Volume 261, number 2, 350–352 FEBS 08169 February 1990

ADP-ribosylated elongation factor 2 (ADP-ribosyl-EF-2) is unable to promote translocation within the ribosome

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Received 28 December 1989

Elongation factor 2 (EF-2), ADP-ribosylated in vitro by the A-fragment of diphtheria toxin, can (in the presence of GMPPCP) form stable complexes with ribosomes regardless of whether the ribosomes are empty or carrying poly(U) and Phe-tRNA in the A-site. Despite its efficient binding to ribosomes, ADP-ribosyl-EF-2, in contrast to the non-modified EF-2 is unable to promote the shift of Phe-tRNA from the A-site to the P-site of the ribosome as determined by the puromycin reaction, i.e. it is incapable of promoting the translocation reaction within the ribosome.

Elongation factor 2; ADP-ribosylation; Diphtheria toxin; Elongation factor 2-promoted translocation

1. INTRODUCTION

Protein biosynthesis at the elongation stage in eukaryotes is mediated by two elongation factors, EF-1 and EF-2. EF-1 is responsible for codon-dependent binding of aminoacyl-tRNA to the A-site of the ribosome. EF-2 promotes the translocation of peptidyl-tRNA from the A-site to the P-site, coupled with the displacement of deacylated tRNA and the mRNA shift by one codon (reviewed in [1]).

EF-2 can be ADP-ribosylated in the reaction with NAD^+, catalyzed by the A-fragment of diphtheria toxin [2,3]. ADP-ribosylation occurs at a unique aminoacyl residue – diphthamide [4] – and results in inactivation of the factor and complete inhibition of protein synthesis [2,3,5].

Promotion of translocation includes several EF-2 partial reactions: pretranslocation binding of the EF-2/GTP complex to a ribosome, translocation reaction itself, ribosome- and EF-2-dependent hydrolysis of GTP to GDP and inorganic phosphate, dissociation of the EF-2/GDP complex and the exchange of GDP bound to EF-2 to GTP (see review [1]). It is of interest to know which of the enumerated partial reactions of EF-2 is actually blocked by ADP-ribosylation. Previous reports in the literature did not clarify this problem, only negative or contradictory results were obtained [6–12]. In one of the first reviews [6] devoted to this problem it was summarized that all the partial functions of EF-2 studied do not seem to be affected by ADP-ribosylation. It was even reported that ADP-ribosyl-EF-2 is able to promote a single translocation act [7]. The mechanism by which ADP-ribosylation inhibits EF-2 functioning was called 'a mystery' [6]. Later it was reported that ADP-ribosylation partially [11], or even completely [12], inhibits pretranslocation binding of EF-2 to ribosomes. However, these results contradict a number of earlier studies [8–10].

In this paper we present a study on the effect of ADP-ribosylation at two consecutive steps of EF-2 functioning: the binding of the factor to ribosomes (empty or carrying poly(U) and Phe-tRNA) and the shift of Phe-tRNA from the A-site to the P-site of the ribosome as tested by puromycin reactivity. From the data obtained we conclude that ADP-ribosylation makes EF-2 inactive to promote translocation without affecting its ability to bind to ribosomes.

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Abbreviations: EF, elongation factor; ADP-ribosyl-EF-2, ADP-ribosylated EF-2; GMPPCP, guanosine 5′-(β,γ-methylene)triphosphate; NAD, nicotinamide adenine dinucleotide; poly(U), polyuridylic acid

2. MATERIALS AND METHODS

EF-1 and EF-2 were obtained from a rabbit reticulocyte ribosome-free extract as reported previously [13].

ADP-ribosylation of EF-2 was done as in [14], with minor modifications. The EF-2 preparation (260 μg, 200 μl) was incubated for 10 min at 37°C with 10 nmol of non-radioactive NAD^+ (Boehringer) or nicotinamide-[U-^{14}C]adenine dinucleotide ([^{14}C]NAD^+, 260 mCi/mmol, Amersham) and 10 μg of diphtheria toxin (Calbiochem).

40 S and 60 S ribosomal subunits were isolated from rabbit reticulocytes as in [15].

Ribosomes carrying poly(U) and [^{3}H]Phe-tRNA in the A-site (A-ribosome) were prepared in the presence of EF-1 and GTP as in [13]. The translocation reaction was performed as follows. The incubation mixture (50 μl) contained 3.5 pmol of A-ribosome, 0.2 mM of GTP or GMPPCP and varying amounts of EF-2 or ADP-ribosyl-EF-2 in

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40 mM Tris-HCl, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$ and 2 mM dithiothreitol. After 15 min of incubation at 37°C puromycin was added and the amount of [^{3}H]Phe-puromycin formed was determined [13].

3. RESULTS AND DISCUSSION

3.1. *ADP-ribosylation does not affect pretranslocation binding of EF-2 to ribosomes*

As mentioned above, the data concerning the effect of ADP-ribosylation on pretranslocation binding of EF-2 to ribosomes are contradictory [8–12]. Here we reinvestigated this effect.

