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Heterogeneity of native rat liver elongation factor 2

Abdelkader Marzouki, Jean-Pierre Lavergne, Jean-Paul Reboud and Anne-Marie Reboud

Laboratoire de Biochimie Médicale, Université Lyon 1, UM CNRS 24, 43, Boulevard du 11 Novembre 1918, 69622-Villeurbanne, France

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The high heterogeneity of native rat liver EF-2 prepared from either 105000 × *g* supernatant or microsome high-salt extract was detected by two-dimensional equilibrium isoelectric focusing-SDS-polyacrylamide gel electrophoresis in the presence of 9.5 M urea. Five spots were always detected, all of M_r 95000, which were not artefactual for their amount varied when EF-2 was specifically ADP-ribosylated by diphtheria toxin in the presence of NAD^+, and/or phosphorylated on a threonine residue by a Ca^{2+}/calmodulin-dependent protein kinase (most likely Ca^{2+}/calmodulin-dependent protein kinase III described by others [(1987) J. Biol. Chem. 262, 17299–17303; (1988) Nature 334, 170–173]). Results of ADP-ribosylation and/or phosphorylation experiments with either unlabeled or labeled reagents ([^{14}C]NAD and [^{32}P]ATP) strongly suggest that our preparation contained native ADP-ribosylated and native phosphorylated forms which could be estimated at about 20% and 40% of the whole EF-2. Phosphorylated and ADP-ribosylated forms of EF-2 could be ADP-ribosylated and phosphorylated, respectively, but a native form both ADP-ribosylated and phosphorylated was not detected. Our results also suggest the existence of a minor native form of EF-2 and of its phosphorylated and ADP-ribosylated derivatives.

Elongation factor 2; ADP-ribosylation; Phosphorylation; (Rat liver)

1. INTRODUCTION

Elongation factor 2 (EF-2), a single polypeptide of 857 amino acids (95 kDa), catalyzes the last step of the elongation cycle, translocation, in the course of protein biosynthesis. EF-2 is the only known protein which has the feature of being ADP-ribosylated and inactivated by diphtheria toxin in the presence of NAD^+. ADP-ribosylation occurs at a unique post-translationally modified histidine residue (diphtamide 715) only found in EF-2 and conserved throughout eukaryotic evolution (see [3]). Diphtamide 715 whose importance for the function of EF-2 has recently been stressed [4] is most likely a site of regulatory modifications of the factor because an ADP-ribosyltransferase, with the same mechanism of action as diphtheria toxin, and its inhibitor has been recently identified in different cells [5,6]. However, identification of an in vivo ADP-ribosylated EF-2 has only been done in polyoma-virus-transformed baby hamster kidney cells, very recently [7]. On the other hand, phosphorylation of a maximum of 2 threonine residues, one that lies between residues 51–60 of rat pancreas EF-2, specifically by the Ca^{2+}/calmodulin-dependent protein kinase III, has been shown to inactivate the factor, while dephosphorylation restored its activity [1,2]. Whether a relation exists between phosphorylation and ADP-ribosylation is still unknown. In the present paper, we attempted to identify the 5 different forms of rat liver EF-2 that we were able to separate using an O'Farrell two-dimensional electrophoretic system [8].

Correspondence address: A. Marzouki, Laboratoire de Biochimie Médicale, Université Lyon I, UM CNRS 24, 43, Boulevard du 11 Novembre 1918, 69622 Villeurbanne, France

2. MATERIALS AND METHODS

2.1. *Materials*

Pure diphtheria toxin was a gift from Professor Relyveld (Pasteur Institute, Paris); nicotinamide [^{14}C]adenine dinucleotide ([^{14}C]NAD^+, 10.5 GBq/mmol) and [γ-^{32}P]ATP

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(110 TBq/mmol) were purchased from Amersham; NAD^+ and ATP from Sigma.

2.2. *Preparations*

EF-2 (95% pure) was prepared from a rat liver microsome high-salt extract according to [9], or from rat liver supernatant by a method adapted from the preceding one, with the same basic purification steps. Such preparations incorporated from 0.6 to 0.8 mol of [^{14}C]ADP-ribose per mol of EF-2, in the presence of diphtheria toxin.

