FORMATION OF A COMPLEX INVOLVING ADP-RIBOSYLATED HUMAN TRANSLOCATION FACTOR, GUANOSINE NUCLEOTIDE AND RIBOSOMES

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1. Introduction

The mammalian translocation factor is specifically inactivated by diphtheria toxin in the presence of NAD^+ [1]. This inactivation has been shown to be due to the transfer of the ADP-ribose* moiety of NAD^+ by diptheria toxin into a covalent linkage with the translocation factor [2]. The reaction is prevented or reversed by nicotinamide [3]. Using NAD^+, radioactively labelled in its adenosine or phosphate moiety, it has been possible to label the translocation factor radioactively [2, 4] and to study different aspects of its mode of action [4–10]. Approximately 1 mole of ADP-ribose group is covalently bound to 1 mole of the factor [11].

The exact mode of inhibition of mammalian polypeptide-chain elongation, due to ADP-ribosylation of the translocation factor remains unknown. The formation of a translocation factor–GTP complex does not appear to be affected by the ADP-ribosylation of the factor [7, 8]. On the other hand, ADP-ribosylation of rat liver aminoacyltransferase II has been shown to result in an inhibition of translocation of peptidyl-tRNA into a puromycin reactive ribosomal site [12] as well as in the inhibition of ribosome- and translocation factor-dependent GTP hydrolysis [4, 13]. Several findings have implied that inhibition of polypeptide-chain elongation by diptheria toxin and NAD^+ might be due to inhibition of the formation of a GTP-dependent intermediate complex between the translocation factor and ribosomes [8, 13, 14]. In order to clarify this question, work was initiated to study the effect that ADP-ribosylation of the human translocation factor TF II had upon the formation of the complex involving TF II, GTP and ribosomes [15]. Results reported below suggest that the ADP-ribose derivative of TF II is capable of forming a complex with ribosomes in the presence of GTP.

2. Materials and methods

Elongation factors and ribosomes were prepared from human tonsils as previously described [16, 17]. TF I used corresponded to fraction IV [16, 17]. TF II used was purified from fraction V [17] by phosphocellulose chromatography [15]. This fraction after the phosphocellulose chromatography step will be designated as fraction VI. Ribosomes were purified in addition to the normal purification procedure [16] by including a 1 M KCl washing step. NAD^+, labelled with ^3H in the adenosine moiety, ^3H-NAD^+, specific activity 591 Ci/mole, was purchased from New England Nuclear Chemicals. ^{14}C-GTP, specific activity 36 Ci/mole, and ^3H-Phe, specific activity 1000 Ci/mole and ^{14}C-Phe, specific activity 513 Ci/mole, were products of the Radiochemical Centre, Amersham. Unlabelled NAD^+ and nicotinamide were obtained from Merck, poly U, GTP, and GDP from Boehringer, Mannheim. Highly purified diptheria toxin was a gift from Behring Werke, Marburg.

Binding of ^{14}C-GTP to ribosomes was performed as described [15]. Amounts of TF II, ribosomes, and, if present, of diphtheria toxin and NAD^+ were as indicated in the legends. The assays for ^3H-ADP-ribosylation of TF II, contained in 100 µl 8 mM $MgCl_2$, 60 mM KCl, 50 mM Tris-HCl pH 7.4, 10 mM 2-mercapto-

* Abbreviations used are: ADP-ribose, adenosine diphosphoribose; poly Phe, polyphenylalanine; TF I, mammalian aminoacyl-tRNA binding factor; TF II, mammalian translocation factor.

