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# Synthesis and Characterization of a Heterobifunctional Mercurial Cross-Linking Agent: Incorporation into Cobratoxin and Interaction with the Nicotinic Acetylcholine Receptor†

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ABSTRACT: The heterobifunctional organomercurial reagents 3-(acetoxymercurio)- and 3-(chloromercurio)-5-nitrosalicylaldehyde were prepared, characterized in model studies, and used to probe the interaction between cobratoxin, purified from the venom of the Thailand cobra (*Naja naja siamensis*), and the affinity-purified nicotinic acetylcholine receptor (AcChR) from *Torpedo californica* electroplax. These reagents may also be useful in introducing chemically well-defined heavy metal atoms into proteins containing no reactive thiols. Model reagent adducts were prepared in situ by reductive amination with *N*-butylamine and $N^{\alpha}$-acetyllysine-*N*-methylamide. The nitrophenolic p$K_a$s of the amine adducts were similar to those of the aldehyde reagents though reduced by 1.3–1.5 units when compared with the hydroxylmethyl reduction product. Reaction of either mercuriosalicylaldehyde with cobratoxin led to a single major modification product incorporating 1 mol of the reagent into cobratoxin at Lys 23. The Lys 23 modified toxin had a reduced binding affinity for the AcChR over that of the native toxin ($K_d$ 2.75 nM cf. 0.3 nM). Reduction of the purified AcChR with 1 mM dithiothreitol (DTT) followed by removal of excess thiol led to cross-linking reactions with the Lys 23 modified cobratoxin to both the $\alpha$ and $\beta$ subunits of the AcChR complex. Reaction of DTT-treated AcChR with *N*-ethylmaleimide (NEM) blocked cross-linking, while treatment of the initially cross-linked toxin–AcChR complex with mercaptoethanol leads to reversal of cross-linking. These observations strongly support cross-linking mediated by the formation of a mercury–sulfur bond and further lend support the identity of the respective interacting sites in AcChR and toxin.

Multifunctional reagents have been extensively employed over the past two decades to probe the chemical reactivity of biopolymer surfaces, to study the origins of protein–protein interactions, and, in particular, to examine intrasite relationships at enzyme active centers. Here, we report the synthesis, characterization, and demonstration of the utility of 3-(acetoxymercurio)- and 3-(chloromercurio)-5-nitrosalicylaldehyde. These reagents contain a nitrophenolic group that can be used to follow protein modification by either the aldehyde or mercurial reactivities and to report on the microenvironment at the sites of modification (Hille & Koshland, 1967). Also, proteins or protein complexes thus modified may provide useful heavy metal derivatives for X-ray analysis of protein structure [cf. Edwards et al. (1974)].

These reagents may be employed in multiple though restricted ways [a preliminary report is given in Wohlfeil et al. (1985)]. The aldehyde may be used to reductively alkylate lysine or N-terminal amino groups. Intramolecular cross-

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linking mediated by a reactive organomercurial may occur with free sulfhydryl groups or, secondarily, may be initiated with sulfhydryl groups produced by selective reduction of neighboring cystines. Alternatively, cross-linking of protein modified by reductive alkylation to other proteins bearing free sulfhydryls may be useful in identifying the interacting surfaces of a protein–protein complex. Modification of one protein at or near the interacting site with one of the two reactivities of the reagents would yield a derivative that would act as an affinity reagent for a reactive center located near or within the interacting site of the second protein.

Our long-standing interest in the molecular basis for the action of the curarimimetic neurotoxins (Tsernoglou et al., 1978), and, in particular, the molecular identity of the interaction surfaces within the complex formed between these toxins and the nicotinic acetylcholine receptor (AcChR),[1] led us to develop these and other heterobifunctional reagents (Nickoloff et al., 1985; Wohlfeil et al., 1985) to probe the interacting sites of neurotoxin and AcChR. The application of these mercurial reagents to the study of the toxin–AcChR interaction is reported here. Many of the convenient features as well as the problems, which may accompany the more general use of these reagents, are illustrated.

The curarimimetic neurotoxins are small molecular weight (6–8 kDa) basic proteins from the venoms of Elapid and Hydrophidae snakes [for a review see Karlsson (1979)]. These toxins mimic the action of curare (*d*-tubocurarine) by specific binding to the nicotinic AcChR of the muscle motor end plate, thereby preventing the depolarizing action of acetylcholine (Lee, 1972). The nicotinic AcChR [for reviews see Karlin (1983), Maelicke (1984), and McCarthy et al. (1986)] is now the most intensely studied protein receptor found in excitable membranes. The 280-kDa complex derived from the *Torpedo* electroplax contains five membrane-spanning subunits with the stoichiometry $\alpha_2\beta\gamma\delta$. Each $\alpha$ subunit binds one molecule of acetylcholine (AcCh). The neurotoxin binds competitively with AcCh.

Early crystallographic analyses of toxin structure (Tsernoglou & Petsko, 1976; Low et al., 1976) led us to propose that a cluster of amino acid residues located at the tip of an extended $\beta$-sheet were intimately involved in AcChR binding (Tsernoglou et al., 1978). Nearby but not within this proposed AcChR binding surface was located Lys 23, reported to be the most reactive of the 5–6 lysines typically present in representative neurotoxins (Karlsson, 1979). Selective modification of this residue by a heterobifunctional reagent was thus designed to convert the toxin into an affinity reagent for the AcChR.

Placement of a reactive mercurial near the toxin–AcChR interacting surface by reductive alkylation of lysine 23 would be expected to allow affinity labeling of AcChR sulfhydryls near the site of binding of modified toxin. It has been known for some time that one of the two sites for the toxin binding within the AcChR pentamer could be easily reduced with DTT and affinity alkylated (Weill et al., 1974, Chang et al., 1977, Damle and Karlin, 1978, Moore and Raftery, 1979). Importantly, no affinity labeling occurred with the unreduced AcChR while the reduced AcChR was exclusively labeled on the $\alpha$ subunit. Thus, by prereduction of the AcChR with 1 mM DTT, we believed a Lys 23 mercurial modified toxin would affinity label the AcChR with formation of a mercury–sulfur bond, which would demonstrate the proximal relationship between the region of the toxin neighboring Lys 23 and that region in the $\alpha$ subunit of the AcChR neighboring the easily reduced disulfide.

Here, the preparation of the heterobifunctional mercurial reagents, their reactive properties in model systems, and their use in protein modification are demonstrated with the preparation of the Lys 23 derivative of the representative toxin, cobratoxin. Cross-linking studies with the Lys 23 modified cobratoxin and the DTT-reduced AcChR are also reported.

## Experimental Procedures

*Synthesis of 3-(Acetoxymercurio)- and 3-(Chloromercurio)-5-nitrosalicylaldehyde.* Salicylaldehyde was obtained from Aldrich and used without further purification. All other chemicals were reagent grade. Elemental analysis was performed by Galbraith Laboratories, Knoxville, TN. Melting points were determined on a Thomas-Hoover capillary melting point apparatus. Spectrophotometric data were acquired on Varian DMS-200 UV/vis, Varian 400 MHz FTNMR, and Perkin-Elmer 1600 FTIR spectrometers.

(*A*) *5-Nitrosalicylaldehyde* was prepared by a method only briefly described by Harrison and Diehl (1947). Concentrated nitric acid, 70% (24 g, 0.34 mol), was added dropwise to a cooled (15 °C) and well-stirred mixture of salicylaldehyde (30 g, 0.25 mol) and glacial acetic acid (120 g, 2.00 mol). When the addition was complete, the temperature was allowed to rise to 40 °C over 35 min, at which time the mixture was poured into distilled water (600 mL) and crushed ice (50 g). The deep orange precipitate, a mixture of 3- and 5-nitrosalicylaldehyde isomers, was filtered. The product (33 g) was then taken up in sodium hydroxide (400 mL, 0.8 M) by gentle heating. After filtration to clarify the solution, the 5-nitro isomer recrystallized upon cooling. Recrystallization was repeated twice to yield a total of 15.3 g of the sodium salt of 5-nitrosalicylaldehyde The sodium salt (10 g) was taken up in acetic acid (400 mL, 0.1 M) by warming. Pale yellow needles were obtained on cooling. Recrystallization was repeated twice to give 4 g of 5-nitrosalicylaldehyde, mp 126–127 °C [lit. (*Beilstein*, 1925) mp 126 °C]; IR (KBr) $\nu_{max}$ 3070, 1474, 1343, 719 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.2 (1 H, s, CHO), 8.7 (1 H, s, H6), 8.4 (1 H, d, H4), 7.2 (1 H, m, H3). [Note: 5-nitrosalicylaldehyde has recently become commercially available (Aldrich Chemical Co.)].

