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Interaction of phosphorylated elongation factor EF-2 with nucleotides and ribosomes

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Abstract The intrinsic fluorescence emission spectrum of elongation factor EF-2 due to the 7 Trp residues was not modified after complete phosphorylation of the factor by the specific Ca^{2+}/Calmodulin-dependent kinase III. The effect of nucleotide binding on this fluorescence revealed differences between phosphorylated and unmodified EF-2. Low concentrations of GTP had a smaller quenching effect on the fluorescence of phosphorylated EF-2 than on the fluorescence of unmodified EF-2, whereas GDP had exactly the same quenching effect on the fluorescence of both samples. These results suggest that phosphorylation of EF-2 decreased its affinity for GTP but not for GDP. Ability of phosphorylated EF-2 to form a ternary complex with ribosomes in the presence of a non-hydrolysable GTP analog and its ability to protect ribosomes against ricin-inactivation were both decreased to the same extent. The lower affinity of phosphorylated EF-2 for GTP could be responsible for a weaker and/or incorrect interaction of the factor with the ribosome, in particular with the ricin-site of the 28-S rRNA assumed to be involved in translocation initiation.

Key words: Elongation factor EF-2; Phosphorylation; Guanylic nucleotide; Fluorescence

1. Introduction

Elongation factor EF-2, like other translation factors, belongs to the superfamily of proteins which are activated by GTP. It catalyses, like its counterpart in prokaryotic cells EF-G, the translocation of the peptidyl tRNA from the A to the P site of ribosome, which is followed by the hydrolysis of GTP into GDP and P_i. We have recently shown that interaction of EF-2 with GTP (as well as with other nucleotides) could be studied by measuring the quenching of the intrinsic fluorescence of the factor due to its 7 Trp residues, in the presence of nucleotides [1]. It was possible to demonstrate by this technique that GTP binding induces a conformational change of the EF-2 molecule which unmasks a tryptophan residue. This residue, which was identified as Trp^{221}, is required for biological activity since its oxidation inactivates the factor [2]. Subsequent results [3] suggested that this residue could interact with the 28S RNA loop including the GAGA sequence which is the target of ricin and which is assumed to be involved in initiating the translocation process [4].

It is now well established that EF-2 can be inactivated by phosphorylation by a specific kinase which is Ca^{2+}-calmodulin dependent [5–7]. Two Thr residues can be phosphorylated, Thr^{56} first and then Thr^{58} [8]. The primary mechanism of this inactivation has not been clearly identified yet. It was suggested either that the mechanism of translocation itself was blocked [9], or that the affinity of EF-2 for ribosomes was reduced [10]. The effect of phosphorylation on the interaction of EF-2 with GTP has not been studied directly. However, it has been shown that phosphorylated EF-2 behaves differently from unmodified EF-2 in cross-linking experiments with ^{32}P-labeled guanylic nucleotides using irradiation at 254 nm [11]. Since measurements of the intrinsic fluorescence represent a very sensitive method to study protein-nucleotide interactions, we used this method to compare the affinity of phosphorylated EF-2 for nucleotides with that of unmodified EF-2. We also tested the ability of phosphorylated EF-2 to protect ribosomes against ricin, in order to see if this modification of the factor would alter its interaction with the GAGA-containing loop of the 28S rRNA.

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2. Materials and methods

2.1. Materials

GTP and GDP were purchased from Boehringer, ATP from Pharmacia, and the micro BCA protein assay reagent from Pierce. [8-^{3}H]GuoPP[NH]P (9.25 GBq/mmol) was obtained from Amersham. Okadaic acid was from Calbiochem.

2.2. Preparations

Rat liver eEF-2 (>95% pure) was purified as described previously [12]. Preparation of rat liver ribosomes has also been described [13]. Purified ricin A-chain was a gift from Dr. Cazellas and purified EF-1α was a gift from Dr. Crechet. EF-2 specific calcium-and calmodulin-dependent protein kinase III was prepared as described by Nygård et al. [14]. Protein concentration was determined with the Micro BCA protein assay [15].

