STUDIES ON THE BINDING OF ADP-RIBOSYLATED HUMAN TRANSLOCATION FACTOR TO RIBOSOMES

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Translocation factor TF II is one of the two soluble proteins required for the polypeptide chain elongation on the mammalian ribosome. It is involved in the translocation of peptidyl-tRNA from the so-called acceptor site to the donor site of the ribosome (1). TF II promotes the binding of GTP to the ribosome (2–5); furthermore, it itself binds to the ribosome in the presence of GTP, forming thereby a ternary complex (2–6). GTP bound to the ribosome is cleaved into GDP and Pi, and the energy released is utilized for the translocation step (1).

For sometime, we have been studying the interactions of TF II with the ribosome in a purified cell-free system developed from the human lymphatic tissue. This report will describe a recent approach using a modified and radioactively labelled form of the factor to gain insight into the mechanisms of TF II-ribosome interactions.

TF II represents the unique protein in the mammalian cell, known to be attacked by diphtheria toxin (7, 8). The inactivation of the factor by the toxin results in inhibition of protein synthesis. Diphtheria toxin requires the presence of NAD^+ for its effect (7, 8). The reactions resulting in the inactivation of TF II by diphtheria toxin and NAD^+ can be summarized as follows:
Diphtheria toxin + NAD^+ + TF II $\leftrightharpoons$ (Diphtheria toxin • NAD^+ • TF II) $\leftrightharpoons$ Diphtheria toxin + ADP-ribosyl-TF II + nicotinamide + H^+ (9). ADP-ribosyl-TF II represents the translocation factor carrying one residue of covalently bound adenosine diphosphoribose (10). It is inactive in polypeptide synthesis, but still capable of binding to the ribosome (11). Using NAD^+, radioactively labelled in its adenosine, ribose or phosphate moiety, it is possible to label the factor radioactively (9. 10). This makes direct studies on the binding of the factor to ribosomes possible.

The binding of ADP-ribosyl-TF II to the ribosome is demonstrated in Table 1. As can be seen, the binding of radioactivity to ribosomes was strictly dependent upon the presence of both diphtheria toxin and TF II; it was more than three-fold stimulated by the addition of GTP as well as GDP.

The binding of ADP-ribosyl-TF II seemed to be of specific nature as the unmodified factor competed both in the presence or in the absence of GTP apparently for the same binding site(s) on the ribosome. As can be seen in Table 2, increasing amounts of the unmodified factor inhibited the binding of ADP-ribosyl-TF II to the ribosome. As TF II fraction used in this experiment represented a partially purified protein fraction, protein fractions (12) of different TF II-content were added as source of unmodified factor. The total protein amount added was kept constant throughout the

Table 1: Binding of ^{3}H ADP-ribosyl-TF II to ribosomes, as assayed by the recovery of the bound radioactivity on pelleted ribosomes

System	Ribosome-bound ^{3}H ADP-ribosyl-TF II (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 (11).

Table 2. Effect of the addition of TF II – protein fractions of different purity on the binding of ADP-ribosyl-TF II to the ribosome

Unmodified TF II protein fraction added	pmoles ^{14}C ADP-ribosyl-TF II bound/48.6 pmoles ribosomes		pmoles ^{3}H Phe incorp./ 96 pmoles ribosomes 1 ml, 15 min, 37 °C	
	+ GTP	– GTP	+ GTP	– GTP
None	4.0	2.6	6.0	3.0
117 µg fract. II proteins (= 5.6 pmoles TF II)	4.9	3.2	10.0	2.0
114 µg fract. IV proteins (= 18.2 pmoles TF II)	3.9	2.5	22.0	3.0
114 µg fract. V proteins (= 44.0 pmoles TF II)	2.0	0.9	43,0	6.5
117 µg fract. VI proteins (= 128.0 pmoles TF II)	1.1	0.8	36.0	4.0