(*B*) *3-(Acetoxymercurio)-5-nitrosalicylaldehyde.* Mercuric acetate (2.86 g, 8.98 mol) in acetic acid (25 mL, 0.08 M) was added dropwise over 30 min to a well-stirred solution of 5-nitrosalicylaldehyde (1.50 g, 8.98 mol) in aqueous potassium hydroxide (26.2 mL, 0.30 M) at 70 °C. A yellowish-brown precipitate formed, was filtered from the hot solution, and was washed successively with 0.08 M acetic acid, water, methanol, and diethyl ether. The crude product (2.04 g) was recrystallized from acetic acid (450 mL, 7.0 M) and gave white needles, 1.85 g, mp > 310 °C (53% yield). Anal. Calcd for $C_9H_7HgNO_6$: C, 25.37; H, 1.66; Hg, 47.12; N, 3.29. Found: C, 25.50; H, 1.73; Hg, 46.83; N, 3.36. IR (KBr) $\nu_{max}$ 3423, 1658, 1309, 720 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.4 (1 H, s, CHO), 8.3 (2 H, m, H4,6).

(*C*) *3-(Chloromercurio)-5-nitrosalicylaldehyde.* The acetate salt of 3-(acetoxymercurio)-5-nitrosalicylaldehyde (1.85 g) was taken up in aqueous potassium hydroxide (900 mL, 0.03 M) by gentle warming. The solution was cooled to room temperature, and concentrated hydrochloric acid was added dropwise until all of the product had precipitated. The product was filtered, washed with water, and dried at 75 °C for 24

[1] Abbreviations: DTT, dithiothreitol; NEM, *N*-ethylmaleimide; AcCh, acetylcholine; AcChR, acetylcholine receptor; IP, isosbestic point; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; OPA, *o*-phthalylaldehyde; $^{125}$I-BuTx, $^{125}$I-$\alpha$-bungarotoxin.

h to give 1.55 g (42% overall yield from the parent salicylaldehyde); mp 252 °C (decompn). Anal. Calcd for $C_7H_4ClHgNO_4$: C, 20.89; H, 1.00; Cl, 8.82; Hg, 49.89; N, 3.48. Found: C, 20.98; H, 1.00; Cl, 8.49; Hg, 50.06; N, 3.44. IR (KBr) $\nu_{max}$ 3422, 1662, 1563, 1105 $cm^{-1}$; $^1H$ NMR (DMSO-$d_6$) $\delta$ 10.2 (1 H, s, CHO), 8.6 (1 H, s, H4), 8.4 (1 H, d, H6).

*Reductive Amination of 3-(Chloromercurio)-5-nitrosalicylaldehyde.* (*A*) *2-[(1-Butylamino)methyl]-6-(chloromercurio)-4-nitrophenol (N-Butylamine Adduct).* 3-(Chloromercurio)-5-nitrosalicylaldehyde (53.2 mL, 94 μmol in 0.001 M sodium hydroxide, pH 11.0) was adjusted to pH 9.0 with concentrated hydrochloric acid, and *N*-butylamine (50 μL, 507 μmol) was added. The mixture was allowed to stand for 2 h at room temperature. Sodium borohydride (2 mg, 50 μmol) was added. After 30 min, the pH was adjusted to 3.0 and the solution was extracted three times with equal volumes of anhydrous ether. These washes were combined and saved. The aqueous layer containing the adduct was brought to pH 10.0 with 2.0 N sodium hydroxide and extracted three times with ether. The aqueous solution contained the *N*-butylamine adduct (24% yield); $\lambda_{max}$ (pH 10) 400 nm, $\lambda_{max}$ (pH 3) 285, 313 nm; IP 345 nm; $\epsilon$ = 4500 $M^{-1}$.

(*B*) *2-(Chloromercurio)-6-(hydroxymethyl)-4-nitrophenol.* The combined low-pH ether washes from the above preparation were extracted with one equal volume of saturated brine, dried over magnesium sulfate, and evaporated in vacuo. The residue was redissolved in dilute sodium hydroxide (pH 10.0) to give a pale yellow solution. Spectra were identical with that observed for the hydroxymethyl reduction product prepared by direct reduction of the chloromercury aldehyde in the absence of 1-butylamine; $\lambda_{max}$ (pH 10) 414 nm, $\lambda_{max}$ (pH 3) 316 nm; IP 366 nm; $\epsilon$ = 14800 $M^{-1}$.

*$N^{\alpha}$-Acetyl-$N^{\epsilon}$-[2-hydroxy-3-(chloromercurio)-5-nitrobenzyl]lysine-N-methylamide ($N^{\alpha}$-Acetyllysine-N-methylamide Adduct).* *N*-α-Acetyllysine-*N*-methylamide (109.6 mg, 500 μmol) was added to the 3-(chloromercurio)-5-nitrosalicylaldehyde solution, and the product was worked up in the same manner as described for the *N*-butylamine adduct (51% yield); $\lambda_{max}$ (pH 10) 400 nm, $\lambda_{max}$ (pH 3) 310 nm; IP 345 nm; $\epsilon$ = 11020 $M^{-1}$.

*Titration Procedures in the Characterization of Mercurionitrophenols.* (*A*) *Mercury Titration.* [See Wohlfeil et al. (1985).] Stock solutions of pyridine-2-azo-4′-(*N*′,*N*′-dimethylaniline) ($2 \times 10^{-4}$ M), an indicator dye that reacts with free mercurial groups, mercaptoethanol ($6 \times 10^{-6}$ M) and 3-(chloromercurio)-5-nitrosalicylaldehyde ($4 \times 10^{-5}$ M) were prepared in sodium phosphate buffer (0.1 M, pH 5.8). The mercaptoethanol solutions were freshly prepared. The titrations were performed by using a series of tubes into which fixed amounts of thiol (15 nmol) and dye (25 nmol) were added. Varying dilutions of mercurial reagent stock solution were then added to a series of tubes to cover a range of concentrations from 2-fold higher to more than 10-fold lower than the fixed thiol concentration. Appropriate amounts of buffer were then added to equalize the total volume of each tube. After 5 min, absorbances were recorded at 470 nm.

(*B*) *Spectrophotometric pH Titration.* Stock solutions of phenolic mercurials and the model amine adducts were prepared unbuffered at pH 11.00 to give concentrations with absorbance maxima between 0.2 and 0.8. The pH was adjusted with 3 N HCl or NaOH. Total volume changes were less than 0.5%. Ten or more ultraviolet spectra were recorded between pH 3 and pH 10. A graphical form of the Henderson–Hasselbalch equation was used to determine the p*K*.

*Purification of Cobratoxin from Naja naja siamensis: Preparation and Characterization of a Derivative Subsequent to Reaction with 3-(Chloromercurio)-5-nitrosalicylaldehyde.* Neurotoxin 3 (cobratoxin) was purified from *N. naja siamensis* by the method of Karlsson et al. (1971) from the lyophilized venom, which was obtained from the Miami Serpentarium, Salt Lake City, UT. Amino acid analysis was determined and was identical with that reported by Karlsson et al. (1971). The binding constant ($K_d$ = 0.3 nM) and the toxin's toxicity in mice ($LD_{50}$ = 1.4 μg) were determined and were found to be in good agreement with values previously reported (Karlsson et al., 1971; Klett et al., 1973).

(*A*) *Neurotoxin Modification with 3-(Chloromercurio)-5-nitrosalicylaldehyde.* Purified cobratoxin (20 mg) was taken up in sodium phosphate buffer (6 mL, 0.05 M, pH 9.0) to a final concentration of 0.325 mM. 3-(Chloromercurio)-5-nitrosalicylaldehyde (5.94 mL of 0.87 mM stock solution in 5 mM NaOH) was then added to achieve a 2.65:1 molar excess of reagent. The final pH of the reaction mixture was then adjusted to 9.0 with 0.1 M phosphoric acid. After incubation at room temperature for 90 min, sodium borohydride (65 μL, 5.20 μmol, 0.08 M solution in dimethylformamide) was added and the solution was gently stirred at room temperature for 10 min. The pH was adjusted to 3.5 by the addition of 1 M phosphoric acid and then to 4.80 with 0.5 M NaOH. The reaction mixture was then loaded directly onto phosphocellulose for chromatographic resolution as described below.

