Chemical and Immunochemical Studies on the Receptor Binding Domain of Cholera Toxin B Subunit*

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The contributions of various amino acids to the structure and function of cholera toxin B subunit were assessed with quantifiable, chemically conservative, reversible derivatizations, and sensitive assays of activity. A panel of monoclonal antibodies was employed to monitor the conformational integrity of modified protein and help distinguish the direct from indirect effects of chemical derivatization. We describe a novel monoclonal antibody, which competes with the receptor GM1 for binding to cholera toxin B subunit, and use this reagent to help identify critically located residues.

Our data support the hypothesis that tryptophan participates directly in binding GM1. In addition, we propose a dual role for lysine: first, these basic residues maintain an electrostatic attraction vital to receptor recognition; second, at least 1 lysine resides near the receptor binding domain and may interact with GM1.

The influence of arginyl and tyrosyl residues upon activity is re-examined. Finally, we present data which suggest, in variance with previous studies, that the intramolecular disulfide bond is vital to the structure and function of cholera toxin B subunit.

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The clinical manifestations of cholera are attributable to the physiologic effects of a toxin elaborated by Vibrio cholerae (1-3). Cholera toxin, an 85,000-dalton protein, is composed of two subunits, A and B (4, 5). The A subunit contains two polypeptide chains, A1 (Mr, 22,000) and A2 (Mr, 5,000), linked by a disulfide bond (6). A1 is an enzyme which can penetrate the plasma membrane of susceptible cells and catalyze the transfer of an ADP-ribose moiety from NAD to the N₂ regulatory protein component of adenylate cyclase. As a result, cAMP is produced constitutively (7, 8).

The B subunit consists of a noncovalent pentameric aggregate of identical polypeptide chains (6), the primary structure of which has been determined (9, 10). Each chain is composed of 103 amino acids; an intrachain disulfide bridge links Cys⁶⁶ and Cys⁸⁶. The B subunit's specific receptor is a glycosphingolipid, the monosialoganglioside GM₁. The dissociation constant for this interaction is approximately 10⁻⁷ M (11). The oligosaccharide moiety of GM₁, devoid of ceramide (OS-GM₁), exhibits similar affinity for CT-B (12, 13). Studies reveal that five OS-GM₁ molecules can simultaneously bind the B pentamer (13).

Although much is known about CT-B, the chemical basis of its biologic functions has not been fully elucidated. Specifically, the amino acids which mediate receptor recognition remain largely undefined. Consequently, CT-B constitutes an interesting model with which not only molecular pathogenesis, but also protein-carbohydrate interaction can be examined.

Physical studies of the B subunit have shown that the binding of GM₁, but not related gangliosides, induces a shift in the fluorescence emission spectrum maximum of cholera toxin from 342 nm to 330 nm (14). This "blue shift," which results from interaction between the B subunit and the oligosaccharide moiety of GM₁ (15, 16), reflects a conformational change affecting the single tryptophan residue of CT-B. Using solute quenching and fluorescence energy transfer studies, De Wolf et al. (17) found that this tryptophan is located in a positively charged microenvironment near the GM₁ binding site.

Several investigators have modified CT-B with chemical reagents designed to derivatize specific amino acids. Lonrho and Holmgren (18) assessed the effects of 20 such modifications on the antigenicity, toxicity, and receptor binding activity of holotoxin. Markel et al. (19) found that lysine-directed acylation abolished the GM₁ binding properties of CT-B, but reduction and alkylation of the disulfide bond had no significant effect. Duffy and Lai (20), using the guanidino-specific reagent cyclohexanediol, implicated Arg⁸⁵ as critical to the antigenicity and function of CT-B. Support for the involvement of tryptophan in receptor recognition stems from a study by De Wolf et al. (21) employing 2,4-dinitrophenylsulfonyl chloride.

Chemical modification studies indeed provide important data regarding the role of various amino acids in the structure and function of proteins. However, interpretation of such data is limited by a fundamental ambiguity—difficulty in distinguishing the direct effects of site-specific derivatizations from allosteric effects, conformational changes, and side-reactions.