Pretranslocation binding of EF-2 to ribosomes can be studied in a model system with empty ribosomes (reassociated from ribosomal subunits) in the presence of GMPPCP, a non-hydrolyzable analog of GTP. Ribosomal subunits were mixed with ADP-ribosylated or native EF-2 and various guanyl nucleotides under conditions of reassociation of ribosomes and specific complex formation with EF-2. Complexes of EF-2 with ribosomes were detected by sucrose gradient centrifugation.

As shown in fig.1C, ADP-ribosyl-EF-2 as well as the native factor (fig.1A) formed stable complexes with empty ribosomes in the presence of GMPPCP. According to our calculations in both cases these complexes are rather stoichiometric. No complexes were detected when there were no nucleotides (fig.1, panel B and D) or with GTP or GDP in the reaction mixtures (data not shown). These data are in good agreement with the results in [8,10] but contradict the data in [11,12].

An experiment on the competition of ADP-ribosyl-EF-2 and native EF-2 for binding to a limited amount of ribosomes in the presence of GMPPCP showed that the share of ADP-ribosyl-EF-2 in the fraction of the ribosome-bound factor is the same as that in the initial mixture (table 1). Thus, even under conditions of competition no difference in the affinity of ADP-ribosyl-EF-2 or EF-2 for empty ribosomes is observed.

In the process of peptide elongation EF-2 does not bind to empty, but to translating ribosomes with mRNA, peptidyl-tRNA in the A-site and deacylated tRNA in the P-site. To simulate this situation we prepared ribosomes, not less than 80% of which carried poly(U) and [^{3}H]Phe-tRNA in the A-site (A-ribosomes). A comparison of binding of ADP-ribosyl-EF-2 and native EF-2 to A-ribosomes in the presence of GMPPCP or GDP did not reveal any dissimilarity of the two forms of the factor (fig.2). Both forms of EF-2 bind to A-ribosomes in the presence of GMPPCP and practically no complexes are detected when GDP is added to the reaction mixture (fig.2).

3.2. *Effect of EF-2 ADP-ribosylation on Phe-tRNA translocation from the A-site to the P-site of the ribosome*

As early as 1976 it was shown that ADP-ribosyl-EF-2

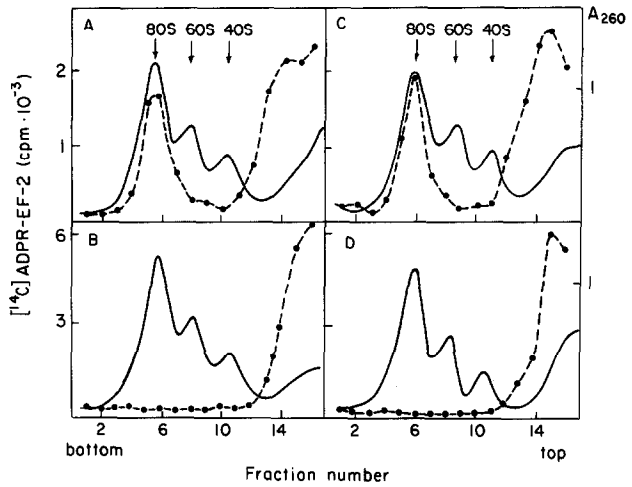

Fig.1. Effect of EF-2 ADP-ribosylation on its binding to empty reconstituted ribosomes. 20 pmol of ribosomes (equimolar mixture of 40 S and 60 S ribosomal subunits) were mixed with 50 pmol of EF-2 (A,B) or 50 pmol of [^{14}C]ADP-ribosyl-EF-2 (C,D) in 200 μl of 30 mM Tris-HCl, pH 7.6, 80 mM KCl, 1 mM $MgCl_2$, 2 mM dithiothreitol and 0.2 mM GMPPCP (A,C) or without GMPPCP (B,D). After incubation for 5 min at 37°C the mixture was applied to a 15–33.5% exponential sucrose gradient prepared in the same solution (without GMPPCP) and centrifuged for 3 h in a SW-41 rotor at 40000 rpm at 4°C. After centrifugation, the gradients were fractionated. EF-2 was detected by TCA-precipitated radioactive material either directly (C,D) or after incubation with diphtheria toxin and [^{14}C]NAD^+ in the presence of 1 mM EDTA (A,B). (——) Absorbance at 260 nm; (●---●) radioactivity.

at a high concentration in the presence of GTP is able to promote at least a single translocation act [7]. However, the authors [7] failed to obtain this effect when GTP in the reaction mixture was substituted by GMPPCP. It is well-known now that EF-2, taken in substrate amounts, can promote the translocation act also in the presence of non-hydrolyzable analogs of

Table 1

Competition of ADP-ribosyl-EF-2 and EF-2 for binding to empty ribosomes in the presence of GMPPCP

