RECOGNITION OF ELONGATION FACTOR 2 BY DIPHTHERIA TOXIN IS NOT SOLELY DEFINED BY THE PRESENCE OF DIPHTHAMIDE

Brian G. VAN NESS*, Barbara BARROWCLOUGH and James W. BODLEY
Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455, USA

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1. Introduction

Diphtheria toxin kills cells by inhibiting protein synthesis through the ADP-ribosylation of protein synthesis elongation factor 2 (EF-2) [1,2]. In this reaction, the catalytically active fragment of the toxin, fragment A, exibits exceedingly narrow specificity. The only known substrate for the enzyme under the usual reaction conditions is EF-2. Not only is EF-2 the only substrate in toxin-sensitive cells but apparently toxin will ADP-ribosylate EF-2 from all eukaryotic sources.

We have found [4–6] that all cases the ADP-ribose is attached by diphtheria toxin to EF-2 via an unusual amino acid, diphthamide, which is not known to occur in other proteins. Diphthamide appears to be an unusual derivative of histidine for which we have proposed the structure 2-[3-carboxyamide-3-(trimethylammonio)propyl] histidine [3]. These findings raise the possibility that toxin recognizes EF-2 simply by the presence of this unusual amino acid derivative. In the course of examining the interaction of toxin with EF-2 we have observed that toxin does not ADP-ribosylate the trypsinderived peptide which contains diphthamide. Hence it would appear that toxin recognizes some additional feature(s) of EF-2 besides the presence of diphthamide in peptide bond. We report these findings here.

2. Materials and methods

[*adenylate*-^{32}P]NAD$^+$ and [*adenine*-2,8-^{3}H]NAD$^+$ were purchased from New England Nuclear Corp.

* Present Address: Institute for Cancer Research, Fox Chase, Philadelphia, PA 19111, USA

Yeast EF-2 was prepared (~10% pure) as in [6] and in some cases was further purified (~40% pure) by chromatography on DEAE-cellulose and Sephadex G-150. The trypsin-derived [^{32}P]ADP-ribosyl peptide was also prepared as in [4]. Published methods were used for the toxin-dependent ADP-ribosylation, trypsin digestion reactions [4] and for sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [7].

3. Results

EF-2 from various sources is a single peptide chain of M_r ~100 000 [2]. In rat liver extracts the protein can be acted upon by endogenous proteases to significantly reduce the size of the ADP-ribose acceptor when analyzed in denaturing systems [8]. We have observed similar results with the yeast protein but in no case did we observe fragments smaller than ~25 000 M_r. Fig.1 shows an autoradiogram of an SDS gel of yeast EF-2 which was [^{32}P]ADP-ribosylated before and after various times of treatment with trypsin. Under these conditions the intact chain was rapidly cleaved so that the major [^{32}P]ADP-ribose acceptor exhibited ~ 25 000–35 000 M_r. Prolonged treatment did not produce smaller labeled fragments.

The experiment shown in fig.2 examines the effects of trypsin digestion on the amount of ADP-ribosylation of EF-2 by toxin. The open symbols describe the results of experiments in which [^{3}H]ADP-ribosyl-EF-2 formation was determined as a function of the time of treatment with two concentrations of trypsin (trypsin:protein ratios of 1:50 and 1:1, respectively). Most of the radioactive material remained insoluble in acid with both trypsin levels.

In contrast, the closed symbols show that very little acid-insoluble material was observed when EF-2 was

