Effect of Elongation Factor 2 and of Adenosine Diphosphate-Ribosylated Elongation Factor 2 on Translocation

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1. The effect of elongation factor 2 (EF 2) and of adenosine diphosphate-ribosylated elongation factor 2 (ADP-ribosyl-EF 2) on the shift of endogenous peptidyl-tRNA from the A to the P site of rat liver ribosomes (measured by the peptidyl-puromycin reaction) and on the release of deacylated tRNA (measured by aminoacylation) was investigated. 2. Limiting amounts of EF 2, pre-bound or added to ribosomes, catalyse the shift of peptidyl-tRNA in the presence of GTP; when the enzyme is added in substrate amounts GMP- $P(\text{CH}_2)_P$ [guanosine (β,γ -methylene)triphosphate] can partially replace GTP. ADP-ribosyl-EF 2 has no effect on the shift of peptidyl-tRNA when present in catalytic amounts, but becomes almost as effective as EF 2 when added in substrate amounts together with GTP; GMP- $P(\text{CH}_2)_P$ cannot replace GTP. 3. The release of deacylated tRNA is induced only by substrate amounts of added EF 2 and also occurs in the absence of guanine nucleotides. In this reaction ADP-ribosyl-EF 2 is only 25% as effective as EF 2 in the absence of added nucleotide, but becomes 60–80% as effective in the presence of GTP or GMP- $P(\text{CH}_2)_P$. 4. The results obtained on protein-synthesizing systems are consistent with the hypothesis that ADP-ribosyl-EF 2 can operate a single round of translocation followed by binding of aminoacyl-tRNA and peptide-bond formation. 5. From the data obtained with the native enzyme it is concluded that the two moments of translocation require different conditions of interaction of EF 2 with ribosomes; it is suggested that the shift of peptidyl-tRNA is catalysed by EF 2 pre-bound to ribosomes, and that the release of tRNA is induced by a second molecule of interacting EF 2. The hydrolysis of GTP would be required for the release of pre-bound EF 2 from ribosomes. 6. The inhibition of the utilization of limiting amounts of EF 2 on ADP-ribosylation is very likely the consequence of a concomitant decrease in the rate of association and dissociation of the enzyme from ribosomes.

Eukaryotic translocation factor EF 2* is specifically inactivated by diphtheria toxin in the presence of NAD^+ . The inhibition is the consequence of the covalent linkage to the enzyme of the ADP-ribose moiety of NAD^+ (Honjo *et al.*, 1968), catalysed by the fragment A of the toxin (Collier, 1975). Early reports showed that ADP-ribosylation of EF 2 induced inhibition of translocation, as measured by the increase in reactivity of ribosomal peptidyl-tRNA with puromycin (Schneider *et al.*, 1968), and greatly decreased the ribosome-dependent GTPase activity of the enzyme (Raeburn *et al.*, 1968; Kloppstech *et al.*, 1969).

* Abbreviations: designation of the elongation factors of eukaryotic origin as EF 1 and EF 2 and of the respective prokaryotic factors as EF T (EF Tu and EF Ts) and EF G conforms to currently accepted nomenclature (Caskey *et al.*, 1972); A site, aminoacyl-tRNA site; P site, peptidyl-tRNA site; GTPase, guanosine triphosphatase; GMP- $P(\text{CH}_2)_P$, guanosine (β,γ -methylene)triphosphate.

Although it is beyond doubt that ADP-ribosylation of EF 2 results in its inactivation in protein synthesis, more recent investigations on the EF 2-catalysed partial reactions of elongation have shown that ADP-ribosyl-EF 2 shares many properties with the native enzyme: ADP-ribosyl-EF 2 binds GTP with the same affinity as EF 2 (Montanaro *et al.*, 1971); the native and the modified enzyme interact with ribosomes competing for the same site (Bermek *et al.*, 1974); when the interaction with ribosomes occurs in the presence of GTP the nucleotide is found in the complex in the form of GDP (Bermek, 1972). Since as the study of elongation proceeds in the details of the partial reactions, the differences between EF 2 and ADP-ribosyl-EF 2 become less and less clear-cut, a reinvestigation on the effect of ADP-ribosylation on translocation has been undertaken.

According to the two-site model for ribosomes, bacterial EF G- and mammalian EF 2-catalysed translocation involves the conversion of acceptor

ribosomes (with deacylated tRNA in the P site and peptidyl-tRNA in the A site, unreactive with puromycin) into donor ribosomes (with peptidyl-tRNA in the P site, reactive with puromycin), the release of deacylated tRNA (from the P site of acceptor ribosomes in their conversion into donor ribosomes) and the movement of codons in mRNA relative to ribosomal sites. In partial reactions two assays of translocation can be performed: the first measures the increase in reactivity with puromycin, which follows the shift of peptidyl-tRNA from the A to the P site of ribosomes; the second consists of the determination of the amount of deacylated tRNA released. In the present work the effect of EF 2 and ADP-ribosyl-EF 2 on translocation has been investigated by both procedures.

