Functional groups of elongation factor 2 involved in interactions with guanosine nucleotides and ribosomes

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Treatment of rat liver EF-2 with *N*-ethylmaleimide (MalNEt) did not affect the direct interactions of the factor with guanine nucleotides or with ribosomes, but inhibited the binding of guanosine 5'-(β,γ-methylene)triphosphate (GuoPP(CH_2)P) to the EF-2–ribosome complex. The amino group reactive reagent 2,4,6-trinitrobenzenesulfonate (TNBS), however, inhibited specifically the direct interactions of EF-2 with guanine nucleotides, but not the binding of GuoPP(CH_2)P to the EF-2–ribosome complex. The different sensitivities of EF-2 to MalNEt and to TNBS suggested that the binding sites involved in the binary *vs.* ternary complex might correspond to different conformational states or might even be distinct physical entities.

Elongation factor 2 *Ribosomal interaction* *Guanosine nucleotide* *Sulfhydryl group* N-*ethylmaleimide* *Amino group specific reagent*

1. INTRODUCTION

Eukaryotic elongation factor 2 (EF-2) possesses cysteine residues [1–3] some of which appear to be in the reduced state, essential for its activity in protein synthesis [4,5]. Treatment of EF-2 with sulfhydryl group reactive reagents [6,7] or lack of a sulfhydryl group reducing agent in the medium [6] results in the inhibition of the EF-2-promoted binding of guanine nucleotides to the ribosome. These findings have previously implied that some sulfhydryl groups are essential for the interaction of EF-2 with guanine nucleotides and/or ribosomes. Nevertheless, the role of sulfhydryl groups in these interactions has not been sufficiently defined. Our knowledge concerning the functional groups other than the sulfhydryl groups has also remained very scant. Hence, we investigated by equilibrium dialysis or sedimentation methods the role of sulfhydryl and of amino groups of EF-2 in the interaction of this factor with ligands. Blocking of the sulfhydryl groups of EF-2 by MalNEt did not affect the direct interaction of the factor with guanine nucleotides or with ribosomes, but inhibited binding of GuoPP(CH_2)P to the EF-2–ribosome complex. On the other hand, the amino group reactive reagent TNBS inhibited specifically the direct interaction of EF-2 with guanine nucleotides, but not the binding of GuoPP(CH_2)P to the EF-2–ribosome complex.

Abbreviations: ADPR-EF-2, adenosine diphosphate ribosylated elongation factor 2; GuoPP(CH_2)P, guanosine 5'-(β,γ-methylene) triphosphate; MalNEt, *N*-ethylmaleimide; TNBS, 2,4,6-trinitrobenzenesulfonate; DMA., dimethyladepimidate; DMS, dimethylsuberimidate

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2. MATERIALS AND METHODS

2.1. *Materials*

Ribosomes and EF-2 were prepared as in [8]. [U-^{14}C]GTP, spec. act. 35 Ci/mol, [8-^{3}H]GDP, spec. act. 10 Ci/mmol, [8-^{14}C]GDP, spec. act. 33 Ci/mol, [8-^{3}H]GuoPP(CH_2)P, spec. act. 12.2 Ci/mmol, and [U-^{14}C]NAD^+, spec. act. 281 Ci/mol or 286 Ci/mol were obtained from the

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Radiochemical Center (Amersham). Unlabelled guanine nucleotides and MalNEt were products of Boehringer (Mannheim) and of Serva (Heidelberg), respectively. Two, 3-butandione was obtained from Pierce, and TNBS from Serva. Oxidized guanine nucleotides [8], DMA and DMS [9,10] were prepared by the methods described.

2.2. *Assays*

EF-2 was determined by ADP-ribosylation [8,11]: EF-2 protein fractions [8] modified by the covalent binding of the radioactive ADP-ribose group has a spec. act. ≃ 4100 dpm/μg protein. Equilibrium dialysis and quantitative binding of ADPR-EF-2 to the ribosome were assayed and the data evaluated as in [8]. Whenever indicated, 38 mM 2,3-butandione [12] or 1 mM of one of the imidates (DMA or DMS) were included in the equilibrium dialysis medium. Equilibrium dialysis in the presence of 2,3-butandione was carried out in the dark.

2.3. *Treatment with MalNEt*

EF-2 in dialysis buffer [8] was incubated in the presence of a 3 mM excess MalNEt for 5 min at 37°C. (Incubation of EF-2 at 37°C for 5 min does not seem to affect the activity of the factor [7].) Thereafter, unreacted MalNEt was neutralized by the addition of 2-mercaptoethanol to a final concentration of 20 mM. ADPR-EF-2 was treated with MalNEt similarly [8,11,13].