74 pmoles ^{14}C ADP-ribosyl-TF II (in 58 µg fraction VI proteins) were incubated in the presence or absence of 1 mM GTP with 3.8 A_{260} units (= 72 pmoles) ribosomes and with or without indicated TF II-containing protein fractions. The reaction mixtures contained the salt concentrations as described (11). TF II-content of the protein fractions added had been determined prior to the experiment by ADP-ribosylation. After the incubation, the ribosomes were isolated by centrifugation and ribosome-bound radioactivity determined as described (11). 0.5 A_{260} units out of resuspended ribosomes were assayed under standard conditions of poly Phe synthesis (11) for ribosome-bound TF II activity. Specific activity of ^{14}C NAD^{+}, used for ADP-ribosylation of TF II was 136 Ci/mole; specific activity of ^{3}H Phe was 1150 Ci/mole. Counting efficiencies for ^{3}H and for ^{14}C were 20 percent and 48 percent, respectively.

assay. The addition of TF II fraction VI proteins containing a nearly two-fold excess of the unmodified factor caused approximately 73 percent inhibition of GTP-dependent binding of ADP-ribosyl-TF II. In the absence of GTP, the inhibition of the binding of ADP-ribosyl-TF II by the unmodified factor amounted to 69 percent. The inhibition of the binding of ADP-ribosyl-TF II by unmodified TF II was accompanied

by an increase of ribosome-bound TF II activity, assayed in poly Phe synthesis. In experiments not shown, the unmodified factor compared to ADP-ribosyl-TF II displayed a significantly higher affinity for its ribosomal binding site. Its binding to the ribosome in the presence of GTP, assayed in poly Phe synthesis, was not inhibited even by the addition of a great excess of ADP-ribosyl-TF II.

Recent studies from several laboratories suggest the existence of overlapping or identical binding site(s) for both AA-tRNA and the translocation factor (13–18). ADP-ribosyl-TF II and Phe-tRNA also displayed a competition for apparently the same or overlapping binding site(s) (Fig. 1). Increasing amounts of Phe-tRNA, prebound non-enzymatically to the acceptor site, significantly reduced the extent of binding of ADP-ribosyl-TF II, again both in the presence or in the absence of GDP which was used instead of GTP in order to avoid any translocation of bound Phe-tRNA. This result appears to suggest that both ADP-ribosyl-TF II and Phe-tRNA bind to a great extent, if not to the same site, at least to the same ribosomes. This