Experimental

Materials

Diphtheria toxin fragment A was prepared as described by Drazin *et al.* (1971) from diphtheria toxin, purified by the method of Pope & Stevens (1958) and obtained from the Istituto Sieroterapico e Vaccinogeno Toscano 'Sclavo' (Siena, Italy). [8-³H]-Puromycin (3.7 Ci/mmol), [adenosine-¹⁴C]NAD⁺ (260 mCi/mmol) and U-¹⁴C-labelled protein hydrolysate (57 mCi/mg-atom of C) were from The Radiochemical Centre, Amersham, Bucks., U.K. tRNA charged with L-[¹⁴C]phenylalanine and with the other 19 non-radioactive amino acids was from New England Nuclear Corp., Boston, MA, U.S.A. (0.167 μ Ci of [¹⁴C]phenylalanine/mg of tRNA; 461 mCi/mmol of L-phenylalanine). GTP, GMP-*P*(CH₂)*P* and aminoacyl-tRNA synthetase [14 units/mg of protein (where 1 unit catalyses the activation of 1 nmol of arginine in 10 min at 37°C)] were from Miles Laboratories, Kankakee, IL, U.S.A. ATP, NAD⁺ and puromycin dihydrochloride were products of Sigma Chemical Co., St. Louis, MO, U.S.A. Yeast tRNA was from Calbiochem, San Diego, CA, U.S.A. Creatine phosphate and creatine kinase were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Sucrose-washed ribosomes were prepared from the liver of starved rats as described by Staehelin & Falvey (1971). Ribosomes thus prepared contain loosely bound EF 2, which can be removed by high concentrations of NH₄⁺ (Felicetti & Lipmann, 1968). NH₄Cl-washed ribosomes were prepared by centrifuging twice sucrose-washed ribosomes through discontinuous sucrose gradients (0.5–2.0 M) containing 0.5 M-NH₄Cl, 5 mM-MgCl₂ and 50 mM-Tris/HCl buffer, pH 7.8, as described by Steinert *et al.* (1974); NH₄Cl was omitted from the 2 M-sucrose layer of the second washing. Both sucrose-washed and NH₄Cl-washed ribosomes were stored in small batches at

–80°C suspended in 20 mM-Tris/HCl buffer, pH 7.5, containing 0.1 M-NH₄Cl, 5 mM-magnesium acetate and 1 mM-dithiothreitol. All samples were thawed only once before use. Ribosome concentration was calculated from the *E*₂₆₀ with the following assumptions: *E*_{1 cm}^{1%} = 125; 1 mg of ribosomes = 250 pmol.

EF 1 and EF 2 were obtained from rat liver 'pH 5 supernatant' and were resolved from each other by adsorption on hydroxyapatite and chromatography on DEAE-cellulose, as previously described (Montanaro *et al.*, 1973). The protein content of the enzyme preparations was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The molar concentration of EF 2 was determined by [¹⁴C]ADP-ribosylation in 0.1 ml reaction mixtures containing, besides EF 2, 50 mM-Tris/HCl buffer, pH 7.5, 10 mM-dithiothreitol, 5 μ M-[¹⁴C]NAD⁺ and 6 μ g of diphtheria toxin fragment A. After 15 min of incubation at 20°C, 0.1 ml of 10% (w/v) trichloroacetic acid was added; the acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% (w/v) trichloroacetic acid and measured.

Methods

Translocation reaction. The amounts of EF 2 or ADP-ribosyl-EF 2 indicated in the legends to Tables were present in 0.1 ml of ADP-ribosylation mixtures containing 50 mM-Tris/HCl buffer, pH 7.5, 10 mM-dithiothreitol and 1 mM-[¹²C]NAD⁺; diphtheria toxin fragment A (6 μ g) was either absent (with EF 2) or present (with ADP-ribosyl-EF 2). After 15 min of incubation at 20°C, the volume was adjusted to 0.2 ml by adding ribosomes (50–80 pmol) and the other components of the translocation reaction. The final concentrations were 45 mM-Tris/HCl buffer, pH 7.5, 150 mM-NH₄Cl, 9 mM-magnesium acetate, 6 mM-dithiothreitol, 0.5 mM-NAD⁺ and 2 mM-GTP or 2 mM-GMP-*P*(CH₂)*P* where indicated. Incubation was at 37°C for 10 min.