(*B*) *Preparation and Specific Activity of $^3H$-Radiolabeled Cobratoxin.* The above procedure was modified to introduce a radiolabel into the derivative as follows: Tritiated sodium borohydride (10 Ci/mmol) in DMF was substituted for nonradiolabeled borohydride while the same molar proportions and concentrations of cobratoxin to borohydride and cobratoxin to the mercurial reagent were used. The specific activity of the mercurial toxin derivative was determined by directly counting aliquots of purified $^3H$-radiolabeled cobratoxin samples of known concentration.

(*C*) *Purification of the 3-(Chloromercurio)-5-nitrosalicylaldehyde Cobratoxin Derivative.* P-11 cellulose phosphate cation exchanger was precycled from the fibrous form with 0.5 M NaOH, allowed to settle for 30 min, and subsequently washed with water until equilibrated to pH 8.0. The resin was then redispersed in 250 mL of 0.5 M HCl, allowed to stand for 10 min, and washed successively with water until equilibrated to pH 6.0. The resin was suspended in 5 volumes of sodium phosphate buffer (0.1 M, pH 4.80) and titrated to pH 4.80 with 0.1 M phosphoric acid. This process was repeated until the pH no longer changed after 30 min. The suspension was shaken by hand only.

A linear gradient of 0.01–0.25 M sodium phosphate was employed in eluting a single protein peak corresponding to the derivative formed. Once the major protein peak eluted, the gradient was discontinued and 0.6 M buffer was applied to elute any remaining adsorbed material from the column. The major protein containing peak fractions except for the trailing fractions were collected and desalted by using a Sephadex G-25 column prepared in 0.2 M ammonium acetate, pH 6.5. Protein containing fractions were then pooled and lyophilized.

*Characterization of the Modified Cobratoxin.* (*A*) *Elemental Microanalysis.* Quantitative elemental analysis of % Hg, % C, and % H was performed, and the results were compared with calculated values for various samples of derivatized toxin. The % C and % H values were normalized by assuming both to be depressed by water of hydration associated with the lyophilized protein sample. The apparent

percentage of hydration was then added to calculate the apparent % Hg from the observed % Hg. Calcd for one mercurial group added to cobratoxin: Hg, 2.50; C, 50.89; H, 6.75. Found: Hg, 2.29; C, 39.22; H, 7.12 (corrected by assuming 20% water of hydration: Hg, 2.92; C, 51.0; H, 6.8).

(*B*) *Spectrophotometric Titration.* The nitrophenolic hydroxyl of the mercuriotoxin derivative was titrated in the same manner as for the model amine adducts. By use of the molar absorptivity for the phenol anion in the model adduct with $N^{\alpha}$-acetyllysine-*N*-methylamide, the molar incorporation of 3-(chloromercurio)-5-nitrosalicylaldehyde per mole of protein was estimated.

*Reduction and S-Carboxymethylation of Cobratoxin and Derivatized Cobratoxin.* Proteins were reduced and carboxymethylated by the method of Crestfield et al. (1963).

*Trypsin Digestion.* The reduced carboxymethylated toxins were taken up in 200 mM ammonium bicarbonate, pH 8.0, to a final protein concentration of 0.5 mg/mL. Trypsin (diphenylcarbamyl chloride treated, Type XI, Sigma, 1100 units/mg), was prepared in a 1.0 mg/mL solution in the same buffer, was then added to achieve a final trypsin concentration of 6 μg/mL (Lobel et al., 1985). Trypsin activity was assayed by using $N^{\alpha}$-benzoyl-DL-arginine-*p*-nitroanilide as substrate by the method of Erlanger et al. (1961). The mixture was incubated at 37 °C for 2 h. A second equal aliquot of trypsin was then added and the incubation repeated. Immediately following the second incubation, the samples were freeze-dried and stored at −20 °C. This limited digest procedure, modeled after that of Lobel et al. (1985), was used in order to obtain comparable digestion conditions and thereby generate similar HPLC separations of the tryptic fragments [cf. those reported by Lobel et al. (1985)].

*HPLC Separation of Tryptic Fragments.* The lyophilized peptide fragments were dissolved in 0.1% (v/v) trifluoroacetic acid (TFA) to a final concentration of 1.0 mg/mL. The fragments were separated on a 0.4 × 15 cm Micro-Pak MCH-5 column. The peptides were eluted at a flow rate of 0.6 mL/min with a gradient consisting of 0.1% (v/v) TFA in deionized water (buffer A) and acetonitrile with 0.1% (v/v) TFA (buffer B). The gradient program was [time (min), % buffer B) 0, 0; 8, 0; 16, 3; 56, 5; 36, 4; 44, 17; 104; 21; 92, 20.

After peak J eluted the gradient was held (usually $t = 92$, % B = 20) until peak K,L began to elute (approximately 10 min), at which time the final peaks are eluted by increasing the % B to 30 over 15 min. Up to 2.5 mg of tryptic fragments could be loaded on the column per run. Peaks detected at 206 nm were pooled individually and stored at −20 °C for amino acid analysis.

*Amino Acid Analysis of Tryptic Peptides.* Isolated tryptic fragments (5–10 μmol) were taken up in prescored 2.0-mL borosilicate ampules with 1.0 mL of constant-boiling 6 N HCl. Samples were vacuum degassed, sealed, and protected with electrophilic chlorination of the tyrosine by the addition of one crystal of phenol (approximately 0.5 mg) per sample. Hydrolysis was carried out for 24 h at 110 °C. Samples were then taken to dryness under nitrogen at room temperature and analyzed.

*HPLC Analysis of Tryptic Peptides.* HPLC amino acid analysis was performed by using a precolumn derivatization method employing *o*-phthalaldehyde (OPA). A Varian MCH-5 N-cap silanol C-18 column (0.4 × 15 cm) with a 0.4 × 4 cm guard column was used on a Varian 5500 HPLC equipped with Fluorochrom detector and autointegrator. The solvent system consisted of 50 mM sodium acetate in 1% (v/v) triethylamine, pH 6.12 (solvent A), and 28% (v/v) solvent A in methanol (solvent B). Both solvents and sample solutions were thoroughly degassed and passed through a 0.45-μm Millipore filter prior to use. Amino acid standards were made fresh and were typically 0.05 M in 2 mL total volume. The MCH-5 column was equilibrated with solvent A for 5 min. The gradient program to elute OPA-derivatized amino acids was [time (min), % solvent A] 0, 100; 2, 100; 8, 80; 15, 71; 28, 71; 37, 58; 43, 35; 65, 0.

Samples and standards were prederivatized by the 2:1 addition (v/v) of OPA to sample. The mixtures were vortexed continuously for exactly 30 s and loaded onto the column exactly 1 min after OPA addition. Typically, the sample volume was 100 μL or less.

*Nitrosalicylated Lysine Determination.* The presence of nitrosalicylated lysine in specific peptides was estimated during amino acid analysis by comparing HPLC amino acid retention times and peak integrations with those of a hydrolyzed nitrosalicylated lysine standard, prepared from the $N^{\alpha}$-acetyllysine-*N*-methylamide adduct as described above. The adduct was hydrolyzed with 6 N HCl at 110 °C for 24 h. The standard was then chromatographed in an identical fashion (cf. the tryptic fragments). The nitrosalicylated lysine gave a characteristic peak retention time of 67.8 min.

*AcChR: Purification and $^{125}$I-α-Bungarotoxin Assay.* AcChR was purified from the electric organ of *Torpedo californica* by neurotoxin–Sepharose affinity chromatography by a method similar to that of Froeher and Rafto (1979) with additional modification as reported by Nickoloff et al. (1985). Some AcChR preparations were concentrated on Bio-Gel HTP hydroxyapatite. Triton X-100 solubilized and purified AcChR was analyzed by the method of Klett et al. (1973) or Schmidt and Raftery (1973). The separation of AcChR from acetylcholine esterase was followed by the method of Ellman (1961). Protein was determined by the method of Lowry (1951). $^{125}$I-α-Bungarotoxin was purchased from New England Nuclear. Some samples were passed through a short column of DEAE-cellulose to minimize background counts (Lukasiewicz et al., 1978).

