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INVOLVEMENT OF ARGININE RESIDUES IN THE BINDING SITE OF CHOLERA TOXIN SUBUNIT B

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SUMMARY: Modification of one or two of three guanido groups in the binding subunit (B) of cholera toxin was achieved at pH 9 with cyclohexanedione at 50 mM or 150 mM concentration, respectively. No change in the helix content or the pentameric structure was observed in the process. The ability to form precipitate with ganglioside G_{m1} or anti-cholera toxin antibody was abolished only when two of the three arginine residues were modified. Analyses of Arg-containing peptides revealed that reaction with cyclohexanedione occurred first with Arg-73 and then with Arg-35. This suggests that Arg-35 or the region in proximity is involved in the interaction of subunit B with ganglioside G_{m1} or the antibody. A diagram of secondary structure as predicted by the Chou-Fasman rule indicates that this region is within a long stretch of β-sheet configuration.

Cholera toxin is a multi-subunit protein consisting of two types of subunits, A and B, in a molar ratio of 1:5 [for a review, see (1)]. Subunit A is involved in the activation of adenylate cyclase, while subunit B serves to attach the toxin to cell surfaces during the intoxication process (1-3). The cell surface substance reponsible for the binding of cholera toxin, through the subunit B, has been identified as ganglioside G_{m1} (4,5). Recently, the primary structure of the subunit B has been elucidated (6,7), and its limited analogy to β-chains of glycoprotein hormones discussed (8).

Lönnroth and Holmgren surveyed the effects of various protein reagents on the activity of cholera toxin (9,10), and found that reagents which modified guanido groups in protein decreased the toxicity, but not the G_{m1} binding or antibody binding of the toxin. The identity of the subunit affected and extent of chemical modification were not determined, however.

In this communication, we report the effect of arginine modification in abolishing the G_{m1}-binding and antibody-binding capacity of cholera toxin subunit B. Evidence suggests that Arg-35 is near or at the binding site of subunit B, within a stretch of random coil between residues 21 and 52, as predicted from the Chou-Fasman rule (11).

MATERIALS AND METHODS

The binding subunit (B) of cholera toxin was prepared from the latter by gel filtration on Sephadex G-75 in 5% formic acid as described previously (2,6). The lyophilized subunit was dissolved in 8 M urea and dialyzed against a gradient of urea from 4 M to 0 M in 0.05 M Tris-HCl buffer (pH 7.5) containing

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0.1 M NaCl, 1 mM EDTA and 0.3 mM NaN_3 at 3° to effect its renaturation. Antiserum to cholera toxin was prepared in the rabbit using purified cholera toxin as previously described (2).

For reaction with cyclohexanedione (12), subunit B was dialyzed overnight against a large volume of 0.1 M [Na] buffer (pH 8.5) at 3°. One volume of dialyzed subunit B (1-2 mg/ml) adjusted to the specified pH was treated with 0.33 volume of cyclohexanedione in the borate buffer at room temperature for 1 hr (see Fig. 1). The modified protein was precipitated with 5% trichloroacetic acid, washed with cold acetone, suspended in H_2O and lyophilized. The protein dissolved readily in 0.1 M borate [Na] buffer (pH 8.5) and was used in the binding experiments.

Sedimentation equilibrium and velocity measurements were made with a Beckman Model E Ultracentrifuge equipped with UV absorbance monitoring. Circular dichroism spectra were measured with a Cary 61 Spectropolarimeter equipped with a difference accessory.

RESULTS

Reaction of cyclohexanedione with cholera toxin subunit B - The reaction between cyclohexanedione and the subunit B of cholera toxin appeared to occur within 1 hr ($t_{1/2} \cong 10$ min) at pH 9 at room temperature (Fig. 1a). The extent of the arginine modification was found to be strongly influenced by the pH of the reaction medium; virtually no reaction took place at pH 7.5 with 50 mM cyclohexanedione and almost all arginine residues in subunit B were modified at pH 11 (Fig. 1b). At pH 9, the extent of modification did not exceed 65% even with a high concentration of cyclohexanedione (Fig. 1c). Under this condition, one of the three arginine residues in subunit B was modified with cyclohexanedione at 50 mM and two of the three at 150 mM. The subunit with one arginine modified was designed CHD_1-B and that with two modified, CHD_2-B.