In some experiments sucrose-washed ribosomes were preincubated, in the absence and in the presence of diphtheria toxin fragment A (6 μ g), for 10 min at 37°C in 0.05 ml of 20 mM-Tris/HCl buffer, pH 7.5, containing 150 mM-NH₄Cl, 9 mM-magnesium acetate, 1 mM-dithiothreitol and 2 mM-NAD⁺. The other components of the translocation reaction were then added and the volume was adjusted to 0.2 ml.

Peptidyl-[³H]puromycin reaction. At the end of the translocation reaction, the samples were cooled to 0°C in an ice-bath. To each sample 2500 pmol of [³H]puromycin (diluted with unlabelled carrier to a specific radioactivity of 900 d.p.m./pmol) was added in 0.05 ml of 120 mM-Tris/HCl buffer, pH 7.5, containing 1 M-NH₄Cl. The final concentration of NH₄Cl was thus 320 mM and that of [³H]puromycin 10 μ M. After 20 min of incubation at 0°C, the reaction was

stopped by adding 0.25ml of 10% (w/v) trichloroacetic acid. The samples were kept at 90°C for 15 min and the hot-acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and measured. Blanks, obtained by adding trichloroacetic acid to ribosomes before the incubation with puromycin, were subtracted from all values.

Assay of deacylated tRNA release. At the end of the translocation reaction the mixtures were filtered through nitrocellulose membrane filters (Millipore HA; average pore size 0.45 μ m) pre-soaked in 20mM-Tris/HCl buffer, pH7.5, containing 150mM-NH₄Cl, 9mM-magnesium acetate and 1mM-dithiothreitol. The filters were washed twice with 0.2ml of the same solution, and 0.3ml of the combined filtrate and washings was used to determine tRNA by aminoacylation with purified aminoacyl-tRNA synthetase. To each sample 2.5 μ mol of ATP, 0.5 unit of aminoacyl-tRNA synthetase and 0.75 μ Ci of ¹⁴C-labelled protein hydrolysate (2500pmol of amino acids) were added in 0.2ml of 220mM-Tris/HCl buffer, pH7.5, containing 25mM-KCl, 11mM-MgCl₂ and 8mM-dithiothreitol. After 20min of incubation at 37°C, the samples were cooled to 0°C, and 0.5ml of cold 20% (w/v) trichloroacetic acid was added. The cold-acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and measured. Blanks, obtained by carrying out the aminoacylation reaction on filtrates from samples without ribosomes, in the absence or in the presence of EF 2, were subtracted from all values. The tRNA released is expressed as pmol of [¹⁴C]-aminoacyl-tRNA formed during the aminoacylation reaction.

Protein synthesis. EF 2 was preincubated for 15 min at 20°C in 0.1ml of 50mM-Tris/HCl buffer, pH7.5, containing 10mM-dithiothreitol and 1mM-NAD⁺; diphtheria toxin fragment A (6 μ g) was either absent (control EF 2) or present (ADP-ribosyl-EF 2). After cooling in ice the volume was adjusted to 0.25ml by adding 25pmol of ribosomes, 250 μ g of EF 1 protein, 22pmol of [¹⁴C]phenylalanyl-tRNA (contained in 60 μ g of mixed charged tRNA) and buffer and salts to a final concentration of 45mM-Tris/HCl, pH7.5, 150mM-NH₄Cl, 9mM-magnesium acetate, 6mM-dithiothreitol and 2mM-GTP. After 10min of incubation at 37°C, the hot-acid-insoluble radioactivity was collected on glass-fibre filters (Whatman GF/C), washed with 5% trichloroacetic acid and measured. The total amount of amino acids incorporated was calculated by using the ratio of phenylalanine acceptor tRNA to total tRNA in the original sample added (Baliga *et al.*, 1973).

Reactivity of nascent peptide chains on ribosomes with puromycin during protein synthesis. The protein-synthesizing mixture (2.5ml; see legend to Fig. 1) was similar to that described above, except that EF 2

was not preincubated and that charged tRNA was not limiting, since it was replaced by a regenerating system consisting of uncharged tRNA, ¹⁴C-labelled amino acids, aminoacyl-tRNA synthetase and ATP. At zero time and after 3 min of incubation at 37°C, four 0.1 ml samples were withdrawn. The remaining mixture was fractionated in 0.1 ml samples, half of which received 6 μ g of diphtheria toxin fragment A. Incubation was allowed to proceed at 37°C and samples were withdrawn at selected times. At the end of the incubations all samples were cooled at 0°C. At all times incorporation of ¹⁴C-labelled amino acids was measured by adding to the samples 0.1 ml of 10% (w/v) trichloroacetic acid and counting the hot-acid-insoluble radioactivity retained on glass-fibre filters. Samples withdrawn at zero time and after 3 min and 10 min of total incubation at 37°C were supplemented with [³H]puromycin (diluted with unlabelled carrier to a specific radioactivity of 2200 d.p.m./pmol) and NH₄Cl to a final concentration of 10 μ M and 320 mM respectively. After 20 min of incubation at 0°C, 0.1 ml of 10% (w/v) trichloroacetic acid was added and the hot-acid-insoluble radioactivity was collected and counted for ¹⁴C and ³H. No difference was observed at any time between the incorporation of ¹⁴C assayed directly and that measured after incubation of ribosomes with puromycin at 0°C, indicating that at this temperature elongation factors were completely inactive.