2.3. Phosphorylation of eEF-2

eEF-2 was phosphorylated by incubation in 20 mM HEPES- KOH pH 7.6, 10 mM $MgCl_2$, 0.15 mM $CaCl_2$, 4.8 μM CaM, 0.27 μM okadaic acid, 125 μM ATP, containing 5 pmol eEF-2 and 2 pmol purified kinase. The reaction mixtures, 25 μl, were incubated at 37°C for 60 min. The reaction was stopped by addition of 0.25 μmol EGTA. Under these conditions, 100% of eEF-2 was phosphorylated as shown by isoelectrofocalisation. Phosphorylated eEF-2 was separated from the kinase using a FPLC system (Millipore) fitted with a MonoQ column (Pharmacia). The EF-2 sample and the column were equilibrated with 20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% glycerol containing 50 mM KCl. The proteins were eluted with a linear 50–500 mM KCl gradient. Phosphorylated eEF-2 was eluted at 200 mM KCl and the kinase at 400 mM KCl. Fig. 1 shows the isofocalisation pattern of purified phosphorylated and unmodified eEF-2. This phosphorylated form should correspond to the phosphorylation of both Thr^{56} and Thr^{58} residues. Indeed, when the incubation time was reduced, an additional band was observed with a migration intermediate between those of the two bands represented in Fig. 1.

2.4. Treatment of ribosomes with ricin A-Chain

Ribosomes were treated with ricin A-chain under conditions adapted from Brigotti et al. [16]. Briefly, rat liver ribosomes (10 pmol) were pre-incubated 15 min at 25°C in 45 μl in 80 mM Tris-HCl (pH 7.4),

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120 mM KCl, 7 mM $MgCl_2$, 2 mM dithiothreitol containing 45 nmol GTP and 31.5 pmol of native or phosphorylated eEF-2. Ricin-A chain was then added in 5 μl of buffer A to a final concentration of 0.15 nM and ribosomes were incubated for an additional 5 min at 25°C. Finally, 10 μl were withdrawn from the reaction mixture for protein synthesis analysis.

2.5. Activity measurements

Poly-(U)-directed polyphenylalanine synthesis was measured as described previously [17] in the presence of 6 pmol of eEF-2 and 80 pmol of EF-1α. These conditions designed to test the activity of ribosomes corresponded to an excess of EF-2. Formation of the ribosome-eEF-2-[8-^{3}H]GuoPP[NH]P complex was measured [18] in the presence of 10 pmol of ribosomes, increasing amounts of EF-2 phosphorylated or not and 20 μM [8-^{3}H]GuoPP[NH]P in a final volume of 100 μl.

2.6. Fluorescence measurements

Emission fluorescence spectra of native or phosphorylated protein were recorded at 25.0 ± 0.1°C on a SLM 8000 C spectro-fluorometer equipped with a 450 W Xenon lamp. The cuvette contained 1 ml of 0.08 μM EF-2 in buffer A (20 mM Tris-HCl, pH 7.6, 8 mM $MgCl_2$, 100 mM KCl, 100 mM sucrose, 1 mM DTT and 10% glycerol). Excitation wavelength was 295 nm. Emission and excitation band-path was set at 4 nm to ensure that only Trp residues were excited [19]. Variations of the lamp emission power were automatically corrected with a rhodamine solution used as a standard in the reference channel. Emission spectra were corrected for buffer blank. eEF-2 fluorescence quenching was deduced from the decrease of fluorescence intensity at 332 nm in the presence of increasing amounts of nucleotides [1] after correction for nucleotide inner-effect determined under the same conditions used with bovine serum albumin and *N*-acetyltryptophanamide [20].

3. Results

3.1. Interaction of phosphorylated EF-2 with nucleotides studied by the quenching of its intrinsic fluorescence

The fluorescence emission spectrum of phosphorylated EF-2, using an excitation wavelength of 295 nm, was found to be superposable with that of unmodified EF-2 (Fig. 2). This fluorescence is due to the 7 Trp residues of the molecule [1]. Fluorescence was partly quenched after addition of increasing concentrations of GTP. However, for the low nucleotide concentrations, the quenching observed with phosphorylated EF-2 was significantly less than that observed with control EF-2. Data represented in Fig. 3A indicate that the apparent affinity of phosphorylated EF-2 for GTP deduced from the quenching values was reduced by a factor of 3.7 (K_d = 44 μM instead of 12 μM). On the other hand, the quenching value at saturation was slightly higher with phosphorylated EF-2 than with control (22% instead of 15%). Addition of increasing concentrations of GDP to phosphorylated and unmodified EF-2 produced identical quenching (Fig. 3B). Since we had shown that ATP also partly quenched EF-2 fluorescence [1], we tested the effect of this nucleotide on the fluorescence of phosphorylated EF-2. Fig. 4 shows that with modified EF-2, higher concentrations of nucleotide were required to induce the same quenching. Saturation curves were sigmoidal, as already observed with native EF-2 [1].