2.3. *Phosphorylation and/or ADP-ribosylation of EF-2*

EF-2 (90 pmol) was incubated as indicated in the legend of table 1. Both reactions were performed in 50 μl of buffer A (20 mM triethanolamine, pH 7.4, 8 mM acetate, 150 μM $CaCl_2$, 100 mM KCl, 1 mM dithiothreitol, 100 mM sucrose). In the phosphorylation assays 2 μg of rat liver cytosol [1] and 10 nmol of ATP (with or without 1.53 pmol of [γ-^{32}P]ATP) were used. In the ADP-ribosylation assays, 3.4 μg of diphtheria toxin, 434 pmol of NAD^+ or [^{14}C]NAD^+ were used. Control experiments performed by incubating the factor with [γ-^{32}P]ATP, diphtheria toxin, [^{14}C]NAD separately, showed neither labeling of the factor nor modifications of its electrophoretic pattern compared with that of EF-2 incubated alone in buffer A for the duration of the reactions. Other control experiments have shown that free EF-2 could not be phosphorylated in the presence of EGTA (1 mM) or trifluoperazine (200 μM) indicating that the kinase was Ca^{2+}/calmodulin (CaM)-dependent, most likely CaM kinase III described by others [1,2].

2.4. *2-D electrophoresis*

Two-dimensional gel analysis in an equilibrium pH gradient-SDS system in the presence of 9.5 M urea was performed according to O'Farrell [8]. All samples were analyzed at the same time under identical electrophoretic conditions. Different pH gradients in the first dimension have been used as indicated in the text. The gels stained with Coomassie blue were autoradiographed with different exposure times. Both gels and autoradiograms were photographed with the same magnification. Every EF-2 spot was identified on the stained gel by its position relative to those of two slightly stained proteins always present in our preparation and never phosphorylated nor ADP-ribosylated (X of M_r 62 500, pH_i around 7.1 and Y of M_r 43 000, pH_i around 7.5) and by its pH_i determined according to [8]. Other proteins used as internal controls, such as diphtheria toxin subunits A and B present in ADP-ribosylation experiments, were repeatedly found to have the same relative position and pH_i value from one experiment to another, which confirmed that there was no ambiguity in the matching of any EF-2 spot from one experiment to another. The specific radioactivity (SR) of each spot was determined by direct scanning of photographs using a Vernon photometer and expressed by the ratio of darkness of the labeled spot ([^{14}C] or [^{32}P]): darkness of the stained spot. Each set of photographs of the [^{32}P] and [^{14}C] autoradiograms and of the stained gels was scanned under identical conditions which differed from one set to the other because of the large variations of the darkness of the spots in the different sets. This means that the SR values can be compared only within experiments using the same isotope. This method of SR quantification was preferred to that of direct determination of staining and counting of the labeled spots, not suitable for the slightly stained (labeled) spots.

3. RESULTS AND DISCUSSION

Native EF-2 subjected to O'Farrell two-dimensional separation in the presence of 9.5 M urea always showed 5 spots, all of M_r 95 000 and of pH_i values varying from about 7.3 to 7.7 (These pH_i values are relative, since high concentrations of urea cause large changes in pH_i of proteins [8].): two trailing ones, one near the cathode end (a) and another more acidic (b), and three well resolved spots (c–e) (fig.1, expt 5). These spots were observed whatever the pH gradient used in the first dimension: pH 5–8, as shown in fig.1, or pH 4–9 (not shown). In the latter case, the spot corresponding to (a) was more intense but the other spots were less well resolved. An artefactual heterogeneity of EF-2 could be discarded for two reasons: these forms were found within all EF-2 preparations examined (5 derived from the 105 000 $\times$ g supernatant and 3 from microsome high-salt extract); their amount varied when the factor preparation was submitted to specific reactions of ADP-ribosylation and/or phosphorylation, which suggested that some of the EF-2 forms found 'in vivo' were ADP-ribosylated and/or phosphorylated.