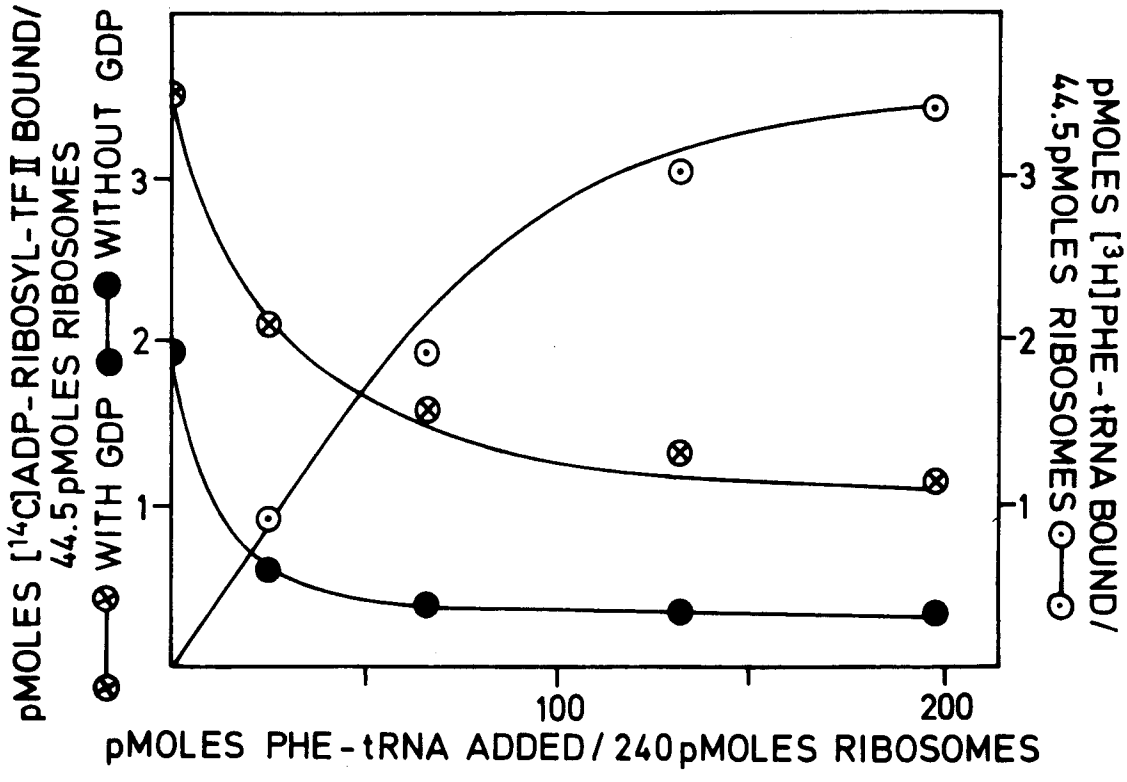

Fig. 1: Effect of Phe-tRNA, bound in the presence of poly U non-enzymatically to the ribosome, on the subsequent ADP-ribosyl-TF II binding. 12.4 A_{260} units (= 240 pmoles) ribosomes were incubated in the presence of 150 μg poly U and, with or without, indicated amounts of $tRNA_{E.coli}$, 2.1 percent charged with ^3H Phe, specific activity 1150 Ci/mole; ionic conditions (15 mM $MgCl_2$, 60 mM KCl, 30 mM Tris HCl pH 7.4) of non-enzymatic binding. Ribosomal complexes were isolated by centrifugation and resuspended in the same medium. 4 A_{260} units (= 74 pmoles) ribosomes were incubated under the same ionic conditions in the presence of 83 pmoles of ^{14}C ADP-ribosyl-TF II and, with or without, 1 mM GDP. Thereafter, ribosomes were centrifuged again through a cushion containing the same salt concentrations plus 0.5 M sucrose. Ribosome-bound ^3H (counting efficiency 20 percent) and ^{14}C (counting efficiency 48 percent) radioactivities were counted in separate channels of a Packard (Tricarb) liquid scintillation spectrometer as described (11).