Radioactivity measurements. Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer. When a single radioactive isotope was present, the filters were counted for radioactivity after addition of 6 ml of methoxyethanol and 10 ml of scintillation fluid [0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene]. The efficiency was approx. 65% for ¹⁴C and 31% for ³H. For dual-labelling counting, the radioactive material present on the filters was solubilized with 0.4 ml of Soluene-350 (from Packard Instrument Co., Downers Grove, IL, U.S.A.), and the samples were counted after addition of 10 ml of scintillation fluid. The efficiency for ¹⁴C was approx. 50% on the ¹⁴C channel and 12% on the ³H channel; the efficiency for ³H on the ³H channel was approx. 44%.

Results

Assay of translocation by the peptidyl-[³H]puromycin reaction

Rat liver ribosomes carry nascent peptides, most of which are also retained when ribosomes are washed free of elongation factors (Schneider & Maxwell, 1973). Some of these chains are in the P site and react promptly with puromycin at 0°C (Inoue-Yokosawa *et al.*, 1974), whereas others are in the A site and require previous translocation to the P site. In our

Table 1. *Effect of guanine nucleotides and of substrate amounts of EF 2 and ADP-ribosyl-EF 2 on the reactivity of nascent peptides of sucrose-washed ribosomes with puromycin*

The experiments were carried out in two steps. In the first step, sucrose-washed ribosomes (50 pmol) were preincubated for 10 min at 37°C in the presence of NAD⁺ alone (Expt. 1) or in the presence of NAD⁺ plus diphtheria toxin fragment A (Expt. 2); in the second step translocation was carried out at 37°C for 10 min and the subsequent peptidyl-[³H]puromycin reaction at 0°C for 20 min as described in the Experimental section. In Expt. 1, 98 pmol of EF 2, and in Expt. 2, 98 pmol of ADP-ribosyl-EF 2, were present where indicated. The data are based on five independent experiments.

Expt.	Preincubation additions	Additions to translocation assay		[³ H]Puromycin reacted (pmol/100 pmol of ribosomes)	
		Nucleotide	Enzyme ...	None	EF 2
1	NAD ⁺	None		5.10	6.23
	NAD ⁺	GTP		11.13	12.11
	NAD ⁺	GMP-P(CH ₂) ₂ P		5.04	8.55
				None	ADP-ribosyl-EF 2
2	NAD ⁺ , fragment A	None		5.03	6.23
	NAD ⁺ , fragment A	GTP		5.79	11.63
	NAD ⁺ , fragment A	GMP-P(CH ₂) ₂ P		5.25	5.73

Table 2. *Effect of guanine nucleotides and of EF 2 and ADP-ribosyl-EF 2 on the reactivity of nascent peptides of NH₄Cl-washed ribosomes with puromycin*

Translocation was carried out at 37°C for 10 min and the subsequent peptidyl-[³H]puromycin reaction at 0°C for 20 min as described in the Experimental section; 54 pmol of NH₄Cl-washed ribosomes were present. The data are based on five independent experiments.

Expt.	Additions to translocation assay			[³ H]Puromycin reacted (pmol/100 pmol of ribosomes)		
	Nucleotide	Enzyme ...		None	EF 2 (13 pmol)	EF 2 (92 pmol)
1	None			3.89	4.56	4.67
	GTP			3.98	8.80	8.72
	GMP-P(CH ₂) ₂ P			3.70	4.07	6.05
				None	ADP-ribosyl-EF 2 (13 pmol)	ADP-ribosyl-EF 2 (92 pmol)
2	None			3.76	4.44	4.90
	GTP			3.85	5.47	7.71
	GMP-P(CH ₂) ₂ P			3.58	3.68	4.89

ribosomal preparations, the reactivity with puromycin increased approximately twofold after translocation with EF 2 and GTP (Tables 1 and 2), indicating that peptide chains were more or less evenly distributed between the A and P sites.

The data reported in Table 1, Expt. 1, show that in sucrose-washed ribosomes GTP alone induced extensive translocation, almost 90% of that observed when EF 2 was added together with GTP. On the contrary, GMP-P(CH₂)₂P had no effect in the absence of EF 2, but could replace GTP to the extent of approx. 50%, when the enzyme was added in molar excess with respect to ribosomes.