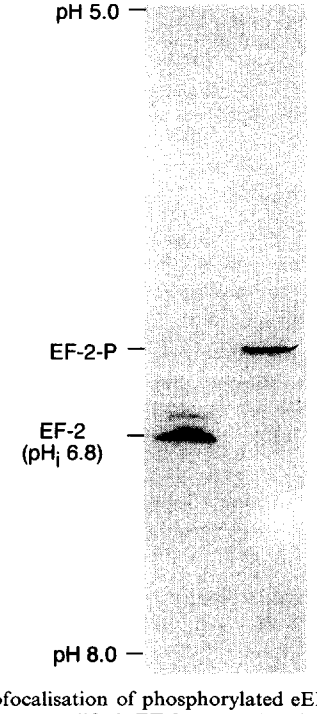

Fig. 1. Isoelectrofocalisation of phosphorylated eEF-2. 1 μg of either phosphorylated or unmodified eEF-2 was analyzed with a PhastSystem (Pharmacia) using a 5–8 pH gradient.

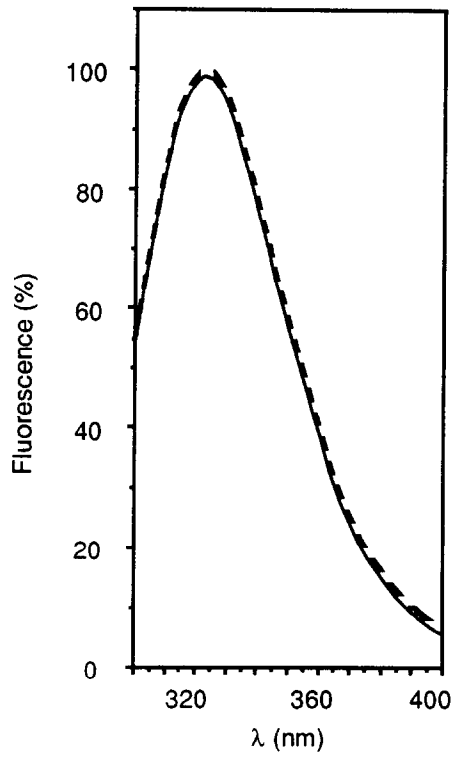

Fig. 2. Fluorescence emission spectrum of phosphorylated eEF-2. This spectrum was determined in buffer A as indicated in section 2. Excitation wavelength was 295 nm. Phosphorylated eEF-2, continuous line; unmodified eEF-2, dashed line.

3.2. Interaction of phosphorylated EF-2 with ribosomes and protection of them against ricin A

The ability of phosphorylated EF-2 to form a complex with ribosomes was measured in the presence of [^{3}H]Guo PP(NH)P, a radioactive derivative of GTP which is not hydrolyzed. As can be seen in Fig. 5A, there is a significant decrease of its ability to form the complex, as compared with that of unphosphorylated EF-2. The decrease corresponds to an affinity reduction of more than 4 fold (K_d value of 0.14 μM instead

