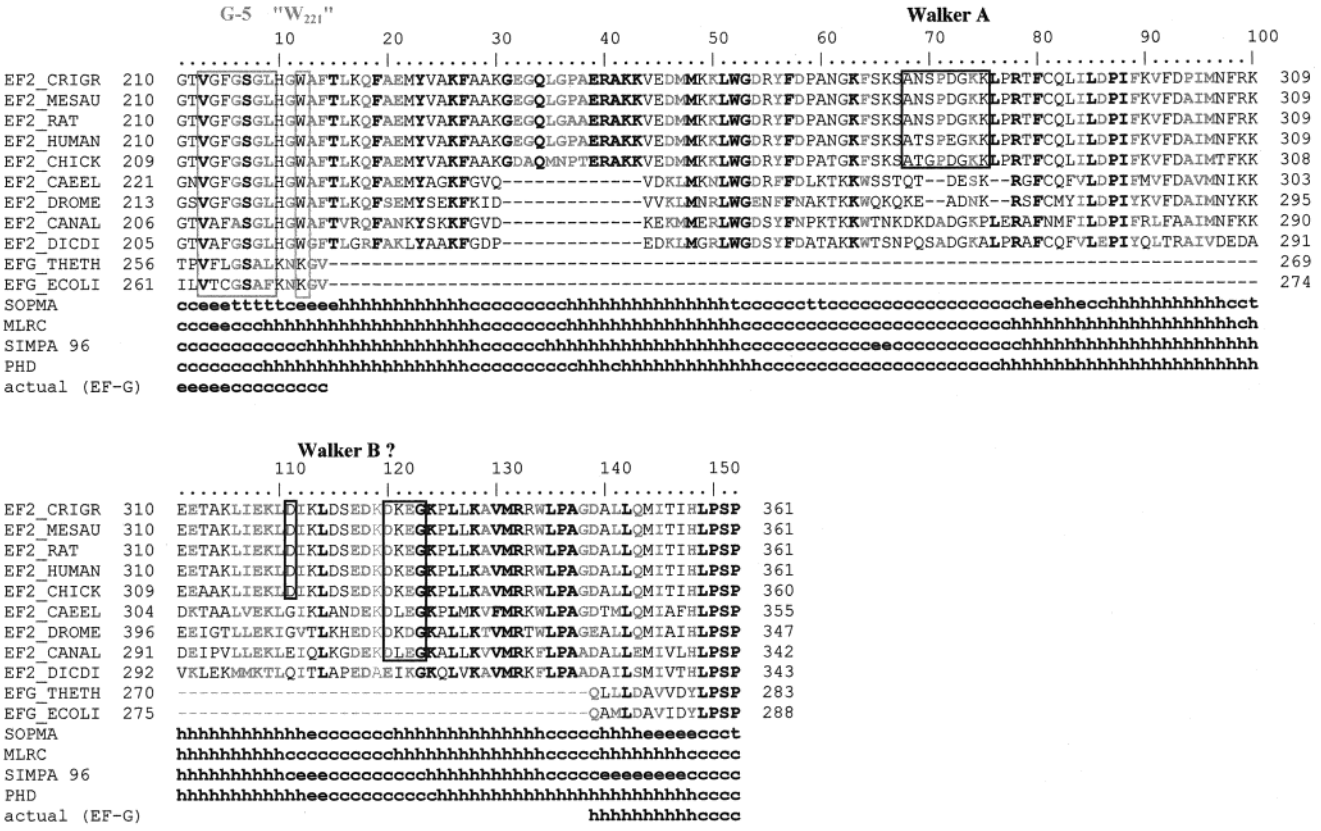

FIGURE 4: Partial amino acid-sequences alignment of G-domain inserts of eEF-2 from several eukaryotes with the corresponding sequences in EF-G. Sequences were obtained from the SWISS-PROT and TrEMBL protein sequence databases (*34*). All the available sequences of eEF-2 from higher eukaryotes are included [CRIGR: *Cricetulus griseus* (Chinese hamster); MESAU: *Mesocricetus auratus* (golden hamster); RAT: *Rattus norvegicus* (rat); HUMAN: *Homo sapiens* (human); CHICK: *Gallus gallus* (chicken)]. The alignment contains also examples of eEF-2 from several classes of other eukaryotes (CAEEL: *Caenorhabditis elegans*; DROME: *Drosophila melanogaster* (fruit fly); CANAL: *Candida albicans* (yeast); DICDI: *Dictyostelium discoideum* (slime mold). Two sequences of EF-G from *Eubacteriae*, the 3-D structures of which are solved, are given for comparison [THETH: *Thermus aquaticus* (subsp. *thermophilus*); ECOLI: *Escherichia coli*]. Primary sequence alignment was performed using CLUSTAL W software (*35*), and optimization of gap insertion was realized with MPSA software (*36*). Fully conserved residues are displayed in black bold letters and amino acids belonging to a conserved group are displayed in gray bold letters. Other amino acids are shown in normal letters. Positions and lengths of the sequences was chosen so as to obtain a highly conserved sequence at each end which allows location of the eEF-2 insert in the G-domain. Residues numbers are given for each sequence. Secondary structure prediction was processed at the NPS@ web server (*37*) located at http://www.ibcp.fr using the following software [SOPMA (*38*), MLRC (*39*), SIMPA96 (*40*), PHD (*41*)]. Secondary structure prediction is made on rat sequence and is identical for all the vertebrate's eEF-2. The secondary structure of EF-G is the actual one and is based on crystallographic 3-D structures (*2*, *4*). Residues involved respectively, in α-helices, *β*-sheets, turns, and coils are represented with **h**, **e**, **t** and **c** letters. The putative second Walker A and B motifs are enclosed with black boxes. The G5 motif and W221 and the corresponding residue in EF-G are displayed in gray boxes. These motifs are indicated above the sequences.

yield of the probe without altering the affinities for the nucleotides is rather unconceivable.

