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Identification of an active-site residue in subunit S1 of pertussis toxin by photocrosslinking to NAD

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The site of interaction of NAD with the isolated S1 subunit of pertussis toxin was investigated by photoaffinity labelling. When S1 was irradiated at 254 nm in the presence of [*carbonyl*-^{14}C]- or [*adenine*-^{14}C]NAD, the uptake of radioactivity was equivalent to 0.75 and 0.1 mol/mol respectively, while the NAD glycohydrolase activity was abolished. Inactivation was thus accompanied by crosslinking of the nicotinamide portion of NAD to the protein. Sequence determination of purified radioactive peptides indicated that Glu-129 was a major site of labelling. This residue is therefore closely associated with either NAD binding or hydrolysis.

Pertussis toxin; Toxin binding; Photoaffinity labeling; Crosslinking; NAD

1. INTRODUCTION

Pertussis toxin (PT), an exotoxin of *Bordetella pertussis*, is believed to be an important pathogen and immunogen associated with whooping cough [1]. Recent achievements in sequencing the PT gene [2,3] have stimulated interest in detoxifying the toxin by genetic engineering [4]. PT is a multimeric protein comprising a B oligomer (subunits S2–S5) that binds to receptors on target cells, and a catalytic A subunit (S1), an ADP-ribosyltransferase that inactivates host regulatory G-proteins [5]. Carroll and Collier [6] have probed the active sites of two other bacterial ADP-ribosyltransferases, diphtheria toxin [6] and *Pseudomonas aeruginosa* exotoxin A [7], by direct photocrosslinking to radiolabelled NAD. In each case a Glu residue labelled with high efficiency was concluded to be a major component of the active site. This report describes the application of photoaffinity labelling to subunit S1 of pertussis toxin, and presents evidence that Glu-129 is closely associated with bound NAD. This residue becomes a prime locus for testing the effects of amino acid substitution on the enzymic activity of pertussis toxin.

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Abbreviations: PT, pertussis toxin; CHAPS, 3-(3-cholamidopropyl)dimethylammoniopropane-1-sulfonate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; RP-HPLC, reverse-phase high-performance liquid chromatography; DTT, dithiothreitol; PTH, phenylthiohydantoin; DT, diphtheria toxin; ET, *P. aeruginosa* exotoxin A; CT, cholera toxin

2. EXPERIMENTAL AND RESULTS

2.1. *Preparation of PT subunit S1*

PT was adsorbed from culture supernatants of *B. pertussis* strain 10536 by passage through a fetuin-agarose affinity medium in 10 mM potassium phosphate, 0.1 M NaCl (pH 7.5). The gel was washed with 0.5 M urea, 50 mM potassium phosphate, 0.1 M NaCl, 1% (w/v) CHAPS (pH 7.5; buffer A), then eluted with buffer A plus 0.5 mM ATP to release S1 [8]. The eluate was passed through fresh fetuin-agarose equilibrated with buffer A plus 0.5 mM ATP to remove residual B oligomer. S1 preparations were shown to be free of other PT subunits by SDS-PAGE, and by RP-HPLC on a Vydac 214TP54 C_4 column eluted with a linear 35–45% acetonitrile gradient in 10 mM trifluoroacetic acid (fig.1). S1 was quantified by HPLC by comparison with the integrated peak intensity at 220 nm of a PT standard (List Biological).