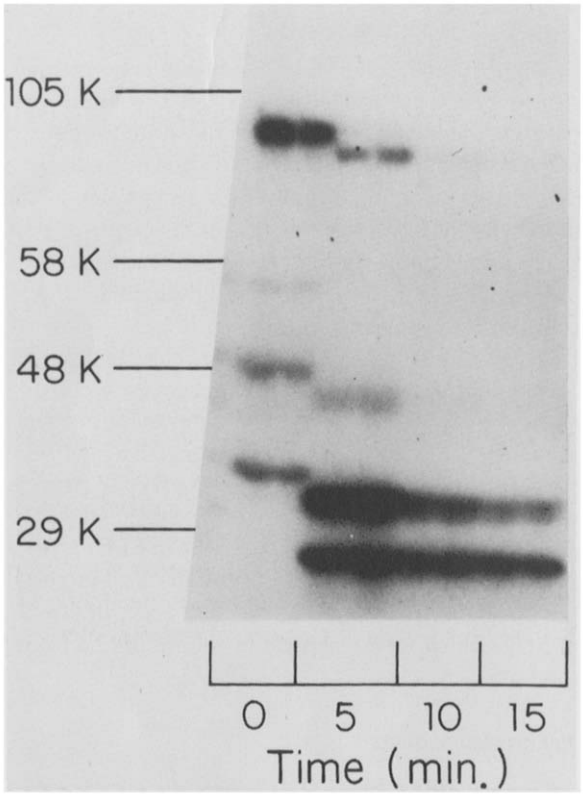

Fig.1. Autoradiogram of an SDS gel of EF-2 which was [^{32}P]-ADP-ribosylated with diphtheria toxin after various periods of digestion with trypsin. EF-2 was digested with trypsin (trypsin:protein ratio, 1:100) for the indicated times at 37°C. The digestion was terminated by the addition of soybean trypsin inhibitor (1 mg/ml). The protein was then [^{32}P]ADP-ribosylated with diphtheria toxin and [^{32}P]NAD$^+$ [4] and subjected to SDS slab-gel electrophoresis [9] and autoradiography. The mobilities of standard proteins and their molecular weights are indicated to the left.

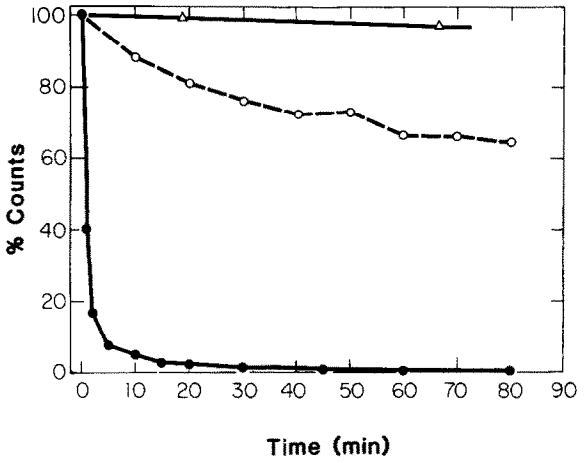

Fig.2. The effect of trypsin digestion of EF-2 before and after its ADP-ribosylation by toxin. In one case [^{3}H]ADP-ribosyl EF-2 was digested for the indicated times at 37°C with either 1:50 trypsin (△) or 1:1 trypsin (○) and the reactions were precipitated with 10% trichloroacetic acid and counted. In comparison EF-2 (●) was first digested with trypsin (1:50) and the reaction was stopped with soybean trypsin inhibitor (1 mg/ml). Diphtheria toxin and [^{3}H]NAD$^+$ were added and after 15 min incubation at 37°C the samples were precipitated with 10% trichloroacetic acid and counted. In each case the results are expressed as the percent of radioactivity precipitated when trypsin inhibitor was added prior to trypsin.

treated with trypsin (1:50) prior to [^{3}H]ADP-ribosylation with toxin. These latter results indicate that either the [^{3}H]ADP-ribose is attached to an acid-soluble fragment of EF-2 or that trypsin cleavage has altered the protein so that it is not recognized by the toxin.

We have observed that the ADP-ribosyl peptide obtained by complete digestion of EF-2 by trypsin is acid-soluble (not shown). Therefore, to distinguish between the two possibilities raised above we employed gel permeation chromatography to determine if the tryptic peptide which contains diphthamide is a substrate for diphtheria toxin. A preparation of partially purified EF-2 was divided in half. One half was [^{3}H]ADP-ribosylated, digested with trypsin under denaturing conditions which lead to complete proteolytic cleavage [4], and the resulting peptides were chromatographed on Sephadex G-15 (fig.3). The radioactive ADP-ribosyl-peptide was observed in the column void volume (open symbols). In contrast, the remaining portion of the EF-2 preparation was first degraded with trypsin and the peptides were reacted with toxin and [^{3}H]NAD$^+$. The closed symbols in fig.3 show that under these circumstances all of the radioactivity was recovered as [^{3}H]NAD$^+$ and none as the [^{3}H]ADP-ribosyl peptide. Employing the rat liver protein Maxwell (personal communication) has also observed that the trypsin-derived peptide is not ADP-ribosylated by toxin.

