CONFORMATION OF DIPHTHERIA TOXIN AND AN ENZYMICALLY-ACTIVE FRAGMENT

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1. Introduction

Diphtheria toxin is synthesized by strains of *C. diphtheria* which are lysogenic for bacteriophage carrying the *tox* gene [1–3]. The toxin, which contains two disulfide bonds, is released into the extracellular medium as a single-chain polypeptide with mol. wt ~60 000 [4,5]. In this form, toxin is inactive [6,7] but can be activated by mild proteolysis and reduction [8]. This yields two fragments, designated A and B, with respective mol. wt ~21 000 and ~38 000 [1–7]. Fragment A, which is derived from the amino terminus of toxin and has been sequenced [9], catalyzes the covalent linkage of the adenosine diphosphate ribose moiety of NAD^+ to elongation factor EF-2 [10,11]. This inactivates EF-2 and thus terminates protein biosynthesis in cell-free extracts of eukaryotes [1–3].

The present study is aimed at elucidating certain conformational aspects of diphtheria toxin and fragment A, both of which are soluble in aqueous solution; in contrast, fragment B is relatively insoluble [2,3]. Circular dichroic (CD) spectra have been obtained for both toxin and fragment A in the near (250–310 nm) and far (200–250 nm) ultraviolet (uv), where the ellipticity is strongly influenced by tertiary and secondary structure, respectively. Analysis of the toxin CD spectrum gave 20% α-helicity and 25–30% β-structure; fragment A exhibited diminished α-helicity and a somewhat elevated content of β-structure. The spectrum obtained for the fragment was in agreement with that for S-carboxymethylated (CM) fragment A [12].

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2. Materials and methods

Cultures of *C. diphtheriae* Park Williams strain 8, kindly provided by the Federal Drug Administration, were grown in C-Y medium [13]. The protein fraction precipitating from the culture medium between 40% and 60% saturation with ammonium sulfate was chromatographed [5] using DEAE-cellulose and gel exclusion chromatography (Sephadex G-100). Semi-purified material (Connaught Laboratories) was also subjected to the same purification procedure and an apparently identical product was obtained. Toxoid was prepared using a formalin treatment as in [13].

Toxin was fragmented by limited trypsin hydrolysis [7]. A 2 ml vol. containing 10 mg diphtheria toxin in 50 mM Tris–HCl, pH 8.2 and 1 mM EDTA was incubated for 30 min at 25°C with 5 μg/ml trypsin (Worthington Biochemical Corp., Freehold, NJ). Proteolytic action was terminated by the addition of 10 μg soybean trypsin inhibitor. The mixture was made 0.1 M with DTT and incubated for 90 min at 37°C. A precipitate containing fragment B was removed by centrifugation, and the clear supernatant containing fragment A was subjected to gel filtration on Sephadex G-100 (two columns of 0.9 × 60 cm in series), which was equilibrated and developed with 50 mM Tris–HCl, pH 8.2, 1 mM EDTA and 1 mM DTT. The fragment eluted as a single symmetrical peak.

The enzymic activities of fragment A and toxin were determined as in [4] with minor modifications.

Elongation factor-2 was kindly provided by Dr Michael Haralson.

The methods used for immunodiffusion, immunoelectrophoresis, polyacrylamide gel electrophoresis and sedimentation equilibrium have been described [14]. Amino acid compositions of acid hydrolysates were determined on a Beckman 120C Analyzer; tryptophan was determined following hydrolysis in methane sulfonic acid and cysteine was measured as cysteic acid.

CD spectra were obtained at 25°C using a Cary 60 spectropolarimeter equipped with a CD attachment using essentially the methods in [14]. The following buffers were used: 50 mM Tris–HCl, pH 7.0, 1 mM EDTA for toxin, and 0.15 M NaCl, 5 mM phosphate, pH 7.0, 1 mM DTT for fragment A; these buffers produced optically clear solutions for both toxin and the fragment. Buffer changes following gel exclusion chromatography were made by dialysis. Near- and far-uv CD spectra were obtained using protein concentrations of 1 mg/ml (1 cm cell) and 0.3 mg/ml (0.5 mm cell), respectively. Protein concentrations were determined as in [15] with bovine serum albumin as standard; checks were also made using quantitative amino acid analysis with norleucine as internal standard. The spectra are reported in terms of mean-residue ellipticity, $[\theta]_r$, for the far-uv CD spectra and molar ellipticity, $[\theta]_m$, for the near-uv CD spectra. $[\theta]_r$ is based on mean-residue mol. wt 107 and 108 for toxin and the fragment, respectively; these were obtained from the amino acid compositions.

3. Results

The toxin preparations appeared homogeneous by immunodiffusion and immunoelectrophoresis (fig.1) and by polyacrylamide gel electrophoresis. Toxin was found to be mol. wt 62 000 (± 10%) in good agreement with the results [1–3]. The amino acid composition of toxin was in agreement with [16]. About 50 ng toxin was lethal when given subcutaneously to adult guinea pigs and 5 ng produced typical skin ulcers.

