Electrostatic Potential of the Acetylcholine Binding Sites in the Nicotinic Receptor Probed by Reactions of Binding-Site Cysteines with Charged Methanethiosulfonates†

David A. Stauffer‡ and Arthur Karlin*

Center for Molecular Recognition and Departments of Biochemistry and Molecular Biophysics, Physiology and Cellular Biophysics, and Neurology, Columbia University, New York, New York 10032

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ABSTRACT: All of the potent agonists and competitive antagonists of the acetylcholine receptors are positively charged, onium compounds. Among the interactions involved in the binding of these compounds, electrostatic forces undoubtedly make an important contribution. There is evidence that the acetylcholine binding site contains both acidic and aromatic amino acids. The acidic side chains could provide long-range charge–charge interactions with acetylcholine, while the aromatic side chains could provide short-range cation–π-electron and hydrophobic interactions. To probe the long-range electrostatic interactions in the binding site, the rate constants for the reactions of sulfhydryl-specific reagents with cysteines in the binding site have been determined as a function of ionic strength. The reagents are the positively charged methanethiosulfonate ethylammonium and methanethiosulfonate ethyltrimethylammonium, the negatively charged methanethiosulfonate ethylsulfonate, and the neutral methyl methanethiosulfonate. In addition, the rate constants of the reactions of these methanethiosulfonates with positively charged, negatively charged, and uncharged simple thiol compounds have been similarly determined. An analysis of these rate constants in terms of absolute rate theory and Debye–Hückel theory is consistent with the acetylcholine binding site containing two to three negative charges and an electrostatic potential at zero ionic strength of about −80 mV relative to bulk solution.

The natural ligand for the nicotinic receptors is acetylcholine (ACh),[1] which bears a positively charged quaternary ammonium group. All other potent agonists and competitive antagonists of these receptors are also positively charged, containing at least one quaternary ammonium group or protonated tertiary or secondary amine (Swanson & Albuquerque, 1991). The structures of these ligands suggest that electrostatic, hydrogen bonding, and hydrophobic interactions all contribute to their binding.

Although the detailed, three-dimensional structures of the ACh binding sites in the receptor are not yet known, the locations of these sites and the identities of several of the amino acid residues contributing to binding are known. The subunit composition of the muscle-type ACh receptors is $\alpha_2\beta\gamma\delta$ [reviewed in Karlin (1993)]. There are two ACh binding sites, one in the interface between the first α and the γ subunit and the other between the second α and the δ subunit (Kurosaki et al., 1987; Blount & Merlie, 1989; Pedersen & Cohen, 1990; Sine & Claudio, 1991; Czajkowski & Karlin, 1991; Czajkowski et al., 1993). Residues at the ACh binding sites have been identified by affinity labeling and by site-directed mutagenesis: In α, two adjacent disulfide-linked cysteines (Kao et al., 1984; Mishina et al., 1985; Kao & Karlin, 1986) and four aromatic residues (Abramson et al., 1989; Galzi et al., 1990, 1991; Cohen et al., 1991; Middleton & Cohen, 1991; Tomaselli et al., 1991; O'Leary & White, 1992) contribute to the ACh binding sites. In δ (Czajkowski et al., 1993) and in γ (Martin, Czajkowski, and Karlin, unpublished results), an aspartyl residue and a glutamyl residue likely contribute charge–charge, electrostatic attraction to the binding of ACh. Other residues have been identified in γ and δ that influence the binding of the competitive antagonist *d*-tubocurarine (Sine, 1993).

The participation of aromatic residues in the binding of ACh in the receptor is consistent with the apposition of ligand quaternary ammonium groups to aromatic rings in acetylcholinesterase (Harel et al., 1993) and in an anti-phosphocholine antibody (Satow et al., 1986; Glockshuber et al., 1991) and with the binding of quaternary ammonium groups by synthetic macrocycles (Dougherty & Stauffer, 1990). The affinity of quaternary ammonium groups for aromatic rings is likely based on cation–π-electron and hydrophobic interactions (Kier & Aldrich, 1974; Petti et al., 1988; Dougherty & Stauffer, 1990). The cation–π-electron interaction should fall off rapidly with distance, and the hydrophobic effect requires close enough contact to displace water molecules from the interacting surfaces; neither is likely to be much affected by ionic strength, especially at or below physiological salt concentrations (Tanford, 1980).

By contrast, the attractive Coulombic force between positively charged ligands like ACh and negatively charged carboxylate groups would be longer range and would be sensitive to ionic strength [e.g., Gilson and Honig (1987);

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* Author to whom correspondence should be addressed.

‡ Present address: CAChe Scientific, Woodbridge, NJ 07095.

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[1] Abbreviations: ACh, acetylcholine; AChR, acetylcholine receptor; BgTX, α-bungarotoxin; DTNB, 5,5′-dithiobis(2-nitrobenzoate); DTT, dithiothreitol; GABA, γ-aminobutyric acid; MA, 2-mercaptoethylamine; MBTA, 4-(*N*-maleimido)benzyltrimethylammonium; ME, 2-mercaptoethanol; MS, 2-mercaptoethanesulfonate; MMTS, methyl methanethiosulfonate; MTS, methanethiosulfonate; MTSEA, methanethiosulfonate ethylammonium; MTSES, methanethiosulfonate ethylsulfonate; MTSET, methanethiosulfonate ethyltrimethylammonium; MTSX, any one of MTSEA, MTSES, MTSET, and MMTS; NaMTS, sodium methanethiosulfonate; NEM, *N*-ethylmaleimide; PYS, 2-mercaptopyridine; TNB, 5-thio-2-nitrobenzoate.

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Sternberg et al. (1987), and Sharp and Honig (1990)]. The dependence of the rate constant for the association of the positively charged *Naja nigricollis* α-neurotoxin with ACh receptor is consistent with a negatively charged toxin-binding site (Weber & Changeux, 1974). In acetylcholinesterase, the dependence on ionic strength of ligand association rates is consistent with an effective charge at the active site of −6 to −9 (Nolte et al., 1980). In fact, these charges are not localized in the catalytic site but are distributed throughout the protein (Radic et al., 1992; Ripoll et al., 1993). Although the effective charge at a site can be deduced from the dependence of a reaction on ionic strength, the determination of the actual charge distribution requires a high-resolution structure.

The electrostatic potential at a site is in principle deducible from the rates of reactions of charged reagents at the site [e.g., Legler (1975), MacKinnon et al., (1989), and Shin and Hubbell (1992)]. In this paper we calculate the electrostatic potential at the ACh binding site of the nicotinic receptor from the rates of reaction of charged reagents with sulfhydryls on Cys192 and Cys193 in the binding site. The reagents we used are methanethiosulfonate (MTS) derivatives, which react specifically and rapidly with thiols to form mixed disulfides (Kenyon & Bruice, 1977; Roberts et al., 1986). We report here on the reactions of MTS ethylammonium (MTSEA), MTS ethyltrimethylammonium (MTSET), MTS ethylsulfonate (MTSES), and methyl MTS (MMTS) with the receptor binding site and with five simple thiols. These MTS reagents were previously used to probe the structures of the ACh receptor channel (Akabas et al., 1992), of the $GABA_A$ receptor channel (Xu & Akabas, 1993), and of the lactose permease (Dunten et al., 1993).