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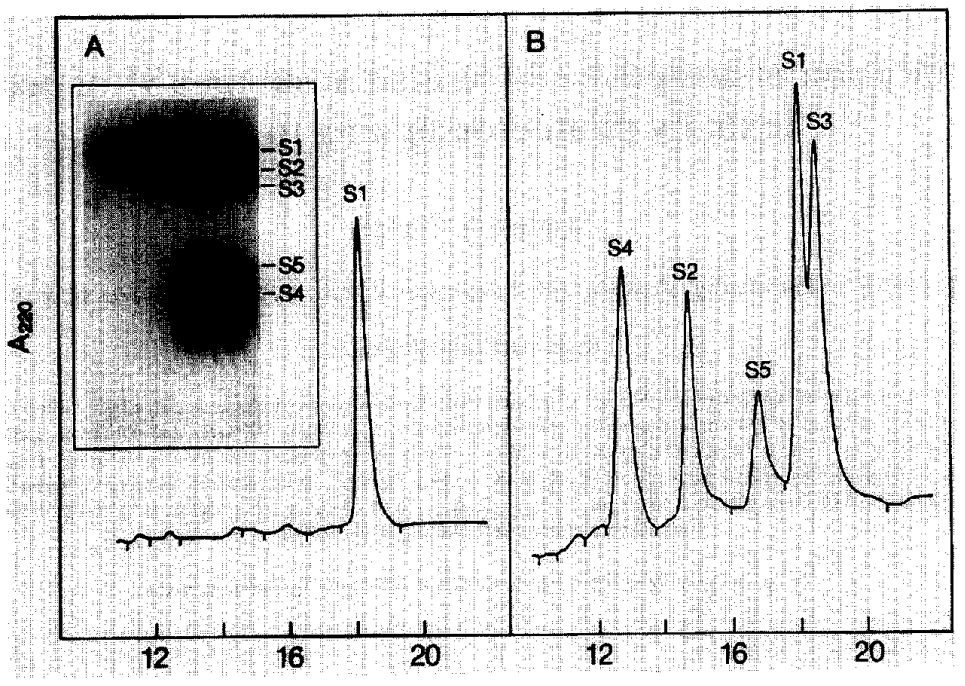

Fig.1. RP-HPLC and SDS-PAGE of subunit S1 and whole PT. HPLC: S1 (A) and PT (B); SDS-PAGE: S1 (inset, left) and PT (inset, right).

2.2. *NAD glycohydrolase assay*

S1 (10–50 μg/ml) in buffer A containing 10 mM DTT was preincubated for 10 min at 25°C, then incubated for 30 min with 1 mM NAD. Acidified reaction mixtures were loaded on a Vydac 201HS54 C_{18} RP-HPLC column and eluted with 0.1 M potassium acetate (pH 7.4) containing 10% (v/v) acetonitrile. Free nicotinamide eluting at 6 min was detected at 264 nm and quantified. Specific activities were in the range 11–15 pmol nicotinamide/min per μg S1, and the Michaelis constant for NAD was about 25 μM.

2.3. *Photocrosslinking of NAD to subunit S1*

Reaction mixtures (100 μl) containing 50 μg/ml S1, 10 mM DTT and 50 μM NAD in buffer A in the wells of a microtitre plate were preincubated at 0°C for 30 min, then irradiated for up to 3 h at 254 nm with a 9 W mercury lamp. Samples were warmed to 25°C, incubated for 20 min with fresh NAD and assayed as above. NAD hydrolase activity was completely abolished after 2 h irradiation, whereas the extent of photoinactivation in the absence of NAD was only 40%. To characterize the NAD-dependent photochemical events, S1 was irradiated under identical conditions with [*carbonyl*-^{14}C]- and [*adenine*-^{14}C]NAD (20 Ci/mmol). The protein was precipitated with trichloroacetic acid to 10% (w/v), washed and measured in a scintillation counter. Radiolabel was incorporated effectively from the nicotinamide moiety (0.75 mol/mol) but not from the adenine moiety (0.1 mol/mol) (fig.2). The extent of crosslinking was decreased by 50% in the presence of 1 mM NAD, owing to optical shielding effects.

2.4. *Identification of the photocrosslinking site*

Reaction mixtures (3 ml) containing 0.1 mg/ml S1, 10 mM DTT and 50 μM [*carbonyl*-^{14}C]NAD (20 Ci/mol) in buffer A were placed in a Petri dish to give a 1 mm layer, then irradiated at 0°C for 2 h. The protein was *S*-alkylated with 4-vinylpyridine, precipitated with 20% (w/v) trichloroacetic acid, redissolved in 2 M urea, 0.2 M ammonium bicarbonate to 0.5 mg/ml, and digested with 50 μg/ml trypsin for 20 h at

