CROSS-LINKING OF ADP-RIBOSYLATED HUMAN TRANSLOCATION FACTOR TO RIBOSOMES

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1. Introduction

It has been recently shown that ADP-ribosylated human translocation factor, otherwise inactive in polypeptide synthesis, is still capable of binding to the ribosome [1]. The binding of ADP-ribosyl-TF II* is significantly increased in the presence of GTP [1]. Moreover, the modified factor can promote the binding of GTP to the ribosome [1–2]. Unmodified TF II, but also Phe–tRNA prebound to the ribosome in the presence of poly U, inhibit the binding of ADP-ribosyl-TF II to the ribosome [3, 4]. These findings appear to suggest that ADP-ribosyl-TF II binds to a ribosomal site specific for TF II. The use of radioactively labelled ADP-ribosyl-TF II [5, 6] might, therefore, provide a suitable assay system for studies on the binding of translocation factor to the ribosome. As will be demonstrated in the present report, ADP-ribosyl-TF II can be cross-linked to the ribosome by incubation in the presence of bifunctional reagents, OPDM or FFDNB. Various other bifunctional reagents tested have displayed no significant coupling effect.

2. Materials and methods

Elongation factors, ribosomes and ribosomal subunits were prepared from human tonsils as previously described [1, 7, 8]. Diphtheria toxin was provided by Behringwerke, Marburg. NAD^+, labelled with ^{14}C in the adenosine moiety, specific activity = 167 Ci/mole or 136 Ci/mole, was purchased from the Radiochemical Centre, Amersham. OPDM and PPDM were obtained from Aldrich Chemical Co., FFDNB and F, F-sulfone from Schuchardt. DEM, DMA and DMS were synthesized according to the methods described [9]. OPDM, PPDM, FFDNB and F, F-sulfone were freshly dissolved before use in acetone, the imidates in 50 mM phosphate buffer pH 8.5.

The binding of [^{14}C]ADP-ribosyl-TF II to ribosome was performed as previously described [1]; Mg^{2+} and K^+ were omitted in the ADP-ribosylation of TF II fraction VI proteins. [^{14}C]ADP-ribosyl-TF II–ribosome complexes isolated by centrifugation [1] were resuspended in homogenization buffer [7] containing neither sucrose nor 2-mercaptoethanol. For the coupling reaction, these ribosomal complexes were incubated for 2 hr at 30°C in the presence of OPDM or PPDM in 50 mM Tris–HCl pH 7.4; in case of FFDNB and F, F-sulfone, the incubation was performed for 8 hr at 20°C in 50 mM Tris–HCl pH 8.5. Incubation in the presence of imidates was done for 20 hr at 0°C in 50 mM phosphate buffer pH 8.5. These incubation conditions were chosen according to studies described [10–21]. Throughout the incubation, the salt concentrations of the homogenization buffer were maintained; the reagent concentration was adjusted to 1 mM, if not indicated otherwise. The ribosomes were then collected by centrifugation through homogenization buffer containing 45% sucrose and 0.5 M NH_4Cl. Ribosome-bound radioactivity was determined as described [1].

* *Abbreviations:* OPDM, *N,N'*-(1,2-phenylene) dimaleimide; PPDM, *N,N'*-(1,4-phenylene) dimaleimide; FFDNB, 1,5-difluoro-2,4-dinitrobenzene; F, F-sulfone, 4,4'-difluoro-3,3'-dinitrodiphenylfulfone; DEM, diethyl-malonimidate; DMA, dimethyl-adipimidate; DMS, dimethyl-suberimidate; ADP–ribose, adenosine diphosphoribose; TF II, human translocation factor, corresponds to EF II; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

Table 1
Breakdown of the ADP-ribosyl-TF II–ribosome complex during centrifugation through high concentrations of sucrose and salt.