† The abbreviations used are: GM₁, galactosyl-[-N-acetylgalactosa-mi]-[N-acetylenuraminyl]galactosylglucosylceramide; CT-B, cholera toxin B subunit; OS-GM₁, the oligosaccharide moiety of GM₁ mAb, monoclonal antibody; TNBS, trinitrobenzenesulfonic acid; PBS, phosphate buffered saline, pH 7.4; BSA, bovine serum albumin; hLT or pLT, labile toxin produced by enterotoxigenic strains of E. coli isolated from humans or swine, respectively.
This ambiguity is compounded by failure of the above-cited chemical studies to demonstrate one or more of the following: (a) reversibility, confirming the chemical specificity of modification; (b) preservation of higher order protein structure, thereby eliminating conformational distortion as a possible cause of biologic inactivity; (c) functional sequence of derivatization with sensitive assays which differentiate between minor decreases in binding affinity and major disruption of activity.

The present study examines the contribution of various amino acids to the receptor binding function of CT-B using quantifiable, reversible derivatizations and sensitive assays of binding. A panel of monoclonal antibodies is employed as reporter groups to monitor the conformational integrity of modified protein and thus help differentiate between the direct and indirect effects of chemical derivatization. In addition, we use a monoclonal antibody that competes with & reporter groups to monitor the conformational integrity of modified protein and thus help differentiate between the amino acids to the receptor binding function of CT-B using quantifiable, reversible derivatizations and sensitive assays of activity.

EXPERIMENTAL PROCEDURES

Materials—Cholera toxin B subunit from V. cholerae strain 569B, purified by affinity chromatography (22), was a generous gift from Institut Merieux, Marcy, France. All chemicals obtained commercially were of the highest grade available unless otherwise stated. The following reagents were purchased from Sigma: dithiothreitol, 2-iodoacetic acid, mercaptoacetic acid, 100% (w/v) aqueous trichloroacetic acid, hydroxylamine, Polin and Clozolin's phenol reagent, 2,4,6-trinitrobenzenesulfonic acid, 2-thiobarbituric acid, orcin ferric chloride, and bovine serum albumin. Formic acid (95-97%), 2,4-cyclohexanedione, and succinic anhydride were purchased from Alrich. Acetic anhydride, formaldehyde, and sodium borohydride were obtained from J. T. Baker Chemical Co. Citric acid monohydrate (Sigma), urea, ammonium chloride, pH 9.6, containing 1% mercaptoacetic acid (33). Reduction and alkylation of the disulfide bond, based on the procedure of Crestfield et al. (35), was conducted in 6 M guanidine HCl or 8 M urea at pH 8.1 with dithiothreitol (100 mol/mol of Cys) and iodoacetic acid or iodoacetamide (2.5-fold molar excess over dithiothreitol). Denaturation was removed by dialysis against 4, 2, 1, and 0.5 M guanidine HCl.

Preparation of OS-GM1-The oligosaccharide moiety was cleaved from GM1 by osmolyzing and alkaline fragmentation, purified by iso-exchange chromatography, and assayed by thin layer chromatography according to the method of Wiegandt and Bücker (23) as modified by Fishman et al. (13). The concentration of OS-GM1 was determined by the tiobarbituric acid assay of Aminoff (24) as adapted by Lee and Eun (25).

Preparation of Monoclonal Antibodies (mAbs)—Methods for the preparation of mAbs 2BD3, 4D9, 4E2, 22C6, and 15C11 were previously described (26). Two additional clones were obtained with differences in the receptor binding activity. After subtraction of the background of the myeloma bank, mAb 35G8 (isotype IgG1) was detected by screening for clones producing antibodies which bound toxin but not the GM1 toxin complex, and mAb 35G8 (isotype IgG1) by screening for cross-reactivity with toxin from V. cholerae strain 569B but not 3082.