*Determination of Equilibrium Binding Constant for 3-(Chloromercurio)-5-nitrosalicylaldehyde-Modified Cobratoxin and AcChR.* A series of samples [4.0 mL, in a buffer containing 100 mM NaCl, 10 mM Tris-HCl, and 0.2% (w/v) Triton X-100, pH 7.4] containing approximately 1.25 pmol of AcChR neurotoxin binding sites and 0.2–25 pmol of [$^3$H]-3-(chloromercurio)-5-nitrosalicylaldehyde-modified cobratoxin were prepared. Bound toxin was determined after 30 min at room temperature by using the DE81 filter-disk assay described by Klett et al. (1973). Prior to being counted, the disks were washed with buffer [10 mM NaCl, 10 mM Tris-HCl, and 0.2% (w/v) Triton X-100 pH 7.4], partially vacuum dried for 1 min, and counted. To convert the observed radioactivity to picomoles of [$^3$H]mercuriotoxin bound, known amounts of [$^3$H]mercuriotoxin were spotted on filters and counted under identical conditions. To determine nonspecific binding of [$^3$H]mercuriotoxin to filters, a similar filtration was performed in which the AcChR was omitted from the reaction mixture.

*AcChR Cross-Linking Studies.* Purified AcChR, typically 50–100 pmol, was reduced with 1 mM dithiothreitol (DTT) in receptor buffer [10 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 units/mL aprotinin, and 0.1% (w/v) Triton X-100, pH 7.4) for 30 min at room temperature. Excess DTT was removed from AcChR either by dialysis against thoroughly degassed receptor buffer to a final DTT

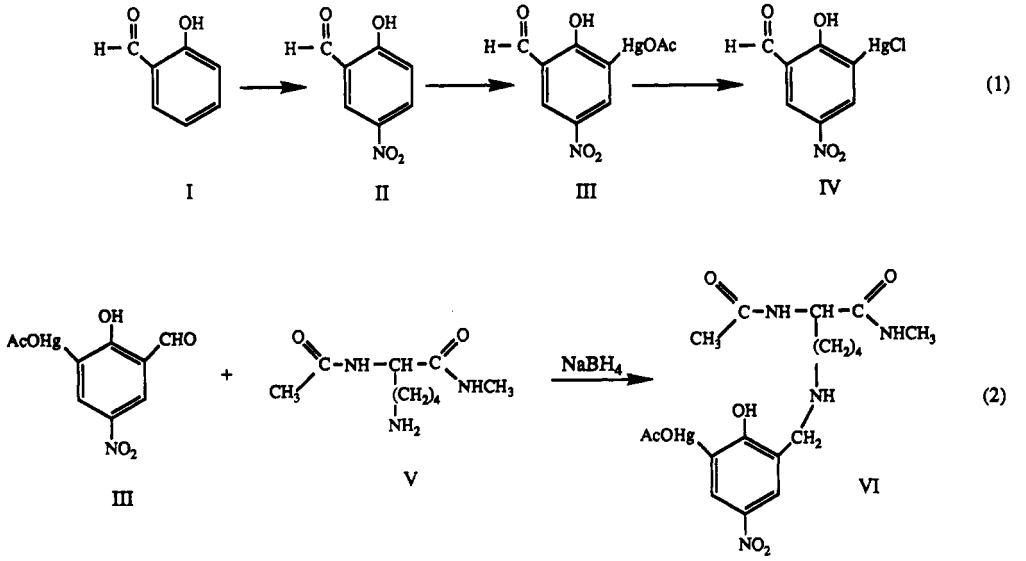


FIGURE 1: (1) Reaction scheme for synthesis of 3-(acetoxymercurio)-5-nitrosalicylaldehyde, III, and 3-(chloromercurio)-5-nitrosalicylaldehyde, IV, from salicylaldehyde, I, and 5-nitrosalicylaldehyde, II. (2) $N^{\alpha}$-Acetyl-$N^{\epsilon}$-[2-hydroxy-3-(acetoxymercurio)-5-nitrobenzyl]lysine-$N$-methylamide, VI (model $N^{\alpha}$-acetyllysine-$N$-methylamide adduct) synthesis from parent compounds III and $N^{\alpha}$-acetyllysine-$N$-methylamide, V.

concentration of less than 0.3 nM or by passing the incubation mixture through a Sepharose 6B column preincubated with receptor buffer. Gel column fractions (0.5 mL) were collected and assayed for AcChR by removing a 5-$\mu$L aliquot and adding it to 5 $\mu$L of $^{125}$I-BuTx (0.3 pmol in receptor buffer). After 15 min at room temperature, the volume was adjusted to 3 mL with buffer and the solution was filtered through one DE81 filter disk prewashed with 3 mL of the same buffer. Each tube was then washed with 3 mL and the washings were gravity-filtered through the disk. The disks were then washed with a final 10-mL aliquot, vacuum dried for 1 min, and counted. Immediately following elution from the column, the 5-$\mu$L aliquot was removed and [$^3$H]mercuriotoxin was added. AcChR:[$^3$H]toxin ratios varied from 1:100 to 4:1. The [$^3$H]toxin was incubated with the reduced AcChR for 30 min at room temperature, and the reaction was quenched with the addition of NEM to a concentration of 5 mM.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* All samples were prepared for $NaDodSO_4$–polyacrylamide gel electrophoresis with a minimum of dilution by adding reagent to achieve the following $NaDodSO_4$ sample buffer concentrations: 2.0% (w/v) $NaDodSO_4$, 62.4 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue. Samples were then boiled for 3 min in the absence of any thiol. Aliquots (25–350 $\mu$L) of each sample were applied to 3% stacking gels over $NaDodSO_4$–10% polyacrylamide resolving gels prepared and run as described by Laemmli (1970). A constant current of 25 mA was used to pull the samples through the stacking gel and a current of 20 mA was then used to complete the electrophoresis. Gels were stained with Coomassie Brilliant Blue to locate AcChR subunit bands prior to fluorographic analysis in which the procedures of Lasky and Mills (1975) were employed.

## Results

*Preparation of Mercuriosalicyldehyde Reagents, Model Adducts, and Reaction with Cobratoxin.* 3-(Acetoxymercurio)-5-nitrosalicylaldehyde was synthesized by nitration and subsequent mercuration of salicylaldehyde, as shown in Figure 1. 3-(Chloromercurio)-5-nitrosalicylaldehyde was prepared directly from the acetoxymercury reagent. Salicylaldehyde (I) was nitrated to yield a mixture of 3- and 5-nitro isomers of nitrosalicylaldehyde (Harrison & Diehl, 1947), which was then separated by successive fractional recrystallization. Infrared and NMR spectra were consistent with the identification of the higher melting isomer with nitration in the 5 position. The 5-nitro isomer was obtained in 37% yield from salicylaldehyde.

The 5-nitro isomer was mercurated quantitatively in acetic acid to yield the expected 3-(acetoxymercurio)-5-nitrosalicylaldehyde (III). The chloromercury compound (IV) formed subsequently was obtained in 53% yield from the parent 5-nitrosalicylaldehyde. Elemental analysis and mercury titration both confirmed the monomercurated nature of the chloride and acetate salts of 3-mercurio-5-nitrosalicylaldehyde, both of which were quite stable and could be stored at room temperature. Long-term storage of the acetoxymercurial was preferred as this compound may be easily recrystallized from dilute acetic acid, whereas the chloromercury reagent was not as easily repurified. NMR spectra were consistent with 5-position nitration and 3-position mercuration of the ring structure.

Model adducts of III or IV were formed and characterized in situ after sodium borohydride reduction of the Schiff bases formed with $N$-butylamine and $N^{\alpha}$-acetyllysine-$N$-methylamide (see Figure 1). The model compounds were prepared to assess the expected chemical and spectrophotometric properties, as these would be predicted to be similar to those observed in reactions of the mercurial reagents with protein amino groups. The amine derivatives and the hydroxymethyl derivative obtained as a byproduct (or by direct reduction of the aldehyde) were separated by differential extraction and their $pK_a$s determined by spectrophotometric titration. A representative spectroscopic plot is shown in Figure 2. The $N$-butylamine and $N^{\alpha}$-acetyllysine-$N$-methylamide adducts were formed in 24% and 51% overall yields from 3-(acetoxymercurio)-5-nitrosalicylaldehyde under the conditions described. Hydrolysis of the lysine adduct gave an altered OPA derivative (cf. lysine). Though difficult to quantitate, this observation was useful in the detection of modified lysine in the trypsin peptides (vide infra).