Concentrations of sucrose and (or) NH_4Cl	pmoles [^{14}C]ADP-ribosyl-TF II/19.3 pmoles (1 A_{260} unit) ribosomes
None (control)	3.07
0.5 M NH_4Cl	0.40
1.75 M sucrose (60%)	0.68
0.5 M NH_4Cl, 1.32 M sucrose (45%)	0.04

5 A_{260} units ADP-ribosyl-TF II–ribosome complexes (= 18.3 pmoles [^{14}C]ADP-ribosyl-TF II) isolated by centrifugation and resuspended in homogenization buffer without sucrose were layered in a volume of 30 µl on 1.8 ml homogenization buffer containing the sucrose and NH_4Cl concentrations indicated. In samples containing no sucrose, 30 µl aliquots were instead mixed with 1.8 ml homogenization buffer with or without 0.5 M NH_4Cl. After centrifugation, radioactivity present in the ribosomal pellets was determined as described [1]. Counting efficiency for ^{14}C was 79%.

For SDS-gel electrophoretic analysis, ribosomal proteins were extracted according to the method of Spitnik-Elson [22]. Electrophoresis was performed in 5% gels for 8 hr at 8 mA/gel; all solutions contained 6 M urea, all other conditions of the method of Weber and Osborn [23] were maintained. β-Galactosidase (Boehringer) and BSA (Merck) were used as reference proteins. After staining with Coomasie blue and destaining, gels were cut into 2 mm slices and incubated with 200 µl 30% H_2O_2 at 50°C overnight. The radioactivity was counted in 4 ml Unisolve solution (Koch–Light Laboratories) in a Packard (Tricarb) liquid scintillation spectrometer.

3. Results

Human translocation factor can be separated from ribosomes by centrifugation through high concentrations of sucrose and NH_4Cl [7]. In order to assay the cross-linking of ADP-ribosyl-TF II to ribosomes in a suitable system, we studied at first the effect of high sucrose and salt concentrations upon ADP-ribosyl-TF II–ribosome complexes. As shown in table 1, centrifugation of the complexes through high concentrations of sucrose and salt resulted in their almost complete (98%) breakdown.

Table 2
Effect of some bifunctional reagents on breakdown of the ADP-ribosyl-TF II–ribosome complex.

Reagent added	pmoles [^{14}C]ADP-ribosyl-TF II/19.3 pmoles (1 A_{260} unit) ribosomes
No reagent (control 1)	5.60
No reagent (control 2)	0.09
DEM	0.26
DMA	0.07
DMS	0.18
FFDNB	6.00
F,F-sulfone	0.71
OPDM	5.85
PPDM	0.35

5 A_{260} units ADP-ribosyl-TF II–ribosome complexes (= 35.6 pmoles [^{14}C]ADP-ribosyl-TF II) were incubated in 30 µl with or without the reagents indicated under the conditions described under Materials and methods. Samples without reagents were incubated for 8 hr at 20°C in homogenization buffer without sucrose and without 2-mercaptoethanol. Control 1 was centrifuged through homogenization buffer containing only 0.25 M sucrose, control 2 like all samples with reagents through homogenization buffer containing 0.5 M NH_4Cl and 1.32 M (45%) sucrose. After centrifugation, radioactivity present in the ribosomal pellets was determined. Counting efficiency for ^{14}C was 79%.

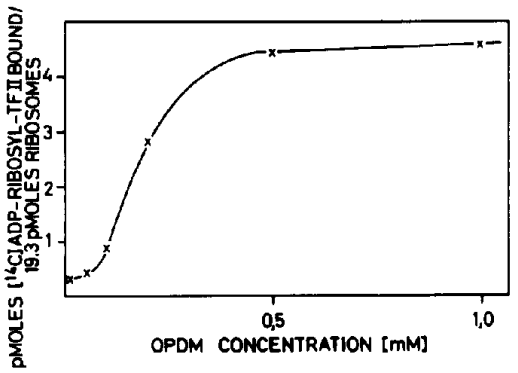

Fig. 1. OPDM concentration-dependence of cross-linking of [^{14}C]ADP-ribosyl-TF II to ribosomes. 10 A_{260} units [^{14}C]ADP-ribosyl-TF II–ribosome complexes (= 82 pmoles [^{14}C]ADP-ribosyl-TF II) isolated by centrifugation were incubated for 2 hr at 30°C at the OPDM concentrations indicated. The reaction was terminated by the addition of 560 mM 2-mercaptoethanol. Cross-linkage was assayed as described under Materials and methods.