In order to characterize every native EF-2 form, EF-2 was submitted to ADP-ribosylation and/or phosphorylation with either labeled or unlabeled reagents as indicated in table 1 (column 2), then the factor submitted to O'Farrell two-dimensional gel electrophoresis and every native EF-2 form was identified with regard to the labeled ones. The stained electrophoretograms and their corresponding autoradiograms are shown in fig.1 and the results of these summarized in table 1. As indicated in section 2 there was no possibility of mismatching any EF-2 spot from one experiment to another. Results of experiments 1–4, repeated 5 times, showed that of the spots (a–e) seen in native EF-2, c and e (or at least part of them) subsisted to all reactions while a, b and d disappeared after ADP-ribosylation (expt 2) and a fortiori after ADP-ribosylation + phosphorylation (expts 3,4). Moreover, the spots (a–e) seen in native EF-2 (expt 5) could be characterized as follows: a was never ADP-ribosylated nor phosphorylated and there-

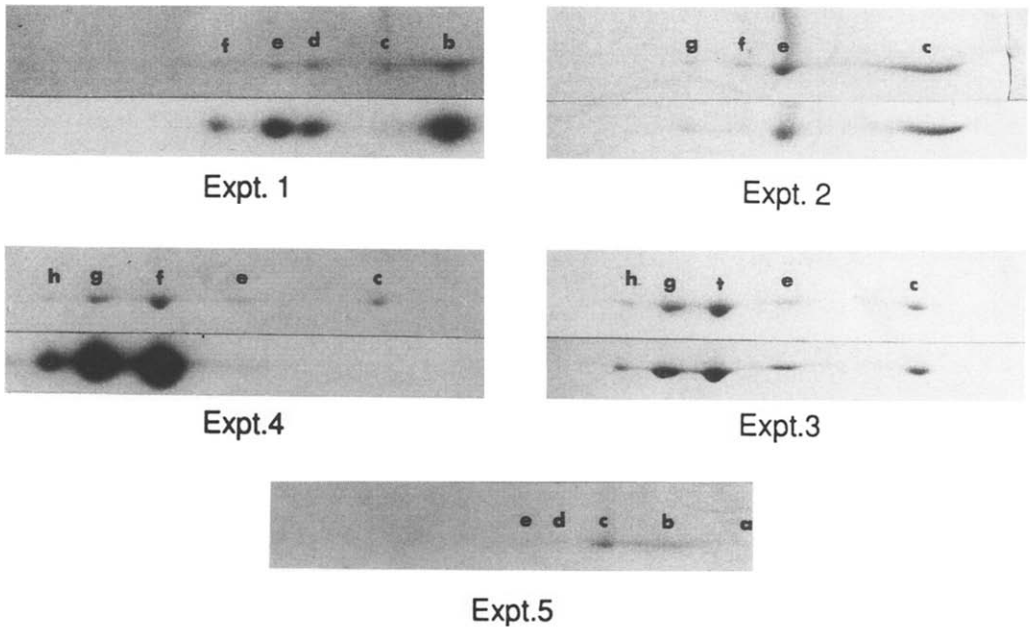

Fig.1. Two-dimensional equilibrium isoelectric focusing-SDS-polyacrylamide gel electrophoresis of EF-2 after phosphorylation and/or ADP-ribosylation. EF-2 was submitted to [^{32}P]phosphorylation (expt 1), [^{14}C]ADP-ribosylation (expt 2), phosphorylation + [^{14}C]-ADP-ribosylation (expt 3), and [^{32}P]phosphorylation + ADP-ribosylation (expt 4) as indicated in the legend of table I and in section 2 before being electrophoresed according to [8] in the presence of 9.5 M urea and using a pH 5–8 gradient. For the control in expt 5, EF-2 was incubated in buffer A for 15 min at 37°C + 2 min at 30°C. Each gel stained with Coomassie blue (top of each set of photographs) was dried and autoradiographed 16 h at −70°C (bottom).

fore represented unmodified EF-2; b and d were phosphorylated (expt 1); c was ADP-ribosylated (expt 2), which is a new finding; e was a mixture of two forms: one ADP-ribosylated (e_1) and one phosphorylated (e_2). Indeed e was either ADP-ribosylated or phosphorylated during ADP-ribosylation or phosphorylation experiments (expts 2,1), but only ADP-ribosylated and no longer phosphorylated after both ADP-ribosylation + phosphorylation (expts 3,4): the phosphorylated form (e_2) seen in expt 1 had undergone ADP-ribosylation in expt 4 and had then migrated to a more acidic region with either f, g or h. These 3 forms were labeled in both expt 3 and expt 4 and therefore were both ADP-ribosylated and phosphorylated. None of these forms were detected in vivo even after repeated silver staining. The appearance of f and g only after the phosphorylation reaction (expt 1) (g becoming clearly visible after a longer exposure) strengthened our conclusion that the EF-2 preparation contained in vivo ADP-ribosylated forms and indicated that these were capable of being phosphorylated. Likewise the appearance of f, g, h only after the ADP-ribosylation reaction (expt 2) (h becoming visible after a longer exposure) agreed with the existence of an endogenously phosphorylated EF-2 and indicated that these were capable of being ADP-ribosylated.