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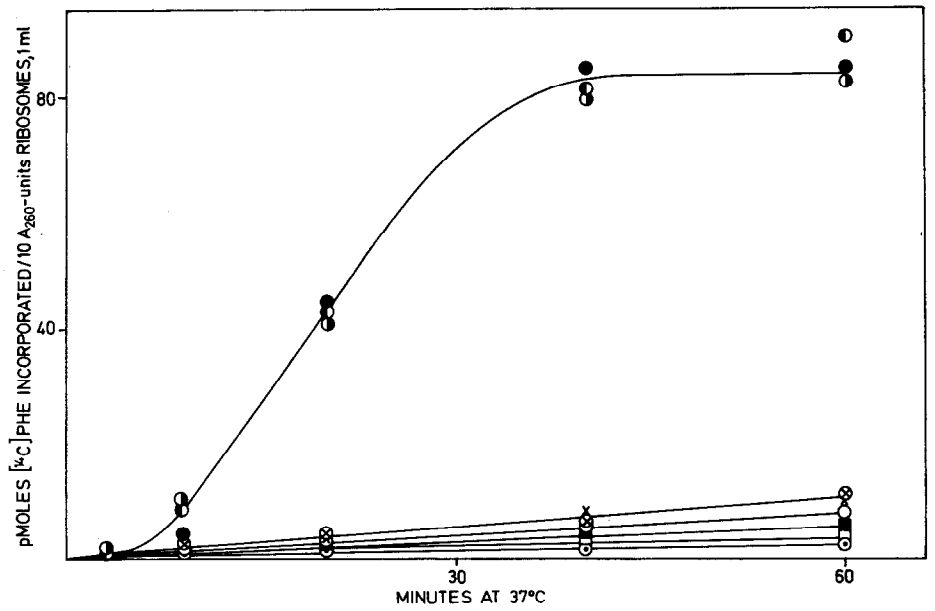

Fig. 1. Reactivation of ribosome-bound ADP-ribosyl-TF II in the presence of diphtheria toxin and nicotinamide, assayed in poly Phe synthesis. First incubation (200 μl, 15 min, 20°): 42 μg TF II fraction VI proteins were incubated in presence or absence of 17 μg diphtheria toxin and 10 μM NAD^+. Conditions otherwise as described under Methods. Second incubation (250 μl, 5 min, 37°): 8 A_{260} units ribosomes and 1.0 mM GTP were added; ionic conditions of the first incubation were maintained. Thereafter, 240 μl were layered on 1.8 ml homogenization buffer and ribosomes were centrifuged and resuspended as described under Methods. Third incubation (70 μl, 15 min, 20°): 3 A_{260} units ribosomes were incubated in presence of 17 μg diphtheria toxin with or without 20 mM nicotinamide. Ionic conditions as above. Fourth incubation (300 μl, 60 min, 37°): 150 μg TF I fraction IV proteins, 60 μg poly U, 180 μg tRNA (*E. coli*), 1.5% charged with ^{14}C-Phe, and 2 mM GTP were added and ionic conditions adjusted to those for poly Phe synthesis [16]. At indicated times incorporated ^{14}C-Phe was determined as described [16]. Counting efficiency for ^{14}C was 67%. (●–●–●): toxin and NAD^+ absent in first incubation; no nicotinamide in third incubation. (◐–◐–◐): toxin and NAD^+ absent in first incubation; nicotinamide present in third incubation. (○–○–○): toxin and NAD^+ present in first incubation; no nicotinamide in third incubation. (●–●–●): toxin and NAD^+ present in first incubation; nicotinamide present in third incubation. (⊗–⊗–⊗): toxin and NAD^+ absent in first incubation; GTP omitted in second incubation; nicotinamide absent in third incubation. (x–x–x): toxin and NAD^+ absent in first incubation; GTP omitted in second incubation; nicotinamide present in third incubation. (■–■–■): toxin and NAD^+ present in first incubation; GTP omitted in second incubation; nicotinamide present in third incubation. (□–□–□): toxin and NAD^+ present in first incubation; GTP omitted in second incubation; nicotinamide absent in third incubation. (⊙–⊙–⊙): no TF II run in this assay.

Table 1
Effect of diphtheria toxin and NAD^+ upon human poly Phe synthesis.