Fragment A chromatographed as a single component during gel exclusion chromatography, and a single precipitin band was observed in immunodiffusion which appeared identical to that produced by toxin. Also, a single component was observed in

Fig.1A

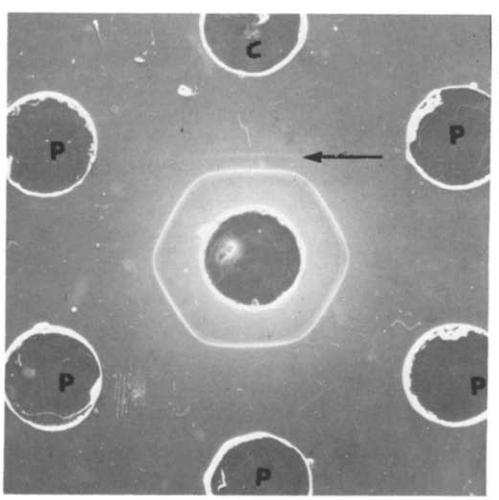

Fig.1B

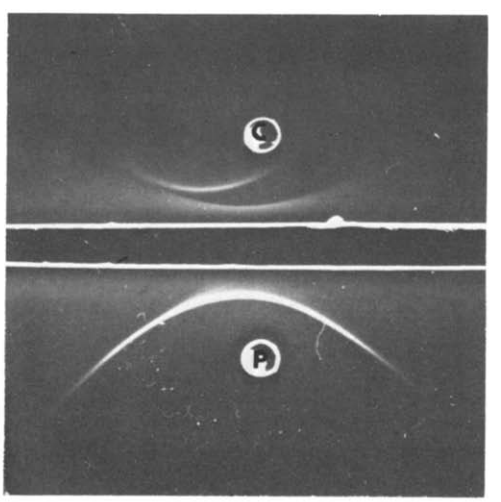

Fig.1. A. Left panel. Immunodiffusion of diphtheria toxin. The center well contained antiserum to crude toxoid; the well labeled C contained crude toxin preparation; the other wells (labeled P) contained purified toxin. The arrow indicates an extra precipitin band in the crude preparation. B. Right panel. Immunoelectrophoresis of diphtheria toxin. The central trough contained antiserum to crude toxoid; the upper well (designated C) contained crude toxin; and the lower well (labeled P) contained purified toxin. (Note the single component in purified toxin and the extra component in crude toxin.)

polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. The amino acid composition of the fragment was in general agreement with that expected from the sequence [9]; however, some differences were noted particularly in histidine and glycine.

Fragment A and toxin were found to have specific activities of 12.8 units/mg and 1.4 units/mg, respectively. The latter presumably arises from the presence of 'nicked' toxin which is present in most preparations [1–3].

The CD spectra of toxin and fragment A are shown in fig.2. The far-uv CD spectra were analyzed by least-squares analysis in order to estimate the fractions of α-helicity (f_α), β-structure (f_β) and nonhelical (i.e., aperiodic) structure (f_{nh}). The data were analyzed at 1 nm intervals using standard protein CD spectra as reference [17], and the results are given in table 1. The computer program for this analysis was kindly provided by Mr R. G. Hammonds jr and the methods have been described [18].

A solution of CM–fragment B was also analyzed by CD spectroscopy. Whereas the presence of opalescence prohibited any quantitative interpretation of the apparent ellipticity, the shape and magnitude of the CD spectrum of CM–fragment B above 250 nm resembled that of CM–fragment A (data not shown,

Table 1
Estimates of secondary structure[a] in diphtheria toxin and fragment A

	f_α	f_β	f_{nh}	Mean deviation
Toxin	0.20	0.31	0.49	467
Fragment A	0.10	0.39	0.51	1322

[a] Based on constrained (i.e., $f_\alpha + f_\beta + f_{nh}$ forced to be unity) least-squares analysis [18] of the far-uv CD spectra in fig.1 with reference proteins [17]. The spectra were also analyzed by non-constrained analysis: toxin yielded $f_\alpha = 0.20$, $f_\beta = 0.25$ and $f_{nh} = 0.45$, with a sums test of 0.90 and a mean deviation of 327 deg·cm²/dmol; the sums test of fragment A was only 0.58, thus making estimates of the individual f_i values unreliable. Units of the mean deviation are deg·cm²/dmol.