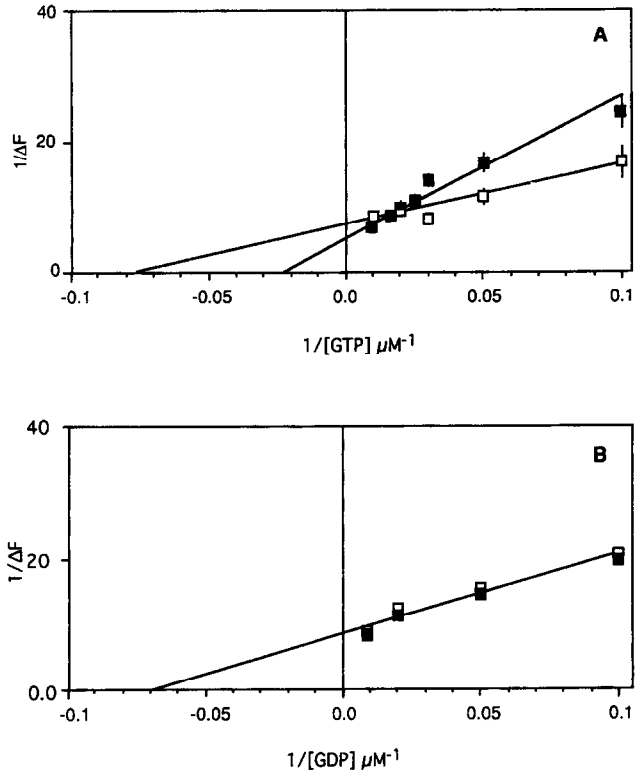

Fig. 3. Effect of guanylic nucleotides on the intrinsic fluorescence of phosphorylated EF-2. Phosphorylated (■) and non-phosphorylated (□) eEF-2 were incubated in buffer A in the presence of increasing concentrations of GTP (A) or GDP (B). Measurements were carried out as described in section 2. The reciprocal of the fluorescence decrease ΔF is plotted versus the reciprocal of the nucleotide concentration. In this figure as in the two following ones each point represents the mean of the results obtained in 3 experiments. Standard deviation is indicated by bars when exceeding the width of the points.

of 0.03 μM). This result is at least qualitatively in agreement with results obtained by Carlberg et al., using a different technique: centrifugation through a sucrose gradient of mixtures containing ^{125}I-labeled EF-2 (phosphorylated or not), Guo PP(NH)P and a mixture of 40S and 60S subunits [10]. It is well established that the toxin ricin inactivates the ribosome by specific depurination of the A_{4324} nucleotide in the 28-S rRNA [21]. We concluded from previous results that an EF-2 amino-acid sequence containing Trp^{221} can protect the ribosome against ricin inactivation [3]. Therefore it was interesting to investigate whether phosphorylated EF-2 could prevent ribosome inactivation by ricin. Indeed, the decreased affinity of the modified factor for ribosome could affect the presumed interaction between Trp^{221} and the GAGA target of Ricin, and this could account for the total inactivity of phosphorylated EF-2. Fig. 5B shows that protection of ribosomes against ricin was lower with phosphorylated EF-2 than with unmodified EF-2. This decrease paralleled that of phosphorylated-EF-2 affinity for ribosomes, the K_d values determined in both cases being almost identical (0.16 and 0.14 μM, respectively, instead of 0.03 μM for unmodified EF-2).

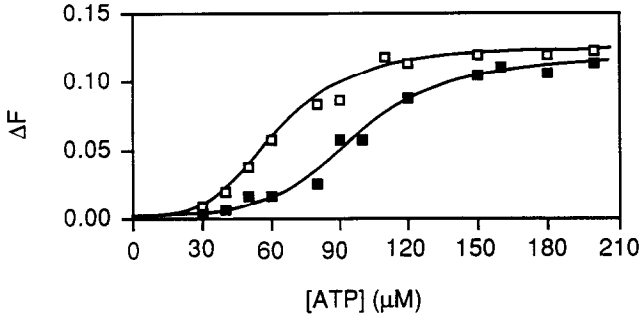

Fig. 4. Effect of ATP on the intrinsic fluorescence of phosphorylated EF-2. The fluorescence decrease of phosphorylated (■) and unphosphorylated (□) EF-2 is plotted as a function of increasing concentrations of ATP. The saturation curve is fitted to the Hill equation which can be formulated : $\Delta F = \Delta F_{max}[S]^n/(K'+[S]^n)$ using the Multifit 2.0 program (Day Computing, Cambridge, UK). ΔF is the fluorescence decrease of EF-2 in the presence of ATP, [S] represents the nucleotide concentration, n is the Hill coefficient and K' a constant.