MATERIALS AND METHODS

Methanesulfonyl chloride, sodium sulfide nonahydrate, 2-bromoethylamine hydrobromide, (2-bromoethyl)trimethylammonium bromide, methyl methanethiosulfonate (MMTS), dithiothreitol (DTT), *N*-ethylmaleimide (NEM), 1-octanol, and 2-bromoethanesulfonic acid, sodium salt, were obtained from Aldrich. 5,5′-Dithiobis(2-nitrobenzoate) (DTNB), 2-mercaptoethylamine (MA), and 2-mercaptoethanesulfonic acid, sodium salt (MS), were from Sigma. 2-Mercaptoethanol (ME) was from BDH Chemicals. 2-Mercaptopyridine (PYS) was from Fluka. [^{125}I]-α-Bungarotoxin ([^{125}I]BgTx) was from New England Nuclear. Scintisol radioassay medium was from Isolab.

Sodium methanethiosulfonate (NaMTS) was synthesized from sodium sulfide nonahydrate and methanesulfonyl chloride according to the method of Shaked et al. (1980). *N*-(4-Maleimido)benzyltrimethylammonium iodide (MBTA) was synthesized as in Karlin (1977).

Buffers frequently used and their designations are as follows: 50 mM NaCl, 10 mM Tris, 3 mM NaN_3, 1 mM EDTA, pH 8.1 (NT50); 100 mM NaCl, 10 mM $NaPO_4$, 1 mM EDTA, 3 mM NaN_3, pH 7.0 (NP100); 10 mM NaCl, 10 mM $NaPO_4$, pH 7.4, 0.2% Triton X-100 (TNP10); 50 mM NaCl, 10 mM $NaPO_4$, pH 7.0, 0.2% Triton X-100 (TNP50); 100 mM NaCl, 10 mM $NaPO_4$, pH 7.0, 0.2% Triton X-100 (TNP100); 600 mM NaCl, 50 mM $NaPO_4$, pH 7.4, 2.0% Triton X-100 (TNP600). In addition, buffers A–D each contained 1.0 mM $NaPO_4$, 0.3 mM NaN_3, and 0.1 mM EDTA, pH 7.00 (adjusted daily prior to use), with the following concentrations of NaCl: A, 1.4 mM; B, 21.4 mM; C, 71.4 mM: D, 131.4 mM. The ionic compositions of $NaPO_4$ and EDTA at pH 7.00 were estimated from the pK_a values (Segel, 1976). The calculated ionic strengths of the buffers are as follows: A, 0.005 M; B, 0.025 M; C, 0.075 M; D, 0.135 M.

Sodium (2-Sulfonatoethyl)methanethiosulfonate (MTSES). A mixture of NaMTS (4.82 g, 36 mmol) and 2-bromoethanesulfonate, sodium salt (6.46 g, 30 mmol), in 125 mL of absolute ethanol was stirred and heated to reflux. The white suspension was heated at reflux for 4 days. Acetone–water (5:1 v/v, 125 mL) was added dropwise to the refluxing solution; a further addition of 25 mL of water dissolved all solids. This solution was concentrated by rotary evaporation until a cloudy white precipitate formed. The initial precipitate was discarded. Absolute ethanol (100 mL) was added, and after 16 h at room temperature, MTSES was recovered as fine white crystals. The yield was 2.33 g (30%). It was 93% pure by TNB assay and had a melting point of 214–216 °C (dec): NMR (500 MHz, D_2O, TSP internal standard) δ 3.38 (t, 2H), 3.57 (s, 3H), 3.58 (t, 2H). Elemental analysis (Galbraith Labs) of a sample 93% pure by TNB assay, calculated for $C_3H_7NaO_5S_3$: C, 14.87; H, 2.91; S, 39.70. Found: C, 13.99; H, 2.88; S, 39.47.

(2-Aminoethyl)methanethiosulfonate Hydrobromide (MTSEA). MTSEA was synthesized from NaMTS and 2-bromoethylamine hydrobromide according to the method of Bruice and Kenyon (1982). The yield in our hands, following recrystallization from absolute ethanol, was 30–45%; the purity was >95% by TNB assay. MTSEA has a melting point of 107–109 °C: NMR (500 MHz, D_2O, TSP internal standard) δ 3.43 (t, 2H), 3.56 (t, 2H), 3.58 (s, 3H). Elemental analysis of a pure sample by TNB assay, calculated for $C_3H_{10}BrNO_2S_2$: C, 15.26; H, 4.27; Br, 33.84; N, 5.93; S, 27.15. Found: C, 15.20; H, 4.30; Br, 34.02; N, 5.91; S, 26.77.

[2-(Trimethylammonium)ethyl]methanethiosulfonate Bromide (MTSET). A solution of NaMTS (2.41 g, 18 mmol) and (2-bromoethyl)trimethylammonium bromide (3.70 g, 15 mmol) in 40 mL of absolute ethanol was stirred and heated at reflux overnight. The solution was cooled, filtered, and concentrated. After 16 h at 4 °C, MTSET was recovered as fine white needles, which were washed with cold ethanol. The yield was 2.91 g (70%). According to the TNB assay, this preparation was 94% pure. Recrystallization from absolute ethanol afforded pure material. MTSET has a melting point of 155–157 °C (dec): NMR (500 MHz, D_2O, TSP internal standard) δ 3.22 (s, 9H), 3.22 (t, 2H), 3.61 (s, 3H), 3.66 (t, 2H). Elemental analysis of a pure sample, calculated for $C_6H_{16}BrNO_2S_2$: C, 25.90; H, 5.80; Br, 28.72; N, 5.03; S, 23.05. Found: C, 25.92; H, 5.88; Br, 29.07; N, 4.97; S, 23.38.

TNB Assay for MTS Reagents (Bloxham & Cooper, 1982). The methanethiosulfonate functional group in the MTSX was assayed by the formation of a mixed disulfide with 5-thio-2-nitrobenzoate (TNB). (This was not useful, however, for assaying NaMTS.) TNB (100 μM) was generated by the action of DTT (50 μM final) upon excess DTNB (2.0 mM) in NT50. To 900 μL of TNB (100 μM) in a quartz cuvette was added 100 μL of H_2O (blank) or 100 μL of MTS reagent in H_2O (ca. 800 μM). The difference in absorbance at 412 nm ($\epsilon = 13.7\ cm^{-1}\ mM^{-1}$) gave the concentration of the MTS reagent. The half-times for hydrolysis of the MTS reagents in NP100, pH 7.0, at room temperature, determined by the TNB assay were 144 min for MMTS, 58 min for MTSES, 40 min for MTSEA, and 15 min for MTSET. These reagents were stable for a day or more in distilled water at 4 °C.