Protein Modifications—Tryptophanyl residues were formylated in HCl-saturated formic acid according to Previore et al. (27), as modified by Holagren (28). Formic acid was distilled prior to use. The extent of derivatization was calculated from the absorbance at 298 nm using a molar extinction coefficient of 4880 cm⁻¹ (27). Deformylation was conducted by incubation in 0.2 M phosphate buffer at pH 11.5 for 30 min or 0.5 M ammonium chloride, pH 9.6, containing 8 M urea, overnight. Quantitative citraconylation of lysine was performed with a 100-fold excess of anhydride (moles/mole amino group) in 0.2 M borate buffer, pH 8.2, 0 °C (29). After 1 h, the solution was divided in half. One portion was dialyzed against borate buffer, pH 8.0, 0 °C for 3 h and assayed for citraconylation by amino acid analysis. The second portion was dialyzed against 2% cold trichloroacetic acid, washed with 8 M urea, dried in vacuo, and resuspended in borate buffer at pH 8.0.

The extent of modification was assessed by TNBS assay (30). Reduction and alkylation were done at 0 °C in 0.2 M borate buffer, pH 9.0, with formaldehyde or acetone and sodium borohydride according to Means and Feeney (32). The reaction was terminated after 1 h by trichloroacetic acid precipitation, and free amino groups were assayed as described above. Arginine-specific derivatization was accomplished by incubation in 0.5 M hydroxyamine, pH 7.0, overnight. Acylation of tyrosine was performed with a 60-fold molar excess of acetylation in borate buffer at pH 7.5 according to the procedure of Siu and Lai (30). Reduction and alkylation of the disulfide bond, based on the procedure of Crestfield et al. (35), was conducted in 6 M guanidine HCl or 8 M urea at pH 8.1 with dithiothreitol (100 mol/mol of Cys) and iodoacetic acid or iodoacetamide (2.5-fold molar excess over dithiothreitol). Denaturation was removed by dialysis against 4, 2, 1, and 0.5 M guanidine HCl.

Preparation of mAbs—Preparation of mAbs involves affinity chromatography on solid-phase is expressed as % maximum binding = (counts bound [competitor = X]) / (counts bound [competitor = 0]) × 100%.

Receptor binding activity is defined as the ratio of IC₅₀ (native CT-B) to IC₅₀ (modified CT-B) multiplied by 100%, where IC₅₀ is the concentration of competitor which produces a 50% reduction of cross-reactivity. The antiguicity of derivatized protein was assessed by a modification of the assay described above: CT-B (5 µg/ml) was coated onto microtiter wells by overnight incubation in PBS. The wells were washed with PBS/BSA, serial dilutions of modified protein were added, and monoclonal antibody (15000 dilution of culture supernatant) was introduced. After 12 h at 37 °C, the wells were washed with PBS/BSA, serial dilutions of modified protein were added, and monoclonal antibody (15000 dilution of culture supernatant) was introduced. After 12 h at 37 °C, the wells were washed and bound radioactivity was determined. The ability of modified protein, at concentration X, to block binding of ¹²⁵I-CT-B to solid-phase GM1 is expressed as % maximum binding (see above).

RESULTS

Seven Monoclonal Antibodies Recognize at Least Five Distinct Conformation-sensitive Epitopes on CT-B—The preparation and binding specificity of five anti-CT-B monoclonal antibodies have been reported (28). Two additional mAbs (40B10 and 15C11) were obtained by alternative screening methods (see "Experimental Procedures"). These antibodies recognize at least three different epitopes based on cross-reaction patterns with the CT homologues hLT and pLT produced by enterotoxigenic strains of Escherichia coli (Table 1). Cross-reactivity with chemically modified CT-B indicates the presence of two additional epitopes (discussed below and summarized in Table 1). Several observations suggest that these antigenic determinants are composed of residues non-contiguous in sequence but juxtaposed in space by virtue of protein folding. First, none of these mAbs binds any of eight
The antigenicity of the CT homologues hLT and pLT produced by enterotoxigenic strains of E. coli, and chemically modified CT-Bs were assessed by competitive radiobiinding assay (see "Experimental Procedures") or solid-phase radiobiinding assay as described below. Polyvinyl microtiter wells were sensitized with antigen (5 μg/ml in PBS overnight), washed with 0.2% (w/v) BSA in PBS, and incubated with serial dilutions of monoclonal antibody for 12 h at 37°C. The wells were washed and approximately 50,000 cpm of 125I-labeled protein A was added. One h later, the plates were washed and bound radioactivity was determined by γ-counting.

