ISOLATION AND CHARACTERIZATION OF A PRECURSOR FORM OF THE 'A' SUBUNIT OF CHOLERA TOXIN

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1. Introduction

Cholera toxin is a protein of M_r 84 000 [1], composed of 2 functional subunits, A and B. The cell binding subunit (B) is an aggregate of 5 β-chains (β_5), each of M_r 11 604 [2]. The A subunit (M_r 29 500) possesses the NAD^+ nucleosidase activity that is responsible for the adenylate cyclase stimulation of cells and cell lyzates [4,5]. The A subunit is composed of 2 chains, α (M_r 24 000) and γ (M_r 5500), that are held together by a single disulfide [2,5,6].

Previous reports [7,8] have provided strong evidence that the A subunit of cholera toxin has in fact a precursor single chain form (M_r 29 500) that is cleaved by limited proteolysis to give the disulfide bonded α- and γ-chains. More recently, studies of cholera toxin biosynthesis [9] gave evidence of 2 polypeptide forms, M_r 52 000 and M_r 45 000, that could be precipitated from in vitro translation mixtures by anti-A subunit suggesting the possibility of an even larger precursor form for the A subunit. Evidence for the occurrence of the M_r 29 500 precursor of A subunit has been indirect and was obtained primarily from gel electrophoretic studies and no chemical characterization has been reported. We report here the isolation and chemical characterization of the precursor A subunit of cholera toxin and present chemical evidence for the positioning of the amino acid sequences of the α- and γ-chains within its single chain structure.

2. Materials and methods

Fermenter cultures of *Vibrio cholerae* (Inaba strain 569B) were grown as in [2]. The toxin was purified by precipitation with sodium hexametaphosphate followed by column chromatography on phosphocellulose Whatman P11 [8]. The separation of A and B subunits of cholera toxin was achieved by gel filtration on Sephadex G-75 eluted with 5% formic acid [10]. Precursor A subunit, α-chain, and γ-chain were also separated on the G-75 column after reduction with 2-mercaptoethanol and alkylation with iodo[1-^{14}C]acetic acid [2]. Amino acid compositional analysis of precursor A subunit, α-chain, and γ-chain was determined from 24, 48, and 96 h hydrolyzates as in [2,11]. Protein hydrolysis with carboxypeptidase A and automated amino-terminal sequence analysis were performed according to [2]. Residues obtained from the 890B Beckman sequencer were identified by high performance liquid chromatography (HPLC) employing C18 reversed-phase chromatography (Beckman/Altex) [12], by gas chromatography, and by amino acid analysis after back-hydrolysis of the phenylthiohydantoin (Pth) amino acids [13]. Sodium dodecyl sulfate(SDS)–polyacrylamide gel electrophoresis was essentially according to [6]. The preparation of anti-subunit A and anti-toxin sera and the method of Ouchterlony double diffusion were as in [14]. Total carbohydrate analysis was achieved by reaction with anthrone [15]. Standard cholera toxin was lot no. EZ3439 obtained from Schwarz/Mann (Spring Valley NY).

3. Results

3.1. *Isolation of single chain precursor A subunit*

Cholera toxin eluted from the phosphocellulose column gave a reaction of identity with standard cholera toxin as determined by Ouchterlony double dif-

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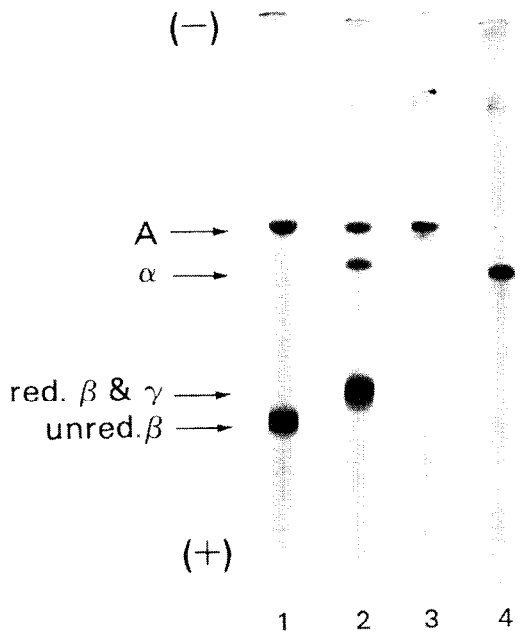

Fig.1. SDS–polyacrylamide gel electrophoresis of purified toxin and subunits. Proteins were heated at 100°C for 3 min in sample buffer prior to electrophoresis: (1) P-11 purified toxin, unreduced, (2) P-11 purified toxin, reduced; (3) carboxymethylated precursor A subunit, pool B2, fig.2B; (4) carboxymethylated α-chain, pool B3, fig.2B.

