Analysis of the catalytic site of the actin ADP-ribosylating *Clostridium perfringens* iota toxin

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Abstract The enzyme component of the actin ADP-ribosylating *Clostridium perfringens* iota toxin was affinity labelled by UV irradiation in the presence of [carbonyl-^{14}C]NAD. A peptide containing the radiolabel was generated by CNBr cleavage and subsequent proteolysis with trypsin. Its amino acid sequence is Gly-Ser-Pro-Gly-Ala-Tyr-Leu-Ser-Ala-Ile-Pro-Gly-Tyr-Ala-Gly-X-Tyr-Glu-Val-Leu-Leu-Asn-His-Gly-Ser-Lys corresponding with the region Gly-363 through Lys-388 in the *C. perfringens* iota toxin. Mass spectrometric data as well as the results of the PTH-amino acid analysis are in line with a modification of a glutamic acid side chain located at position 378. Therefore, in addition to Glu-380, as could be concluded by analogy with other ADP-ribosyltransferases, Glu-378 may play a pivotal role in the active site of *C. perfringens* iota toxin.

1. Introduction

Clostridium perfringens iota toxin belongs to a family of actin-ADP-ribosylating toxins [1]. Other members of this family are *C. botulinum* C2 toxin [2] and *C. spiroforme* toxin [3,4] (for review see [5,6]). These toxins are binary in structure and consist of a binding component and an enzyme component that interact at the surface of target cells. It is suggested that the toxins enter cells by receptor-mediated endocytosis [7,8] followed by a cascade of processes which, finally, result in translocation of the enzyme components into the cytosol. In the target cell, the enzyme component ADP-ribosylates monomeric G-actin at Arg-177 [9,10]. ADP-ribosylation inhibits actin polymerisation [1,2], actin ATPase activity [11] and turns actin into a capping protein that binds to the barbed ends of actin filaments inhibiting fast polymerisation [12]. Although both iota toxin and C2 toxin modify actin at the same amino acid residue, both toxins differ in their substrate specificity. Iota toxin modifies all actin isoforms studied so far, whereas C2 toxin ADP-ribosylates non-muscle β/γ actins and γ-smooth muscle actin but not α-actin isoforms [13].

Significant progress in the understanding of the action of bacterial ADP-ribosylating toxins and of the underlying molecular mechanisms were obtained by crystal structure analyses of *Pseudomonas* exotoxin A [14], diphtheria toxin [15], *E. coli* heat labile toxin [16] and pertussis toxin [17]. These studies suggested that despite rather poor similarity in the primary sequence, ADP-ribosylating toxins share a common active site structure.

Here we have located the active site sequence of the enzyme component of iota toxin using photo-affinity labelling in the presence of [carbonyl-^{14}C]NAD and microsequencing analysis. The amino acid sequence around the labelled active site shows a striking similarity with corresponding regions in the C3-like ADP-ribosyltransferases. Our studies support the importance of two glutamic acid residues in the active site.

2. Materials and methods

2.1. Materials

NAD was from Boehringer Mannheim (Germany). [carbonyl-^{14}C]NAD (54 mCi/mmol) was obtained from Amersham Corp. All other reagents were from commercial sources and analytical grade. Rabbit skeletal muscle actin was purified as described [18].

2.2. Purification of C. perfringens iota toxin

The ADP-ribosylating component of iota toxin was purified by a two step procedure from the culture supernatant of *C. perfringens* type E strain CN 5063, which was kindly donated by Dr. S. Thorley (Wellcome Beckenham, Kent, UK). The culture supernatant was precipitated with ammonium sulphate (70% saturation). The collected pellets were extracted with 50 mM Tris-HCl, pH 7.5, followed by dialysis against buffer containing 10 mM Tris-HCl, pH 7.5. The ADP-ribosylating component was recovered in the flow-throw of a DEAE Sephadex anion exchange chromatography and was subsequently concentrated by membrane filtration (Amicon). The enzyme component of iota toxin was more than 95% pure (Fig. 1).

2.3. ADP-ribosylation assay

ADP-ribosylation reactions were carried out essentially as described [1,19]. Briefly, iota toxin (enzyme component, 1 μg/ml) was incubated in a medium containing 1 mM dithiothreitol, 50 μM $MgCl_2$, 50 μM [^{32}P]NAD (0.5 μCi), 50 mM triethanolamine, pH 7.5, and 5 μM rabbit skeletal muscle α-actin for 45 min at 37°C. For determination of the specific activity the incubation time was kept at 4 min. ADP-ribosylated actin was separated on 11% SDS-PAGE and detected with a Phosphorimager (Molecular Dynamics).

