

Clostridium perfringens iota toxin ADP-ribosylates skeletal muscle actin in Arg-177

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Clostridium perfringens iota toxin ADP-ribosylates actin. Substrates of *C. perfringens* toxin are both non-muscle β/γ -actin and skeletal muscle actin. This finding suggests that *C. perfringens* iota ADP-ribosylates the same amino acid in skeletal muscle and non-muscle actin as does *C. botulinum* C2 toxin in non-muscle actin. Protein chemical analysis involving thermolysin cleavage on [³²P]ADP-ribosylated actin or tryptic digestion followed by a secondary thermolysin cleavage of the radiolabelled fragments showed one major site of ADP-ribosylation. From its amino acid composition and sequence, the radiolabelled peptide was identified as peptide 175–177, locating the acceptor ADP-ribosyl amino acid as Arg-177.

ADP-ribosylation; Actin; Iota toxin; C2 toxin; (*C. perfringens*, *C. botulinum*)

1. INTRODUCTION

Various microbial toxins such as diphtheria, cholera and pertussis toxins interfere with eukaryotic cell functions by ADP-ribosylation of regulatory GTP-binding proteins [1–3]. Recently, it has been shown that *Clostridium botulinum* C2 toxin and *C. perfringens* iota toxin belong to a new class of microbial ADP-ribosyltransferases, whose eukaryotic substrate is actin [4–7]. Both clostridial toxins show striking similarities: they are binary in structure and consist of a low- and a high-molecular-mass component [8,9]. In both cases, the high-molecular-mass components (iota toxin, 78 kDa; C2 toxin, 88 kDa) are apparently involved in binding of the toxins to eukaryotic cell membrane, while the low-molecular-mass components (~50 kDa) possess ADP-ribosyltransferase activity [6,10,11]. Both toxins apparently modify G- but

not F-actin and ADP-ribosylation caused by either toxin severely reduces the ability of actin to polymerize [4,5,7]. However, both toxins are different in their substrate specificity. Whereas iota toxin ADP-ribosylates non-muscle and skeletal muscle actin with similar efficiency, the latter is almost not modified by C2 toxin [7]. Recently, it has been shown that C2 toxin ADP-ribosylates platelet β/γ -actin in Arg-177 [12]. In order to gain more insight into the substrate specificity of the toxins, in the present study we analyzed the ADP-ribose acceptor amino acid of skeletal muscle actin modified by iota toxin.

2. MATERIALS AND METHODS

2.1. Materials

C. perfringens iota toxin and *C. botulinum* C2 toxin were purified from culture medium of *C. perfringens* type E strain CN 5063 and *C. botulinum* type C strain 9243, kindly donated by Drs S. Thorley (Wellcome Biotech, Beckenham, England) and S. Nakamura (Kanazawa, Japan), respectively, essentially as described [9]. All other

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reagents were of analytical grade and obtained from commercial sources.

2.2. Purification of actin isoforms

Human platelet cytoskeletal actin was purified according to [13] with an additional gel filtration step as in [12]. Rabbit skeletal muscle was purified as described [14].

2.3. SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed according to [15]. Gels were stained with Coomassie brilliant blue, destained, and autoradiographed on Kodak X-Omat AR films.

2.4. ADP-ribosylation assay

ADP-ribosylation was performed as in [5]. Briefly, about 1 mg rabbit skeletal actin (in G-buffer [14]) was ADP-ribosylated in a medium containing 10 mM thymidine, 10 mM dithiothreitol, 1 mM $MgCl_2$, 1 mM EDTA, 1–4 μg iota toxin, 10 μM [^{32}P]NAD (about 10 μCi) and triethanolamine-HCl (pH 7.5) in a total volume of 1 ml for 30 min at 37°C. Thereafter, unlabelled NAD was added to a final concentration of 0.5 mM and the reaction was continued for a further 2 h. Unreacted NAD was separated by centrifugation through centricon 30 (Amicon, Witten, FRG). Thereafter, actin was denatured in 3 M guanidine-HCl and precipitated in ethanol.

2.5. Protein-chemical analysis of ADP-ribosylated actin

The identification of ADP-ribosylated amino acid in actin was carried out essentially as described for *C. botulinum* C2 toxin-ADP-ribosylated actin [12]. Briefly, 1 mg [^{32}P]ribosylated actin ethanol precipitate was dissolved in formic acid (98%) at 0°C and dialyzed against water with three changes over 4 h. The actin solution was made 0.5% in ammonium bicarbonate and digested with trypsin (25 μg) for 2 h at 37°C and finally lyophilized. Peptides were resuspended in 0.5 ml of pH 6.5 buffer (10% pyridine, 0.5% acetic acid) and the soluble and insoluble peptides separated by centrifugation in Eppendorf tubes. For further cleavage, the insoluble fragments were redissolved in 3 M NH_4OH , diluted in 10 vols water, adjusted to pH 8–9 with acetic acid and digested with thermolysin (1:50 enzyme/substrate

ratio) for 3 h at 55°C. The soluble tryptic peptides and the thermolysin secondary peptides were separated by a 'three-dimensional' combination of paper electrophoretic and chromatographic techniques, and detected by diluted fluorescamine staining [16]. The radioactive peptides were located by autoradiography, recovered from the paper by elution with pH 6.5 buffer, identified by amino acid analysis using a Biotronik (FRG) amino acid analyzer and sequenced with a gas-phase sequenator [17] equipped with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, USA).