The effect of GTP alone is clearly due to contamination of ribosomes with EF 2, since it is abolished by preincubation of ribosomes with NAD⁺ and diphtheria toxin fragment A (Table 1, Expt. 2) or by washing ribosomes free of elongation factors through sucrose gradients containing NH₄Cl (Table 2).

Schneider & Maxwell (1973), by titration of EF 2 with [³H]NAD⁺ in the presence of diphtheria toxin, showed the presence of 4 pmol of enzyme in 100 pmol of sucrose-washed ribosomes. This value has been confirmed by us for our preparations of sucrose-washed ribosomes. The results reported in Table 1 indicate that GTP allows the recycling of such limiting amounts of EF 2 so that translocation can spread over most of the active ribosomal population, but that the catalytic utilization of the enzyme becomes impossible when GTP is replaced by its non-hydrolysable analogue GMP-P(CH₂)₂P or when EF2 has been ADP-ribosylated. On the contrary, when EF 2 is added in substrate amounts to ribosomes, GMP-P(CH₂)₂P becomes partially effective in translocation. Moreover, substrate amounts of ADP-ribosyl-EF 2 in the presence of GTP, but not of GMP-P(CH₂)₂P, do support translocation (Table 1, Expt. 2).

The data obtained with NH_4Cl -washed ribosomes agree with the above results and conclusions. Table 2 shows that: (i) GTP together with EF 2 supports full translocation independently of the concentration of added enzyme; (ii) $\text{GMP-P}(\text{CH}_2)\text{P}$ can partially replace GTP when EF 2 is present in substrate amounts; (iii) ADP-ribosyl-EF 2 does not induce translocation at low concentrations, but becomes 80% as effective as EF 2 when added in substrate amounts together with GTP; (iv) with ADP-ribosyl-EF 2, GTP cannot be replaced by $\text{GMP-P}(\text{CH}_2)\text{P}$.

The results obtained with ADP-ribosyl-EF 2 might be explained by the presence of trace amounts of native enzyme in the ADP-ribosylated preparations. However, if both forms of enzyme compete for the same ribosomal binding site (Bermek *et al.*, 1974), a catalytic reutilization of a few molecules of EF 2 in the presence of a large excess of ADP-ribosyl-EF 2 appears unlikely. Moreover, as shown in Table 3, the conditions of ADP-ribosylation were such as to ensure, when ADP-ribosyl-EF 2 was present in four-fold excess with respect to ribosomes, an inhibition

of protein synthesis which was 95% of the maximum value, obtained with EF 2 equimolar to ribosomes. According to the linear relationship below this value between protein synthesis and EF 2 added (Table 3), the incorporation given by 100 pmol of ADP-ribosyl-EF 2 would correspond to that obtained with 1.5 pmol of EF 2. However, it should be considered that this value of incorporation is only about three times that observed with EF 1 alone. The incorporation given by EF 1 is due to the binding of aminoacyl-tRNA to donor ribosomes (56% of the active ribosomal population, see Table 2) followed by peptide-bond formation via peptidyltransferase; in this process donor ribosomes are changed into acceptor ribosomes (Baliga & Munro, 1972). If ADP-ribosyl-EF 2 translocates at least once (as suggested by the data of Tables 1 and 2) the acceptor ribosomes originally present in the ribosomal preparation (44%) and those newly formed (56%), the binding of aminoacyl-tRNA to these ribosomes, followed by peptide-bond formation, will make the total incorporation 150% of that observed with EF 1 alone. Therefore the residual incorporation in the presence of 100 pmol of ADP-ribosyl-EF 2 is not inconsistent with complete ADP-ribosylation of the enzyme.

Table 3. *Effect of EF 2 and of ADP-ribosyl-EF 2 on amino acid incorporation by NH_4Cl -washed ribosomes*

For experimental details see the text; 25 pmol of NH_4Cl -washed ribosomes were present. The data are based on three independent experiments.

Enzymes present in incubation mixtures	Amino acids incorporated (pmol/100 pmol of ribosomes)
EF 1	12
EF 1+EF 2 (5 pmol)	92
EF 1+EF 2 (12.5 pmol)	188
EF 1+EF 2 (25 pmol)	434
EF 1+EF 2 (100 pmol)	434
EF 1+ADP-ribosyl-EF 2 (100 pmol)	34

Assay of translocation by the measurement of tRNA release

The results obtained by this method are shown in Table 4. The amount of tRNA released by substrate amounts of EF 2 and GTP was of the same order of magnitude as that of the peptide chains, which became reactive with puromycin under similar conditions of incubation. Rather surprisingly, however, no tRNA was released when GTP alone was added to sucrose-washed ribosomes (Table 4) or when GTP was added together with catalytic amounts of EF 2 (13 pmol) to NH_4Cl -washed ribosomes (results not shown),

Table 4. *Effect of guanine nucleotides, EF 2 and ADP-ribosyl-EF 2 on the release of deacylated tRNA from sucrose-washed and from NH_4Cl -washed ribosomes*

Translocation was carried out at 37°C for 10 min, and the tRNA released was assayed as described in the Experimental section. In Expt. 1, 82 pmol of sucrose-washed ribosomes, and in Expt. 2, 81 pmol of NH_4Cl -washed ribosomes were present. In both experiments 186 pmol of either EF 2 or ADP-ribosyl-EF 2 were present where indicated. The data are based on six independent experiments.