The mercury group was titrated by using the mercury indicator dye pyridine-2-azo-4′-($N'$,$N'$-dimethylaniline) (Koltz & Carver, 1961). Mercurial reagents were reacted with known amounts of 2-mercaptoethanol. The azo dye reaction detected mercurial in excess. The reaction is accompanied by an ab-

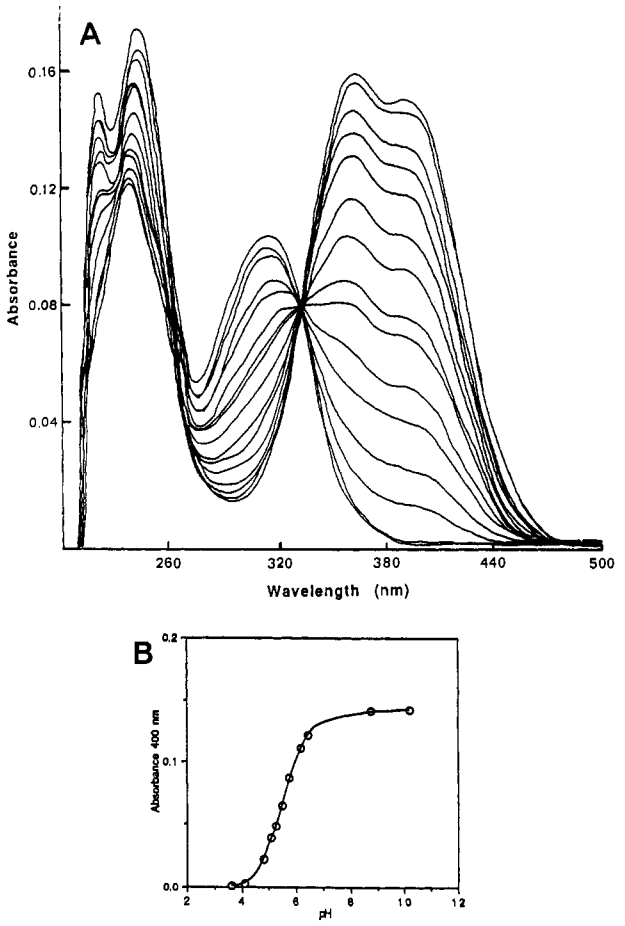


FIGURE 2: (A) Spectrophotometric pH titration of 3-(acetoxymercurio)-5-nitrosalicylaldehyde. Individual spectra generated at varying pH values from 3 to 10. (B) Absorbance at maxima plotted vs respective pH reveals standard titration curve.

sorbance change in the visible region. Absorbance at 470 nm was plotted versus amount of mercurial added. The breakpoint of the two linear portions of the plot established an end point where the molar amounts of mercurial and thiol are equal. From the amount of mercurial added, the apparant molecular weight could be calculated. These values, the results of direct chemical analysis, and the spectroscopic properties were all consistent with the structures given.

Cobratoxin was purified from the venom of *N. naja siamensis*, the Thailand cobra. The identity of the purified cobratoxin was confirmed by amino acid analysis, determination of molar absorptivity at 280 nm, toxicity determined in mice ($LD_{50}$ = 1.4 $\mu$g), and binding analysis with nicotinic AcChR ($K_d$ = 0.3 nM), all of which fit previously reported values for this toxin (Karlsson et al., 1971).

Purified cobratoxin was modified by using 3-(chloromercurio)-5-nitrosalicylaldehyde in a 2.65:1 molar excess of reagent to toxin. The aldehyde function reacts with cobratoxin lysine(s) to form Schiff base intermediates at pH 9.00. The imine was then reduced with sodium borohydride with a 1:1 molar ratio of borohydride to total reagent employed. The reaction was also employed to conveniently radiolabel the cobratoxin derivative by using titrated sodium borohydride. A radiolabeled derivative with 2.15 Ci/mmol specific activity was prepared by using 10 Ci/mmol sodium borohydride. The derivatized toxin was then separated from the reduced reagent chromatographiically on phosphocellulose. The single protein peak was subsequently desalted on Sephadex G-25. The reaction at 2.65:1 reagent to protein ratio gave product apparently quantitatively substituted at position 23 and not elsewhere

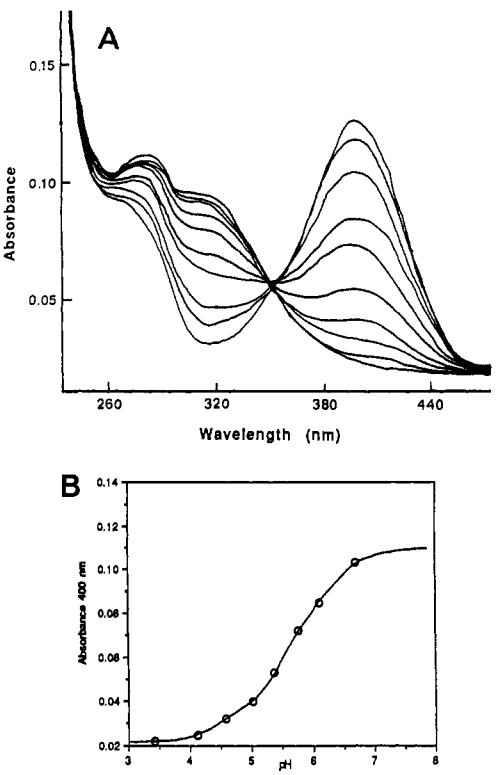


FIGURE 3: Spectrophotometric pH titration of Lys 23 modified mercuriotoxin (A) and graphical representation of data for p$K$ determination (B) as in Figure 2.

(vida infra). This may be due in part to the reactivity of the lysine 23 amino group and in part to the precipitation of the intermediate Schiff base prior to addition of sodium borohydride, after which the precipitated complex redissolves. Prior to addition of borohydride and after brief equilibration of reagent with toxin, the precipitated complex could be centrifuged.

*Characterization of Mercuriotoxin.* The specific activity of the [$^3$H]mercuriotoxin derivative relative to that of the [$^3$H]sodium borohydride employed and the spectroscopically estimated mercurionitrosalicylate incorporation into cobratoxin were consistent with 1 mol of reagent incorporated/mol of toxin. Mercurionitrosalicylate was estimated by using the molar absorptivity at 400 nm for the model lysine adduct at high pH. The modified toxin derivative lost reactive mercury when dialyzed in standard dialysis membranes. When analyzed after desalting on Sephadex, samples contained an amount of mercury consistent with monoincorporation.

The results of spectrophotometric titrations of the mercuriotoxin derivative are shown in Figure 3. The spectra were characteristic of the introduction of 1 mol of reagent/mol of protein. The p$K_a$ of the cobratoxin derivative was essentially identical with that of the model *N*-acetyllysine-*N*-methylamide adduct. A number of still incompletely characterized mercurial adducts separated at other reaction ratios of mercurial reagent to protein have in some cases different p$K_a$s, which may, in part, account for their separation on phosphocellulose (Wohlfeil et al., 1985).