The binding properties of the cyclohexanedione-modified subunit B - Subunit B of cholera toxin in its natural polymeric form (2) [choleragenoid] formed a precipitin line with ganglioside G_{m1} in a Ouchterlony double-diffusion

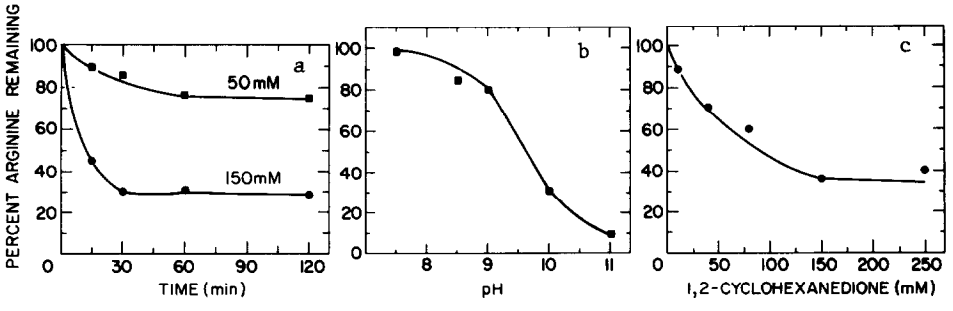


Fig. 1. Reaction of cyclohexanedione with cholera toxin subunit B. The reactions were performed as described in "Methods". After the reaction, samples were hydrolyzed in 5.7 N HCl containing 0.1% v/v mercaptoacetic acid (12) under vacuum for 22 hr at 110° and analyzed for unmodified arginine using a Jeol 6AH Amino Acid Analyzer. Percentages of arginine remaining were calculated in relation to Lys and His from the same hydrolysate. [a] Time course of the reaction. [b] pH dependence at 80 mM cyclohexanedione. [c] Extent of reaction at various reagent concentrations at pH 9.0.

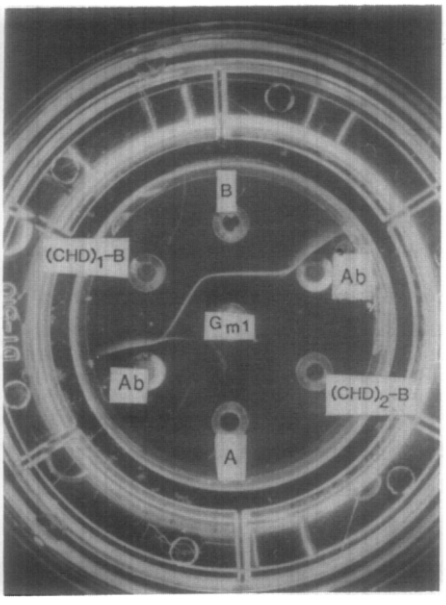
plate as with anti-cholera toxin antibody (Fig. 2). When 35% of arginine residues were reacted with cyclohexanedione, the modified subunit B (CHD_1-B) still retained the binding activity towards ganglioside G_{m1} as well as antibody. This property was lost when two-thirds of the arginines were modified (CHD_2-B).

Physicochemical properties - In its native state, subunit B is a polymer with a molecular weight of 55,200, as determined by the sedimentation equilibrium method (2). No change in molecular weight was observed after the arginine modification was performed. It is suggested that CHD_1-B and CHD_2-B retained the polymeric structure of the subunit B.

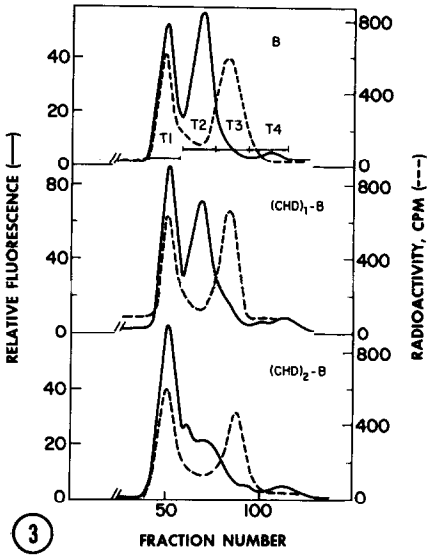
The circular dichroism spectrum of cholera toxin subunit B showed a single negative trough at 218 nm, in agreement with that reported by Fishman et al. (15). After reaction with cyclohexanedione, slight increases in the mean residue ellipticity were observed: $[\theta]_{218}$ for subunit B was 10,200, while that of CHD_1-B and CHD_2-B were 11,100 and 13,080, respectively. However, there were no changes in the shape and position of the trough, indicating that no significant change in conformation had occurred.