Expt.	Ribosomes	Additions to translocation assay			tRNA released (pmol/100 pmol of ribosomes)		
		Nucleotide	Enzyme	...	None	EF 2	ADP-ribosyl-EF 2
1	Sucrose-washed	None			0.03	4.82	1.10
		GTP			0.22	5.33	3.65
		$\text{GMP-P}(\text{CH}_2)\text{P}$			0.00	4.13	2.70
2	NH_4Cl -washed	None			0.06	4.64	1.29
		GTP			0.29	5.93	3.47
		$\text{GMP-P}(\text{CH}_2)\text{P}$			0.01	3.86	3.05

conditions which ensure extensive translocation as assayed by the peptidyl-puromycin reaction (Tables 1 and 2). On the contrary, substrate amounts of EF 2 also induced a significant release of tRNA in the absence of added nucleotides. The results were similar with both sucrose-washed and NH_4Cl -washed ribosomes and indicate that the two moments of translocation, the shift of peptide chains from the A to the P site and the release of deacylated tRNA, are not concomitant, but require different conditions of interaction of EF 2 with ribosomes. As shown in the last column of Table 4, ADP-ribosyl-EF 2 was only 25% as effective as EF 2 in the absence of added

nucleotide, but became 60–80% as effective in the presence of GTP or GMP- $P(\text{CH}_2)_3\text{P}$.

Position of nascent peptides after inhibition of protein synthesis by diphtheria toxin fragment A and NAD^+

The finding that ADP-ribosyl-EF 2 is effective in translocation when added in stoichiometric excess with respect to ribosomes gives rise to the question whether the inhibition of protein synthesis which follows ADP-ribosylation of substrate amounts of EF 2 leads to a predominant location of the peptide chains in the P or in the A site. Chains will remain confined to the P site if ADP-ribosyl-EF 2 remains bound to ribosomes after translocation so as to prevent the subsequent EF 1-catalysed binding of aminoacyl-tRNA. A confinement in the A site will occur if after translocation induced by ADP-ribosyl-EF 2, the EF 1-catalysed binding of aminoacyl-tRNA followed by peptide-bond formation occurs normally, but a second translocation is forbidden. To decide between the two alternative hypotheses an experiment was designed in which normal protein synthesis was allowed to proceed for a short time in the presence of EF 1 and substrate amounts of EF 2; then EF 2 was ADP-ribosylated and, after further incubation, the number of peptide chains in the P site was determined by means of the puromycin reaction at 0°C . The results are shown in Fig. 1. The reactivity of nascent peptides with puromycin increased during the first 3 min of incubation, reaching a value which was not modified in the control system by further incubation. At 7 min after the addition of diphtheria toxin fragment A in the presence of NAD^+ , protein synthesis had completely stopped. In parallel a fall in the reactivity of nascent peptides with puromycin was observed, the value returning to that measured before ribosomes were engaged in protein synthesis. The results show that under our experimental conditions a steady state is quickly reached in protein synthesis in which most of the peptide chains are in the P site. The shift back to the A site on ADP-ribosylation of EF 2 indicates that the halt in protein synthesis is not associated with inhibition of the binding of aminoacyl-tRNA.

Discussion

The shift of peptidyl-tRNA from the A to the P site measured by means of the puromycin reaction has been extensively investigated both in prokaryotic (Tanaka *et al.*, 1968; Brot *et al.*, 1968; Kaji *et al.*, 1969; Skoultchi *et al.*, 1969) and in eukaryotic systems (Skogerson & Moldave, 1968; Schneider *et al.*, 1968; Hardesty *et al.*, 1969; Schneider & Maxwell, 1973). Attempts to correlate the release of deacylated tRNA

Fig. 1. Effect of ADP-ribosylation of EF 2 on protein synthesis and on the reactivity of ribosomal nascent peptides with puromycin