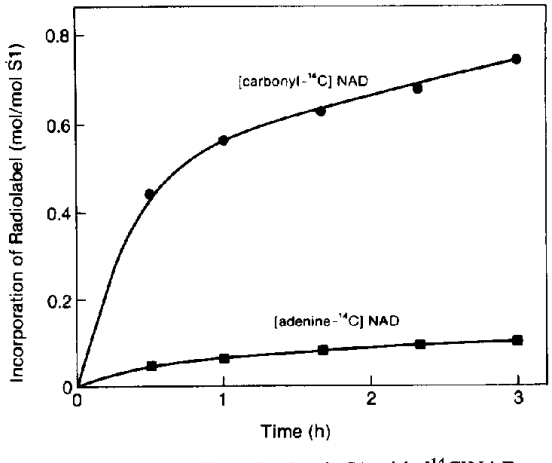

Fig.2. Photolabelling of subunit S1 with [^{14}C]NAD.

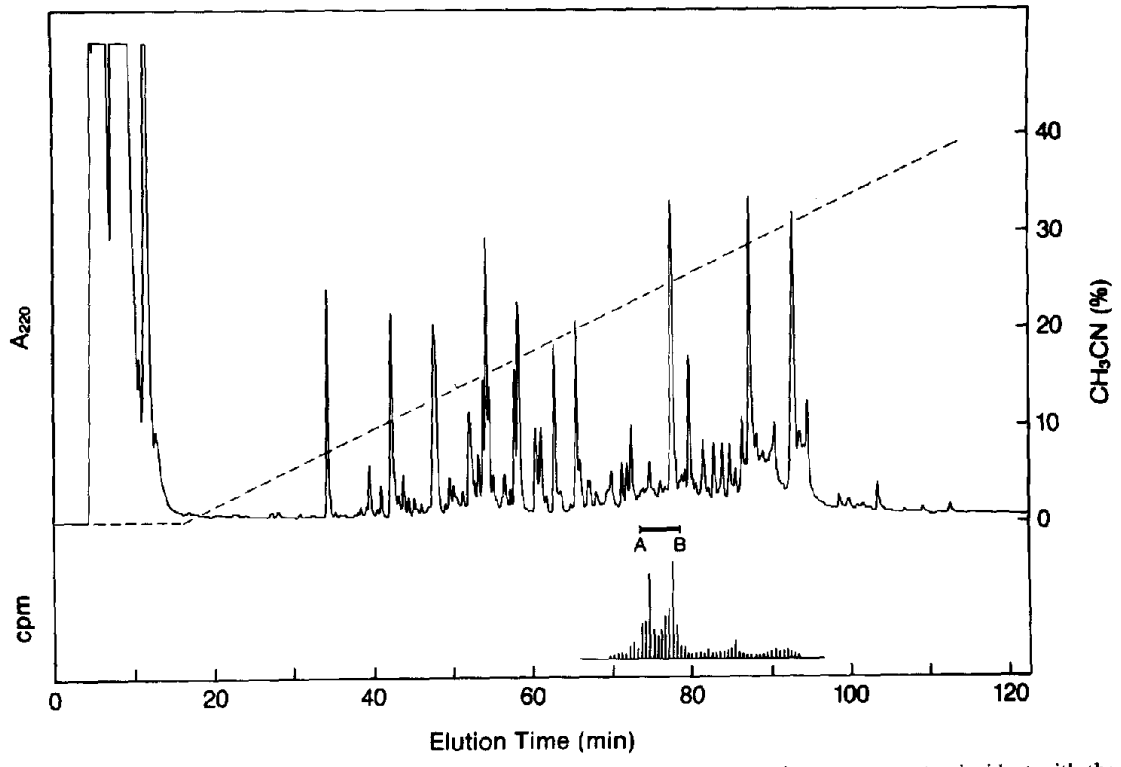

Fig.3. Fractionation of S1 tryptic peptides by RP-HPLC. Radioactive peptide B was a minor component coincident with the major peak seen in this chromatogram. Radioactivity was not detected outside the region shown.

