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Specific binding of nucleotides and NAD^+ to *Clostridium difficile* toxin A

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Binding of nucleotides, a tetrapolyphosphate, and NAD^+ to purified toxin A of *Clostridium difficile* was determined by monitoring changes in intrinsic fluorescence following excitation at 280 nm, and recording emissions at 340 nm. Binding was specific for concentrations over the range 5 to 100 μM for ATP, GTP, and their respective non-hydrolysable analogues AMP-PNP and Gpp(NH)p, tetrapolyphosphate and NAD^+.

Clostridium difficile toxin A; Nucleotide; Nucleoside; Phosphate binding

1. INTRODUCTION

Clostridium difficile is a well established cause of pseudomembranous colitis and also accounts for a significant proportion of cases of antibiotic-associated diarrhoea [1,2]. Of the known and putative virulence factors described [3] the most important in terms of contributing to the features of the disease are the two major toxins, designated A and B, and in particular toxin A [4,5]. Our understanding of the structure and functions of these two toxins is limited, particularly of any structure-function relationships [6]. It has been demonstrated that toxin B has a polyphosphate binding site and will specifically bind ATP [7]. Whether or not toxin A also has this function is unknown. We therefore undertook a study to determine this and to examine the possibility of an NAD^+ binding site based on the method of monitoring protein conformational changes by following changes in intrinsic tryptophan fluorescence.

2. MATERIALS AND METHODS

2.1. *Preparation of toxin A*

Toxin A was prepared in pure form from *C. difficile* VPI 10463 as described in detail previously [8]. Briefly, the procedure consisted of passing crude culture filtrate through a bovine thyroglobulin affinity column ad 4°C (which retains toxin A) and thermal elution at 37°C. This product is further purified by two sequential anion-exchange chromatographic steps (Q Sepharose FF and Mono Q) by fast protein liquid chromatography (Pharmacia). The purified toxin A preparation was dialysed at 4°C overnight against 20 mM Tris-buffer at pH 7.5, and kept stored at 4°C.

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2.2. *Fluorescence difference spectrum of toxin A*

Fluorescence was determined with a Perkin-Elmer LS-5 Luminescence Spectrometer containing a thermostatically controlled cuvette holder. The excitation and emission slits were set at 20 nm and 10 nm respectively. The fluorescence difference spectrum of a solution of toxin A in 20 mM Tris versus toxin A-free buffer was performed between 290 and 380 nm following excitation of the samples at 280 nm. The emission spectrum of 10 μM free tryptophan (Sigma) was also performed at the same wavelengths.

2.3. *Titration of toxin A intrinsic fluorescence with ligand*

Titration of the intrinsic fluorescence of toxin A with ATP and GTP (sodium salts, Sigma), their respective non-hydrolysable analogues AMP-PNP, and Gpp(NH)p (lithium salts, Boehringer Mannheim), tetrapolyphosphate (hexaammonium salt, Sigma), and β-nicotinamide adenine dinucleotide (NAD^+, Boehringer Mannheim), were performed by adding 5–25 μl increments of 1 mM substrate in 20 mM Tris, pH 7.5, to a 1 ml solution of approximately 100 μg/ml of toxin A in the same buffer. The samples were carefully stirred after each addition, ensuring that light was always excluded from the system. Titrations were performed at 30°C. The decline in protein fluorescence following excitation at 280 nm was measured at 340 nm emission. In order to correct for inner filter effects, a solution of tryptophan, of the same initial fluorescence as the protein solution, was similarly titrated with all substrates and corrections made according to the method of Honore and Pedersen [9]. Measurements were corrected for dilution.

2.4. *Binding of toxin A to ATP-agarose*

Toxin A (0.2 ml of a 100 μg/ml solution) was applied to a column of ATP-agarose (1 cm × 0.5 cm, Sigma) containing 7 μmol ATP, the column left for 2 h at room temperature, then washed with 20 mM Tris, pH 7.4. The amount of toxin A bound to the column was determined by estimating the amount of toxin A that eluted. The experiment was conducted 3 times and the triplicate mean recorded.

2.5. *Protein estimation*

Protein concentrations were determined by the method of Lowry et al. [10].