The protein-synthesizing system contained, in 2.5 ml, 50 mM-Tris/HCl buffer, pH 7.5, 150 mM- NH_4Cl , 9 mM-magnesium acetate, 1 mM-dithiothreitol, 1 mM-ATP, 0.4 mM-GTP, 10 mM-creatine phosphate, 125 μg of creatine kinase, 1.25 mg of uncharged tRNA, 1.5 units of aminoacyl-tRNA synthetase, 12.5 μCi of ^{14}C -labelled protein hydrolysate (41 650 pmol of amino acids), 2.5 mg of EF 1 protein, 540 pmol of EF 2, 360 pmol of NH_4Cl -washed ribosomes and 0.4 mM- NAD^+ . At zero time and at different times of incubation at 37°C in the absence (●) and in the presence (○) of diphtheria toxin fragment A (6 $\mu\text{g}/0.1$ ml), 0.1 ml samples were analysed for incorporation of ^{14}C -labelled amino acids (—) and for reactivity of ribosomal nascent peptides with ^3H puromycin at 0°C (----), as described in the Experimental section. Each point is the mean of duplicate assays from two experiments. The s.e.m. was less than 2% for each point and is omitted for clarity.

with the translocation of peptidyl-tRNA have been carried out on prokaryotic ribosomes pre-charged with exogenous tRNA (Kuriki & Kaji, 1968; Ishitsuka *et al.*, 1970), diphenylalanyl-tRNA (Kuriki & Kaji, 1968; Ishitsuka *et al.*, 1970) or *N*-acetyl-diphenylalanyl-tRNA (Lucas-Lenard & Haenni, 1969; Inoue-Yokosawa *et al.*, 1974). From the evidence obtained it has been concluded that both translocation and release are catalysed by the bacterial translocation factor EF G and that the two events take place concomitantly (Lucas-Lenard & Haenni, 1969), the release of tRNA being a consequence of the movement of peptidyl-tRNA (Ishitsuka *et al.*, 1970).

The results reported in the present paper indicate that in eukaryotic systems the release of deacylated tRNA depends on EF 2, the elongation factor that corresponds to bacterial EF G. However, with rat liver ribosomes carrying endogenous peptidyl-tRNA two experimental situations occur in which translocation of peptidyl-tRNA and release of deacylated tRNA do not run in parallel: (i) catalytic amounts of EF 2 in the presence of GTP induced translocation but not the release of tRNA; (ii) substrate amounts of EF 2 in the absence of guanine nucleotides were more effective in tRNA release than in translocation.

It has been suggested that bound peptidyl-tRNA and tRNA may have an important regulatory role in the interaction of elongation factors with ribosomes and that considerable differences may exist in this respect between bacterial and mammalian systems (Chinali & Parmeggiani, 1973): whereas in bacterial systems charged tRNA bound at either the A or the P site prevents the formation of a stable EF G-ribosome complex even in the presence of GMP- $P(\text{CH}_2)P$, in eukaryotic systems EF 2 and GTP interact preferentially with donor ribosomes, giving a stable complex in which the guanine nucleotide is in the form of GDP (Baliga & Munro, 1972). On the basis of this finding and other evidence Baliga & Munro (1972) suggested that in the mammalian elongation cycle EF 2 binds to ribosomes when they are in the post-translocation state with the A site vacant. The subsequent EF 1-catalysed binding of aminoacyl-tRNA locks EF 2 in the ribosomal structure, the enzyme becoming operative in translocation when peptide-bond formation has brought peptidyl-tRNA into the A site. After or during translocation, EF 2 is released from ribosomes and is replaced by a new molecule of enzyme (Moldave, 1972).

The data reported in the present paper confirm earlier evidence (Skogerson & Moldave, 1968; Schneider *et al.*, 1968; Schneider & Maxwell, 1973) that ribosomes washed free of elongation factors can be converted from the acceptor into the donor type by the addition of EF 2 and GTP (Table 2). Since translocation occurs, an interaction of the enzyme with acceptor ribosomes must take place, even though

a stable EF 2-ribosome complex is not formed (Baliga & Munro, 1972). Apparently when translocation occurs in the presence of GTP the complex is strongly driven towards dissociation, and this might well represent the moment at which in the repetitive cycle of elongation pre-bound EF 2 is released from ribosomes. The role of GTP in removing EF 2 from ribosomes is supported by the observation that the addition of GTP allows the recycling of catalytic amounts of EF 2 bound to sucrose-washed ribosomes (Table 1). When, however, the conversion of ribosomes from the acceptor into the donor type is induced by limiting amounts of EF 2, pre-bound or added to ribosomes, a release of deacylated tRNA is not observed (Table 4). Since the release event apparently depends on the availability of excess of EF 2, we tentatively suggest the following modification of the partial model of the function of EF 2 proposed by Baliga & Munro (1972): translocation operated by EF 2 pre-bound to ribosomes only involves the movement of peptidyl-tRNA from the A to the P site; in this process EF 2 is released from ribosomes as a consequence of the hydrolysis of GTP; the ejection of deacylated tRNA occurs in a subsequent step, when donor ribosomes interact with a new molecule of EF 2. The hypothesis requires, however, that the release of deacylated tRNA observed under our experimental conditions occurs from the same ribosomes that have undergone the A $\rightarrow$ P site translocation, whereas we are dealing with a mixed population, in which different ribosomes might compete for available EF 2; the presence of acceptor ribosomes, which do not carry deacylated tRNA but can undergo translocation, and of otherwise inactive ribosomes, which carry ejectable tRNA, cannot be excluded.