Cycle				5				10				15				
Pep A	Ile	Leu	Ala	Gly	Ala	Leu	Ala	Thr	Tyr	Gln	Ser	X	Tyr	Leu	Ala	...
Pep B	Ile	Leu	–	Gly	Ala	Leu	Ala	Thr	Tyr	Gln	Ser	X	Tyr	Leu	Ala	...
S1	Ile	Leu	Ala	Gly	Ala	Leu	Ala	Thr	Tyr	Gln	Ser	Glu	Tyr	Leu	Ala	His Arg Arg
Residue			120					125					130			135

37°C. The acidified mixture was fractionated on a Vydac 218TP510 C_{18} RP-HPLC column using a linear 0–40% acetonitrile gradient in 10 mM trifluoroacetic acid, with flow rate 2.5 ml/min. Two major radioactive peptides (A,B) accounted for 50% of the eluted radioactivity, with the remainder being widely dispersed (fig.3). Peptides A and B were purified to homogeneity on a C_{18} column by applying a 20–30% acetonitrile gradient in 10 mM trifluoroacetic acid, followed by a similar gradient in 20 mM ammonium acetate (pH 6.5).

The peptides (about 1 nmol) were sequenced on an Applied Biosystems 470A gas-phase sequenator; a portion of the effluent was diverted for monitoring of radioactivity. Up to cycle 15, the sequences were identical (except for a missing residue at cycle 3 in peptide B), and corresponded uniquely to S1 residues 118–132 (below). In both peptides, radioactivity was associated only with an unidentified amino acid (X) at cycle 12, equivalent to position 129. Radioactivity was not detected at cycles beyond 15, where no amino acids were positively identified. Thus, Glu-129 was the predominant site of photocrosslinking. However, disparity in the RP-HPLC retention times of the radiolabelled PTH-amino acids released from the two peptides indicated two modes of crosslinking between the Glu side chain and the NAD fragment. This feature might account for the difference in retention times of peptides A and B, though it is also possible that the peptides were of unequal length, since their C-termini were not established.

3. DISCUSSION

UV irradiation of S1 in the presence of its substrate NAD generated photoproducts in which the nicotinamide but not the adenine portion was efficiently incorporated into the protein, accompanied by loss of enzymic activity. Glu-129 was a specific site of crosslinking and is therefore likely to be an important component of the nicotinamide interaction site. The lack of a derivative carrying

the adenine moiety of NAD might indicate either weaker binding to the toxin, or simply less efficient photoexcitation of the adenine ring. Carroll and Collier [6,7] reported similar selective photolabelling of Glu residues in catalytically active fragments of diphtheria toxin (DT) and *P. aeruginosa* exotoxin A (ET). The Glu-directed photoproduct from DT was shown to contain only the nicotinamide ring of NAD, thus placing the carboxylate side chain very close to the nicotinamide–ribose bond ruptured during ADP-ribosylation [9]. The active-site geometry of S1 may be somewhat different, since two distinct radiolabelled amino acids 'X' from Glu-129 were observed. As an approach to the design of genetically inactivated forms of PT for vaccine purposes, we are currently studying the functional role of Glu-129 in more detail by site-directed mutagenesis, and a series of Glu mutants with 500–1000-fold lower catalytic activity has been isolated and characterized (Loosmore, S.M. et al., in preparation). A recently reported PT mutant in which four extra amino acids were inserted between Tyr-107 and Val-108 exhibited a 100-fold drop in ADP-ribosyltransferase activity [4], possibly as a result of a conformational change affecting the nicotinamide site around Glu-129.

An alignment of local sequences surrounding the functional glutamates in ET, DT and S1 is shown below. Matched residues are scored for identity and for a greater-than-even chance of evolutionary relatedness. Clearly, there is little common homology. However, a scan of the cholera toxin sequence reveals a five-residue homology when Glu-47 is aligned with Glu-129 of S1: it remains to be seen whether this proves to be a similar functional site.

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ET (546–560) E E E G G R L E T I L G W P L
                .       .   .     .
                .   .   .   .     .
DT (141–155) A E G S S S V E Y I N N W E G
                .           .   .
                .   .   .       .   .   .
S1 (122–136) A L A T Y Q S E Y L A H R R I
                        .   .   .   .
                .   .       .   .   .   .
CT (40–54)   L M P R G Q S E Y F D R G T Q
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