Rates of Reaction of MTS Reagents with Thiols in Solution. The reactions were monitored in a Hi-Tech Scientific PQ/SF-53 rapid-kinetics spectrophotometer. The reaction temp-

erature was maintained at 25.0 (±0.1) °C with a Hi-Tech C-400 circulator and FC-200 flow cooler. The spectrophotometer unit was controlled and the data were analyzed by On-Line Instruments Systems, Inc., 4120AT computer software, run on an IBM-AT.

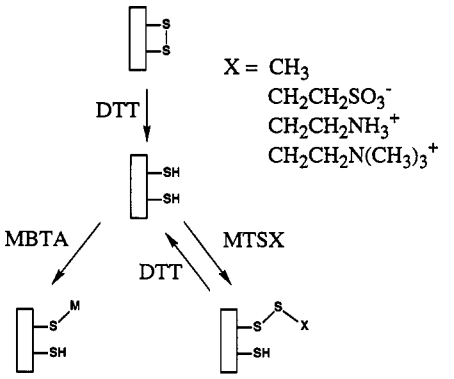
In a typical experiment, the first syringe was loaded with ca. 200 μM thiol in twice-concentrated buffer A, B, C, or D at pH 7.0, while the second syringe was loaded with ca. 2.0 mM MTS reagent in water. (The pH values of the corresponding mixtures were measured after a series of kinetics runs and were typically found to be pH 7.10 ± 0.05.) The paired syringes were driven by about 3 bar of compressed air. Equal volumes (100 μL) of solutions passed through a ball mixer into an optical cell. The dead-time of the apparatus under these conditions was 2–3 ms. The change in absorbance due to the conversion of the sulfhydryl to a disulfide was monitored at the following wavelengths (nm): ME, 236; MA, 220; MS, 236; PYS, 343; TNB, 440. The data were fit to give the pseudo-first-order rate constant, and the second-order rate constant was calculated as the pseudo-first-order rate constant divided by the concentration of MTS reagent. For each condition, the reported rate constant is the mean ± SEM (n = 5).

ACh receptor-rich membrane (fraction B2) was isolated from *Torpedo californica* electric tissue according to the method of Czajkowski et al. (1989). The specific activity of receptor binding sites was 2–3 nmol/mg, assayed by the binding of [^{125}I]BgTx (Czajkowski et al., 1989). Receptor-rich membrane (400–800 pmol of toxin sites/200 μL) was suspended in different buffers by centrifugation in a Beckman Airfuge at 130000g for 3 min at room temperature; the supernatant was carefully removed, and the membrane was suspended in buffer by passing it in and out 10 times through a Becton-Dickinson U-100 insulin syringe.

Reduction of ACh Receptor Binding-Site Sulfhydryls. Receptor-rich membrane in 200 μL of NT50 was treated with 0.2 mM DTT for 30 min. The membranes were sedimented in a Beckman Airfuge three times to remove DTT and were resuspended each time in 200 μL of buffer A.[2]

Rates of Reaction of MBTA with the Reduced Receptor Binding-Site Sulfhydryls. The assay is based on the irreversible inhibition of toxin binding by the affinity labeling by MBTA of one of the two reduced ACh binding sites (Damle & Karlin, 1978). An aliquot (30 μL) of DTT-treated receptor-rich membrane was mixed with 5.0 mL of the appropriate ionic strength buffer (A, B, C, or D) to give 1–2 nM toxin sites. Two 500-μL aliquots were removed for the determination of specific toxin binding at time zero. To the remaining 4.03 mL was added 40 μL of 1.0 μM MBTA. An aliquot (500 μL) was removed at each time point and added to 500 μL of 20 mM NEM in TNP100 to quench the reaction. The number of toxin binding sites remaining in each aliquot was determined as in Czajkowski et al. (1989). Rate constants were evaluated by fitting the pseudo-first-order kinetic equation to the disappearance of toxin binding sites due to the reaction with MBTA. The maximum decrease in the number of toxin binding sites was about 50%, which corresponded to the alkylation by MBTA of one of two cysteines in one of two sites. The reported rate constant at each ionic strength is the mean ± SEM (n = 4).

Scheme 1



Rates of Reaction of MTS Reagents (MTSX) with the ACh Binding Site. The rates were determined by competition of MTSX with MBTA (Scheme 1). The binding-site disulfide was reduced with DTT, and the reduced cysteines were treated with a mixture of MBTA and MTSX, each in excess over the thiols (downward arrows in Scheme 1). DTT was added in excess to stop the reactions and to reduce the mixed disulfide formed by the reaction of MTSX and cysteine (upward arrow in Scheme 1). The thioether bond formed by the reaction of MBTA and cysteine is not cleaved by DTT, and the alkylation of a binding-site cysteine by MBTA prevents the binding of toxin to that site. Thus, we determined the extent of reaction of MBTA by measuring the extent of block of toxin binding. At the concentrations of MBTA used, it alkylated just one of the two ACh (and toxin) binding sites and one of two cysteines at that site (Karlin et al., 1976), and the rates of reaction of MTSX refer to that site.

Just as for the reaction with MBTA alone, membrane was reduced, washed, and diluted into buffer A, B, C, or D. Solutions in the appropriate buffer containing different combinations of MBTA and MTSX concentrations were prepared as follows: 20 nM MBTA and 20 nM to 20 μM MMTS or MTSES; 200 nM MBTA and 20 nM to 20 μM MTSEA or MTSET. The competition reaction was initiated by the mixing of 500 μL of membrane and 500 μL of the solution containing MBTA and MTSX. After 5 min, 100 μL of 5.5 mM DTT in NT50 was added both to stop the reactions and to reduce the mixed disulfides formed by the reaction of the MTS reagents with the binding-site sulfhydryls. After 10 min, all remaining free sulfhydryls in the receptor and in DTT were alkylated by the addition of 100 μL of 24 mM NEM in TNP600. (Reduction of the receptor and alkylation with NEM have only a small effect on toxin binding.) The toxin sites remaining, namely those sites not blocked by MBTA, were assayed as above.

The reactions of MTSX with the reduced ACh binding sites (AChRSH) susceptible to labeling by MBTA were analyzed according to the following equations, in which [AChRS–SX] and [AChRS–MBTA] are the concentrations of the adducts of MTSX and MBTA with AChRSH, respectively, k_{MBTA} is the second-order rate constant for the reaction of MBTA with AChRSH, and k_{MTSX} is the second-order rate constant for the reaction of MTSX with AChRSH.

$$d[AChRS–MBTA]/(k_{MBTA}[MBTA]) = d[AChRS–SX]/(k_{MTSX}[MTSX]) = [AChRSH]dt \quad (1)$$

[2] Membrane-bound receptor (30–40% pure) was used instead of solubilized and purified receptor to allow us to lower by repeated sedimentation and suspension of the membrane the concentration of dithiothreitol from 200 μM in the initial reduction step to below 10 nM prior to the addition of MBTA and MTSX. The presence of nonreceptor proteins in the membrane has no effect on specific toxin binding or on the inhibition of toxin binding by the affinity reaction of MBTA at the ACh binding site (Damle & Karlin, 1978).