2.4. *Treatment with TNBS*

EF-2 in dialysis buffer [8] was incubated for 5 min at 4°C in the presence of 1 mM TNBS and then applied to a Sepadex G-50 column (1 × 25 cm) equilibrated with dialysis buffer. The EF-2 eluting with the void volume was pooled.

3. RESULTS

3.1. *Formation of binary complex*

As shown in table 1, EF-2 could bind GTP or GDP despite treatment with MalNEt. Similarly the omission of 2-mercaptoethanol during equilibrium dialysis did not abolish the interaction between EF-2 and GDP, but elevated the K_d-value. The interaction of EF-2 with guanine nucleotides was likewise unaffected by the presence of 2,3-butandione. However, in the presence of one of the imidates or of TNBS no interaction between EF-2 and guanine nucleotides could be detected. Moreover, TNBS completely inhibited the stable linkage between oxidized GuoPP(CH$_2$)P and EF-2 [8] as indicated by gel filtration on Sephadex G-50 (fig. 1). These findings suggested that the amino but not the sulfhydryl groups may be involved in direct interaction of EF-2 with guanine nucleotides.

Table 1

Effects of different reagents on the quantitative binding of guanine nucleotides to EF-2

System	K_d (μM)	Binding site/EF-2 (n)
EF-2 + GDP	0.54	0.67
+ MalNEt	1.2	0.6
− 2-mercapto-ethanol	3.3	1.0
+ TNBS	–	– (no binding)
+ DMA	–	– (no binding)
+ DMS	–	– (no binding)
+ 2,3-butan-dione	1.2	1.0
EF-2 + GTP	2.0	0.76
+ MalNEt	2.0	0.6
+ TNBS	–	– (no binding)
+ DMS	–	– (no binding)
+ 2,3-butan-dione	1.93	0.8

The experimental procedure for equilibrium dialysis was as indicated in section 2. The EF-2 concentration was kept at 0.3 μM, the GDP concentration was varied betweeen 0.2 and 2 μM and the GTP concentration between 0.2 and 9 μM. The equilibrium dialysis medium corresponded to the dialysis buffer [8]. Whenever present, the concentration of MalNEt was 10 mM (a 3 mM excess over 2-mercapthoethanol), that of TNBS or of one of the imidates 1 mM and that of 2,3-butandione 38 mM

3.2. *Binding of ADPR-EF-2 to ribosomes*

Besides retaining the activity for binary complex formation, ADPR-EF-2 treated with MalNEt could also interact directly with ribosomes, as indicated by its presence in the ribosomal complexes obtained by centrifugation (table 2). When compared to the untreated control, this interaction revealed a decreased affinity for ribosomes (K_d = 0.99 μM *vs.* K_d = 0.25 μM). Moreover, whereas untreated ADPR-EF-2 displayed in the

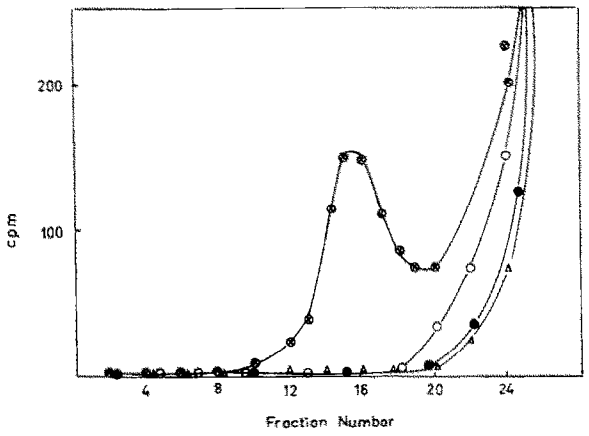

Fig. 1. Effect of treatment of EF-2 with TNBS on the formation of the linkage between EF-2 and oxidized GuoPP(CH$_2$)P. Twenty-five EF-2 was preincubated with or without 1 mM TNBS for 5 min at 0°C. After addition of 0.3 mM GuoPP(CH$_2$)P or GuoPP(CH$_2$)P$_{ox}$ incubation was continued for 5 min at 37°C: 75 μl of reaction mixture containing EF-2 and [^{3}H]GuoPP(CH$_2$)P (○—○); EF-2 + TNBS and [^{3}H]GuoPP(CH$_2$)P (●—●); EF-2 + TNBS and [^{3}H]GuopPP(CH$_2$)P$_{ox}$ (⊗—⊗); or EF-2 + TNBS and [^{3}H]GuoPP(CH$_2$)P$_{ox}$ (△—△) were applied to a Sephadex G-50 column equilibrated with dialysis buffer. Fractions of 300 μl were collected and counted in 3 ml of Bray's solution in a Packard (Tricarb) liquid scintillation counter.