System	pmoles ^{3}H-Phe incorporated per 1 ml, 30 min, 37°
Control	166
–TF II	3
+Diphtheria toxin	167
+NAD^+	174
+Diphtheria toxin, + NAD^+	11

Control system contained all the components of the standard system of human poly Phe synthesis [16]: 10 A_{260} units/ml of ribosomes, 215 μg/ml TF I fraction IV proteins, 26 μg/ml TF II fraction VI proteins, 600 μg/ml tRNA (*E. coli*) 0.9% charged with ^{3}H-Phe, and 2 mM GTP were used. If present, 170 μg/ml diphtheria toxin and 5 μM NAD^+ were used. Counting efficiency for ^{3}H was 20%..

ethanol, 5 μM ^{3}H-NAD$^+$, 17 μg diphtheria toxin and TF II fraction VI proteins as indicated in the legends. The assays were incubated for 15 min at 20° (the ADP-ribosylation reaction reaches a plateau under these conditions after ca. 10 min). After the incubation 75 μl aliquots were plated on GF/A filters (Whatman) and radioactivity precipitable in cold CCl_3COOH was counted in 2 ml of toluene containing 0.4% 2,5-diphenyloxazole in a Packard (Tricarb) liquid scintillation spectrometer. Alternatively, ^{3}H-ADP-ribosylated TF II was bound to ribosomes in a second incubation (5 min, 37°) after ADP-ribosylation assay. In this case, the assays were first chilled on ice and the assay volumes were, thereafter, increased to 150 μl by the addition of ribosomes (4–6 A_{260} units) and 1.0 mM GTP or 1.0 mM GDP. The ionic milieu was kept constant. After the second incubation, the assays were chilled again and 140 μl were carefully layered on 1.8 ml homogenization buffer [16] and centrifuged in an MSE Type 10 × 10 rotor for 2.5 hr at 150,000 *g*. Ribosomal pellets were resuspended in 70 μl homogenization buffer and from each sample 2.5–3.5 A_{260} units of ribosomes were plated on GF/A filters and the ribosome bound radioactivity was counted in a Packard liquid scintillation spectrometer as above. Poly U-dependent poly Phe synthesis was performed as previously described [16]. Amounts of elongation factors used were as indicated in the legends.

3. Results

In the presence of 5 μM NAD$^+$, diphtheria toxin inhibited human poly Phe synthesis more than 90% (table 1). The same and even 10-fold higher concentrations of diphtheria toxin and NAD$^+$ failed, however, to display a similar inhibitory effect on the TF II-mediated binding of GTP to ribosomes (table 2).

Moreover, ADP-ribosyl-TF II was apparently capable of binding to ribosomes in the presence of GTP or GDP, as demonstrated by the recovery of radioactivity in the pellets of ribosomes, incubated with ^{3}H-ADP-ribosyl-TF II (table 3). GDP appeared to be at least as effective as GTP in the promotion of the binding of ^{3}H-ADP-ribosyl-TF II to ribosomes. Some binding of the radioactivity to ribosomes could be observed also in the absence of a guanosine nucleotide (32% of the value observed in the presence of GTP).

Alternatively, the GTP-promoted binding of ADP-ribosyl-TF II to ribosomes could be demonstrated by incubation of ADP-ribosyl-TF II–ribosome complexes, purified by centrifugation, in the presence of diphtheria toxin and of nicotinamide (fig. 1): Incubation of ADP-ribosyl-TF II–ribosome complexes with diphtheria toxin and 20 mM nicotinamide resulted in a nearly complete reactivation of the modified and ribosome-bound factor, as assayed in poly Phe synthesis. Such ribosomes carrying ADP-ribosyl-TF II displayed, however, almost no TF II activity in poly Phe synthesis, if they were incubated in the absence of nicotinamide. The incubation in the presence of diphtheria toxin and nicotinamide had no effect upon the activity of the unmodified factor, bound to ribosomes in the presence of GTP.

4. Discussion

The results show that TF II-mediated binding of GTP to ribosomes is not significantly affected by diphtheria toxin and NAD$^+$ concentrations otherwise sufficient to inhibit poly Phe synthesis almost completely. The ADP-ribosylation reaction has been previously observed to result in a slight inhibition of the rat liver transferase II (T_2) promoted binding of GTP to ribosomes [4]. ADP-ribose derivative of TF II appears not only to catalyze the binding of GTP to ribosomes, but also to bind itself to ribosomes. The binding of ADP-ribosyl-TF II is stimulated several fold in the presence of GTP or GDP. These observations imply that ADP-ribosyl-TF II is capable of forming a complex together with GTP and ribosomes, just as it has been shown to be the case with the unmodified form of the factor [15]. Different lines of evidence have previously implied that ADP-ribosylated mammalian translocation factor is not capable of interacting with ribosomes [8, 13, 14]. The difference between these previous observations and those reported in this communication might be explained by the milder centrifugation conditions of ribosomal complexes used in our case.