When [MBTA] and [MTSX] are much greater than [AChRSH] and, therefore, constant, integration of eq 1 yields

$$[\text{AChRS–MBTA}]/[\text{AChRS–SX}] = (k_{\text{MBTA}}[\text{MBTA}])/(k_{\text{MTSX}}[\text{MTSX}]) \quad (2)$$

After sufficient time, all AChRSH will have reacted either with MBTA or with MTSX. From eq 2, the fraction of AChRSH reacted with MBTA is

$$f_{\text{MBTA}} = 1/\{1 + (k_{\text{MTSX}}[\text{MTSX}])/(k_{\text{MBTA}}[\text{MBTA}])\} \quad (3)$$

Experimentally

$$f_{\text{MBTA}} = (y_{\max} - y)/(y_{\max} - y_{\min}) \quad (4)$$

where $y_{\max}$ is the toxin binding when no MBTA or MTSX is added, $y_{\min}$ is the toxin binding when only MBTA is added, and y is the toxin binding when a mixture of MBTA and MTSX is added. Given [MTSX], [MBTA], and k_{MBTA}, determined as described above, k_{MTSX} was determined by a nonlinear least-squares fit of the experimentally determined values of f_{MBTA} to eq 3. The reported rate constant for each MTS reagent at each ionic strength is the mean ± SEM (n = 4).

RESULTS

Theory. We apply absolute-reaction-rate theory and Debye–Hückel theory to obtain rate constants as a function of the electrostatic potential, the charges of the reactants, and the ionic strength of the solution (Laidler, 1965). The reaction of A and B is assumed to go through an activated complex, AB‡, and the rate constant is given by

$$k = \nu \exp(-\Delta G^\ddagger/RT) \quad (5)$$

where ν is the vibrational frequency of the activated complex in the degree of freedom leading to its decomposition and $\Delta G^\ddagger = G^\circ_{AB^\ddagger} - G^\circ_A - G^\circ_B$ is the standard Gibbs free energy difference per mole. We consider separately the long-range electrostatic contribution to $\Delta G^\ddagger$, which we call $\Delta G^\ddagger_{lr}$, and the sum of all other contributions (including interactions with solvent), which we call the short-range contributions, $\Delta G^\ddagger_{sr}$.

$$\Delta G^\ddagger = \Delta G^\ddagger_{sr} + \Delta G^\ddagger_{lr} \quad (6)$$

The reactants, A and B, and the activated complex, AB‡, have algebraic charges, z_A, z_B, and $z_A + z_B$, respectively, and their electrostatic energies include the energies of the ionic atmospheres surrounding them. The change in electrostatic energy, due to long-range electrostatic forces, associated with the formation of the activated complex can be calculated as the sum of energies associated with the uncharging of the ionic atmospheres of the reactants, with the approach of the reactants so that their centers are a reaction radius apart, and with the charging of the ionic atmosphere of the activated complex:

$$\Delta G^\ddagger_{lr} = z_A F\psi_B + RT \ln[\gamma_{AB^\ddagger}/(\gamma_A\gamma_B)] \quad (7)$$

ψ_B is the electrostatic potential due to B at the reaction radius, at zero ionic strength, and the γ are the molar activity coefficients of the indexed species. The first term on the right is the electrostatic free energy for the formation of the activated complex in the absence of ionic atmospheres, and the second term on the right is the difference in the free energies of the charging of the ionic atmospheres of the complex and of the reactants (Kortüm & Bockris, 1951).

Combining eqs 5–7, we obtain

$$\log(k) = \log(k_{sr}) - [0.434F/(RT)]z_A\psi_B + \log(\gamma_A\gamma_B/\gamma_{AB^\ddagger}) \quad (8)$$

where the logarithm is base 10 and $k_{sr} = \nu \exp[-\Delta G^\ddagger_{sr}/(RT)]$.

Under a number of simplifying assumptions, including spherically symmetrical ions, and in the limit of low ionic strength

$$\log(\gamma) = -Qz^2\sqrt{u} \quad (9)$$

where $Q = 1.826 \times 10^6/(\epsilon T)^{1.5}$, ϵ is the dielectric constant, and u is ionic strength (Kortüm & Bockris, 1951, pp 170–177). Combining eqs 8 and 9, we obtain

$$\log(k) = \log(k_{sr}) - [0.434F/(RT)]z_A\psi_B + 2Qz_Az_B\sqrt{u} \quad (10)$$

At 25 °C in water, $Q = 0.51\ \text{M}^{-0.5}$; hence, with rounding off

$$\log(k) = \log(k_{sr}) - [0.434F/(RT)]z_A\psi_B + z_Az_B\sqrt{u} \quad (11)$$

where ψ_B is in volts. The dependence of k on the electrostatic potential, as expressed in eq 11, is free of geometrical assumptions; the dependence of k on ionic strength, however, is based on highly simplifying assumptions, including spherical symmetry. With $[\log(k_{sr}) - [0.434F/(RT)]z_A\psi_B] = \log(k_0)$, where k_0 is the rate constant at zero ionic strength, eq 11 is equivalent to the Bronsted–Bjerrum–Debye–Hückel equation [e.g., Laidler (1965), p 220].

When the above equations are applied to the reaction of thiols with electrophiles, and in particular with methanethiosulfonates (Roberts et al., 1986), the reactive species is the thiolate, TS$^-$. The ionization of the thiol, however, is also influenced by ionic strength (Snyder et al., 1981). Let g be the fraction of the total concentration of thiol, [T] = [TSH] + [TS$^-$], that is ionized; i.e., g = [TS$^-$]/[T]. The rate constant calculated in terms of the total thiol concentration, k_T, equals g times the rate constant in terms of the thiolate concentration, k_{TS^-}. Thus, for the reaction of TS$^-$ and MTSX

$$\log(k_{T,X}) = \log(k_{sr,TS^-,X}) - [0.434F/(RT)]z_X\psi_{TS^-} + z_Xz_{TS^-}\sqrt{u} + \log(g) \quad (12)$$

g depends on the pK_a of the thiol at zero ionic strength, the pH, and the ionic strength (Snyder et al., 1981). For receptor, the p$K_{a,0}$ values of the binding-site thiols are not known, and to avoid additional free parameters and approximations, we factor out the function g and the effect of changes in ionic strength on thiol ionization. We take the ratio of the rate constants, $k_{T,X}$ and $k_{T,Y}$, for the reaction of two MTS reagents, MTSX and MTSY, with the same thiol, TSH, at the same pH and ionic strength; thus, eq 12 yields

$$\log(k_{T,X}/k_{T,Y}) = \log(k_{sr,TS^-,X}/k_{sr,TS^-,Y}) - [0.434F/(RT)]\ (z_X - z_Y)\psi_{TS^-} + (z_X - z_Y)z_{TS^-}\sqrt{u} \quad (13)$$

We now assume that the ratio of the constants containing the contribution of short-range interactions to the rate constants, $k_{sr,TS^-,X}/k_{sr,TS^-,Y}$, is characteristic of the two MTS reagents, MTSX and MTSY, and independent of the particular thiol compound. We can consider $k_{sr,TS^-,X}/k_{sr,TS^-,Y}$ the ratio of the intrinsic reactivities of MTSX and MTSY. We assume that this is constant over all thiol compounds; i.e., for any