2.4. Photo-affinity labelling of C. perfringens iota toxin

600 μl of *C. perfringens* iota toxin (1 mg/ml, in 25 mM ammonium bicarbonate) was incubated with 100 μM [^{14}C]NAD for 30 min at 4°C followed by UV irradiation (254 nm, 3 cm distance, 3000 μW/cm^2) for 2 h. Thereafter, the amount of incorporated label was determined by precipitation of iota toxin with trichloroacetic acid (20%, w/v), subsequent filtration onto nitro-cellulose filters and counting for retained radioactivity. Irradiated iota toxin was separated from unreacted NAD by HPLC chromatography using a Sephasil RP-C18 2.1×100 mm column (Pharmacia) and lyophilised.

CNBr-cleavage was performed in 70% formic acid with a >300-fold molar excess of CNBr over protein. The mixture was incubated in the dark for 24 h at room temperature followed by evaporation in a Speed Vac (Savant) apparatus. Remaining CNBr and acid were

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removed by subsequent lyophilization after resuspending in water. The CNBr-fragment mixture was then further digested with trypsin (enzyme substrate ratio: 1/50, by weight) in 0.1 M Tris-HCl buffer (pH 8.5) at 37°C overnight. The resulting peptides were separated by reverse-phase HPLC chromatography using a C-18 Vydac 2.1×250 mm column. Peptides were eluted with a gradient of 0% B to 80% B in 90 min (solvent A: 0.1% TFA in water (v/v); solvent B: 0.1% TFA in 70% acetonitril in water (v/v). Twenty percent of the outlet flow was directed into the ion source of an electrospray ionization mass spectrometer (VG Platform, Fisons Instruments, Manchester, UK), on-line connected with the HPLC system (140A Solvent delivery system, Applied Biosystems). The remaining 80% of the eluate was directed into a 759 A absorbance detector, Applied Biosystems) and peptides were detected at 216 nm and manually collected. Twenty percent of each of the collected peaks was removed, dried in vacuo and the radioactivity measured after mixing with 150 μl scintillation liquid.

2.5. Amino acid sequence analysis

The ^{14}C-labelled peptide peak was selected for sequence analysis. This was carried out with an Applied Biosystems model 477A Protein Sequencer with an on-line model 120A PTH analyzer. The instrument was run with the standard programme according to the manufacturer's instructions. A constant aliquot (40%) of the PTH-derivative formed in each cycle was directed into a fraction collector for radioactivity counting.

3. Results and Discussion

When purified iota toxin was UV-irradiated in the presence of [^{14}C]NAD, about 25–30% of iota toxin was labelled. At this stage the enzyme activity was completely inhibited (not shown). Interestingly irradiation in the absence of NAD also caused full inhibition of transferase activity, suggesting a high sensitivity to UV irradiation, even after short exposure times (e.g. 30 min). To determine the amino acid residue which was affected, the labelled transferase was first cleaved by CNBr followed by trypsin digestion. Peptides formed were separated by reverse phase HPLC. As shown in Fig. 2A, only one major labelled peptide was detected. It was subjected to amino acid sequence analysis and the following results were obtained: Gly-Ser-Pro-Gly-Ala-Tyr-Leu-Ser-Ala-Ile-Pro-Gly-Tyr-Ala-Gly-X-Tyr-Glu-Val-Leu-Leu-Asn-His-Gly-Ser-Lys (Fig. 2B).

This sequence corresponds with residues Gly-363 through Lys-388 of the mature *C. perfringens* enzyme as published [20] and corrected recently [21]. Note that Gly is at position 363 of the iota toxin from the *C. perfringens* strain CN 5063, whereas Asp is at this site in strain 10768 which was used to derive the published sequence [20]. Residue X, carrying the radiolabel, eluted as an 'unconventional' PTH-derivative with a retention time between dimethylphenylthiourea and PTH-Ala (Fig. 3A). This is exactly as previously noted in the course of similar analyses of the ADP-ribosyltransferases of *Clostridium limosum* [22] and of *Bacillus cereus* [23], where sequencing of the unmodified peptide or DNA-sequence predicted a glutamic acid residue. Also in the case of the iota toxin, we notice a glutamic acid in the corresponding DNA-sequence at the site of the modified residue [21].