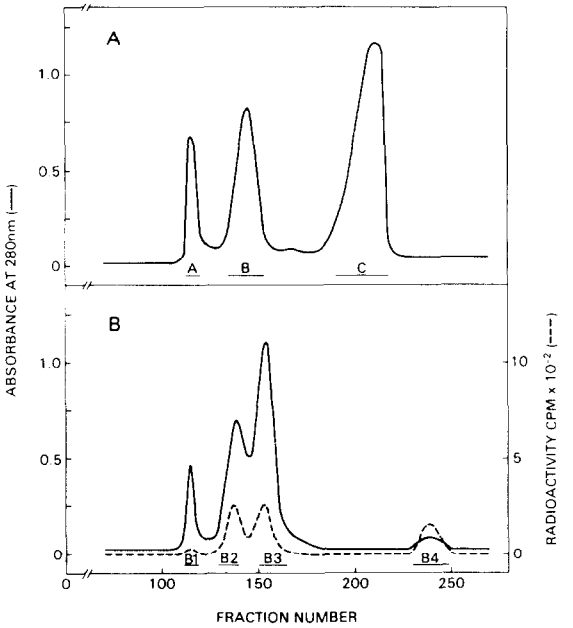

Fig.2. Gel filtration of cholera toxin and A subunit on Sephadex G-75, two columns in tandem (each 2.5 cm × 110 cm) eluted with 5% formic acid. Flow rate was 15 ml/h and 4 ml fractions were collected. (A) ~60 mg P-11 purified toxin applied in 20 ml 5% formic acid containing 5 M guanidine–HCl; (B) ~20 mg A subunit (pool B, fig.2A) was reduced and carboxymethylated (buffer was 5.2 M in guanidine–HCl) and applied to column.

fusion. Compositional analysis of the phosphocellulose purified toxin was comparable to that of standard cholera toxin (not shown). SDS–acrylamide gel electrophoresis under non-reducing conditions revealed a separation pattern identical to that of the standard toxin (fig.1(1)). The toxin was fractionated into subunits on Sephadex G-75 eluted with 5% formic acid as shown in fig.2A. Three peaks were eluted and pooled as shown. The excluded peak (pool A) contained a high molecular mass contaminant, the second peak (pool B) was A subunit, and the third peak (pool C) was β subunit. Subunit A (pool B) was subsequently reduced and carboxymethylated and re-chromatographed on the same G-75 column (fig.2B). Four peaks were obtained. The elution volumes of the third (pool B3) and fourth (pool B4) peaks corresponded to those of the α-chain and γ-chain, respectively. The second peak (pool B2) was the putative precursor of A subunit. Amino acid analysis and acrylamide gel electrophoresis (fig.1) confirmed the identity of these peaks. Strikingly, under reducing conditions, a mass species of M_r 29 500 that was M_r 5500 larger than the α-chain was identified in pool B2 of the G-75 column (fig.2B). Identical treatment of standard toxin preparations did not demonstrate the occurrence of the M_r 29 500 species (not shown; see [6]) The first peak (A or B1) that eluted from the G-75 column was a contaminant that contained 40–60% carbohydrate (by weight), as judged by reaction with anthrone, and 30% protein (uncorrected for water) as determined by amino acid analysis. When tested by Ouchterlony double diffusion the contaminant formed a faint precipitin line with cholera anti-toxin and appeared antigenically identical to cholera toxin. Polyacrylamide gel electrophoresis indicated that this material stained poorly with Coomassie blue and that a small amount of toxin was strongly bound to this component since the toxin subunits were not dissociated in the sample application buffer for the G-75 column that contained 5 M guanidine–HCl and 1% 2-mercaptoethanol. Selected homogeneous fractions of peaks B2 (precursor A subunit) and peak B3 (α-chain) obtained from the fractionation

Table 1
Amino acid composition[a] of the precursor A subunit of cholera toxin

Amino acid	Precursor A subunit[b]		α-Chain[c]	γ-Chain[c]
CM-Cys	2.2 ± 0.2	(2)	1	1
Asp	33.0 ± 0.1	(33)	26	7
Thr[d]	10.8 ± 0.5	(11)	7	3
Ser[d]	20.0 ± 0.4	(20)	12	5
Glu	28.7 ± 0.3	(29)	22	7
Pro	16.2 ± 0.8	(16)	19	0
Gly	20.8 ± 0.4	(21)	23	2
Ala	16.7 ± 0.1	(17)	18	0–1
Val[e]	10.7 ± 0.1	(11)	10	2
Met	3.3 ± 0.4	(4)	3	1
Ile[e]	14.0 ± 0.5	(14)	10	3
Leu	16.1 ± 0.2	(16)	15	3–4
Tyr	17.8 ± 0.1	(18)	16	2
Phe	7.2 ± 0.2	(7)	5–6	2
His	10.8 ± 0.1	(11)	10–11	1
Lys	9.4 ± 0.6	(9)	3	5
Arg	15.8 ± 0.3	(16)	14	2
Try	n.d.	(2)[f]	2	0
Total		257	214–216	46–48

[a] Values are given in residues/molecule (values in parentheses are best or nearest integers)
[b] Time-course hydrolysis: 24, 48 and 96 h (based on M_r 29 500)
[c] Taken from [11]; values for the γ-chain were recalculated on the basis of 2 arginines/molecule established by sequence analysis (L. K. D., A. K., in preparation)
[d] Extrapolated to zero time of hydrolysis
[e] Average of 48 h and 96 h values
[f] Not determined, value of 2 taken from α-chain

of A subunit (fig.2B) all reacted positively with anti-subunit A as judged by Ouchterlony double diffusion. Amino acid compositional analyses of the component chains of A subunit are listed for comparison in table 1.