### Cross-reactivity of the anti-CT-B monoclonal antibodies

The antigenicity of the CT homologues hLT and pLT produced by enterotoxigenic strains of E. coli, and chemically modified CT-Bs were assessed by competitive radiobiinding assay (see "Experimental Procedures") or solid-phase radiobiinding assay as described below. Polyvinyl microtiter wells were sensitized with antigen (5 μg/ml in PBS overnight), washed with 0.2% (w/v) BSA in PBS, and incubated with serial dilutions of monoclonal antibody for 12 h at 37°C. The wells were washed and approximately 50,000 cpm of 125I-labeled protein A was added. One h later, the plates were washed and bound radioactivity was determined by γ-counting.

### Table I

| Monoclonal antibody | 32D3 | 40D9 | 4E2 | 22C6 | 15C11 | 30G8 | 40B10 |
|---------------------|------|------|-----|------|-------|------|-------|
| CT-B               | +    | +    | +   | +    | +     | +    | +     |
| CT-B + GmI         | +    | +    | +   | +    | +     | +    | +     |
| hLT                 | +    | +    | +   | +    | +     | +    | +     |
| pLT                 | +    | +    | +   | +    | +     | +    | +     |
| CT-B modified with |      |      |    |      |       |      |       |
| HCl/formic acid    | +    | +    | +   | +    | +     | +    | +     |
| Citraconic anhydride| -   | -    | +   | -    | -     | -    | -     |
| C6H2O/NaBH4         | +    | +    | +   | +    | +     | +    | +     |
| Cyclohexanediene    | +    | +    | +   | +    | +     | +    | +     |
| Acetylirimidazole   | +    | +    | +   | +    | +     | +    | +     |
| Dithiothreitol/ICH2COOH | -  | -    | +   | -    | -     | -    | -     |

* Cross-reactivity was determined by competitive radiobiinding assay.
* Cross-reactivity was determined by solid phase radiobiinding assay.
* CT-B was bound to solid-phase GmI.
* Minus signifies less than 5% binding in comparison to native CT-B.

### Fig. 1. Competition between mAb 40B10 and OS-GmI for binding to CT-B.

Serial dilutions of OS-GmI were mixed with each of the seven monoclonal antibodies (see Table I) at 1:5000 dilution of culture supernatant, and the resulting solutions were added to polyvinyl microtiter wells containing CT-B in the solid phase. After 12 h at 37°C, the wells were washed and approximately 50,000 cpm of 125I-protein A was added. One h later, the plates were washed and bound radioactivity was determined by γ-counting. Nanomolar concentrations of OS-GmI competitively inhibited the binding of mAb 40B10 to CT-B (○—○). All other antibodies, as represented by mAb 29C6 (O—O), were unaffected by OS-GmI.

### Recognition of Tryptophan—The B subunit contains a single tryptophan (Fig. 2) whose participation in receptor

The eight synthetic peptides correspond to the following CT-B residues: 1-9, 9-21, 26-38, 40-50, 50-61, 62-73, 73-86, and 86-97. The three proteolytic fragments (residues 1-37, 38-68, and 69-101) were produced by cyanogen bromide cleavage of reduced and carboxymethylated CT-B.
mylation and the concomitant reversal of spectrophotometric changes occur at high pH (Fig. 3B). Formylated CT-B does not bind GM1, while deformylated protein regains partial activity (Fig. 4A). Complete functional reversal was not achieved owing, perhaps, to degenerative changes during incubation at high pH. Nevertheless, partial restoration of activity confirms the importance of tryptophan in binding GM1. Formylation reversibly disrupts the binding of mAb 40B10 (Fig. 4B), but does not affect binding of the other monoclonal antibodies (Table I). These findings suggest that tryptophan does indeed reside near the receptor binding domain of CT-B and that functional perturbations associated with formylation result not from gross conformational distortion, but rather the direct and local effects of covalent derivatization.