Cobratoxin modification at a unique lysine residue, in principle, eliminates one potential trypsin cleavage site. In place of the two peptides generated from tryptic cleavage of the reduced carboxymethylated native cobratoxin, one new peptide should appear containing reagent. However, the modified lysine $\epsilon$-amino reaction product will remain positively charged and therefore may be amenable to proteolytic digestion by trypsin, although at an arguably slower rate due

Table I: Amino Acid Analysis Data of Tryptic Peptides Generated from Cobratoxin and Mercuriotoxin[a]

| amino acid | F | G, H | J | I | K, L | M | F + M[b] | N[c] | F[c] | G[c] |
|---|---|---|---|---|---|---|---|---|---|---|
| G | 0.1 (1) | 0.2 (1) | | 0.2 (1) | 0.2 (1) | | 0.2 (2) | 0.2 (1) | 0.2 (1) | 0.2 (1) |
| H | 0.3 (1) | | | | 0.4 (1) | | 0.3 (1) | 0.2 (1) | 0.6 (1) | |
| R | | 0.3 (1) | | 0.4 (1) | | 0.3 (1) | 0.5 (1) | 0.4 (1) | | 0.23 (1) |
| D, N | 1.8 (2) | 1 (1) | 1 (1) | 3.8 (4) | 3 (3) | 0.9 (1) | 3 (3) | 3.2 (3) | 2.4 (2) | 1.4 (1) |
| T | 1 (1) | 2.2 (2) | 2.4 (2) | 2.6 (3) | 2.9 (3) | 1.3 (1) | 2.2 (2) | 2.6 (2) | 1 (1) | 2.2 (2) |
| S | | | 0.4 (1) | 0.7 (1) | 0.6 (1) | 0.5 (1) | 0.6 (1) | 0.3 (1) | | |
| A | | 1.8 (2) | | | | 0.7 (1) | 0.4 (1) | 0.5 (1) | | 1.3 (2) |
| C | 1.6 (2) | 2.2 (2) | 0.8 (1) | 3.3 (3) | 3.1 (3) | 2.6 (2) | 4.9 (4) | 3.2 (4) | 2.6 (2) | 3.1 (2) |
| V | 0.8 (1) | 3 (2) | | 0.8 (1) | 0.8 (1) | | 0.7 (1) | 1 (1) | 1 (1) | 2.6 (2) |
| F | | | 0.7 (1) | 1 (1) | 1.1 (1) | 1.2 (1) | 1 (1) | 1.3 (1) | | |
| I | | | 0.9 (1) | 1.1 (1) | 2.1 (2) | 1.4 (1) | 1.1 (1) | 0.7 (1) | | |
| L | | 0.8 (1) | | | | | | | | 0.8 (1) |
| E, Q | | | | 0.7 (1) | | | | | | |
| Y | (1) | | | | (1) | | | | | |
| K | (1) | (1) | | | (2) | | | | | |
| W | | | | | | (1) | (1) | (1) | | |

[a] Data are presented as observed number of residues followed by predicted number of residues (in parentheses). [b] For comparison with peak N. [c] Mercuriotoxin tryptic peaks.

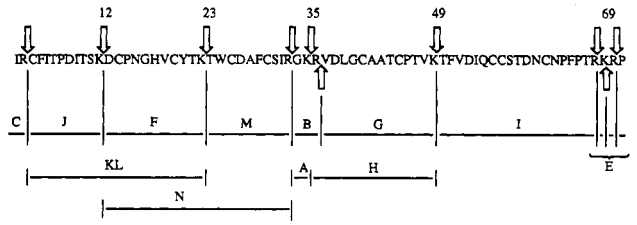


FIGURE 4: Amino acid sequence map of cobratoxin. Arrows and vertical lines define possible tryptic cleavage points. Tryptic peptides are identified by uppercase letters and correspond to Table I. Lysine residue positions are indicated by Arabic numerals.

to steric hinderance and a partial negative charge on the nitrophenolate. For this reason a limited tryptic digestion was employed.

An amino acid sequence map of cobratoxin showing the predicted trypsin cleavage points and peak assignments is given in Figure 4. The reduced and carboxymethylated native cobratoxin and the reduced and carboxymethylated mercurial–toxin tryptic peptides were separated by reverse-phase high-pressure liquid chromatography as shown in Figure 5, under similar conditions to those employed by Lobel et al. (1985). Individual peaks were identified by amino acid analysis. Table I lists the amino acid composition of the peaks.

Peptides obtained from the toxin derivative were consistent with the modification of Lys 23. The new peak generated by the eliminated tryptic cleavage site, peak N, had an amino acid composition identical with that of native cobratoxin fragments F plus M. Also, peaks F and M were appreciably attenuated and peak KL was absent, as compared with native cobratoxin. KL was observed in native digests due to the inherently slow tryptic cleavage of the Asp–Lys peptide bond. The absence of KL in derivatized toxin and the appearance of a new later eluting peptide, peak O, suggested that O was the expected KL–M peptide generated from a Lys 23 modification. Nitrosalicylated lysine was found in hydrolysate from peaks F and N of mercuriotoxin derivative digests by comparison with a standard derived from the $N^{\alpha}$-acetyllysine-$N$-methylamide model adduct, which eluted characteristically at 67.8 min in the HPLC chromatogram. The chromatographic pattern obtained for the trypsin peptides of the toxin derivative closely matches the pattern reported by Lobel et al. (1985) for a Lys 23 biotinylated cobratoxin derivative.

*Interaction of the Mercuriotoxin with AcChR.* The reversible binding of the [$^3$H]cobratoxin derivative to purified AcChR was characterized by measuring the formation of complexes as a function of toxin concentration, after the

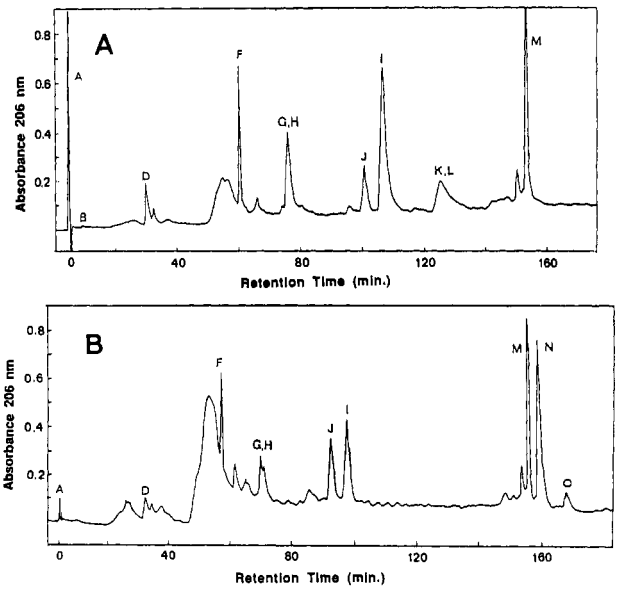


FIGURE 5: HPLC separation of peptides generated from tryptic digestion of cobratoxin (A) and mercuriotoxin (B). Peptides are identified by uppercase letters (Table I, Figure 4).

binding reaction reached equilibrium. This was a modified version of the procedure described by Klett et al. (1973) for titrated pyridoxal phosphate derivatives of cobratoxin. The analysis of these results by the method of Scatchard (1949) yielded calculated dissociation constants for the mercuriotoxin–AcChR complex to be $K_d = 2.75 \times 10^{-9}$ M, which is consistent with that reported elsewhere for a Lys 23 modified toxin (Lobel et al., 1985).

Cross-linking of radiolabeled and nonradiolabeled mercuriotoxin occurs with DTT-reduced AcChR from *T. californica* electroplax. After reduction of the AcChR complex with 1 mM DTT, measurable cross-linking into both the $\alpha$ and $\beta$ subunits of the AcChR was observed when the gels were examined by fluorographic analysis (Figure 6). The cross-linking yields are low. Reactions carried out with approximately equivalent concentrations of AcChR toxin binding sites and mercuriotoxin always showed unreacted toxin as the major product.

If, after cross-linking, the AcChR complex was treated with mercaptoethanol prior to electrophoretic analysis, no cross-links of mercuriotoxin to $\alpha$ and $\beta$ subunits were observed. Further, treatment of the AcChR as isolated and purified with the mercuriotoxin led, as expected, to no observed cross-linking. Also, if after reduction of AcChR with 1 mM DTT the re-

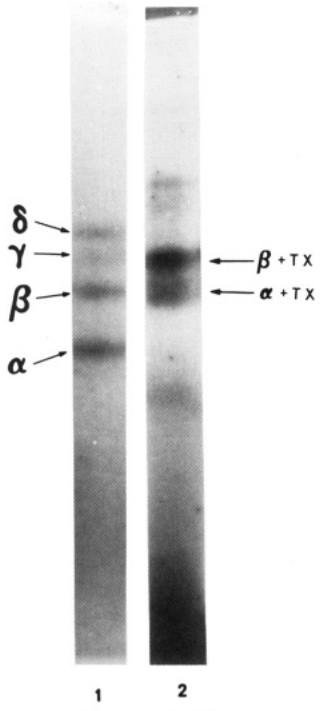


FIGURE 6: Autoradiograph of $NaDodSO_4$–polyacrylamide gel electrophoretic resolution of [$^3$H]mercuriotoxin cross-linking into AcChR. Resolved AcChR subunit positions, as indicated by a Coomassie Blue stained dried gel as prepared for fluorography, are shown (lane 1). Presumptive cross-linked complexes are indicated by a fluorogram of a gel generated in identical fashion (lane 2). A mercuriotoxin, covalently cross-linked into an AcChR subunit, would add ca. 8000 daltons to that subunit's apparent molecular weight, resulting in the electrophoretic pattern shown.

duced AcChR was treated with NEM prior to treatment with the mercuriotoxin, no cross-linking was observed. We conclude that mercuriotoxin is able to cross-link to sulfhydryl groups on the AcChR that become free or accessible as a result of DTT reduction.