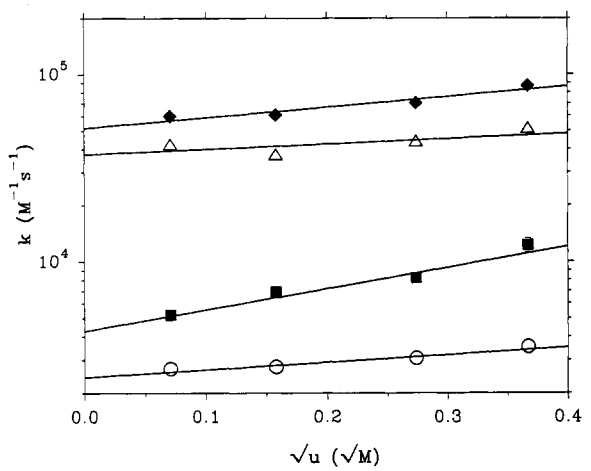


FIGURE 1: Rate constants for the reactions of methanethiosulfonates with 2-mercaptoethanol as a function of ionic strength. The reactions were monitored in a stopped-flow spectrophotometer, and the second-order rate constants were calculated, as described under Materials and Methods. Each rate constant is the weighted mean of five determinations; in each case the standard error of the mean is smaller than the symbol. The data are fit by the equation $\log(k) = \log(k_0) + m(u)^{1/2}$, where k is the second-order rate constant, k_0 is the rate constant at zero ionic strength, m is the slope, and u is the ionic strength. Symbols: (unfilled circles) MMTS; (filled squares) MTSES; (upward-pointing, unfilled triangles) MTSEA; (filled diamonds) MTSET.

thiol compounds, TSH and VSH

$$k_{sr,TS^-,X}/k_{sr,TS^-,Y} = k_{sr,VS^-,X}/k_{sr,VS^-,Y} \quad (14)$$

(This assumption is equivalent to the symmetrical assumption

$$k_{sr,TS^-,X}/k_{sr,VS^-,X} = k_{sr,TS^-,Y}/k_{sr,VS^-,Y}$$

i.e., this ratio is characteristic of the two thiols and independent of the MTS reagent.)

These ratios can be factored out, therefore, by taking the ratio of ratios

$$\rho = (k_{T,X}/k_{T,Y})/(k_{V,X}/k_{V,Y}) \quad (15)$$

Equations 13–15 imply

$$\log(\rho) = -[0.434F/(RT)](z_X - z_Y)(\psi_{TS^-} - \psi_{VS^-}) + (z_X - z_Y)(z_{TS^-} - z_{VS^-})\sqrt{u} \quad (16)$$

where $[0.434F/(RT)] = 17$ at 25 °C. Equation 16 relates the observed rate constants (in terms of the total concentrations of the thiols) to the ionic strength and to the differences in the charges of the MTS reagents, the differences in the electrostatic potentials at the thiolates, and the differences in the charges of the thiolates (or, equivalently, of the thiols).

Rate Constants for the Reactions of MTSX with Simple Thiols. We determined the rate constants for the reactions of the MTS reagents with a number of simple thiol comopunds to test the applicability of the above equations to the reactions of these relatively small molecules and to compare these rate constants to the rate constants for the reactions of the MTS reagents with the receptor. The rate constants were determined at four ionic strengths, and the rate constant at zero ionic strength was obtained by extrapolation (Figure 1). The rate constants at zero ionic strength for the reactions of the four MTS reagents with each of the simple thiols increased in the order MMTS < MTSES < MTSEA ≤ MTSET, with MTSET reacting 13–81 times more rapidly than MMTS (Table 1).

Table 1: Ionic Strength Dependence of the Reactions of Simple Thiols, and of the ACh Receptor Binding-Site Cysteines, with MTS Reagents[a]

thiol	z_{TS^-}[b]	MTSX	z_X	slope[c]	log(k_0)[d]	rel k_0[e]
ME	−1	MMTS	0	0.39 ± 0.08	3.38 ± 0.02	1
		MTSES	−1	1.10 ± 0.18	3.63 ± 0.05	1.8
		MTSEA	1	0.27 ± 0.20	4.57 ± 0.05	15
		MTSET	1	0.55 ± 0.13	4.71 ± 0.03	21
MA	0	MMTS		0.45 ± 0.11	4.59 ± 0.03	16
		MTSES		0.15 ± 0.27	5.15 ± 0.06	59
		MTSEA		0.60 ± 0.42	5.37 ± 0.10	98
		MTSET		−0.57 ± 0.71	5.72 ± 0.17	220
MS	−2	MMTS		1.36 ± 0.31	3.55 ± 0.10	1.5
		MTSES		1.73 ± 0.20	3.71 ± 0.06	2.1
		MTSEA		0.20 ± 0.07	5.08 ± 0.02	50
		MTSET		0.61 ± 0.32	5.00 ± 0.09	42
PYS	−1	MMTS		−0.40 ± 0.42	2.35 ± 0.09	0.093
		MTSES		0.08 ± 0.21	2.69 ± 0.05	0.20
		MTSEA		−0.58 ± 0.29	3.43 ± 0.06	1.1
		MTSET		−0.47 ± 0.29	3.74 ± 0.07	2.3
TNB	−2	MMTS		0.14 ± 0.13	4.24 ± 0.04	7.2
		MTSES		0.50 ± 0.14	4.43 ± 0.04	11
		MTSEA		−0.75 ± 0.08	5.71 ± 0.02	210
		MTSET		−1.02 ± 0.07	6.15 ± 0.02	590
AChR		MMTS		1.24 ± 0.20	4.49 ± 0.06	13
		MTSES		2.08 ± 0.45	3.87 ± 0.15	3.1
		MTSEA		−0.75 ± 0.39	6.55 ± 0.09	1500
		MTSET		−1.89 ± 0.34	7.35 ± 0.05	9300
		MBTA		−1.33 ± 0.08	6.96 ± 0.02	3800

[a] The second-order rate constant, k, for the reaction of each thiol and each MTS reagent was determined in terms of the total concentration of thiol as under Materials and Methods, and the linear least-squares fit of log(k) (weighted by mean/SEM) vs $(u)^{1/2}$ was calculated. [b] The net charge of the thiolate form is given. [c] The observed slope is that of the linear least-squares fit. [d] The intercept of the fitted line is log(k) at zero ionic strength. Errors in the values of the parameters for each pair of thiol and MTSX are the errors of the least-squares fit. [e] The k_0 values are divided by k_0 for the reaction of ME and MMTS.

The rate constants depend on the charges of the reactants. Positively charged MTSEA reacted more rapidly than negatively charged MTSES with all of the simple thiols; however, the ratio of the rate constants (at zero ionic strength), $k_{0,MTSEA}/k_{0,MTSES}$, was greater the greater the negative charge on the thiolate compound. The net charges on the thiolates (including the $-S^-$) and the ratios of the rate constants were as follows: MA, charge 0, ratio 2; PYS and ME, charge −1, ratios 6 and 9; MS and TNB, charge −2, ratios 19 and 23.