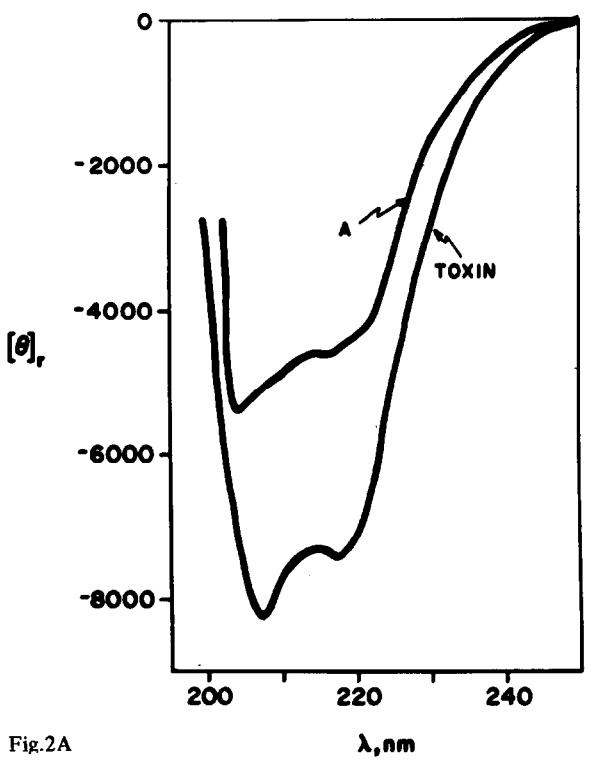

Fig.2A

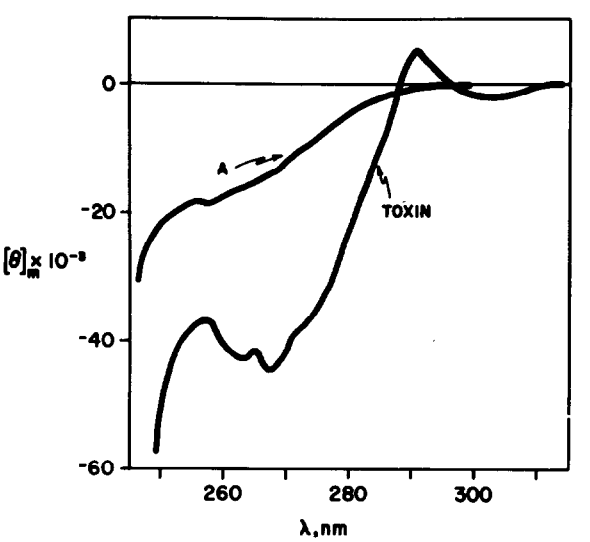

Fig.2B

Fig.2. CD spectra of diphtheria toxin and fragment A. A. Left panel. Far-uv CD spectra; $[\theta]_r$ denotes mean-residue ellipticity. B. Right panel. Near-uv CD spectra; $[\theta]_m$ refers to molar ellipticity.

although the spectrum of CM–fragment A was similar to that given in fig.2). The far-uv CD spectrum of CM–fragment B was intermediate to that of fragment A and toxin. Quantitatively, this indicates that fragment B, like fragment A, maintains some ordered secondary structure in aqueous solution.

4. Discussion

There is a paucity of information on the conformational aspects of diphtheria toxin and its fragments. As suggested by [2], one would expect a rather dramatic conformational change to accompany the activation of the toxin since this involves the scission of two covalent linkages, one peptide and one disulfide. Our results are fully consistent with this hypothesis. Because fragment B has only limited solubility in aqueous solutions, the differences in the CD spectrum of toxin and the sum of the spectra of fragments A and B cannot be quantitated. Nevertheless, our tentative results, both in the near- and far-uv spectral region, indicate that the sum of the CD spectra of CM–fragments A and B, when corrected for the respective molecular weights, do not yield the observed spectrum of toxin. This, in turn, suggests that conformational changes do occur upon fragmentation of diphtheria toxin.

To our knowledge, this is the first report on the CD properties of diphtheria toxin. The present CD results below 250 nm indicate that about 50% of the toxin residues are in some form of ordered secondary structure. Fragment A has an apparent lower content of α-helicity (~10% as opposed to 20% in toxin) and an apparent slightly elevated percentage of β-structure as judged by constrained least squares analysis. The only other pertinent CD report is the recent study on the far-uv CD spectrum of CM–fragment A before and after modification with 2-hydroxy-5-nitrobenzyl bromide [12]. The CD spectrum reported herein was quite similar to that reported, although the conditions differed appreciably. It is of interest that our estimates of f_α, f_β and f_{np} on fragment A agree with the values calculated using semi-empirical rules for prediction of secondary structure from sequence data [12].

The near-uv CD spectrum of diphtheria toxin is characterized by a positive extremum at ~291 nm which can safely be assigned to tryptophan [19]. Interestingly, neither fragment A nor (opalescent) CM–fragment B exhibited positive ellipticity in this spectral region, thus indicating some alteration in the microenvironment of one or more tryptophans upon fragmentation. Toxin is also characterized by negative extrema between 260 nm and 270 nm, presumed to arise mainly from phenylalanine [19], while fragment A exhibits only shallow, negative ellipticity with little, if any, fine structure. Toxin and fragment A contain about 7.5 mol % and 9.8 mol % of aromatic amino acids, respectively [9,16]. Thus, the reduced ellipticity of fragment A relative to toxin suggests that the average local environment of the aromatic residues differ in the two proteins. This could arise if there is less constraint of these groups in fragment A than in toxin.

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