Location of the modified arginine residues in subunit B - The subunit B contains a Lys-Arg sequence at positions 34-35 and Lys-Lys at positions 62-63, and thus yields one each of free arginine and lysine per mole upon complete digestion with trypsin (6). To determine whether Arg-35 was affected by the modification reaction, samples were digested with chymotrypsin, then with trypsin and the digest analyzed for free lysine and arginine residues. The results indicated that Arg-35 was modified in CHD_2-B but not in CHD_2-B (Table I).

In an attempt to isolate peptides containing the modified arginine, tryptic digests of CHD_1-B and CHD_2-B were gel filtered on Sephadex G-25, and the elution patterns compared with that from native subunit B (Fig. 3a). The arginine-containing peptides, Ala-Ile-Glu-Arg (64-67), Met-Lys-Asn-Thr-Leu-Arg (68-73) and Ile-Phe-Ser-Tyr-Thr-Glu-Ser-Leu-Ala-Gly-Lys-Arg (24-35), from incomplete trypsinolysis were all found in the T2 fraction from unmodified subunit B (6). Progressive decreases in the T2 fraction and corresponding increases in T1 were observed with samples from CHD_1-B and CHD_2-B. This indicated that cleavage of an arginyl bond by trypsin was inhibited on its modification with cyclohexanedione. Chromatography of T2 fractions from CHD_1-B and CHB_2-B (Figs. 3b and 3c) separately on an Aminex-A5 column both yielded significant amounts of peptide, Ala-Ile-Glu-Arg (64-67) [Table I], indicating that Arg-67 remained unmodified after two other arginines had reacted (in CHD_2-B). By inference, Arg-73 should be the residue modified in CHD_1-B, and Arg-73 and Arg-35, in CHD_2-B.



(2)



(3)

Fig. 2. Interaction of native or modified subunit B with ganglioside G_{m1} and anti-cholera toxin antibody in a double diffusion plate (IDF Cell I, Cordis Lab., Miami, FL). Native subunits A and B of cholera toxin, modified subunit B [CHD_1-B and CHD_2-B], ganglioside G_{m1} (G_{m1}, .21 mg/ml) and anti-cholera toxin antibody [AB] were placed in the designated wells, and incubated for 24 hr at 37°. All protein concentrations were adjusted to 1 mg/ml with 50 mM Tris-HCl (pH 7.5) and 0.1 M NaCl

Fig. 3: Tryptic peptide patterns of native and modified subunit B on Sephadex G-25. Samples were reduced and S-carboxymethylated with [^{14}C]iodoacetic acid essentially as described previously (2), except for the use of borate [Na] buffer instead of Tris-HCl buffer. These were treated with 2% (by weight) of TPCK-trypsin (Sigma Chemical Co., St. Louis, MO) in 50 mM borate [Na] buffer (pH 8.5) for 6 hr at 37°, then gel filtered on a Sephadex G-25 column (1.5 × 200 cm) in the same buffer. The flow rate was 5 ml/ hr and 1.0 ml fractions were collected. Aliquots were analyzed for peptides with fluorescamine after alkaline hydrolysis (6) and for cysteine-containing radiolabeled peptides. Fractions designated with T1-T4 were combined for further separation or analyses.

TABLE I. Determination of unmodified arginine after tryptic digestion.

	% Yield on Trypsinolysis			
	Lys-36[a]	Arg-35[a]	Peptide 64-67[b] (Arg-67)	Peptide 68-73[b] (Arg-73)
Subunit B	96.6 (1)[c]	70 (1)	90 (1)	46 (1)
CHD_1-B	96.4 (1)	72 (1)	68 (0.8)	0 (0)
CHD_2-B	96.4 (1)	25 (0.3)	61 (0.7)	0 (0)

[a] On exhaustive digestion with trypsin of the subunit B (see text), Lys-63 and Arg-35 are released from the -Gln-Lys-Lys-Ala- and the -Gly-Lys-Arg-Glu-sequences, respectively. The digests were analyzed directly on an amino acid analyzer.

[b] The peptides containing Arg-67 and Arg-73 were isolated from fraction T2 (Fig. 3) by ion-exchange chromatography (6) and quantitated by amino acid analysis. The overall yields are shown.

[c] The ratio to the value from unmodified subunit B.