If these reactions are influenced by long-range electrostatic interactions, then the rates should be affected by changes in the ionic strength of the medium. If the electrostatic interaction between the reactants is attractive, then the rate should decrease with increasing ionic strength; if the electrostatic interaction between the reactants is repulsive, then the rate should increase with increasing ionic strength. (As discussed above, there is a secondary effect of ionic strength on the rate of reaction: the thiolate is the reactive species and, at a pH less than the pK_a of the thiol dissociation, thiolate concentration can vary significantly with ionic strength.)

For the reactions of the TNB thiolate (net charge −2), the rate constant for the reaction with MTSES (charge −1) increased with ionic strength; i.e., the slope of the plot of log(k) vs $(u)^{1/2}$ was positive (Table 1). For the reactions with MTSEA and with MTSET (net charges +1), the slopes were

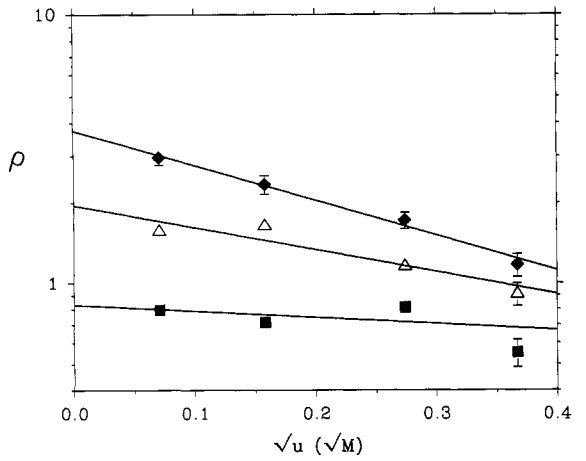


FIGURE 2: Normalized rate constants of the reactions of TNB with MTSX as a function of ionic strength. The data are plotted according to eq 16, where $\log(\rho) = \log(k_{TNB,MTSX}/k_{TNB,MMTS}) - \log(k_{ME,MTSX}/k_{ME,MMTS})$. Symbols: (filled diamonds) MTSET; (upward-pointing, unfilled triangles) MTSEA; (filled squares) MTSES.

negative. These are relatively simple cases, since the TNB is fully ionized at pH 7 (Roberts et al., 1986). In general, however, the slope of $\log(k)$ vs $(u)^{1/2}$ is not well predicted by $z_A z_B$ (eq 11). Better agreement between theory and experiment is obtained when the secondary effect of ionic strength is eliminated by our taking ratios of rate constants.

Electrostatic Potentials and Charges of the Simple Thiols. For each simple thiol and each MTSX, we formed the ratio of ratios, ρ, as in eq 15, where V is ME, T is any of the other simple thiols, Y is MMTS, and X is MTSEA, MTSET, or MTSES. We plotted $\log(\rho)$ vs $(u)^{1/2}$ (Figure 2) and obtained the intercept and the slope. According to eq 16, the intercept at zero ionic strength yields the difference between the electrostatic potential at the reacting thiolate, TS^-, and the electrostatic potential at the ME thiolate, and the slope depends on the differences in the charges of the reactants. Since the only charge on the ME thiolate is the $-S^-$ itself, the difference in the charges of each thiol and ME is simply the charge of the undissociated thiol (TSH), i.e., not including the charge on the $-S^-$. Similarly, the difference in the electrostatic potentials between each thiol and ME is the electrostatic potential of each thiol, not including the potential due to the $-S^-$.

For each simple thiol, we calculated its charge from the slope (Table 2). Although these varied, the average of the three estimates of the charge of each of the thiols had the expected sign and, for PYS and TNB, close to the expected magnitude. Also, the estimates of the electrostatic potential of the thiols were consistent: for MA, MS, and TNB, with charges of +1 or −1 (excluding the charge on the S^-), the absolute values of the potentials varied around an overall mean of 19 ± 5 mV. For PYS, with zero charge, the electrostatic potential was close to zero (Table 2).

[3] This indirect method to determine the rate constants for the MTS reagents was used for two reasons: (a) The read-out of the reaction as inhibition of toxin binding was absolutely specific for the binding site. (b) Direct determination of the reaction of a binding-site cysteine with MTSX is difficult because it appears that $-SCH_2CH_2X$ is eliminated slowly from the initially formed mixed disulfide with one of the binding-site cysteines, by exchange with the adjacent second binding-site cysteine, to re-form the native binding-site disulfide. The reaction of the binding-site cysteines with MBTA, however, is blocked in either case. As shown in Figure 3 (at high concentrations of MTSX), the final reduction reverses all inhibition of toxin binding due to the reaction with MTSX.

Table 2: Charges and Electrostatic Potentials of Simple Thiols Estimated from the Linear Least-Squares Fit of ρ vs $(u)^{1/2}$ [a]

thiol	z_{TS^-}	MTSX	$z_{TS^-} - z_{VS^-}$ [b] observed	$z_{TS^-} - z_{VS^-}$ [b] expected	$\psi_{TS^-} - \psi_{VS^-}$ [c] (mV)
MA	0	MTSES	1.06 ± 0.21	1	17.8 ± 2.7
		MTSEA	0.12 ± 0.41	1	23.2 ± 5.3
		MTSET	−1.25 ± 0.66	1	11.1 ± 9.9
		wtd mean	0.39 ± 0.67	1	18.3 ± 3.5
MS	−2	MTSES	−0.02 ± 0.38	−1	−11.3 ± 5.3
		MTSEA	−0.98 ± 0.30	−1	−19.5 ± 4.1
		MTSET	−0.77 ± 0.60	−1	−7.4 ± 8.6
		wtd mean	−0.60 ± 0.29	−1	−14.1 ± 3.6
PYS	−1	MTSES	0.38 ± 0.19	0	6.5 ± 2.5
		MTSEA	0.02 ± 0.21	0	6.2 ± 2.8
		MTSET	−0.14 ± 0.14	0	−3.1 ± 1.7
		wtd mean	0.06 ± 0.15	0	2.3 ± 3.2
TNB	−2	MTSES	0.23 ± 0.32	−1	−4.8 ± 4.3
		MTSEA	−0.81 ± 0.23	−1	−17.3 ± 3.2
		MTSET	−1.29 ± 0.10	−1	−33.9 ± 1.4
		wtd mean	−0.90 ± 0.45	−1	−24.4 ± 8.4

[a] The rate constants were determined at four ionic strengths, as described under Materials and Methods, and the data were fitted by eq 16, as in Figure 2. [b] Subscript T indicates the thiol in column 1, V indicates ME, X indicates the MTSX in column 3, and Y indicates MMTS. The observed ($z_{TS^-} - z_{VS^-}$) is the slope of the linear fit divided by ($z_X - z_Y$); since the charge on MMTS is 0, ($z_X - z_Y$) = z_X. The expected ($z_{TS^-} - z_{VS^-}$) values are calculated with the z_{TS^-} in column 2 and taking the charge of ionized ME as −1. [c] The difference in the electrostatic potentials of the thiol in column 1 and of ME is calculated from the intercept of the linear fit divided by $-17(z_X - z_Y)$. Errors in the parameters for each pair of a thiol and a MTSX are the standard errors of the weighted least-squares fit of eq 16 to the data. Errors in the weighted means are SEM.