It is known that the nicotinamide moiety of NAD^+ can be cross-linked by UV-irradiation to the γ-methylene carbon formed by decarboxylation of the pivotal glutamic acid in the active centre of the transferase [24]. This leads to an increase of the mass by 76 Da [24]. Mass spectrometric analysis of the labelled peptide revealed a mass of 2729 Da, corresponding with the expected modification in the Gly-363–Lys-388 peptide (Fig. 3B).

Thus, two lines of evidence support the conclusion that Glu-378 represents the modified residue: first, a characteristic modified PTH-residue is identified at this position and second, the increase in mass of the tryptic peptide corresponds very well with a conjugate formed between glutamic acid and the nicotinamide moiety.

Interestingly, Glu-378 is located within a region of significant homology with a number of ADP-ribosyl transferases. This list includes C3-like transferases [25–29], cholera toxin [30], the family of *Rhodospirillum*-like ADP-ribosyltransferases [31], the T2, T4, T6 bacteriophage transferases [32] and several recently identified eukaryotic glycosylphosphatidylinositol-anchored mono-ADP-ribosyltransferases (or NAD-ases) such as the rabbit muscle ADP-ribosyltransferase [33–35] and the family of RT6-like T-cell alloantigens [36–38].

An alignment of these sequences is shown in Fig. 4. The most conserved residue in this region is a glutamic acid residue consistently followed by a very hydrophobic tripeptide sequence. This Glu-residue seems pivotal for catalysis since it can be cross-linked by UV-irradiation to nicotinamide, concomitant with inhibition of ADP-ribosyltransferase activity. This is the case for Glu-148 of diphtheria toxin [39], Glu-553 of *Pseudomonas* exotoxin A [40], Glu-129 of the S_1 subunit of the pertussis toxin [41] which all seem functionally equivalent. Photoaffinity-labelling and site-directed mutagenesis similarly identified Glu-174 of the C3-like *C.limosum* transferase as part of the active site [22,28]. Finally, it was shown that the C3-like transferase from *B. cereus* could be modified at a functionally and structurally equivalent residue [23].

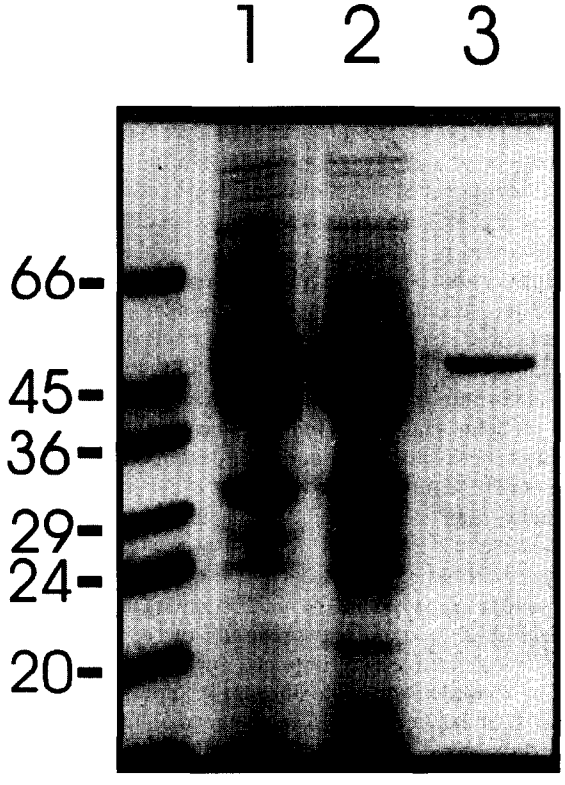

Fig. 1. SDS-PAGE analysis of the ADP-ribosylating component of *C. perfringens* iota toxin. Iota toxin was purified from culture supernatant (lane 1) of *C. perfringens* Type E strain CN5063 by ammonium sulphate precipitation (70%), lane 2) and subsequent DEAE-anion exchange chromatography (lane 3) as described in section 2.