3. RESULTS AND DISCUSSION

Fluorescence spectroscopy has previously been used

to study toxin–ligand interactions and to provide an indirect method for determining fractional saturation. For example the binding of NAD^+ to diphtheria toxin fragment A [11], *Pseudomonas aeruginosa* exotoxin A [12] and pertussis toxin [13], and the interaction of *Schizosaccharomyces pombe* mitochondical F_1-ATPase with phosphate and nucleotide [14], have been investigated by monitoring the quenching of the proteins' intrinsic fluorescence.

The emission spectrum from toxin A following excitation at 280 nm gave 2 peaks, one at 300 nm and the other at 340 nm, the latter corresponding to tryptophan fluorescence, confirmed by the emission spectrum of free tryptophan. Titrations of the intrinsic fluorescence of toxin A with nucleotides, NAD^+ and the tetrapolyphosphate were thus performed at 280 nm excitation, 340 nm emission, and an assumption that the reduced fluorescence can be correlated with fractional saturation was made on the observed saturation at higher concentrations (ca. 100 μM) of each ligand (Figs. 1a–f). It can be seen from Figs. 2a–f that the binding of nucleotides, the tetrapolyphosphate and NAD^+ is specific and of high affinity, and further that binding is greater for the tetrapolyphosphate than the nucleotides and that binding of the adenosine was greater than for the guanosine nucleotides. Of interest in this respect is the observation of Florin and Thelestam [7] that adenosine

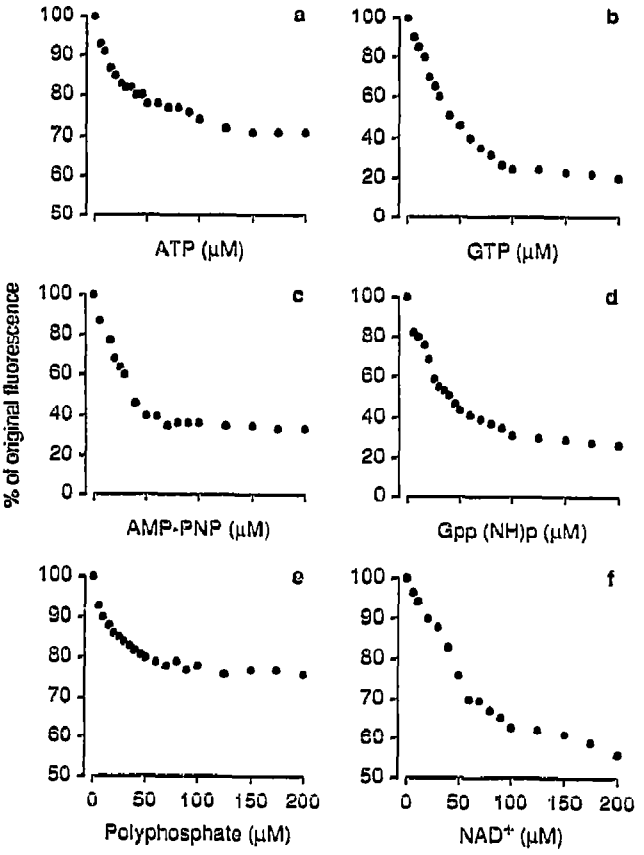

Fig. 1. Fluorescence titration of toxin A with (a) ATP, (b) GTP, (c) AMP-PNP, (d) Gpp(NH)p, (e) tetrapolyphosphate, and (f) NAD^+.