## Discussion

In 1969 McMurry and Trentham described a class of simple organomercurial compounds containing the nitrophenol moiety. These compounds were shown to be useful in the study of thiol groups in proteins, specifically in D-glyceraldehyde-3-phosphate dehydrogenase. Derivatives of nitrophenols contain hydroxyl groups with p*K*s in the pH range of most biochemical systems of interest. The phenolic anions have chromophores in the visible spectrum from 380 to 415 nm and are conveniently followed experimentally. Mercuration of the nitrophenols was of interest because it introduced a thiol-reactive moiety into an already chromophoric probe. In this way, thiol groups of proteins could be targeted for the introduction of these "reporter groups" (Burr & Koshland, 1964), i.e., indicators of perturbation of biological systems to which they are attached. By using D-glyceraldehyde-3-phosphate dehydrogenase as a model system, the authors showed that these reagents were sensitive probes of the microenvironment of an active-site thiol group.

The experimental rationale we adopted was founded in part on these early observations. We sought to synthetically take these reagents one step further and to develop compounds that could act as heterobifunctional cross-linking reagents within the biological system of interest and to incorporate an aldehyde function into the reagents, thereby targeting primary amino functions for reaction and complement the existing thiol-reactive mercurial group.

The two organomercurial derivatives prepared can be incorporated into lysine residues or an N-terminal amino group, with retention of a reactive mercurial function. This reaction may be generally useful in introducing chemically well-defined heavy metal atoms into proteins containing no reactive thiols (Wohlfeil et al., 1985). Isomorphous, heavy metal atom derivatives are required to solve the phase in the X-ray analysis of proteins (Green et al., 1954).

The organomercurial cross-linking reagents have been thoroughly characterized to demonstrate the separate properties of the aldehyde, nitrophenol, and mercurial functional groups. Model adducts of the aldehyde group were synthesized in situ by reaction of the mercurial reagents with the primary amines $N^{\alpha}$-acetyllysine-*N*-methylamide and *N*-butylamine, followed by reduction. The preparation of these adducts allowed determination of the expected p$K_a$s of the nitrophenolic function when reaction of 3-(chloromethyl)-5-nitrosalicylaldehyde is used to reductively alkylate an exposed lysine residue of a protein.

As expected, the p$K_a$s of the acetoxy- and chloromercurial reagents are similar. However, the relatively large p$K_a$ difference between the *o*-hydroxymethyl reduction product and the (alkylamino)methyl-substituted phenols may be associated with the ionic and hydrogen-bonded stabilization of the phenolic anion by the protonated amine as well as with the inductive withdrawing potential of (alkylammonio)methyl compared with the hydroxymethyl. Whatever the exact contribution to the reduced p$K_a$ of the nitrophenol in the aminoalkylated compounds, no major additional perturbations occur in the Lys 23 toxin derivative prepared here; i.e., the p$K$ of the protein derivative is exactly what it would be expected to be on the basis of model chemistry. Indeed, it suggests the modified residue is readily exposed to solvent.

It was our original intention to use the heterobifunctional cross-linking reagents to synthesize and characterize a number of mono- and/or multiderivatized toxins and to subsequently study their individual properties in an effort to probe the toxin's active site. Various toxin residue modifications would have been expected to modify toxin–AcChR binding in different ways, depending on the site of modification into free cysteines or reduced cystines on the AcChR surface, e.g., into the easily reducible disulfide located on the $\alpha$-subunit. The identity of this disulfide has recently been confirmed to be a vicinal cystine connecting the adjacent cysteines Cys 192 and Cys 193 in the $\alpha$ subunit of the AcChR (Kao & Karlin, 1986). This assignment has been further supported by the recent experiments of Kellaris and Ware (1989).

There is some precedent in the literature for using this approach to probe the various toxin residue contributions to AcChR binding. Covalent cobratoxin derivatives have been synthesized and characterized by making use of the toxin's six positively charged amino functions (Ile 1, Lys 12, Lys 23, Lys 35, Lys 49, and Lys 69). Cooper and Reich (1972) prepared two tritiated pyridoxal phosphate derivatives. Both possessed decreased toxicity. Other modifications of the amino groups in cobratoxin have been carried out by carbamylation, acylation, and guanidination (Karlsson et al., 1972; Karlsson & Eaker, 1972). Mono- and diacetyl and up to hexaacetyl (and carbamyl) derivatives have been prepared.

Only two classes of isomeric monosubstituted toxin derivatives have been completely resolved into individual components. Karllsson et al. (1972) reported the complete separation of the six monoacetyl derivatives by gradient chromatography on the cation exchanger Bio-Rex 70. These six derivatives had indistinguishable $LD_{50}$s in mice. The same group of investi-

gators then acylated both free and AcChR-bound cobratoxin in an attempt to identify amino groups taking part in the binding to AcChR (Balasubramaniam et al., 1983). More recently, Lobel et al. (1985) reported the synthesis and separation of six monobiotinylated derivatives. The monobiotinylated derivatives were found to bind the AcChR less avidly than the native toxin in the order Lys 23 > Lys 49 > Lys 35 > Lys 69 = Lys 12 > Ile 1, with Lys 23 binding least avidly. This observation is clearly consistant with an interfacial AcChR recognition site nearest to the Lys 23 residue (Tsernoglou et al., 1978).

The composition of the mercuriotoxin has been confirmed spectroscopically, by nitrophenol incorporation studies, and directly by elemental microanalysis. Mercury could be lost from the derivative under mild conditions of handling. Tryptic digestion of reduced and carboxymethylated toxin followed by HPLC separation and amino acid analysis of the proteolytic fragments generated a peptide map that indicated the mercuriotoxin to be derivatized at Lys 23. Due to the chemical nature of the secondary amine bond formed by mercurial reagent and the toxin amino function, the linkage should remain protonated and thus partially cleavable by trypsin.

Preferential modification of Lys 23 was expected. This residue has been described as being hyperreactive to hydrophobic reagents such as dansyl chloride (Tstelin et al., 1982). Lobel et al. (1985) also reported the hyperreactivity of Lys 23. In their series of experiments the Lys 23 monobiotinylated derivative was the predominant reaction product. Balasubramanian et al. (1983) reported a Lys 23 monoacetylated cobratoxin derivative to dominate toxin modifications, accounting for 56% of total monosubstituted derivatives. In the preparation of various spin-labeled derivatives the hyperreactivity of Lys 23 has also been demonstrated. A piperidyl *N*-hydroxysuccinimide ester Lys 23 derivative accounted for 97–98% of the monolabeled derivative (Ivanov et al., 1980).

During the modification reaction of cobratoxin and the mercurial reagent the toxin–imine intermediate precipitated from solution. The monoderivatized cobratoxin was thereby less available for further reaction with remaining excess reagent and is effectively removed from solution after reaction with 1 mol of reagent. Considering this observation, together with the inherent Lys 23 hyperreactivity in instances where precipitation was presumably not involved, the fact that a single Lys 23 cobratoxin derivative was found to be the predominant reaction product under our conditions was not surprising.