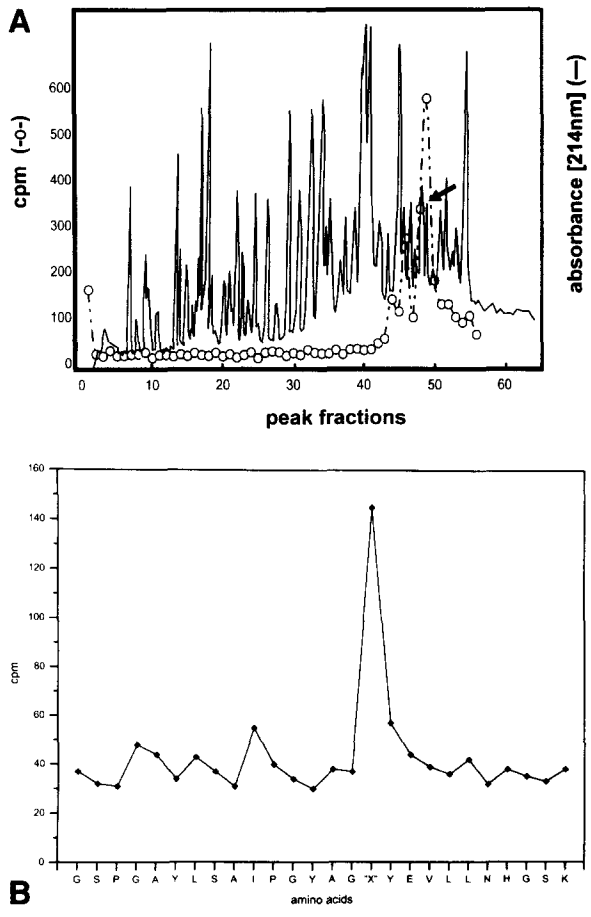

Fig. 2. (A) Elution profile of the peptides from the irradiated enzyme component of *C. perfringens* iota toxin. The enzyme component of *C. perfringens* iota toxin was UV irradiated in the presence of [carbonyl-^{14}C]NAD for 120 min. Thereafter, the labelled transferase was isolated by chromatography and cleaved by CNBr and subsequently digested with trypsin. The peptides were analysed by HPLC chromatography as described. Elution profiles of proteolytic peptides (——); radioactivity (○) was determined in an aliquot of the eluting fractions. Most radioactivity was recovered in fraction 49 (arrow). (B) Amino acid sequence analysis and measurement of radioactivity in the residues of the ^{14}C-labelled proteolytic peptide (fraction 49) from *C. perfringens* iota toxin. The abscissa gives the amino acids determined; the radioactivity is given on the ordinate.

A similar (Glu-380) conserved residue followed by a hydrophobic tripeptide is found in the labelled peptide of the iota-toxin, but unexpectedly not Glu-380 but Glu-378 was now labelled. This observation came to us somewhat as a surprise in view of the constant observations of a functional Glu adjacent to the hydrophobic cluster (see above). Enzymes with a similarly spaced biglutamic acid sequence are the transferases of cholera toxin, the *E. coli* heat-labile toxins, the mosquitocidal toxin from *B. sphaericus*, T2-like transferases, *Rhodospirillum*-like transferases, the rat T cell alloantigen RT6 and rabbit muscle ADP-ribosyltransferase (Fig. 3). For none of them, a similar labelling experiment has been performed, and it is not known if our observation could also apply to the other enzymes.

In answering this question it is interesting that mutant *E. coli* heat labile toxin in which the glutamic acid residues at either position 110 and 112 are replaced with aspartic acid exhibits severely reduced enzyme activity [42]. Furthermore, these findings are in good agreement with mutational analysis carried out on rabbit muscle ADP-ribosyltransferases showing three consecutive Glu-residues (Glu-238, Glu-239, Glu-240) in a region corresponding to that identified in the iota toxin sequence [33,35]. When in this transferase either Glu-238 or Glu-240 is exchanged by Asp, a drastic reduction in enzymatic