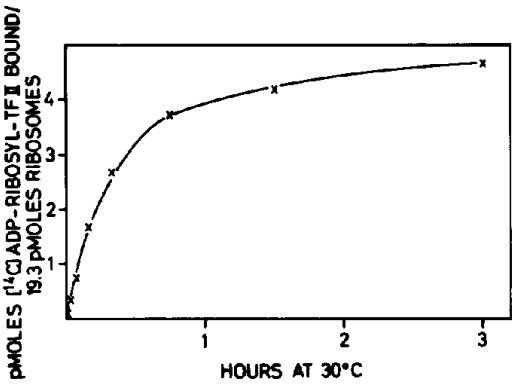

Fig. 2. Kinetics of cross-linking of [^{14}C]ADP-ribosyl-TF II to ribosomes in the presence of OPDM. 100 A_{260} units [^{14}C]ADP-ribosyl-TF II–ribosome complexes (= 562 pmoles [^{14}C]ADP-ribosyl-TF II) isolated by centrifugation were incubated in 500 μl in the presence of 1 mM OPDM at 30°C. At times indicated, 60 μl aliquots were taken out and the unreacted reagent was inactivated by the addition of 560 mM 2-mercaptoethanol. Cross-linkage was assayed as described under Materials and methods.

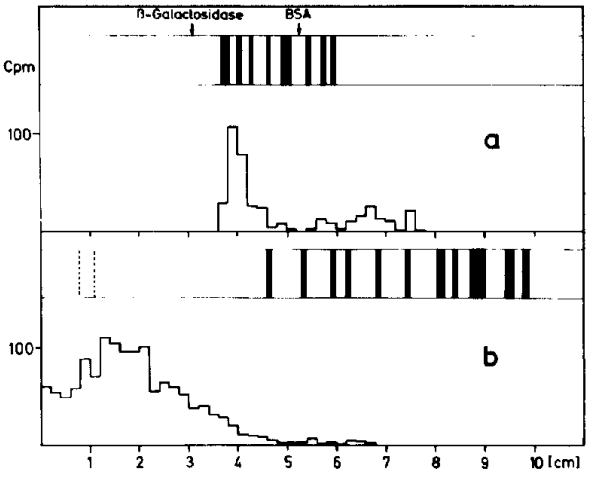

Fig. 3. Analysis of (a) [^{14}C]ADP-ribosyl-TF II-fraction VI proteins and (b) proteins from FFDNB-treated [^{14}C]ADP-ribosyl-TF II–ribosome complexes by electrophoresis in SDS-urea gels.

The effects of various bifunctional reagents on the stability of the complex could be assayed in this system. After incubation with FFDNB or OPDM of [^{14}C]ADP-ribosyl-TF II–ribosome complexes, purified by centrifugation, a quantitative recovery of the complexes was obtained despite the subsequent centrifugation through high sucrose- and salt-concentrations (table 2). Imidates and PPDM under the incubation conditions employed displayed no significant effects on the breakdown of the complex. A slight recovery (13%) of the complex could be observed, however, after incubation in the presence of F,F-sulfone.

As shown in figs. 1 and 2, incubation in the presence of 1 mM OPDM for 2 hr at 30°C appeared to be fully sufficient for a quantitative recovery of the complex after centrifugation through high sucrose- and salt-concentrations. In the presence of 1 mM FFDNB, cross-linkage was apparently complete after 8 hr at 20°C (kinetics not shown).