The crystal structures of a short toxin (Tsernoglou & Petsko, 1976, 1977; Low, 1976) and two long toxins (Walkinshaw et al., 1980; Agard & Stroud, 1982) reveal many common structural features among the neurotoxins. The toxins fold into three major loops and one tail all emerging from one globular head. The protruding central loop (loop II) is composed of residues 21–40 and is flanked on either side by two shorter loops, loop I (residues 4–13) and loop III (residues 44–55). The C-terminal tail portion (residues 63–71), present in the long toxins, is located behind and beneath the loops, as represented in Figure 7A. These residues do not appear to extend the $\beta$-sheet structure of any of the loops. The four invariant disulfides are found in the globular core region and are apparently in the same relative locations in all the known, sequenced variant neurotoxins. The long toxins contain an additional disulfide linking residues 30 and 34 at the tip of the central loop. The spacing between the cysteine residues involved in the disulfides allows a twisting at the tip of the loop, which both preserves the extended $\beta$-sheet structure and aligns

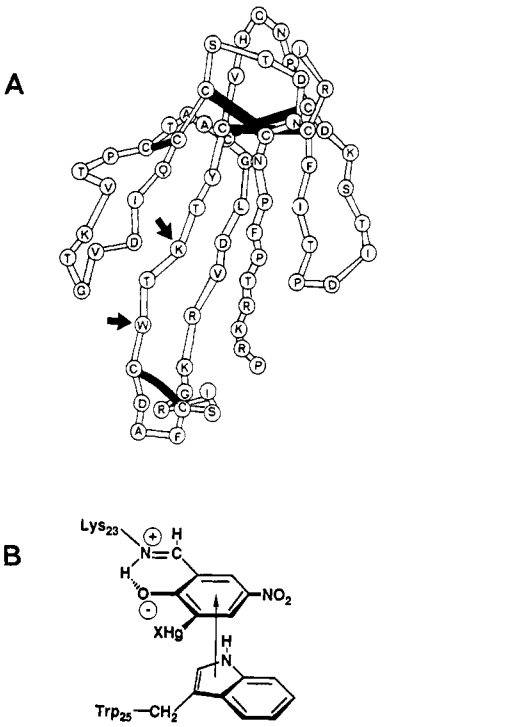


FIGURE 7: (A) Diagram based on that reported by Walkinshaw et al. (1980) of cobratoxin with Lys 23 and Trp 25 indicated by arrows. Residues are identified at approximate positions of $\alpha$-carbons. (B) The postulated charge transfer interaction between the indole ring of Trp 25 and the Lys 23 cobratoxin derivative is illustrated.

the residues thought to play a major role in the binding of toxin to AcChR in a way similar to that found in the short toxins (Tsernoglou et al., 1978). A charge transfer interaction may occur between the indole ring and the Schiff base, stabilizing the transition-state imine and thus partially explaining the hyperreactivity of Lys 23 and the observed precipitation of the adduct as it is formed and its subsequent return to solution after the addition of reducing agent. Figure 7B illustrates the potential proximity of the Lys 23 mercurionitrosalicylate to the indole ring of Trp 25.

The aligned primary sequences of over 60 variant neurotoxins show that approximately one-third of the residues are the same or similar (Karlsson, 1979). The neurotoxins also contain other invariant residues in addition to the eight half-cystines [reviewed in Dufton and Hider (1983) and Low (1979)]. The residues that are invariant, or conservatively substituted, have been assigned either a structural or functional role through numerous residue-specific chemical modification studies [reviewed in Karlsson (1979)]. It has been proposed that some of these residues, primarily belonging to loops II and III and the cavity these two loops create, may mimic acetylcholine (Tsernoglou et al., 1978) or various competitive antagonists (Dufton & Hider, 1983; Menez et al., 1982; Tsernoglou et al., 1978; Walkinshaw et al., 1981). Low (1979) has proposed that the entire surface of the concavity, which includes most of the conserved residues, is involved in the AcChR binding.

The locations of the conserved residues in the three-dimensional structures suggest that one surface of the central loop II and loop III are principally involved in AcChR binding. There is uncertainty about which residues within this region interact directly with AcChR and about the contributions of the various residues that lie outside this region to the binding.

We have found that the mercuriotoxin binds unreduced AcChR approximately 10-fold less avidly than native cobratoxin. The Lys 23 biotinylated derivative of Lobel et al.

(1985) also showed reduced AcChR binding affinity. Lys 23 of cobratoxin is at the edge or within the surface of the toxin important in AcChR binding. Thus, reduced binding affinity for the mercuriotoxin is in agreement with schemes that emphasizes the importance of Lys 23 in AcChR interaction.

Cross-linking of neurotoxin to its binding sites on the AcChR has been used by a number of investigators to demonstrate that the neurotoxin binding sites lie on the $\alpha$ subunits and that the $\beta$, $\gamma$, and $\delta$ chains are near neighbors to the $\alpha$ subunit. Hamilton et al. (1978) showed tritiated methylcobratoxin cross-linked into the $\alpha$ and $\beta$ subunits of the AcChR by using bifunctional cross-linking reactions in the toxin–AcChR complex. Nathanson and Hall (1980) demonstrated photolabeling of the $\alpha$, $\beta$, $\gamma$, and $\delta$ chains with an $^{125}$I-BuTx derivative that contained several azido-modified groups. After specifically reducing one disulfide bond per $^{125}$I-BuTx molecule (presumed to be the one at the tip of loop II), Witzemann et al. (1979) prepared photoaffinity derivatives of the toxin with azidoaryl side chains of various lengths. A 14-Å side chain cross-linked into the $\alpha$ and $\delta$ subunits while a 33-Å side chain cross-linked only with the $\delta$ chain. Oswald and Changeux (1982) cross-linked $^{125}$I-BuTx derivatives into $\alpha$, $\gamma$, and $\delta$ subunits, and Hamilton et al. (1985) have reported cross-linking of a dithiobis(succinimidopropionate) (DTSP) cobratoxin derivative into the $\alpha$, $\beta$, $\gamma$, and $\delta$ chains. More recently, Pedersen and Cohen (1990) have demonstrated photoactivated labeling of the $\alpha$, $\gamma$, and $\delta$ chains of the AcChR with *d*-[$^3$H]tubocurarine. The latter two groups have argued, on the basis of the covalent labeling characteristics of toxin derivatives and small molecule competitive antagonists of AcCh, for an arrangement of the AcChR subunits that places either the $\gamma$ or the $\delta$ subunit between the two $\alpha$ subunits within the membrane rosette.

Experiments here demonstrate that a radiolabeled Lys 23 mercuriotoxin derivative of cobratoxin cross-links into prereduced AcChR. Cross-linking into both $\alpha$ and $\beta$ subunits was observed. No cross-linking was observed if the AcChR was not previously reduced with DTT or if the reduced AcChR–mercuriotoxin cross-linked products were subsequently exposed to excess $\beta$-mercaptoethanol. Similarly, if prereduced AcChR was treated with NEM prior to reaction with mercuriotoxin, no cross-linking was observed. This evidence suggests the mercurial–sulfhydryl nature of the cross-linking. The low cross-linking yields are probably due to a number of factors linked to the inherent instability of the mercurial. In particular, the presence of neighboring free sulfhydryls after DTT reduction of the vicinal disulfide at the AcCh binding site may facilitate demercuration of the toxin derivative and lead to artifactual $\beta$-cross-linking.

These findings are not inconsistent with the notion that $\gamma$ and $\delta$ are located between $\alpha$ subunits. The low cross-linking yield observed would argue in favor of the $\beta$ chain being a near neighbor to only one of the two $\alpha$ chains. However, our findings suggest a $\beta$-chain sulfhydryl in the vicinity of the neurotoxin binding domain on the $\alpha$ subunit that contains the easily reduced disulfide. It is unclear which $\beta$-subunit sulfhydryl is labeled and which of the two $\alpha$ chains is its nearest neighbor (Kellaris & Ware, 1989; Pedersen & Cohen, 1990). The possibility that cobratoxin does not initially label both $\alpha$ and $\beta$ cannot be completely excluded on the basis of our data, but it appears unlikely. We believe that these cross-linking products are formed as a result of primary reactions between the mercuriotoxin and one of the $\alpha$ subunit AcChR sulhydryls formed on reduction with DTT. Subsequently, before or during the experimental procedures used to detect cross-linking, the primary cross-linked product is significantly exchanged with a $\beta$-subunit sulhydryl or alternatively undergoes secondary $\alpha$-subunit group assisted demercuration of the AcChR–Hg-Tx linkage. In conclusion, after prereduction of AcChR with 1 mM DTT, a Lys 23 mercurial modified cobratoxin derivative affinity labels the AcChR through the formation of a mercury–sulfur bond, thus demonstrating the formal proximal relation between the region of the toxin neighboring Lys 23 and that region of the $\alpha$-subunit of the AcChR neighboring the easily reduced disulfide. While the low yield and apparent nature of the cross-linked products can be explained in terms of the lability of the mercury–sulfur bond and the presence a secondary free sulhydryl formed after the DTT reduction of AcChR, the exact mechanism of formation of the final observed cross-linked products remains to be determined.

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