In a separate experiment, [^{32}P]ADP-ribosylated actin (0.5 mg) was also directly digested with ther-

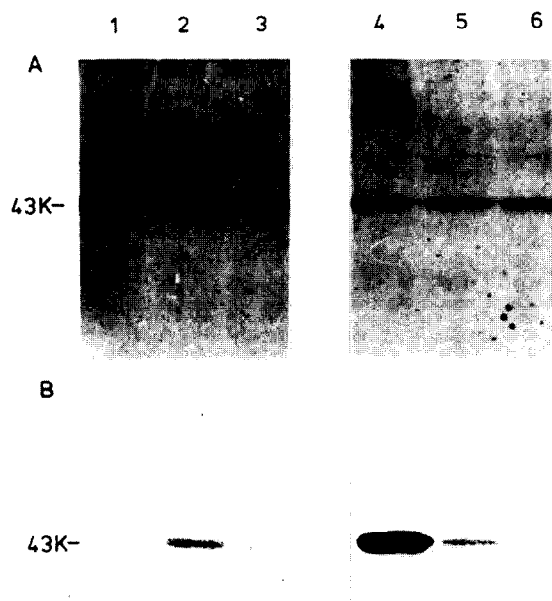


Fig.1. ADP-ribosylation of human platelet and rabbit skeletal muscle actin by *C. botulinum* C2 toxin and *C. perfringens* iota toxin. Rabbit skeletal muscle G-actin (4 μg) (lanes 1–3) and human platelet G-actin (2 μg) (lanes 4–6) were ADP-ribosylated with C2 toxin (1 $\mu g/ml$; lanes 1,4), iota toxin (0.4 $\mu g/ml$; lanes 2,5), and without toxin (lanes 3,6) in the presence of 5 μM [^{32}P]NAD ($\sim 0.2 \mu Ci$) for 30 min as described. Proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by Coomassie staining (A) or autoradiography (B).

molysin. The fragmentation was performed essentially as for the tryptic hydrolysis using an enzyme/substrate ratio of 1:60 (by wt). Thermolysin peptides were separated, detected, and identified as described above.

3. RESULTS

Fig.1 shows that human platelet β/γ -actin was ADP-ribosylated by both *C. perfringens* iota toxin (0.08 $\mu\text{g/ml}$) and *C. botulinum* C2 toxin (1 $\mu\text{g/ml}$). Minor labelling by iota toxin was due to application of a submaximally effective concentration of the toxin. Even at such a low concentration of iota toxin, rabbit skeletal muscle α -actin was also ADP-ribosylated. In contrast, C2 toxin did not ADP-ribosylate skeletal muscle actin at concentrations maximally effective in modifying platelet actin.

In order to locate the site of modification, [^{32}P]ADP-ribosylated skeletal muscle actin was digested with thermolysin. Paper peptide mapping, followed by autoradiography, revealed a single

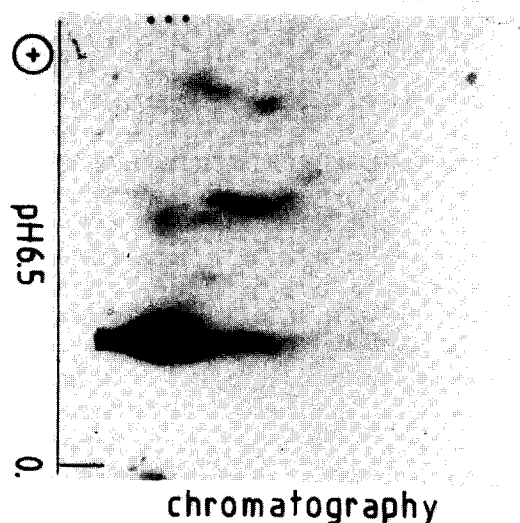


Fig.2. Two-dimensional paper peptide map of the thermolysin peptides of [^{32}P]ADP-ribosylated skeletal muscle actin. The start position is indicated by (0.); the first separation is by horizontal paper electrophoresis at pH 6.5 followed by vertical separation by paper chromatography. Only the anodic side (containing the acidic peptides) of the autoradiogram is shown. Note the presence of a single major radioactive spot.

major radiolabelled spot (fig.2). Staining the peptide fingerprint with diluted fluorescamine showed that no other thermolysin peptide comigrated with the radiolabelled peptide. This was further confirmed from the amino acid composition (Ile, 1.0; Met, 0.8; Arg, 0.7) and consecutive sequence analysis of the labelled peptide concluding for a sequence Ile-Met-X (fig.3). From the composition (the presence of equimolar quantities of arginine in the hydrolysate), the identified sequence (Ile-Met), and the known specificity of thermolysin, the ADP-ribosyl acceptor region could be identified as region 175–177. The presence of arginine in the acid hydrolysate but absence as a phenylthiohydantoin derivative in cycle 3 of the automated gas-phase degradation identifies Arg-177 as the modified residue.