SDS-gel electrophoretic analysis of proteins extracted from cross-linked ADP-ribosyl-TF II–ribosome complexes implied that modified factor was bound to some ribosomal protein(s). Analysis of ADP-ribosylated-TF II-fraction VI proteins suggested that the factor itself could exist, besides the main peak of 100 000 (± 5000), in at least yet another form of mol. wt 43 000 (± 5000) (fig. 3a). Cross-linking in the presence of FFDNB resulted in a shift of radioactivity into a range of mol. wt over 100 000 (fig. 3b). The radioactivity was heterodisperse with somewhat distinct peaks corresponding to around 240 000, 200 000, 170 000 and 140 000. A similar profile was obtained after treatment in the presence of OPDM. The bind-

Table 3
Binding and covalent-linking of ADP-ribosyl-TF II to the ribosomal subunits.

Ribosomes	pmoles [^{14}C]ADP-ribosyl-TF II/100 pmoles ribosomes		
	bound	cross-linked with OPDM	with FFDNB
40 S	5.8	4.1	5.7
60 S	31.3	27.1	21.1
40 S + 60 S	42.0	37.6	37.1
80 S	37.6	36.4	34.1

The binding and cross-linkin of [^{14}C]ADP-ribosyl-TF II to 6 A_{260} units of 40 S, 15 A_{260} units of 60 S, 6 A_{260} units of 40 S + 14 A_{260} units of 60 S or 20 A_{260} units of 80 S were performed as described under Materials and methods. Counting efficiency for ^{14}C was 79%.

ing of ADP-ribosyl-TF II occurred primarily to the large ribosomal subunit (table 3). Nevertheless, binding to 40 S subunit was also observed. The addition of 40 S to 60 S ribosomes appeared to stimulate the binding of ADP-ribosyl-TF II slightly. In the presence of OPDM or FFDNB, ADP-ribosyl-TF II bound could be cross-linked to the subunits as efficiently as to 80 S ribosomes.

4. Discussion

The results suggest that covalent linkage of ADP-ribosyl-TF II to the ribosome can be obtained by treatment with OPDM or FFDNB. OPDM, a bifunctional sulfhydryl reagent, has been found to cross-link the *E. coli* 30 S ribosomal proteins S18 and S21 [10]. Another phenylene dimaleimide, PPDM, proved to be ineffective in our system. Since the distance between the functional maleimide groups varies from 5.2 Å to 12.0 Å in these two reagents (OPDM and PPDM) [10], it might be expected that a rigid conformation of ADP-ribosyl-TF II–ribosome complex permits a cross-linkage only in the former case. The maximum distance (5–6 Å) between the functional arms in FFDNB [19] suggests that the same groups on TF II and the ribosome might be involved in the cross-linking reactions of both OPDM and FFDNB. It is likely, but not yet proven, that the cross-linkage in the presence of OPDM occurs via sulfhydryl groups of the modified factor and the ribosome. FFDNB, on the other hand, has been shown to react preferentially with amino groups and phenolic groups of tyrosine [17]. Nevertheless, its reactions with sulfhydryl groups have been reported as well [17, 18, 24]. The nature of heterodisperse products observed in SDS-gel electrophoresis is unknown. The possibility exists that the modified factor is coupled to different ribosomal proteins. TF II has been shown to undergo a specific breakdown upon aging at 0–5°C into components approximately 64 000, 42 000 and 37 000 [25]. Breakdown of the factor, before or after cross-linkage to the ribosomal protein(s), into such components might also contribute to heterogenity of products.

The large ribosomal subunit appears to be the primary site of ADP-ribosyl-TF II binding. Previously, unmodified TF II has been observed to interact with 60 S particles alone [26, 27], promoting the hydrolysis of GTP [27]. This activity has been shown to be stimulated by the addition of 40 S subunits [27]. Our data appear to be in agreement with these observations. They suggest, however, that an additional interaction between ADP-ribosyl-TF II and 40 S subunits must also exist.

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