4. Discussion

Our present study confirms that phosphorylated EF-2 has a decreased ability to form a ternary complex with ribosomes and a non-hydrolyzable GTP analog. But in addition, using the very sensitive technique of intrinsic fluorescence measurements, our results suggest that even in the absence of ribosomes, phosphorylated EF-2 has a decreased affinity for GTP, but not for GDP. The possibility that EF-2 phosphorylation did not affect GTP binding but rather modified the Trp fluorescence

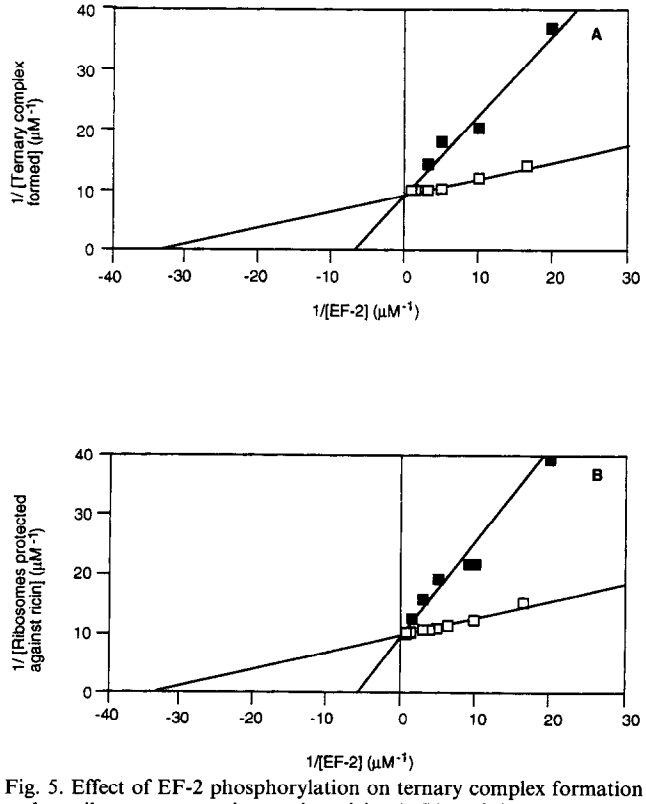

Fig. 5. Effect of EF-2 phosphorylation on ternary complex formation and on ribosome protection against ricin. A: Plot of the reciprocal of the concentration of the ternary complex formed versus the reciprocal of the native (□) or phosphorylated (■) eEF-2 concentration. Reaction was performed as described in section 2. B: Plot of the reciprocal of the ribosome concentration protected against ricin inactivation versus the reciprocal of the native (□) or phosphorylated (■) EF-2 concentration. Treatment of ribosomes is described in section 2.

in such a way that this fluorescence was less quenched by the nucleotide was ruled out, since the emission spectra of phosphorylated and unmodified EF-2 were identical. The phosphorylated amino-acid residues (Thr^{56} Thr^{58}) do not belong to the consensus sequences which are supposed to interact with GTP [22]. Therefore the reduced affinity of phosphorylated EF-2 for GTP is probably due to differences in the conformation of the factor. Activation of EF-2 with GTP is most likely the prerequisite step for its binding to ribosomes. An impaired complex formation between phosphorylated EF-2 and GTP should result in a decreased affinity of the modified factor for the ribosome and, consequently, for the ricin site of 28S rRNA assumed to be involved in triggering the translocation process [4]. The fact that the apparent K_d values found for the different interactions of phosphorylated EF-2 showed a similar increase supports the hypothesis that these variations result from the same cause. However it is not clear whether such an increase (by a factor of 4) can account entirely for the almost total inhibition of protein synthesis observed in the presence of phosphorylated EF-2. It should be noted that, reciprocally, EF-2 cannot be phosphorylated when included in a ternary complex with a GTP analog and ribosome, whereas it is fully reactive when included in a complex with GDP and ribosome [13]. Comparison of the effect of EF-2 phosphorylation with that of the other EF-2 post-translational modification, ADP-ribosylation, is interesting, because in the latter case there is also a decrease in the affinity of EF-2 for ribosome in the presence of a GTP analog but no modification of the interaction of EF-2 with GTP (Sontag et al., unpublished results). The significance of the decreased affinity of phosphorylated EF-2 for ATP is not clear, since the meaning of the interaction of unmodified EF-2 with this nucleotide remains unknown yet. We do not know, in particular, wether the interaction site of guanylic and adenylic nucleotides with EF-2 are different or not. However, the fact that phosphorylation of EF-2 affects the interaction of the factor with both nucleosides tri-phosphate (but not di-phosphate) suggests a common site of interaction for the γ-phosphate of guanylic and adenylic nucleotides.

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