Nucleotide binding to elongation factor 2 inactivated by diphtheria toxin

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Binding of guanosine nucleotides to purified native and ADP-ribosylated wheat germ EF-2 was measured. Both forms of EF-2 bound [^{3}H]GDP to the same extent. [^{3}H]GDP binding to native but not to ADP-ribosylated EF-2 was reduced in the presence of GTP and ribosomes. Binding of [γ-^{32}P]GTP to EF-2 was significantly reduced upon ADP-ribosylation. ADP-ribosylation almost abolished both the stimulatory effect of ribosomes on GTP binding to EF-2 and the ability of EF-2 to form a high-affinity complex with GuoPP(CH_2)P and ribosomes. Low-affinity complex formation between EF-2 · GDP and ribosomes was not influenced by ADP-ribosylation. The results indicate that the inhibition of the elongation process caused by the toxin is probably due to the inability of modified EF-2 to exchange GDP with GTP.

Elongation factor 2 *ADP-ribosylation* *Nucleotide binding* *Diphtheria toxin*

1. INTRODUCTION

Eukaryotic elongation factor 2 (EF-2) from a wide variety of sources such as mammals [1], plants [2] and yeast [3] can be ADP-ribosylated by diphtheria toxin in the presence of NAD^+. This ADP-ribosylation reaction leads to an almost complete inhibition of cytosolic protein synthesis [4]. However, it is not clear why ADP-ribosylated EF-2 is inactive in protein synthesis. Few differences were observed between native and toxin-inactivated EF-2 when the various steps in the EF-2 catalyzed reactions were studied [5]. For example, it has been claimed that native and ADP-ribosylated EF-2 bind GTP with the same affinity [5], and interact with the ribosome competing for the same binding site [6]. It was also reported that at non-limiting concentrations, toxin-inactivated EF-2 catalyzed the shift of peptidyl-tRNA from the A site to the P site on the ribosome [7].

Recently, it was shown [8] that EF-2 interacts with the ribosome in two binding states, namely a high-affinity pre-translocation state specific for EF-2 · GTP and a low-affinity post-translocation state in which EF-2 · GDP is bound in a less stable form. In the light of this finding and the uncertainty of the GTP-binding data of EF-2 [9], we have reinvestigated some of the properties of ADP-ribosylated EF-2. The results indicate a clear difference in the nucleotide- and ribosome-binding capability of toxin-inactivated EF-2.

2. MATERIALS AND METHODS

2.1. *Materials*

Raw wheat germ was obtained from Niblack (Rochester, U.S.A.) and diphtheria toxin from Connaught Laboratories (Ontario, Canada). NAD^+, GDP, GTP and GuoPP(CH_2)P were from Sigma (St. Louis, MO). Radioactive nucleotides were purchased from The Radiochemical Center (Amersham).

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2.2. *Purification of EF-2*

EF-2 was purified from wheat germ extracts as described [10]. The purity of the preparation, determined from the extent of ADP-ribosylation, was 98%.

2.3. *ADP-ribosylation*

50–100 pmol EF-2 containing 50 mM 2-mercaptoethanol, 20 mM Tris-HCl (pH.8), 200 μM NAD^+ and 5 μg diphtheria toxin in a volume of 200 μl was incubated at 37°C for 10 min. In experiments with radioactive NAD^+ the radioactivity incorporated was measured in a liquid scintillation spectrometer after trichloroacetic acid precipitation and washing of the proteins as in [11].

2.4. *Binding of guanosine nucleotides to EF-2*

The nucleotide-binding experiments were carried out using presoaked nitrocellulose membrane filters and radioactive nucleotide as described in [12].

2.5. *Formation of ribosome·EF-2 complex*

The formation of stable ribosome·EF-2 complex was measured essentially as described by Nygard and Nilson [8], using the non-hydrolysable GTP analogue guanosine 5′-(β-γ-methylene)triphosphate {GuoPP(CH_2)P}. Low-affinity complex formation was measured after glutaraldehyde fixation [8].

2.6. *Preparation of salt-washed ribosomes*

Ribosomes were prepared from wheat germ extract containing 0.5 M KCl buffer [13]. The ribosomal pellet was suspended in the same buffer containing 0.6 M KCl and collected by centrifugation through 1 M sucrose [13].

2.7. *Radioactive labeling of EF-2*

Native and ADP-ribosylated EF-2 was dialysed against a buffer containing 100 mM KCl; 20 mM triethanolamine-HCl (pH 7.6), 2 mM DTT, 0.1 mM EDTA and 5% (v/v) glycerol and labelled with ^{125}I according to Bolton and Hunter [14]. The iodination reaction was stopped by adding 1 M Tris-HCl (pH 7.6) to a final concentration of 50 mM. Iodine not covalently bound to EF-2 was removed by two gel filtration steps. The specific activity of the preparations was in the range 6000–7000 cpm/pmol EF-2.