3.2. *Amino- and carboxyl-terminal sequence analysis*

Automated amino-terminal sequence analysis of the M_r 29 500 component of A subunit (peak B2, fig.2B) revealed only a single sequence which was identical to that of the α-chain sequence (table 2). In addition, hydrolysis of the M_r 29 500 component with carboxypeptidase A identified leucine as the carboxyl-terminus (1 mol leucine released/mol protein). Similar hydrolysis of the γ-chain also identified leucine to be carboxyl-terminus.

Table 2
Automated sequence analyses[a] of precursor A subunit and α-chain of cholera toxin

Edman cycle[b]	Precursor A subunit[c]		α-Chain[d]	
0	–	(25.0)	–	(58.0)
1	Asn	(22.5)	Asn	(50.1)
2	Asn	(23.7)	Asn	(45.9)
3	Asp	(18.4)	Asp	(40.2)
4	Lys	(11.2)	Lys	(27.8)
5	Leu	(6.9)	Leu	(35.0)
6	Tyr	(1.1)	Tyr	(28.3)
7	Arg	(1.9)	Arg	(7.3)
8	Ala	(2.2)	Ala	(24.5)
9	Asp	(1.2)	Asp	(25.2)

[a] Pth residues quantitated by amino acid analysis after back-hydrolysis (nmol)
[b] In addition, analyzed by gas chromatography
[c] Initial nmol applied to sequencer
[d] In addition, analyzed by HPLC

4. Discussion

Rapid purification of cholera toxin, even in the absence of proteolytic inhibitors, using hexametaphosphate precipitation and chromatography on phosphocellulose as in [8], resulted in the isolation of a single chain precursor of subunit A in addition to subunit A. The yield of the precursor form was usually ~30% of total cleaved and uncleaved A subunit. Amino-terminal sequence analysis of the uncleaved M_r 29 500 component identified a single sequence which was identical to that obtained for the α-chain of subunit A. The α-chain sequence obtained was essentially identical to that in [16] except that we have identified position 2 to be Asn rather than Asp. The γ-chain amino-terminal sequence is uniquely different from that of the α-chain [6,16]. Moreover, carboxyl-terminal analysis of the M_r 29 500 component gave results which were identical to the γ-chain and identified leucine as carboxyl-terminus. The carboxyl-terminus of the α-chain was reported to be serine [17]. These results established that the primary structure of the uncleaved precursor form of A subunit begins with the α-chain sequence and is followed by the γ-chain sequence, i.e., α-γ. The amino acid composition of the precursor A subunit was comparable to the summation of the compositions of α- and γ-chains (table 1). These results suggested that enzymic hydrolysis of the precursor form probably did not involve any sig-

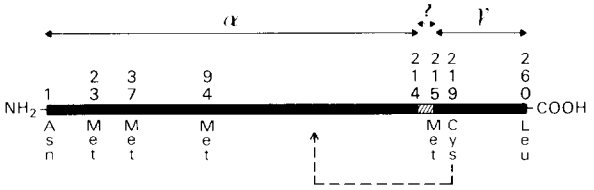

Fig.3. Schematic representation of the primary structure of the precursor A subunit of cholera toxin. Position of methionyl residues from [17]. Cys-219 of γ- chain attaches to an undetermined cysteinyl residue in the carboxyl-terminal CNBr fragment of the α-chain [17]. Hatched region between residues indicates possible removal of residues by limited proteolysis.

nificant reduction of residues; however, it is possible that a few intervening amino acids may have been excised by limited proteolysis (fig.3).

Rapid purification of cholera toxin in essentially two steps and fractionation of the toxin into subunits by gel filtration on Sephadex G-75 identified a high molecular mass contaminant that contained carbohydrate and protein. This material stained poorly with Coomassie blue when compared to pure proteins on a weight basis. A small amount of cholera toxin was bound virtually irreversibly to this component. Our preliminary evaluation is that this material may be lipopolysaccharide and that trace amounts of toxin strongly bound to it. The relative amounts of this material appeared to vary during purification of different batches of toxin.

Indirect evidence had indicated that the A subunit of cholera toxin occurs as a single chain precursor [7,8]. These results provide direct chemical evidence for the occurrence of a single chain A subunit precursor. The purification, amino acid composition, and carboxyl- and amino-terminal analysis are described. These results established that the amino acid sequence of the precursor begins with the α-chain sequence and is followed by the γ-chain sequence. Fig.3 summarizes our present understanding of the structure of the A subunit of cholera toxin. However, this structure also may occur as a result of limited proteolysis of a still larger fragment as suggested by biosynthesis studies [9].

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