The radiolabelled peptide was recovered with a final yield of 20% of total actin digested. Previous

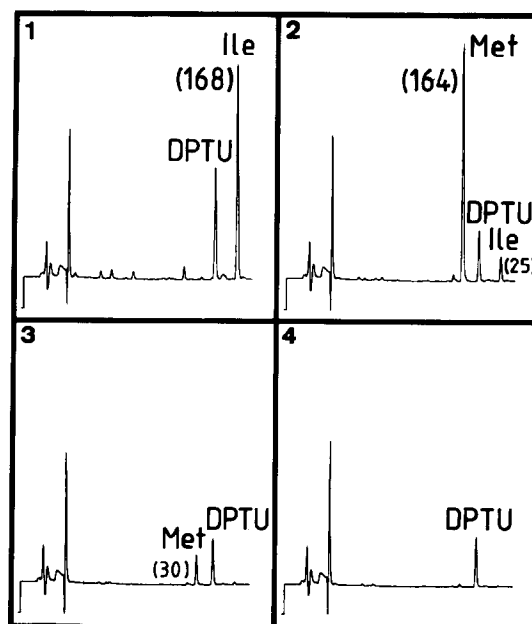


Fig.3. Traces of phenylthiohydantoin amino acid analyses of cycles 1–4 of the ADP-ribosylated thermolysin peptide of skeletal muscle actin. 1 nmol was loaded on the sequenator and 40% of the amount of PTH amino acid was analyzed. The initial coupling yield was 42%. Numbers in parentheses indicate pmol recovered. DPTU indicates the diphenylthiourea noted as the major byproduct and serving as an internal reference.

studies [7] have shown that the extent of in vitro ADP-ribosylation of skeletal muscle actin by the iota toxin amounts to values varying from 25 to 50% of total actin. This means that the radiolabelled fragment was recovered in yields ranging from 80 to 40% of maximally recoverable peptide. Such values are high when paper separation and elution techniques are used for purification. In addition, no other major radiolabelled peptide was observed and the amount of radioactivity recovered in this peptide was as high as 70% of the initial label. These high recovery values strongly argue for the existence of a unique site of ADP-ribosylation. Unlike its nonmodified basic derivative, the ADP-ribosyl Ile-Met-Arg peptide migrates as an acidic peptide. Its relative electrophoretic mobility (0.2 according to the Offord plot) [18] and very low chromatographic mobility are both arguments for a covalently linked ADP-ribosyl group adding two negative charges, a mass of 519 Da and a strong hydrophilic nature to the original tripeptide.

The position of the ADP-ribosylation acceptor amino acid was further confirmed from the results of a tryptic digestion of [32 P]ADP-ribosyl skeletal muscle actin. Such digestion yields a peptide mixture, a fraction of which is soluble at pH 6.5 and another fraction remains insoluble. The radiolabel is equally divided over both fractions. The labelled soluble tryptic peptide was recovered from the paper and identified by sequencing as a mixture of peptide 96–113 and the region covering residues 174–183. Previous actin amino acid studies [16] have shown that fragment 174–183 may be released as a soluble peptide by a partial nonspecific cleavage at the His-173–Ala-174 peptide bond from the insoluble peptide 148–183. When the 174–183 and 96–113 mixture was treated with thermolysin, a single labelled peptide could then be isolated, yielding an unambiguous composition and sequence corresponding with the region 175–177. The same thermolysin fragment was also generated from the insoluble tryptic core fraction, this time originating from peptide 148–183.

These results allow one to conclude that arginine at position 177 of skeletal muscle actin is the only major acceptor amino acid for the ADP-ribosylation by *C. perfringens* iota toxin.

4. DISCUSSION

Here we report that *C. perfringens* iota toxin modifies skeletal muscle α -actin in Arg-177. It has been shown, previously, that *C. botulinum* C2 toxin, which ADP-ribosylates nonmuscle actin, but not that of skeletal muscle, also modifies actin in Arg-177 [12]. Thus, both toxins not only share the same substrate actin but also an identical acceptor amino acid. The modification of the identical amino acid explains the similarities found in the ADP-ribosylation of actin by either toxin, e.g. that phalloidin blocks the ADP-ribosylation and that modified actin loses its ability to polymerize. However, the present data do not explain why skeletal muscle actin can serve as substrate of iota toxin but not of C2 toxin. The difference in substrate specificity is most surprising because skeletal muscle and nonmuscle actin are highly homologous and differ only by about 6% [19]. It has been suggested, recently, that this difference may be due to the amino acid preceding Arg-177, which is leucine in nonmuscle actin and methionine in skeletal muscle actin. However, the data shown here indicate that exchange of leucine for methionine in skeletal muscle actin does not principally prevent an ADP-ribosylation of Arg-177, and suggest that the structural requirements for the ADP-ribosylation of actin by these two toxins reside at another level.

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