PRIMARY STRUCTURE AT THE SITE IN BEEF AND WHEAT ELONGATION FACTOR 2 OF ADP-RIBOSYLATION BY DIPHTHERIA TOXIN

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1. Introduction

Elongation factor 2 (EF-2) is the eukaryotic protein which catalyzes the translocation of the nascent peptide chain on the ribosome during the elongation phase of protein synthesis. EF-2 is selectively inactivated by covalent ADP-ribosylation catalyzed by diphtheria toxin [1,2]. In vitro studies of this reaction show that EF-2 from all eukaryotic sources tested is specifically modified by the toxin while other proteins, including the prokaryotic analog of EF-2, elongation factor G, are not [3]. The site of ADP-ribosylation in EF-2 from rat liver [4] and yeast [5] has been shown to be a new amino acid, amino acid X, which presumably results from a post-translational modification of a standard amino acid. The exact chemical structure of the modified residue or the functional nature of the inactivation has yet to be determined.

In an effort to define the primary sequence requirements in EF-2 for the diphtheria toxin reaction and to make phylogenic comparisons between EF-2s from widely varied sources, the amino acid sequences of the trypsin-derived ADP-ribosyl-peptides of EF-2 from beef liver and wheat germ were examined. These sequences are compared with those reported for rat liver [4] and yeast [5]. All 4 peptides are closely homologous in amino acid sequence and contain amino acid X, the site of ADP-ribose attachment, in the penultimate position.

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2. Materials and methods

2.1. *Purification of the peptides*

The assay and partial purification of EF-2 and the isolation of pure tryptic peptides from beef liver and wheat germ were performed by modifications of the procedure in [5]. Beef liver (600 g) was homogenized with 700 ml of Littlefield's medium (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–Cl (pH 7.8)) [4] in a Waring blender followed by centrifugation at 100 000 × *g* for 10–12 h in Littlefield's medium plus 0.5 M NH_4Cl. The ammonium sulfate precipitation step was omitted. The supernatant was dialyzed into buffer A (20 mM Tris–phosphate (pH 7.8), 0.1 mM EDTA, 5 mM β-mercaptoethanol) and applied to a DEAE–Sephadex A-50 column (6 × 63 cm) equilibrated with buffer A. EF-2 was eluted from the column with buffer B (200 mM Tris–phosphate (pH 7.8), 300 mM KCl, 0.1 mM EDTA, 5 mM β-mercaptoethanol). After dialysis back into buffer A, the EF-2 was chromatographed on a second DEAE–Sephadex A-50 column (4 × 43 cm), which was developed with a 5 l linear gradient of buffer A and buffer B. This yielded EF-2 with a spec. act. 0.1 μmol/mg protein.

EF-2 from wheat germ (300 g) was purified in the same manner with modifications in buffers (breaking buffer, 20 mM Tris · Cl (pH 7.8), 50 mM KCl, 1 mM $MgAc_2$, 2 mM $CaCl_2$, 5 mM β-mercaptoethanol [6]; buffer A, 20 mM Tris · Cl (pH 7.8), 100 mM KCl, 2 mM $MgAc_2$, 5 mM β-mercaptoethanol; buffer B, 20 mM Tris · Cl (pH 7.8), 300 mM KCl, 2 mM $MgAc_2$, 5 mM β-mercaptoethanol) and with appropriately smaller columns and gradient. This yielded EF-2 with a spec. act. 0.7 μmol/mg protein.

Partially purified EF-2 from either source was modified with 2 μg/ml diphtheria toxin (Connaught Med. Res. Labs) and 5–10 μM [^{3}H]NAD [5]. Following proteolytic digestion, the trypsin-derived [^{3}H]ADP-ribosyl peptide was chromatographed on a DEAE–Sephadex A-25 column (2 × 32 cm) equilibrated in 20 mM Tris · Cl (pH 7.4) and eluted with an 800 ml linear gradient of increasing NaCl (final conc. 500 mM for the beef peptide and 250 mM for the wheat peptide). The radioactive peak was lypholyzed, desalted on a Sephadex G-15 column (2 × 32 cm), loaded onto an acetylated dihydroxyboryl-substituted aminoethyl cellulose (acetylated DBAE-cellulose) column (0.7 × 10 cm) in 350 mM NH_4Ac (pH 8.0) and eluted with 350 mM NH_4Ac (pH 5.0). This yielded wheat-germ peptide (0.7 nmol/g wheat germ) that was pure as judged by sequence and amino acid analyses. The beef-liver peptide required further purification by removal of the ADP-moiety with phosphodiesterase and alkaline phosphatase and rechromatography in DEAE–Sephadex A-25 [4] and yielded 0.2 nmol peptide/g liver. Prior to sequence analysis of either peptide, the ADP moiety was enzymatically removed [4].

2.2. *Amino acid and sequence analyses*

Amino acid analyses of the ADP-ribosyl and ribosyl peptides were performed using a single column program on a Beckman model 119 analyzer which utilized an expanded scale and an Infotronics CS210 integrator. Amino acid X was identified by chromatography on a short column using a tryptophan program [4,7]. Automated sequence analyses of the ribosyl peptides were performed on a Beckman Sequencer model 890C using repetitive Edman degradation with a modified 0.1 M Quadrol buffer program [8]. Initial coupling was routinely 85% and repetitive yield 88% with < 30% overlap. The phenylthiozolinone derivatives were converted into phenylthiohydantoin derivatives and identified either by high-pressure liquid chromatography or amino acid analysis following acid hydrolysis [9].