According to current views, in bacterial protein-synthesizing systems the cleavage of the terminal phosphate bond of GTP observed in the reactions catalysed by EF Tu (Yokosawa *et al.*, 1973), EF G (Inoue-Yokosawa *et al.*, 1974) and IF 2 (Mazunder, 1972) is related to the release of the enzymes from ribosomes after they have carried out their individual functions, release which must precede the subsequent step of the elongation cycle. In mammalian systems, Lee *et al.* (1973) have demonstrated that the non-hydrolysable analogue GMP- $P(\text{CH}_2)P$ can significantly replace GTP in the EF 2-dependent formation of phenylalanyl-puromycin catalysed by reticulocyte ribosomes, and Henriksen *et al.* (1975) have suggested that the GDP formed in GTP-promoted translocation induces a conformational change of EF 2, which decreases the affinity of the enzyme for ribosomes. Our data show that, in rat liver ribosomes, GMP- $P(\text{CH}_2)P$ can partially replace GTP when added together with substrate amounts of EF 2, but does not allow the repetitive utilization of catalytic amounts of enzyme in translocation of peptidyl-tRNA. Taken

together all these observations indicate that hydrolysis of GTP is not required for the binding of EF 2 to ribosomes, nor for translocation of peptidyl-tRNA, but rather for the subsequent release of EF 2.

When the effect of ADP-ribosyl-EF 2 on the shift of peptidyl-tRNA from the A to the P site of ribosomes was investigated, a clear-cut difference with respect to EF 2 was evident only in the presence of limiting amounts of enzyme, ADP-ribosyl-EF 2 becoming almost as active as EF 2 when added in concentrations that approached or exceeded that of ribosomes. This behaviour might indicate a decreased affinity of the modified enzyme for the ribosomal binding site and/or a lower rate of release of ADP-ribosyl-EF 2 from ribosomes. The first mechanism is supported by the observation that, although both EF 2 and ADP-ribosyl-EF 2 bind to ribosomes in the presence or absence of GTP (Bermek, 1972) competing for the same site (Bermek *et al.*, 1974), the unmodified enzyme displays a higher affinity for the common site (Bermek, 1974). On the other hand the decreased ribosome-linked GTPase activity of ADP-ribosyl-EF 2 (Raeburn *et al.*, 1968; Kloppstech *et al.*, 1969) probably depends on the formation of a more stable ribosome-GDP-ADP-ribosyl-EF 2 complex, since GDP is even more effective than GTP in stimulating the binding of the modified enzyme to ribosomes (Bermek, 1972; Bermek *et al.*, 1974). Thus the inhibition of the utilization of limiting amounts of EF 2 on ADP-ribosylation is very likely the consequence of a concomitant lowering of both the rates of association and of dissociation of the enzyme from ribosomes.

In bacterial systems, fusidic acid, which stabilizes the ribosome-GDP-EF G complex (Bodley *et al.*, 1970; Brot *et al.*, 1971), has an effect on translocation similar to that induced by ADP-ribosylation of EF 2, since it inhibits the activity of catalytic amounts of enzyme, but does not prevent a single round of translocation with substrate amounts of EF G (Modolell *et al.*, 1973; Inoue-Yokosawa *et al.*, 1974). Because of a common ribosomal site of interaction for EF G and EF Tu, fusidic acid secondarily inhibits the binding of aminoacyl-tRNA to ribosomes and, when acting on protein-synthesizing systems, confines nascent peptides to the P site (Cundliffe, 1972). The results in Fig. 1 show that this is not the case after inhibition of protein synthesis by ADP-ribosylation of EF 2; nascent peptides are apparently confined to the A site as if bound ADP-ribosyl-EF 2 did not prevent EF 1-catalysed binding of aminoacyl-tRNA and subsequent peptide-bond formation. In this regard ADP-ribosyl-EF 2 would behave as the native enzyme (Baliga & Munro, 1972).

When translocation was assayed by measuring the release of deacylated tRNA, ADP-ribosylation of EF 2 chiefly inhibited the reaction observed in the absence of added nucleotide. Further investigation of

this effect may help to clarify the basal differences between the native and the modified enzyme.

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