As suggested by previous findings [4, 13], ADP-ribosylation of the factor might directly result in the inactivation of its ribosome-dependent GTPase activity. However, Tiboni and Ciferri have very recently ob-

Table 2
Effect of diphtheria toxin and NAD^+ upon TF II-promoted binding of ^{14}C-GTP to ribosomes.

Additions	pmoles ^{14}C-GTP bound 0.1 ml, 5 min, 37°
17 μg Diphtheria toxin + 5 μM NAD^+	0.1
170 μg Diphtheria toxin + 50 μM NAD^+	0.5
TF II	2.5
TF II + 17 μg diphtheria toxin + 5 μM NAD^+	2.8
TF II + 170 μg diphtheria toxin + 50 μM NAD^+	2.8
Ribosomes	1.2
Ribosomes + 17 μg diphtheria toxin + 5 μM NAD^+	1.3
Ribosomes + 170 μg diphtheria toxin + 50 μM NAD^+	1.3
TF II + ribosomes	14.8
TF II + ribosomes + 17 μg diphtheria toxin + 5 μM NAD^+	13.3
TF II + ribosomes + 170 μg diphtheria toxin + 50 μM NAD^+	14.1

Experimental conditions were described in detail elsewhere [15]. Assays (100 μl) contained 8 mM $MgCl_2$, 60 mM KCl, 50 mM Tris-HCl pH 7.4, 8–10 mM 2-mercaptoethanol, 15 μM ^{14}C-GTP, and the components added as above. If present, 18 μg of TF II fraction VI proteins and 2,5 A_{260} units ribosomes were used. Bound ^{14}C-GTP was determined by passing assays through nitrocellulose filters. Blank (=0.4 pmoles) determined by filtering a reaction mixture without TF II, ribosomes, diphtheria toxin, and NAD^+, were subracted from all the values above. Counting efficiency for ^{14}C was 67%.

Table 3
Binding of ^3H-ADP-ribosyl-TF II to ribosomes, as assayed by the recovery of the bound radioactivity on pelleted ribosomes.

System	Ribosome-bound radioactivity (pmoles/2.7 A_{260} units)
Complete	8.7
–Diphtheria toxin	0.1
–TF II	0.1
–Diphtheria toxin, –TF II	0.1
–GTP	2.8
–GTP, + GDP	9.7

Assay conditions were as described under Methods. Complete system contained diphtheria toxin and 135 μg TF II fraction VI proteins, equivalent to 123 pmoles ^3H-ADP-ribosyl-TF II, in the first incubation plus 4.5 A_{260} units ribosomes and 1.0 mM GTP (or 1.0 mM GDP) in the second incubation. Minus ribosome blank (=0.05 pmoles) was determined by rinsing the bottom of the centrifuge tube with 70 μl of homogenization buffer and counting 50 μl out of it on a GF/A filter in a Tricarb. This blank was subtracted from all values. Counting efficiency for ^3H was 20%.

served in a cell-free system from *P. zopfii* that (highly purified) translocation factor-dependent hydrolysis of GTP on 80 S ribosomes is not inhibited by diphtheria toxin- and NAD^+ -concentrations otherwise almost completely inhibitory in poly Phe synthesis [18]. In agreement with this observation, our preliminary experiments have shown that the product of ADP-ribosyl-TF II-catalyzed binding of GTP to ribosomes is in the form of GDP, as analyzed by chromatography on poly (ethyleneimine)-cellulose thin layers. These latter data suggest that a function of TF II, tightly coupled with the translocation step, is primarily affected by ADP-ribosylation of the factor.

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