Modification of Lysine—The B subunit contains 9 lysines (Fig. 2). Modification of lysyl residues with a 100-fold molar excess of succinic anhydride in guanidine HCl by Markel et al. (19) abolished receptor binding activity. This acylating agent, however, is not absolutely specific for amino groups and reacts, even near neutral pH, with tyrosyl, histidyl, cysteinyl, seryl, and threonyl residues (39, 40). Moreover, fully succinylated CT-B gains approximately 20 negative charges and may be unable to renature following exposure to guanidine HCl. Here, we examine the importance of lysine with a reversible modification conducted under conditions of physiologic pH and ionic strength.

The amino groups of CT-B react quantitatively with citraconic anhydride in 0.2 M borate buffer, pH 8.0, as determined by TNBS assay (data not shown). Decitraconylation of the N-acyl derivative occurs spontaneously at low pH. O-Acyl groups that may form as side reactions are stable under these conditions (39). Fig. 5 depicts the effects of modification on CT-B with 2,4-cyclohexanedione and found that derivatization of Arg9, second of the 3 residues to react, correlates with loss of receptor binding activity, as determined by an Ouchterlony-type double-diffusion analysis. The double-diffusion analysis, however, does not measure binding directly, relying instead upon visualization of stable CT-B/
Modification of Tyrosine—CT-B was derivatized with N-acetyl-2-imidazole according to the method of Riordan et al. (34) for the determination of surface exposed tyrosyl residues. Spectral changes indicated that 1 of 3 tyrosines in CT-B reacted ($
\delta E_{\text{cm}} = 1160 \text{ M}^{-1} \text{cm}^{-1}$). Modification did not significantly affect receptor binding activity (Fig. 8) or antigenicity (Table I). This finding is in concordance with Markel et al. (19) who employed tetranitromethane.

Modification of the Disulfide Bridge—Reduction and carboxymethylation was conducted with iodoacetamide or iodoacetic acid in guanidine HCl after Crestfield et al. (35). The reaction was quantitative and did not affect other residues, as determined by amino acid analysis. Modification abolishes receptor binding activity (Fig. 8) and antigenicity (Table I), suggesting that the disulfide bond is critical to the maintenance of conformation and function. In control experiments, exposure of CT-B to guanidine HCl per se does not affect activity. Markel et al. (19) argue against the importance of this bond, finding that reduced and alkylated CT-B retains partial receptor binding function in a competitive radiobinding assay. However, their derivatized protein manifests less
2,4-cyclohexanedione. dione under conditions that resulted in the progressive modification of arginyl residues (Table 1). Receptor Binding Activity (see "Experimental Procedures") is depicted as a function of arginine modified.

DISCUSSION

The results of protein modification studies can be difficult to interpret. Chemical reagents seldom display absolute specificity for a single amino acid side chain. Even well described procedures yield atypical reactions due to the unique micro-environment of certain residues within native protein. Furthermore, the direct and local effects of derivatization are often indistinguishable from allosteric influence or the consequences of conformational disruption. This report presents an approach—employing reversible, quantifiable reactions, sensitive assays, and immunologic structural probes—which seeks to minimize the limitations inherent to protein modification investigations.

We describe an interesting monoclonal antibody (40B10) which competes with Gm for binding to CT-B and enlisted the aid of this reagent to identify residues located near the receptor binding domain. We found that formulation of the single tryptophan residue in CT-B reversibly disrupts binding of both GM1 and 40B10 but preserves structural integrity, as monitored by a panel of conformation-sensitive mAbs. This result supports the contention of De Wolf et al. (21) that tryptophan participates directly in the receptor binding event. Lysine is seen to play a dual role. These residues appear to maintain an electrostatic attraction between toxin and receptor without which binding cannot occur. In addition, at least 1 lysyl residue is within the mAb 40B10 epitope and may also interact directly with GM1. We repeated the arginine-specific modification performed by Duffy and Lai (20) but cannot attribute the functional sequelae to derivatization of Arg9.

Finally, our data suggest that a superficial tyrosyl residue is essential, but the intramolecular disulfide bridge is vital to the structure and function of CT-B.

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