In conclusion, our results strongly suggest that eEF-2 possesses two distinct binding sites specific respectively for guanylic and adenylic nucleotides that are either independent or slightly modulated one by each other.

Selectivity of the Guanylic Nucleotide Binding Site and Discrimination against Adenylic Nucleotide Binding. In contrast to ATP binding proteins, the motifs located in the G-core that ensure the selectivity for guanylic nucleotides in the GTPases are easy to identify in the primary sequence. These motifs contribute not only to recognize specifically guanylic nucleotides but also to discriminate strongly against adenylic nucleotides or non-puric nucleotides (*6*). The most conserved of these elements, designated as G-4, possesses the sequence NKXD (X being any amino acid). It has been demonstrated to be mostly responsible for the selectivity toward guanylic nucleotides in the G-core of several GTPases. In the case of Ras p21 and EF-Tu, a difference of affinity of 7 orders of magnitude is reported between GTP and ATP (*24*, *25*). Another motif, designated as G-5, buttresses the guanine base recognition site but has usually a less conserved sequence that makes its identification more difficult in the sequences of GTPases. Nevertheless, by aligning sequences of several factors involved in translation, Ævarsson has demonstrated that G-5 is more conserved in this subfamily of GTPases (*7*). The consensus sequence found is VXXGS(A/G)(L/K) instead of the (T/G)(C/S)A sequence described for other GTPases, which makes its identification in these sequences easier (Figure 4). Both the G-4 (N_{158}KMD$_{161}$) and G-5 (V_{212}GFGSGL$_{218}$) motifs are found in eEF-2, and it can be assumed that they exert the same function in eEF-2 as in other GTPases. Therefore, that the guanylic nucleotide binding site in eEF-2 could also accommodate adenylic nucleotides would have been very unusual.

Putative Location of the Adenylic Nucleotide Binding Site. The region in the vicinity of Trp221 is assumed to interact

with the highly exposed adenine 4224 of the α-sarcin/ricin loop of the 28 S rRNA. Hence, it has been suggested that the ability of eEF-2 to interact with an adenine-containing molecule like ATP might be a consequence of its ability to interact with the α-sarcin/ricin loop (*26*, *27*). However, this hypothesis locating the binding site for adenylic nucleotides in the immediate vicinity of Trp221 appears highly improbable since the transfer efficiency measured for Mant-ATP or Mant-ADP is very low. Moreover, the reasons that have led to this hypothesis turn out not to be in agreement with other experimental data: ATP and ADP bind to eEF-2 with much higher affinities than AMP or adenosine (*10*). Therefore, the adenylic nucleotide binding site is likely to contain structural features involved in the binding of the phosphate moiety.

In contrast to the adenine recognition (*28*−*30*), the binding of the phosphate chain requires usually conserved features. A detailed analysis of several eEF-2 primary sequences (Figure 4) reveals the presence in eEF-2 of higher eukaryotes of potential Walker A and B motifs in addition to the corresponding G-core motifs (G1 and G3). Classically, Walker A motif (G/A)XXXXGK(S/T) is located in a loop and Walker B motif is constituted by a β-strand terminated by an Asp residue. The additional motifs found in eEF-2 are A_{277}NSPDGKK_{283} (Walker A motif) and either D_{320} or D_{329}KEG_{332} (Walker B motif). These two motifs are located in a 120-residue insert of the G-domain that is delimited by two well-conserved regions of the G-domain (Figure 4). D_{320} might be a better candidate than D_{329}KEG_{332} which possesses the signature DXXG found in the G3 motif of GTPases since in D_{329}KEG_{332}, Asp_{329} follows charged residues that are unlikely to be part of a β-strand. On the contrary, D_{320} is predicted in a β-strand topology by most of the secondary structure prediction methods. Furthermore, Asp_{320} is conserved only among the EF-2s which contain also the Walker A motif (Figure 4). The putative Walker A motif found here is not absolutely identical to the classical Walker A motif, but there are several examples of variation among the P-loops (*31*−*33*). It is noteworthy that the additional Walker A motif is correctly predicted in a loop (Figure 4).

The G-core insert of eEF-2 in which the Walker A and B motifs are found might be the equivalent of the G-domain insert of EF-G, although there is no obvious amino acid sequence analogy between the two inserts that are located in different parts of the G-cores of these proteins (*2*). There is no recognizable motif in EF-G sequence for binding a second nucleotide, but it cannot be totally excluded that EF-G does bind ATP since experimental data are currently missing. However, from our results, it seems more likely that EF-G and eEF-2 have diverged one from each other in the course of the evolution and that eEF-2 has acquired an ATP/ADP binding site in the same way it has acquired phosphorylation and ADP-ribosylation sites.

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