3. Results and discussion

Purification of the trypsin-derived ADP-ribosyl peptides from eukaryotic EF-2s is greatly facilitated by the specificity of the diphtheria toxin reaction. Quantitative modification by toxin of partially purified EF-2 (enriched as little as 20-fold) was achieved and a single ADP-ribosyl peptide was recovered from either source following trypsin digestion. The presence of the diols in the ADP-ribosyl peptide also permits the use of acetylated DBAE-cellulose, which retains diols selectively, resulting in a rapid affinity purification step effecting > 100-fold enrichment.

The amino acid analyses of the trypsin-derived ADP-ribosyl peptides from beef liver and wheat germ EF-2s are shown in table 1. For comparison, the numbers in parentheses indicate the quantities of the amino acids derived from sequence analyses of the same peptides. Both peptides contained low levels of contaminating glutamic and aspartic acids. Contaminating lysine was also present in the beef peptide. The yield of serine for the wheat peptide was low presumably due to destruction during hydrolysis. Glycine appeared in a mole ratio of 1 when the ADP-

Table 1
Composition of the tryptic peptides from two sources of elongation factor 2

Amino acid	Residue/molecule[a]	
	Beef liver[b]	Wheat germ[c]
Aspartic acid	3.5 (3)	2.4 (2)
Threonine	1.0 (1)	1.2 (1)
Serine	0	0.6 (2)
Glutamic acid	0.8	2.1 (1)
Glycine	0	1.9 (1)
Alanine	2.1 (2)	1.3 (1)
Valine	2.3 (2)	3.0 (3)
Isoleucine	1.0 (1)	1.8 (2)
Leucine	1.4 (1)	1.2 (1)
Amino acid X	0.8 (1)	0.8 (1)
Phenylalanine	1.0 (1)	0.6 (1)
Histidine	1.8 (2)	1.1 (1)
Lysine	0.3	0
Arginine	1.0 (1)	1.0 (1)
Total	17.0 (15)	19.0 (18)

[a] This ratio represents the average of 2 determinations and was calculated assuming 1.0 residue arginine/molecule. Amino acids present at < 0.2 residue/molecule are not included. The numbers in parentheses were derived from sequence analysis

[b] Amino acid analysis of the ribosyl peptide

[c] Amino acid analysis of the ADP-ribosyl peptide

	1	5	10	15
Rat Liver (4)	Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg			
Beef Liver	Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg			
Yeast (5)	Val-Asn-Ile-Leu-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg			
Wheat Germ	Gly-Ile-Ser-Phe-Glu-Val-Ser-Asp-Val-Val-Leu-His-Thr-Asp-Ala-Ile-X-Arg			

Fig.1. Sequences of trypsin-derived ribosyl peptides of elongation factor 2 from 4 eukaryotic sources.

ribosyl peptide from either source was analyzed, resulting from the acid hydrolysis of adenosine [10].

It was observed in the rat-liver peptide [4] and yeast peptide [5] that an unusual amino acid, amino acid X, was the site of ADP-ribosylation. This residue migrates in the region of tyrosine on the single column amino acid analysis program, but in a unique position on a short column program designed to separate tryptophan and basic amino acids. The peptides from both beef liver and wheat germ contain this residue and sequence analysis confirmed that the penultimate amino acid X is the recipient of ADP-ribose by the criteria in [5].

The primary structure of the trypsin-derived ribosyl peptides from all 4 sources is shown in fig.1. The mammalian peptides have identical amino-acid sequences and all 4 peptides are identical in the pentapeptide region of the C-terminus surrounding amino acid X. The wheat-germ peptide is 18 residues long with the additional amino acids extending from the N-terminus. It has 11 residues in common with the mammalian peptides and 9 in common with the yeast peptide. The underlined residues in fig.1 indicate the locations at which the amino acids differ from those found in the mammalian peptides. Thus, the trypsin-derived ADP-ribosyl peptide sequences from these 4 sources are highly conserved and all contain amino acid X, suggesting that this region serves an indispensable function in protein synthesis in addition to being the site of the diphtheria toxin reaction.

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References

[1] Collier, R. J. (1975) Bacteriol. Review 39, 54–85.
[2] Pappenheimer, A. M. (1977) Ann. Rev. Biochem. 46, 69–94.
[3] Tiboni, O. and Ciferri, O. (1971) FEBS Lett. 19, 174–179.
[4] Robinson, E. A., Henriksen, O. and Maxwell, E. S. (1974) J. Biol. Chem. 249, 5088–5093.
[5] Van Ness, B. G., Howard, J. B. and Bodley, J. W. (1978) J. Biol. Chem. 253, 8687–8690.
[6] Legocki, A. B. and Marcus, A. (1970) J. Biol. Chem. 245, 2814–2818.
[7] Simpson, R. J., Neuberger, M. R. and Liu, T. Y. (1976) J. Biol. Chem. 251, 1936–1940.
[8] Braur, A. W., Margolies, M. N. and Haber, E. (1975) Biochemistry 14, 3029–3035.
[9] Lundell, D. J. and Howard, J. B. (1978) J. Biol. Chem. 253, 3422–3426.
[10] Kruger, M. (1892) Hoppe-Seyler's Z. Physiol. Chem. 16, 160–172.