Rate Constants for the Reactions of MTSX with the Receptor Binding-Site Thiols. The rates of reaction of the MTSX with the receptor binding-site thiols were determined by the competition of MTSX and MBTA for these thiols (Scheme 1 and Materials and Methods). The MTSX protected against the irreversible inhibition of toxin binding by MBTA.[3] Assayed by this protection, the positively charged MTSET and MTSEA reacted with the reduced binding site much more rapidly than did the neutral MMTS or the negatively charged MTSES (Figure 3 and Table 1). The order of the rate constants at each ionic strength, and also of the rate constants extrapolated to zero ionic strength, was MTSET > MTSEA > MMTS > MTSES; i.e., the negatively charged MTSES was the most slowly reacting at the ACh binding site (Figure 4; Table 1). By contrast, the neutral MMTS reacted more slowly than MTSES with all of the simple thiols. Furthermore, at zero ionic strength, positively charged MTSEA reacted 480 times faster than negatively charged MTSES at the ACh binding-site thiol compared to 2–23 times for simple thiol compounds (Table 1). This is an indication that the electrostatic potential at the ACh binding-site thiolate is considerably more negative than the potential at the thiolates of the small compounds.

The reactions of the binding-site thiols with the MTSX were sensitive to changes in ionic strength (Figure 4). The slopes of the linear fit to $\log(k)$ vs the square root of ionic strength were consistent with eq 11 applied to a negatively charged binding site: the slope, predicted to be the product of the charges, was positive for the reaction with the negatively charged MTSES and negative for the reactions with the positively charged MTSEA and MTSET (Table 1). The magnitudes of the slopes were greater for the reactions with the binding site than those for the reactions with the simple thiols, implying that the magnitude of the effective binding-

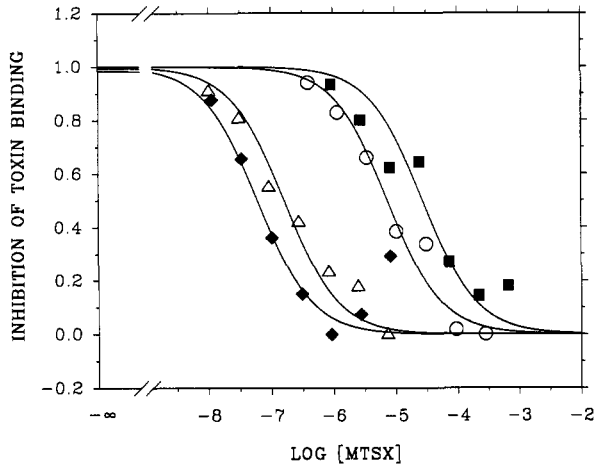


FIGURE 3: Competition of MTSX with MBTA for reaction with the ACh binding-site cysteines. After the reaction of the mixture of MBTA and MTSX with the reduced cysteines of the ACh binding site (Materials and Methods), those cysteines that have formed mixed disulfides with MTSX were re-reduced; thus, the inhibition of toxin binding measures the extent of reaction with MBTA, which is not reversed by reduction (see Scheme 1). The inhibition of toxin binding was scaled so that 1 is the maximum inhibition by MBTA alone, which blocked about half of toxin binding (Damle & Karlin, 1978); the inhibition of 0 is that obtained in the absence of both MBTA and MTSX. For the more slowly reacting MMTS and MTSES, the MBTA concentration was 10 nM, and for the more rapidly reacting MTSEA and MTSET, the MBTA concentration was 100 nM. Since the quotient of the concentrations of MTSX and MBTA determine the relative extents of reaction (eq 2), all of the data are plotted as if the MBTA concentrations were 100 nM; i.e., the MMTS and MTSES concentrations are multiplied by 10. The data shown were obtained at an ionic strength of 0.075 M. Symbols: (unfilled circles) MMTS; (filled squares) MTSES; (upward-pointing, unfilled triangles) MTSEA; (filled diamonds) MTSET.

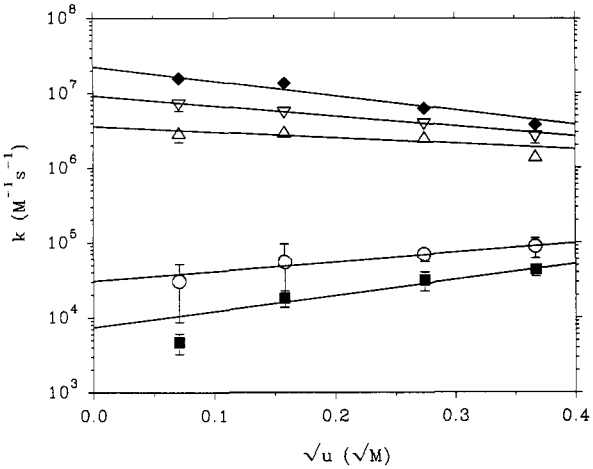


FIGURE 4: Rate constants for the reactions of MBTA and of MTSX with the reduced binding-site cysteines as a function of ionic strength. The rate constants were determined as described under Materials and Methods (also see Figure 3) and plotted as in Figure 1. Symbols: (unfilled circles) MMTS; (filled squares) MTSES; (upward-pointing, unfilled triangles) MTSEA; (downward-pointing, unfilled triangles) MBTA; (filled diamonds) MTSET.

site charge is greater than one. Quantitatively, however, eq 11 did not yield consistent magnitudes for the binding-site charges. Again, theory and experiment were in better agreement when we considered the ratio of the rate constants.

Electrostatic Potential and Charge at the Receptor Binding Site. As with the simple thiols, we formed the ratio of ratios (ρ) of the rate constants for the reaction of the receptor binding-site cysteines with each of the MTSX relative to the reaction with MMTS and the rate constants for the reactions of each

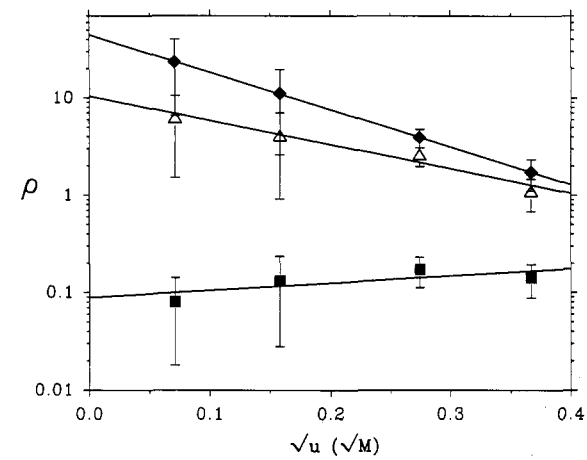


FIGURE 5: Normalized rate constants of the reactions of the reduced receptor binding-site cysteines (AChRSH) with MTSX as a function of ionic strength. The data are plotted according to eq 16, where $\log(\rho) = \log(k_{AChRSH,MTSX}/k_{AChRSH,MMTS}) - \log(k_{ME,MTSX}/k_{ME,MMTS})$. Symbols: (unfilled circles) MMTS; (filled squares) MTSES; (upward-pointing, unfilled triangles) MTSEA; (filled diamonds) MTSET.

of the simple thiols with each of the MTSX relative to the reactions with MMTS. The plots of $\log(\rho)$ vs $(u)^{1/2}$ were well fit by straight lines (Figure 5). The effective charge at the ACh binding-site thiolate, excluding the thiolate charge itself, was calculated from the slope of these lines. The estimates for this charge were, in all cases, negative; the overall average of these estimates was −2.6 (Table 3).