3. RESULTS

3.1. *Guanosine nucleotide binding to native and ADP-ribosylated EF-2*

The binding experiments carried out by the nitrocellulose filter assay [12] at 2 mM Mg^{2+} showed no difference in the GDP-binding ability of native and ADP-ribosylated EF-2 (table 1). A slight but consistent inhibition of GDP-binding by native EF-2 was observed in the presence of ribosomes. A similar effect of ribosomes on GDP-binding by rat liver EF-2 has been reported [12]. Addition of ribosomes did not influence the GDP-binding capacity of ADP-ribosylated EF-2. [^{3}H]GDP binding to native EF-2 was 30% lower when measured in the presence of a 10-fold excess of non-radioactive GTP. Addition of ribosomes lowered this value further (table 1). ADP-ribosylated EF-2 did not show this competitive effect of GTP. EF-2 has been shown to have a common binding site for GDP and GTP and to bind

Table 1

Binding of GDP to native and ADP-ribosylated EF-2

EF-2 state	Additions	Total [^{3}H]GDP bound (pmol)
Native	none	23.3 ± 1.0
	ribosome	19.7 ± 1.8
	GTP (50 μM)	16.1 ± 1.1
	ribosome + GTP (50 μM)	10.3 ± 1.5
ADP-ribosylated	none	24.2 ± 2.0
	ribosome	24.5 ± 2.2
	GTP (50 μM)	23.7 ± 1.7
	ribosome + GTP (50 μM)	22.4 ± 2.3

50 pmol EF-2 was incubated at 25°C with 5 μM [^{3}H]GDP (2×10^5 dpm) in a total volume of 200 μl buffer (50 mM Tris-HCl, pH 7.4; 50 mM KCl; 2 mM Mg^{2+}; 1 mM DTT, 0.1 mM EDTA for 10 min. 180 μl of the incubation mixture was filtered under mild suction through a nitrocellulose filter pre-soaked in the above buffer. The filter was washed 3 times with 200 μl of cold buffer and the bound radioactivity was measured in a liquid scintillation counter. Filter-bound radioactivity in the absence of EF-2 ($\leq$ 1 pmol) was subtracted from all values. The concentration of ribosomes when present was 25 pmol (1.3 A_{260} units)

GDP more strongly than GTP in the absence of ribosomes [9]. The data presented above are in agreement with this finding and indicate that ADP-ribosylation does not affect the GDP-binding conformation of this protein.

Accurate measurement of GTP binding to EF-2 is difficult due to the low affinity of EF-2 for GTP compared to GDP, on the GTPase activity present even in highly purified EF-2 preparations [9]. This problem was partly overcome in the present study by using [γ-^{32}P]GTP. At a concentration of 5 μM GTP, 50 pmol EF-2 bound 3 pmol GTP in the absence of ribosomes (table 2). GTP binding to ADP-ribosylated EF-2 was less than 1 pmol, under similar conditions.

3.2. *Formation of ribosome·EF-2 complex*

As pointed out earlier stable complex formation between EF-2, GTP and the ribosome is difficult to demonstrate due to GTP hydrolysis [8]. Complex formation can, however, be demonstrated with the non-hydrolysable GTP analogue GuoPP(CH$_2$)P. Native EF-2 formed such a stable complex with high efficiency (table 3). More than 75% of the EF-2 present in the assay was bound to the ribosomes. With ADP-ribosylated EF-2 less than 6% of EF-2 radioactivity was associated with the ribosomes. Low-affinity complex formation between EF-2, GDP and ribosomes, detected only after glutaraldehyde fixation [8], revealed no significant difference between native and toxin-inactivated EF-2 (table 3).

Table 2

Binding of GTP to native and ADP-ribosylated EF-2

EF-2 state	Additions	Total [^{32}P]GTP bound (pmol)
Native	none	3.6 ± 0.4
	ribosome	17.8 ± 1.6
ADP-ribosylated	none	0.8 ± 0.2
	ribosome	1.3 ± 0.5

50 pmol EF-2 was incubated at 25°C with 5 μM [γ-^{32}P]GTP (8.8×10^5 dpm) for 3 min. The conditions were as described in table 1 except for the buffer which contained 0.5 mM KH_2PO_4 in addition. The concentration of ribosome when present was 25 pmol (1.3 A_{260} units). Filter-bound radioactivity in the absence of EF-2 ($\leqslant$ 1 pmol) was subtracted from all values

Table 3

Formation of complex with ribosome by native and ADP-ribosylated EF-2

EF-2 state	Nucleotide	EF-2 bound to ribosome (cpm × 10^{-3})	(mol/mol)
Native	GuoPP(CH$_2$)P	31.1 ± 2.8	0.76
	GDP	18.2 ± 2.5	0.45
ADP-ribosylated	GuoPP(CH$_2$)P	2.4 ± 0.5	0.06
	GDP	19.3 ± 2.3	0.48

The incubation mixture contained, in a final volume of 100 μl: 100 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 7.6), 1 mM DDT, 5% (v/v) glycerol, 60 pmol ribosome, 60 pmol ^{125}I-labelled EF-2 (4×10^4 cpm) and 10 nmol GDP or GuoPP(CH$_2$)P. After 5 min incubation at 25°C, the samples were applied to 12 ml of a 10–30% (w/v) sucrose gradient in a buffer containing 100 mM KCl, 3 mM $MgCl_2$, 1 mM DTT and 20 mM Tris-HCl (pH 7.6). The gradient was centrifuged at 200 000 × g_{av} for 2.5 h and harvested after monitoring the absorbance at 260 nm using a flow cuvette. Fractions (0.5 ml) were collected and those corresponding to the ribosome peak were pooled and precipitated with 10% trichloroacetic acid (w/v) in the presence of 100 μg wheat germ extract. The precipitate was collected on glass-fibre filters and counted in a gamma counter. In experiments using GDP, the incubation mixture was fixed with 5% glutaraldehyde before centrifugation [8]

Taken together, these results clearly demonstrate that the toxin-inactivated EF-2 is unable to form a high-affinity pre-translocation complex with ribosome and GTP. The reported GTP binding to ADP-ribosylated EF-2 [5] could be an artifact caused by GDP binding since these authors did not perform the GTP-binding experiments with [γ-^{32}P]GTP. Since ADP-ribosylation did not abolish the GDP-binding ability of EF-2 (table 1), the inhibition of the elongation process caused by the toxin is most probably due to the inability of EF-2 to exchange GDP with GTP, either directly or through GTP-GDP transphosphorylase [8,9].

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