Factor	Conc. (pmol)	Additions	[^{14}C]ADP-ribosyl-EF-2 bound (pmol)
[^{14}C]ADP-ribosyl-EF-2	50	0.2 mM GMPPCP	15.3
		none	0.8
EF-2	25	0.2 mM GMPPCP	7.8
[^{14}C]ADP-ribosyl-EF-2	25	none	1.1

200 μl of the reaction mixture contained 16 pmol of ribosomes (equimolar mixture of 40 S and 60 S ribosomal subunits) in 30 mM Tris-HCl buffer, pH 7.6, 80 mM KCl, 6 mM $MgCl_2$, 2 mM dithiothreitol and other additions, as shown in the table. After 5 min incubation at 37°C the mixture was centrifuged through 3 ml of 10% sucrose, prepared in the same buffer for 2 h in a Type 75 rotor at 45000 rpm at 4°C. Radioactivity of [^{14}C]ADP-ribosyl-EF-2 was measured in ribosomal pellets. An equimolar mixture of EF-2 and [^{14}C]ADP-ribosyl-EF-2 was prepared by incubation of EF-2 with diphtheria toxin in a molar ratio of EF-2 to [^{14}C]NAD^+ 1:0.6

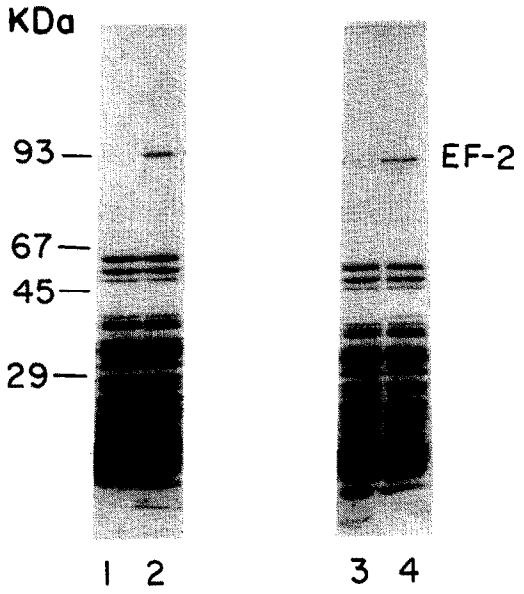

Fig.2. Effect of EF-2 ADP-ribosylation on its binding to A-ribosomes. 7 pmol of A-ribosomes were mixed with 16 pmol of EF-2 (1 and 2) or 16 pmol of ADP-ribosyl-EF-2 (3 and 4) in 100 μl 20 mM Tris-HCl buffer, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol and 0.2 mM GDP (1,3) or 0.2 mM GMPPCP (2,4). After 5 min incubation at 37°C the reaction mixtures were centrifuged for 2 h in Type 75 Beckman rotor at 45000 rpm at 4°C through 3 ml of 10% sucrose, prepared in the same buffer, but without nucleotides. The results of SDS-electrophoresis of pellets are presented.

GTP [16,17]. In the light of these data, translocation at high concentration of ADP-ribosyl-EF-2 in the presence of GTP, but not GMPPCP [7], can be explained by an admixture of the non-modified factor, working catalytically with GTP.

Here we reinvestigated this problem and studied the ability of ADP-ribosyl-EF-2 to promote translocation of Phe-tRNA from the A-site (puromycin non-reactive) to the P-site (puromycin reactive) of the ribosome in the presence of GTP or GMPPCP and tested it by the puromycin reaction. In our experiments ADP-ribosyl-EF-2 in contrast to the native factor was unable to promote transition of [^{14}C]Phe-tRNA to the puromycin reactive state in the presence of GTP or GMPPCP even when ADP-ribosyl-EF-2 was added in a large excess (table 2) or when the time of translocation reaction was prolonged from 15 min up to 2 h (data not shown). Neither diphtheria toxin nor NAD^+ when added to the reaction mixture separately affect the activity of EF-2 (data not shown).

In this study we have demonstrated that ADP-ribosyl-EF-2 can bind to pretranslocated ribosomes and that such a binding does not result in the translocation reaction. One of the explanations of this fact is damage

Table 2

Effect of EF-2 ADP-ribosylation on translocation of [^{3}H]Phe-tRNA

Factor	Conc. (pmol)	Nucleotide	Yield of [^{3}H]Phe puromycin (pmol)
None		GMPPCP	0.10
None		GTP	0.14
EF-2	8	GMPPCP	2.34
	80	GMPPCP	2.66
	1	GTP	3.02
	8	GTP	3.10
ADP-ribosyl-EF-2	8	GMPPCP	0.23
	80	GMPPCP	0.31
	1	GTP	0.20
	8	GTP	0.32

of the EF-2 active center which catalyzes translocation within the ribosome. Another possibility can be that pre-translocation binding of ADP-ribosyl-EF-2 to the ribosome proceeds in an incorrect manner.

Acknowledgements: We wish to thank Dr A.G. Ryazanov for help and fruitful discussions and Professor Boyd Hardesty and Dr Gisela Kramer for valuable advice.

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