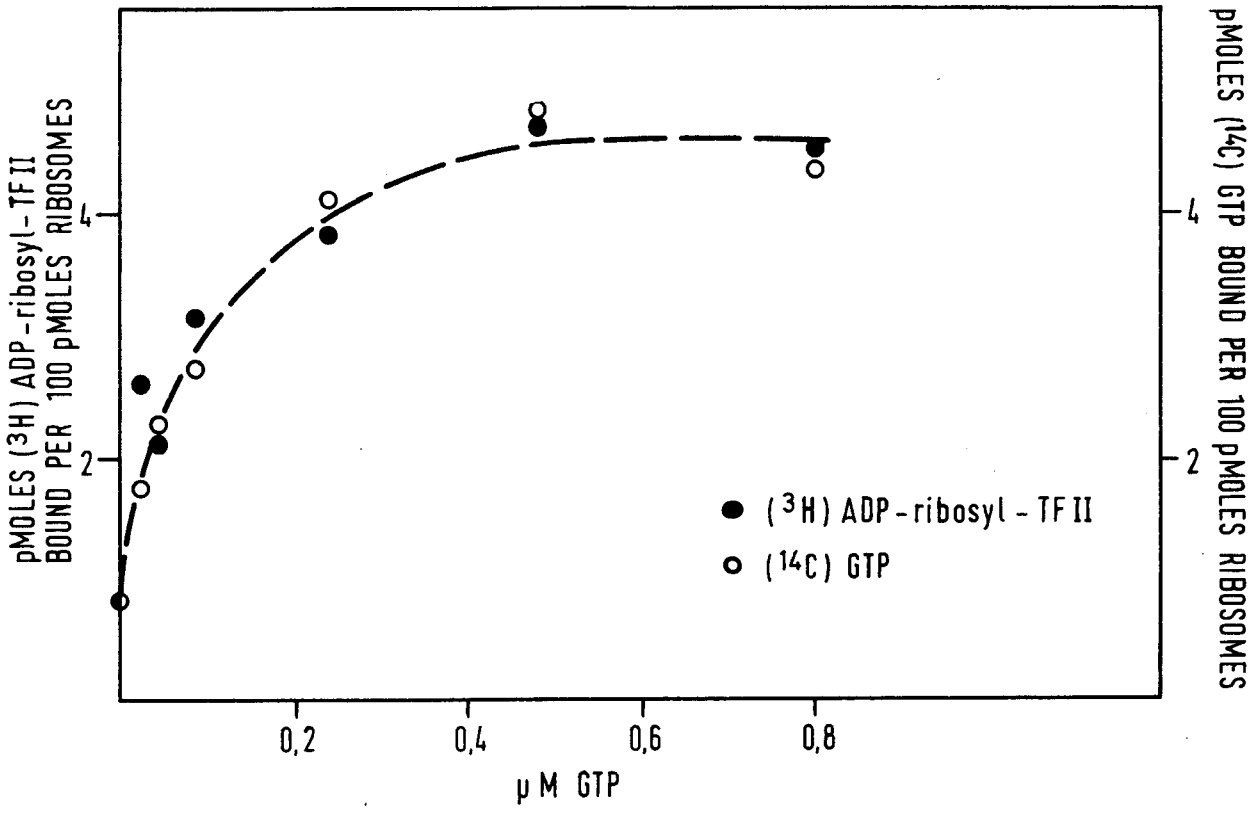

Fig. 2: Effect of increasing concentrations of GTP upon the binding of ADP-ribosyl-TF II to the ribosome. 82 pmoles ^{3}H ADP-ribosyl-TF II were incubated with 6.1 A_{260}units ribosomes and indicated concentrations of ^{14}C GTP, specific activity 36 Ci/mole. The reaction mixtures contained ionic concentrations as described (11). After centrifugation ribosome-bound ^{3}H and ^{14}C radioactivities were determined in separate channels of a Packard (Tricarb) liquid scintillation spectrometer, as described (11).

ribosomal population capable of binding both of the mentioned components of polypeptide synthesis might represent that of „active" ribosomes. Inhibition of the binding of ADP-ribosyl-TF II observed, however, can be explained in terms of some allosteric effects of Phe-tRNA bound as well as by the overlapping or the identity of the binding sites of both of these components.

As demonstrated in Table 3, the modified factor bound primarily to the larger ribosomal subunit. Only slight binding appeared to occur to 40 S particles. Nevertheless, the addition of 40 S to 60 S particles significantly increased the extent of ribosome-bound ADP-ribosyl-TF II, presumably by stabilizing the complex between the modified factor and the larger ribosomal subunit. Similiar results have also been previously obtained with the unmodified factor (20).

Fig. 2 shows the binding of ADP-ribosyl-TF II to the ribosome as a function of GTP. The double labelling used in this experiment (^{3}H ADP-ribosyl-TF II versus ^{14}C GTP) makes the determination of the stochiometry of ribosome-bound factor to GTP possible; approximately 1 mole of the modified factor was bound to ribosomes per 1.4 mole of GTP after subtraction of blanks. In this experiment, 0.4 μM GTP appeared to be nearly sufficient for the maximum stimulation of ADP-ribosyl-TF II-binding to ribosomes.

Table 3. Binding of ADP-ribosyl-TF II to ribosomal subunits

Particles	Ribosome-bound ADP-ribosyl-TF II	
	pmoles/4 A_{260} units	pmoles/100 pmoles ribosomes
40 S	2.9	1.1
60 S	8.1	7.5
40 S + 60 S	14.2	18.5

108 μg TF II fraction VI proteins containing 94 pmoles ^{3}H ADP-ribosyl-TF II were incubated for 5 min at 37 °C in the presence of 1 mM GTP with 5 A_{260} units 40 S or 5 A_{260} units 60 S, or 1.5 A_{260} units 40 S plus 3.5 A_{260} units 60 S particles. The subunits used were less than 3 % cross-contaminated as assayed in poly Phe synthesis (19). Ribosomebound radioactivity was determined as described (11). Counting efficiency for ^{3}H was 20 %. The specific activity of ^{3}H NAD^+ used was 591 Ci/mole. Calculation of molar amounts of ribosomal subunits from A_{260} was based on assumptions described (19).

The results described suggest that use of radioactively labelled ADP-ribosyl-TF II can provide a useful assay system for characterization of the ribosomal binding site of the translocation factor. ADP-ribosyl-TF II has been recently cross-linked by means of bifunctional reagents N,N'-(1,2-phenylene)dimaleimide and 1,5-difluoro-2,4-dinitro-benzene to ribosomal proteins (21). This represents a new possibility for structural work on TF II-binding site on the ribosome.

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