Since there is only one site (diphtamide 715) for ADP-ribosylation on the EF-2 molecule, the multiplicity of the EF-2 forms suggested that some forms could have more than one phosphorylation site, which would be in agreement with [2]. We found that the different forms of EF-2 were phosphorylated on threonine only, in agreement with [1,2]: the same phosphopeptide was found after extensive trypsinolysis of f and g, probably that sequenced by Nairn and Palfrey [1], and a more acidic phosphopeptide was obtained from h treated under the same conditions (results not shown). In order to determine the degree of phosphorylation of each form, specific radioactivity (^{32}P SR) of every spot was determined, as indicated in section 2, and then compared to that of the other EF-2 forms in expt 1 (after rapid ^{32}P-phosphorylation: 2 min at 30°C) and expt 4 (after extensive ^{32}P-

Table 1

Comparison from ADP-ribosylation and/or phosphorylation of the different EF-2 'spots[i]

Expt	Reactions[ii]	Characteristics[iii]	EF-2 spots[iv]								
			a	b	c	d	e		f	g	h
							e_1	e_2			
			(I+II)	(Ip)	(IA)	(IIp)	(IIA)	(Ipp)	(IpA)	(IIpA)	(IppA)
1	[^{32}P]phosphoryl.	SI	68*	54	18	13		9	n.d.		
		^{32}P LI	0	90	0	27		33	21		
		^{32}P SR	0	1.7	0	2.1		3.7	1.9*		
2	[^{14}C]ADP-ribosyl.	SI	0	0	66	0	41		10	8	
		^{14}C LI	0	0	65	0	38		9	9	
		^{14}C SR			1.0		0.9		0.9	1.1	
3	phosphoryl. + [^{14}C]ADP--ribosyl.	SI	0	0	31	0	20		54	47	16
		^{14}C LI	0	0	29	0	21		82	66	23
		^{14}C SR			0.9		1.0		1.5	1.4	1.4
4	[^{32}P]phosphoryl. + ADP-ribosyl.	SI	0	0	18	0	12		46	28	5
		^{32}P LI	0	0	0	0	0		160	109	36
		^{32}P SR			0		0		3.5	3.9	7.2
		^{32}P SR/^{14}C SR (expt 3)							2.3	2.8	5.1

[i]Results were from the two-dimensional electrophoregrams and autoradiograms seen in fig. 1

[ii] Every sample was incubated under the conditions described in section 2, as follows: in expt 1, EF-2 was preincubated in buffer A for 15 min at 37°C (the duration of the ADP-ribosylation assay) before being phosphorylated for 2 min at 30°C; in expt 2, EF-2 was preincubated for 2 min at 30°C (the duration of the phopshorylation assay) before being ADP-ribosylated for 15 min at 37°C; in expts 3 and 4, EF-2 was both phosphorylated (2 min at 30°C) and ADP-ribosylated (15 min at 37°C)

[iii] Staining intensity (SI) and ^{32}P or ^{14}C labeling intensity (^{32}P LI, ^{14}C LI) were determined from direct scanning of the photographs seen in fig.1. Specific radioactivity (^{32}P SR or ^{14}C SR) of every EF-2 spot was determined as indicated in section 2.

[iv] Each EF-2 spot was indicated to be derived from either EF-2 I or EF-2 II and to be ADP-ribosylated (A), monophosphorylated (p), biphosphorylated (pp) or both (pA or ppA) as indicated in the text

* Mean of values obtained from two experiments identical to expt 1 and performed at the same time, during which: form a had penetrated into the first dimension gel (this was not the case in expt 1: there are often slight variations at the basic end of the isoelectric focusing gels, see [8]) and the gel was more uniformly destained around f than in expt 1