Further evidence that the trypsin-derived peptide is not a substrate for toxin was provided by the reversibility of the toxin reaction [1,2]. The [^{3}H]ADP-ribosyl peptide was reacted with toxin and nicotinamide at acid pH, conditions which favor the reverse

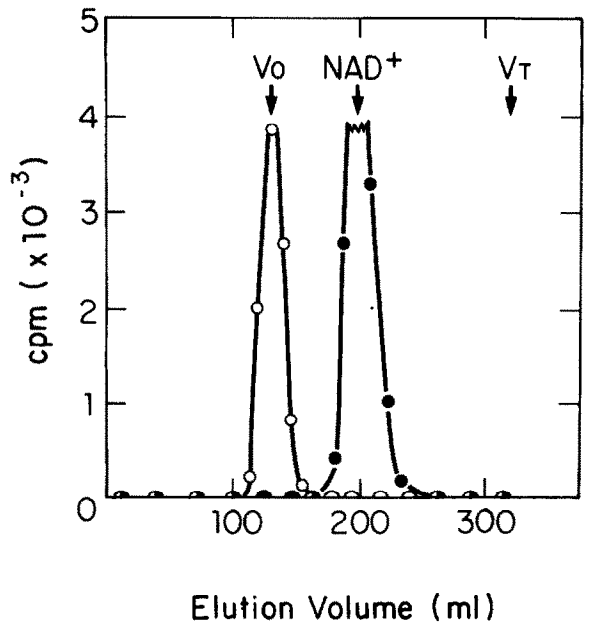

Fig.3. Gel permeation analysis of the [^{3}H]ADP-ribosyl peptide. In the experiment represented by the open circles, 50 ml of an EF-2 preparation (3.2 mg/ml) was [^{3}H]ADP-ribosylated with [^{3}H]NAD$^+$ (10 μM, 1 Ci/mmol) and toxin (30 min at 20°C) and the protein was precipitated with 10% trichloroacetic acid and washed. The precipitate was dissolved in 50 ml buffer and digested at 37°C with two additions of trypsin (1:100) followed by 5 mg soybean trypsin inhibitor. Ten ml of this solution was applied to a 2.6 × 53 cm column of Sephadex G-15 equilibrated and eluted with 20 mM ammonium acetate (pH 8.0). Fractions were collected and aliquots were counted. In the experiment represented by the closed circles, 50 ml of the same EF-2 preparation was first precipitated with 10% trichloroacetic acid and the precipitate was washed and dissolved in 50 ml buffer. The protein was digested with trypsin and the reaction was terminated with trypsin inhibitor as above. Diphtheria toxin and [^{3}H]NAD$^+$, in the levels used above, were then added and the solution was incubated as described. Ten ml of this solution was applied to the same Sephadex G-15 column and aliquots of the eluting fractions were counted. The V_0 and V_T designations in the figure represent the column void and total volumes, respectively.

reaction. Upon analysis by thin-layer chromatography, all of the radioactivity was recovered in the original peptide and none as [^{3}H]NAD$^+$ (not shown). Under comparable conditions using the intact protein the reaction was 50–90% reversible.

4. Discussion

Diphthamide, occurs within a highly conserved sequence in apparently all EF-2 s [5]. The amino acid in this protein is the only known substrate for diphtheria toxin and it is reasonable to conclude that its presence plays an important part in recognition of the protein by the toxin. Indeed, the results in [9] suggest that complete post-translational modification of the presumed histidine progenitor of diphthamide is required for toxin recognition.

These results indicate that EF-2 can sustain only a limited number of proteolytic cleavages before it is no longer recognized by diphtheria toxin. Moreover, the toxin appears to be completely inactive in either the forward or back reaction with the tryptic peptide which contains diphthamide. It thus appears that diphtheria toxin recognizes something in EF-2 beyond the presence of diphthamide. These findings suggest the possibility that diphthamide may occur in other proteins which are not substrates for diphtheria toxin.

Acknowledgements

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References

[1] Collier, R. J. (1975) Bacteriol. Rev. 39, 54–85.
[2] Pappenheimer, A. M. jr (1977) Annu. Rev. Biochem. 46, 69–94.
[3] Van Ness, B. G., Howard, J. B. and Bodley, J. B. (1980a) J. Biol. Chem. in press.
[4] Van Ness, B. G., Howard, J. B. and Bodley, J. W. (1978) J. Biol. Chem. 253, 8687–8690.
[5] Brown, B. A. and Bodley, J. W. (1979) FEBS Lett. 103, 253–255.
[6] Van Ness, B. G., Howard, J. B. and Bodley, J. W. (1980b) J. Biol. Chem. in press.
[7] Skar, D. C., Rohrbach, M. S. and Bodley, J. W. (1975) Biochemistry 14, 3922–3926.
[8] Collins, J. F., Raeburn, S. and Maxwell, E. S. (1971) J. Biol. Chem. 246, 1049–1054.
[9] Moehring, J. M., Moehring, T. J. and Danley, D. E. (1980) Proc. Natl. Acad. Sci. USA 77, 1010–1014.