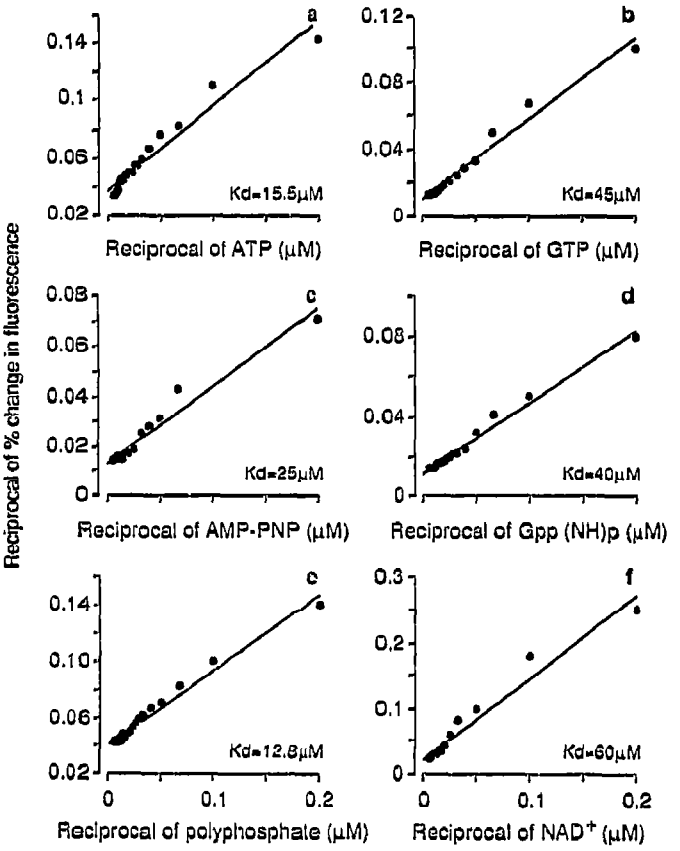

Fig. 2. Double reciprocal plot of % change in fluorescence against concentration of (a) ATP, (b) GTP, (c) AMP-PNP, (d) Gpp(NH)p, (e) tetrapolyphosphate, (f) NAD^+. K_d values are calculated from the intersection on the X-axis.

triphosphates were better able than other nucleotides to delay the cytopathogenic effects induced by *C. difficile* toxin B in MRC-5 cells and that a tetrapolyphosphate was better than the nucleotides. They also showed, as judged by a decrease in cytotoxicity of eluted material, that about 20% of a preparation of toxin B was retained on an ATP-agarose column [7]. We show here that a proportion of toxin A is also retained on an ATP-agarose column, but in this case the proportion retained, 35 ± 6%, is greater than that reported for toxin B. It has been shown that toxin A binds to agarose [15], however others have been unable to confirm this [16]. So, although it is possible that toxin A bound to the agarose, it is more likely that retention on the column was due to interaction of toxin A with ATP.

It is difficult to determine the significance of the ability of toxin A to bind physiological levels of nucleotides. However, that this ability is shared with toxin B is of little surprise due to the high degree of similarity (63.7%) at the molecular level (Seddon, unpublished) based on published sequences [17,18], confirmed by N-terminal analysis of 203 amino acids showing 64% homology [19]. A number of the other bacterial protein toxins have a phosphate binding site [20–23]. In some

cases these represent well-characterised calmodulin-dependent adenylate cyclases, e.g. the edema factor of *Bacillus anthracis* and the adenylate cyclase of *Bordetella pertussis* [20] which share a conserved domain related to the consensus amino acid sequence (Gly-X-X-X-X-Gly-Lys-Ser, X = any amino acid) present in many eukaryotic and prokaryotic ATP and GTP binding proteins [24]. We were unable to find this consensus amino acid sequence in toxin A or in the published sequence for diphtheria toxin [25,26], which has a polyphosphate binding site [22] and shares with toxin A some characteristics related to its cytotoxicity [27]. In these two respects is of note the above-mentioned relatively high degree of homology between the gene sequences for these two toxins. Interestingly, the reverse form of the consensus amino acid sequence Gly-X-X-X-X-Gly-Lys-Ser is present in toxin B localised at amino acid position 359–366 (Seddon and Borriello, unpublished), though neither toxins A or B have adenylate cyclase activity [28].

The binding of polyphosphorylated compounds by toxin A was not restricted to nucleoside phosphates as the inorganic tetrapolyphosphate was also bound, the binding being greater than for any of the nucleotides. This is in keeping with the findings for diphtheria toxin [22] and the indirect evidence for toxin B [7]. It would appear that toxin A has a phosphate binding site analogous to the 'P' site on fragment B of diphtheria toxin [21,22] or the polyanion binding site postulated for toxin B [7]. Of further interest is the demonstration that toxin A binds the nucleoside NAD^+, a further feature in keeping with other bacterial toxins. In diphtheria toxin, the phosphate and nucleoside binding sites are distinct but appear to be close to each other in the tertiary configuration, contributing to the nucleotide binding site [21]. It is unknown if this is also the case for toxin A or whether nucleoside binding is a feature of toxin B.

The functions of a phosphate binding site and, for toxin A a nucleoside binding site, are unknown for either toxin A or B. However our demonstration of a phosphate binding site in toxin A further confirms the similarities between the two toxins, and with a nucleoside binding site, is in keeping with the presence of such sites in other bacterial protein cytotoxins.

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