phosphorylation and ADP-ribosylation: duration of phosphorylation, 2 min at 30°C; duration of ADP-ribosylation, 15 min at 37°C). In expt 4, the ^{32}P SR/^{14}C SR ratio was also determined using the ^{14}C SR values from the corresponding spots in expt 3. Comparison of the SR values listed in table 1, allowed us to complete the characterization of the labeled EF-2 spots (expt 1) as follows: assuming that b and d were monophosphorylated, f was both monophosphorylated and ADP-ribosylated, e_2 was biphosphorylated and its amount was larger than that of e_1 (since the ^{32}P SR value of e was about twice that of b, d and f which would not be the case if e_2 was not largely predominant). d had a ^{32}P SR value 24% higher than that of b for it was less diluted by endogenously phosphorylated EF-2 (cf. b and d in control expt 5, fig.1). That monophosphorylated forms, b and d, had different pH_i values indicating that they were probably derived from two slightly different EF-2 species (I and II). Comparison of ^{32}P SR and ^{32}P SR/^{14}C SR values for the different spots in expts 4 and 3 allowed us to characterize completely the g and h spots. g as f was both monophosphorylated and ADP-ribosylated, and because their different pH_i values, f and g are probably also derived from two different EF-2 species, very likely the I and II species already discussed. h was both biphosphorylated and ADP-ribosylated. Indeed this form was characterized by ^{32}P SR and ^{32}P SR/^{14}C SR values about twice those of f and g. That f and g had a ^{32}P SR value in expt 4 about twice as high as that of f in expt 1 is an indication of a lower dilution by endogenously phosphorylated forms in expt 4 than in expt 1. Indeed the unmodified EF-2 (I + II) forms (a), transformable into the f, g, h ones, had been completely transformed in expt 4 but subsisted in a relatively high amount in expt 1 (see table 1). More-

over an analysis of the staining compared with the labeling intensities of every EF-2 spot, tentatively characterized as indicated in table 1 (3rd line), allowed us to deduce that f, g, h seen in expt 4 derived from a through either b, d and e_2, which totally disappeared from expt 1 to 4, or c and e_1, whose amount diminished from one half to two thirds when comparing expt 2 with expts 3 and 4, but never disappeared. This indicated that phosphorylated forms: b, d and e_2 were totally ADP-ribosylatable. The remaining ADP-ribosylated forms c and e_1 seen in expt 4 might result from the action of phosphatase(s), present in the cytosol extract used for phosphorylation, on part of f, g, h forms. The other possibility that ADP-ribosylated forms c and e_1 were only partly phosphorylatable, although less likely, could not be totally discarded. Comparison of ^{14}C SR values of EF-2 spots in expts 2 and 3 indicated that after extensive phosphorylation (expt 3), f, g and h had an SR value slightly higher than c and e_1 in expts 2 and 3 and higher than f and g in expt 2. This was most likely due to a lower dilution of the ^{14}C labeling by in vivo ADP-ribosylated factor in the first case compared with the other ones. In order to provide supplementary evidence that native EF-2 contained phosphorylated and ADP-ribosylated forms, the factor was treated with alkaline phosphatase or with diphtheria toxin and nicotinamide under conditions of de-ADP-ribosylation [5]. Both treatments considerably decreased the solubility of the factor. Most of this did not penetrate into the gel and the remaining forms were poorly resolved.

The amounts of native ADP-ribosylated and phosphorylated EF-2 forms could be estimated by comparing the staining intensity of the major forms c and b seen in native EF-2 (expt 5) to that of the total EF-2 forms, which was determined in several experiments under different conditions. These estimations were about 20% and 40%, respectively. However, in the cell the amount of endogenous ADP-ribosylated EF-2 is probably higher than that estimated here becaue at each step of EF-2 purification we collected the fractions which were the most active in the [^{14}C]ADP-ribosylation assay with diphtheria toxin. The amount of endogenous phosphorylated forms is probably also underestimated due to the action of phosphatases during the first step of EF-2 preparation. The existence of native ADP-ribosylated EF-2 could explain why, in all our experiments, EF-2 accepted only 0.6 to 0.8 mol of ADP-ribose/mol. The fact that the relative amounts of several EF-2 derivatives probably varied according to the state of the cell could also explain why during the purification steps EF-2 was eluted from ion-exchange columns under slightly different conditions from one experiment to the other. The minor form (II) of native EF-2 could be estimated to represent less than 25% of total EF-2, from the ratio value of the staining intensity of g/staining intensity of whole EF-2 determined in expt 4 (disregarding e_1 which should also be derived from EF-2 II). EF-2 II could be due to a proteolysis of native EF-2 near one end of the molecule, which would not change its M_r significantly, or to the presence of bound GDP, which would resist electrophoresis and modify its pH_i, or to differences in the primary structure of the molecule.

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