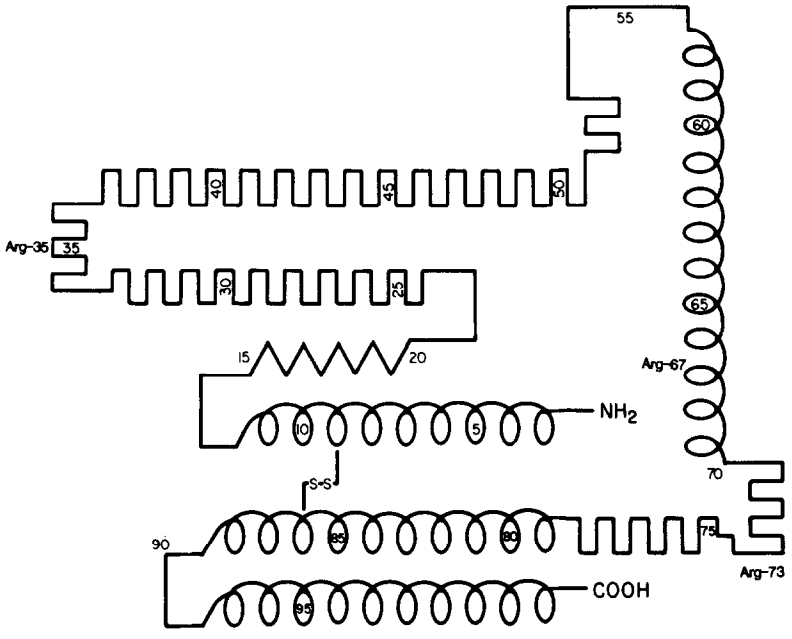


Fig. 4: A schematic diagram of the secondary structure of cholera toxin subunit B predicted by the Chou-Fasman method (11). Reaction with cyclohexanedione occurred first at Arg-73 and then at Arg-35. Arg-67 remained unreacted under the conditions used.

Secondary structure prediction for cholera toxin subunit B - Based on the known primary structure of cholera toxin subunit B (6), regions containing α-helix, β-turn and coil configuration in the polypeptide chain were predicted by the method of Chou and Fasman (11). A schematic diagram was constructed on the basis of the prediction and the known disulfide bridge between Cys-9 and Cys-86 (Fig. 4). Both Arg-73 and Arg-35, the first and second residues to be modified, respectively, are located within the predicted coil region, whereas the unreactive Arg-67 is within an α-helix.

DISCUSSION

The initial observation that G_{m1} inhibits the binding of cholera toxin to membranes suggested a possible binding site for G_{m1} in the subunit B (4). Markel *et al.* suggested that the binding function of the subunit B was dependent on its multivalent nature and that modification of residues committed to subunit association could result in the loss of G_{m1} binding (13).

Recent studies by Rosenblatt *et al.* (14) on parathyroid hormones and by Mahley *et al.* (15) on low-density lipoproteins showed that cyclohexanedione selectively modified arginyl residues in the proteins and abolished their binding to cell surface receptors. They concluded that arginine residues played a role in receptor interaction.

In the present study, selective modifications of arginine residues in the binding subunit of cholera toxin was achieved at pH 9 and appropriate concentrations of cyclohexanedione. When 25% to 35% of the arginines in subunit B (∿ one arginine) were modified, there was no effect on either G_{m1} binding or antibody binding. However, when 60% of the arginines (∿ two arginines) were modified, both G_{m1} and antibody binding were abolished, in contrast to the results of Lönnroth and Holmgren in which the holotoxin was used (9,10). Analyses on peptides containing unreacted arginine indicated that Arg-73 was the first to be modified and Arg-35, the second. The analyses also suggested that Arg-35 or the region nearby was involved in the interaction of subunit B with G_{m1}, cell surface or the antibody. This region consists of random coil configuration as predicted by the method of Chou and Fasman (11). Modification of one or two arginines did not result in changes in the physical properties of the molecule (in polymeric form), as determined by circular dichroism and sedimentation studies.

Peptides containing unmodified arginines were analyzed in this study because methods for a sensitive detection of cyclohexanedione derivatives of arginines, and hence the peptides containing these residues, were not available (12). Attempts to identify modified arginines through regeneration of the residue with NH_2OH (12) only yielded equivocal results. Positive identification of peptides containing modified arginines must await the availability of radiolabeled cyclohexanedione.

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