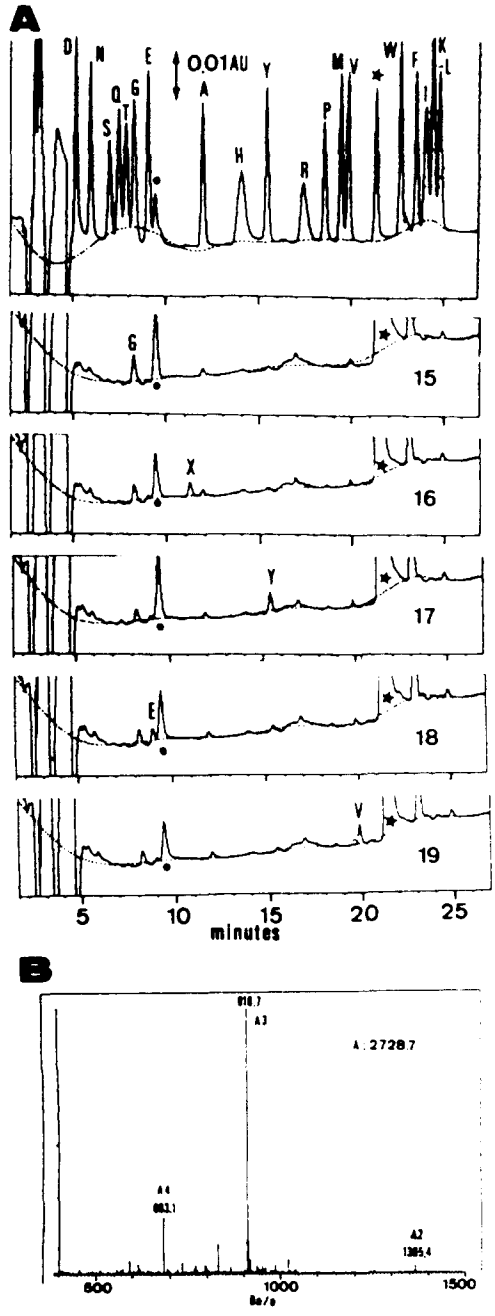

Fig. 3. (A) HPLC-tracing of the separations of the phenylthiohydantoin (PTH) amino acid residues released in cycles 15 through 19 during the automated Edman degradation of peptide collected from 49. The upper panel shows the standard PTH-residues. Amino acid derivatives are indicated by the standard one-letter notation. X in cycle 16 denotes the modified residue: most likely the Glu-nicotinamide conjugate. The asterisk indicates the position of dimethylphenylthiourea; a full circle: the position of diphenylthiourea. (B) Mass spectrum of the peptide nicotinamide conjugate. A2, A3, and A4 indicate the positions of the doubly, triply, and quadruply charged ions, respectively. The deduced molecular mass is shown. The *x*-axis represents the mass over charge ratio (m/z) while the *y*-axis measures the ion intensity.

activity is noticed [35]. In contrast, substitution of Glu-239 by Asp has almost no effect. These studies suggest that the similarly spaced glutamic acid residues participate in the catalytic activity. In the 'mono'-glutamic acid transferases, the second glutamic acid position is often occupied by glutamine, or in individual cases by arginine or serine: residues that may participate in hydrogen bond formation. It is thus possible that this residue also actively participates in the reaction, but only when it is a glutamic acid residue, a covalent link can be formed by UV-irradiation. In view of the above results, it is tempting to conclude that the strictly conserved Glu-residue and the second preceding Glu-residue are important for catalytic activity.

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          378 380
            ↓ ⇓
GYAGEYEVLLNHGSK-388   iota toxin

TFKGQLEVLLPRSST-182   LIM
YFPGQLEVLLPRNNS-181   C3N
AFAGQLEMLLPRHST-182   C3P
AYYGQQEVLLPRGTE-188   EDIN
AYPGQYELLLPR          Cer

PHPDEQEVSALGGIP-120   CT
PHPYEQEVSALGGIP-120   LT
PFPNEDEITFPGGIR-205   MTX
LATYQSEYLAHRRIP-137   PT

GIATEAEVILPRGLM-598   T2

YYTHEEEVLIPGYEV-217   mRT6
FFPGEEEVLIPPFET-248   rMT

EEGGRLETILGWPLA-561   ETA
EGSSSVEYINNWEQA-156   DT
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Fig. 4. Comparison of the amino acid sequences of the region adjacent to a glutamic acid residue suggested to be involved in catalysis of the ADP-ribosylation by various transferases (Lim, *C. limosum* ADP-ribosyltransferase [28]; C3N, *C. botulinum* C3 transferase [26]; C3P, *C. botulinum* C3 transferase [25]; EDIN, *S. aureus* transferase [27]; Cer, *B. cereus* transferase [23]; CT, cholera toxin [30]; LT, *E. coli* heat labile toxin [43]; MTX, mosquitocidal toxin from *B. sphaericus* [44]; PT, pertussis toxin [45]; T2, bacteriophage ADP-ribosyltransferase T2 [32]; mRT6, mouse homologue of the rat T cell alloantigen RT6 [46]; rMT, rabbit muscle ADP-ribosyltransferase [33]; ETA, *Pseudomonas* exotoxin A [47]; DT, diphtheria toxin [48].

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