presence of GuoPP(CH$_2$)P an increased affinity for the ribosome, after treatment with MalNEt, this was no more the case: the K_d in the presence of GuoPP(CH$_2$)P was 1.01 μM for ADPR-EF-2 treated with MalNEt and 0.08 μM for the control.

Table 3

Effect of preincubation of EF-2 with MalNEt or TNBS on the quantitatives (EF-2-promoted) binding of GuoPP(CH$_2$)P to ribosomes

Components present in preincubation	K_d (μM)	Binding sites ribosome (n)
EF-2	0.07	0.55
EF-2, MalNEt	0.03	0.01
EF-2, TNBS	0.17	0.66

The experimental procedure was as indicated in section 2. The EF-2 concentration was kept at 0.15 μM and the ribosome concentration at 0.3 μM. The GuoPP(CH$_2$)P concentration was varied between 5 nM and 75 nM. The ribosomal complexes were isolated by centrifugation and the data evaluated as in [8]. The values are means of 2–4 separate determinations evaluated by the least squares method. The K_d-value found previously for GuoPP(CH$_2$)P by equilibrium dialysis [8] was 0.26 μM

3.3. *Formation of the ternary EF-2*–guanine nucleotide–ribosome complex

GuoPP(CH$_2$)P which, as determined by the sedimentation method, bound with high affinity to the binding site in the ternary complex involving native EF-2 (K_d = 0.07 μM, n = 0.55) revealed negligible binding (n = 0.01) to the corresponding site in the ternary complex involving EF-2 treated with MalNEt (table 3). In contrast, TNBS did not greatly affect the formation of the ternary complex involving GuoPP(CH$_2$)P (K_d = 0.17 μM, n = 0.66).

Table 2

Effect of preincubation with MalNEt on the quantitative binding of ADPR-EF-2 to ribosomes

Additions			
1. Incubation	2. Incubation	K_d (μM)	Binding sites/ribosome (n)
ADPR-EF-2	ribosome	0.25	1.20
ADPR-EF-2	ribosome, GuoPP(CH$_2$)P	0.08	1.20
ADPR-EF-2, MalNEt	ribosome,	0.99	0.82
ADPR-EF-2, MalNEt	ribosome, GuoPP(CH$_2$)P	1.01	1.0

The experimental procedure was as indicated in section 2. The ADPR-EF-2 concentration was varied between 16.5 and 132 nM. The ribosome concentration was 0.3 μM. The concentration of GuoPP(CH$_2$)P, whenever added, was 20 μM. The ribosomal complexes were isolated by centrifugation and the data evaluated as in [8]. The values are means of 4 separate determinations evaluated by the least squares method. The previous K_d-values [8] found for the binding of ADPR-EF-2 to ribosomes by itself and that in the presence of GuoPP(CH$_2$)P were 0.18 μM and 0.05 μM, respectively

4. DISCUSSION

The different sensitivities of EF-2 to MalNEt *vs.* TNBS thus implicate that the binding sites for guanine nucleotides involved in binary and ternary complexes correspond either to different conformational states of the same and single site or are distinct physical entities. The conformation (or site) involved in binary complex formation seems to be selective for GDP [8,14,15] and that involved in the ternary complex for GTP (and analogues) [8]. Qualitative differences between behaviors of GDP and of GTP (analogues) in interaction with EF-2 (and ribosomes) have been previously observed [14,16–18]. Based on equilibrium binding data EF-2–GTP does not appear to be an intermediate in the formation of the ribosomal ternary complex [8]. Our unpublished observations indicate, moreover, that linkage of oxidized guanine nucleotides to EF-2 does not abolish its activity in protein synthesis. These findings together suggest that the guanine nucleotide binding site involved in the binary complex is distinct from that involved in the ternary complex. However, the biological significance of the direct interaction of guanine nucleotides with EF-2 remains obscure. A possible relationship between these interactions and a modulation of EF-2 activity during chain elongation is currently under investigation.

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