The electrostatic potential of the ACh binding site *minus* that of each of the simple thiols was calculated from the intercept at zero ionic strength, according to eq 16. For each simple thiol, the three differences in potential determined with the three charged MTS reagents were fairly consistent, although in each case the magnitude of the potential determined with MTSET was greatest (Table 3). For each simple thiol, we took the mean of these three potential differences and subtracted the mean potential obtained above for the simple thiol (Table 2). Thus, we obtained the electrostatic potential at the reaction radius of the receptor binding-site thiolate, at zero ionic strength and excluding the contribution of the S^- itself (Table 3). The overall average of these potentials was −79 ± 6 mV.

DISCUSSION

Electrostatic Potentials of Simple Thiols. The application of eq 16 to the rate constants for the reactions of the simple thiols tests the assumptions underlying this equation. For each thiol, we obtained three estimates of the electrostatic potential at the $-S^-$, excluding the contribution of the $-S^-$ itself (Table 2). Therefore, considering the charges of the thiols, not including the $-S^-$, MA has a charge of +1, and the mean electrostatic potential was 18 mV. The thiol forms of MS and TNB have charges of −1, and the mean electrostatic potentials were −14 and −24 mV, respectively. The thiol form of PYS has zero charge, and the mean electrostatic potential was close to zero. For the charged thiols, moreover, the mean electrostatic potentials correspond closely to the electrostatic potential expected for a point charge in water. The Coulombic electrostatic potential 10 Å from a single spherically symmetrical charge in water (dielectric constant 78.5) is plus or minus 18 mV, which corresponds closely to the above mean values derived from eq 16. Although the thiols and MTS reagents are not spherical, and the charges are not central,

Table 3: Charge and Electrostatic Potential at the ACh Binding-Site Thiols Relative to Charge and Potentials at the Thiols of the Small Compounds[a]

thiol	z_{VS^-}	MTSX	$z_{AChRS^-} - z_{VS^-}$[b]	z_{AChR}[c]	$\psi_{AChRS^-} - \psi_{VS^-}$[d] (mV)	ψ_{AChR}[e] (mV)
ME	−1	MTSES	−0.72 ± 0.51		−62.3 ± 8.5	
		MTSEA	−2.44 ± 0.45		−60.2 ± 7.5	
		MTSET	−3.77 ± 0.02		−97.6 ± 0.3	
		wtd mean	−3.61 ± 0.88	−3.6	−94.7 ± 12	−95
MA	0	MTSES	−1.97 ± 0.29		−82.4 ± 4.7	
		MTSEA	−2.78 ± 0.94		−87.1 ± 15	
		MTSET	−2.43 ± 0.86		−106 ± 14	
		wtd mean	−2.22 ± 0.23	−1.2	−88.0 ± 7.0	−70
MS	−2	MTSES	−0.85 ± 0.18		−52.8 ± 3.0	
		MTSEA	−1.40 ± 0.10		−39.6 ± 1.7	
		MTSET	−2.89 ± 0.72		−87.7 ± 12	
		wtd mean	−1.34 ± 0.61	−2.3	−47.6 ± 14	−62
PYS	−1	MTSES	−1.00 ± 0.65		−67.9 ± 11	
		MTSEA	−2.50 ± 0.61		−67.4 ± 10	
		MTSET	−3.68 ± 0.16		−95.8 ± 2.7	
		wtd mean	−3.02 ± 0.77	−3.0	−86.3 ± 9.4	−84
TNB	−2	MTSES	−1.17 ± 0.30		−59.9 ± 5.1	
		MTSEA	−1.56 ± 0.42		−42.2 ± 7.0	
		MTSET	−2.45 ± 0.14		−62.9 ± 2.3	
		wtd mean	−1.96 ± 0.38	−3.0	−58.3 ± 6.4	−83
overall means				−2.6 ± 0.4		−79 ± 6

[a] The rate constants were determined as described under Materials and Methods and were fitted by eq 16, as in Figure 5. In eq 16, subscript T indicates the ACh binding site cysteines, V indicates the thiol specified in column 1, X indicates the MTSX in column 3, and Y indicates MMTS. Errors in the parameters for each pair of a thiol and a MTSX are the standard errors of the weighted least-squares fit. Errors in the weighted means are SEM. [b] $(z_{AChRS^-} - z_{VS^-})$ was calculated by dividing the slope of the linear fit by $(z_X - z_Y)$. [c] The charge at the receptor binding site, not including the ionized thiolate, was calculated from $z_{AChRS^-} - z_{VS^-}$ and z_{VS^-}. [d] The difference in the electrostatic potentials of the binding site and of the thiol in column 1 was calculated by dividing the intercept of the fit by $-17(z_X - z_Y)$. [e] The electrostatic potential at the reaction radius of the reacting binding site thiolate, due to all charges except the $-S^-$ itself, is calculated as $[(\psi_{AChRS^-} - \psi_{VS^-}) - (\psi_{VS^-} - \psi_{ME^-})]$, assuming that the electrostatic potential due just to the reacting $-S^-$ in the receptor is equal to that due to the $-S^-$ in ME^-. The values of the term $(\psi_{VS^-} - \psi_{ME^-})$ are the mean values in the last column in Table 2, headed $(\psi_{TS^-} - \psi_{VS^-})$, where T refers to a simple thiol and V refers to ME; for T = ME this term, of course, is zero.

10 Å is roughly the sum of the radii of a MTSX and a thiol compound, i.e., roughly the reaction radius. The consistency and reasonableness of the electrostatic potentials of the simple thiols, estimated using eq 16, support our use of this equation in estimating the electrostatic potential at the ACh binding-site cysteines.

A further test of the consistency of eq 16 and of the assumed constancy of the ratio of intrinsic reactivities expressed in eq 14 is that $\log(\rho)$ should be zero at all values of ionic strength, when two thiols with the same charge are compared; i.e., both $(z_{TS^-} - z_{VS^-})$ and $(\psi_{TS^-} - \psi_{VS^-})$ should be zero. Forming the ratio of the rate constants at zero ionic strength (Table 1), with ME and PYS, which have the same charge, and with MMTS as Y, we calculate, with MTSES as X, $\log(\rho) = -0.09$, with MTSEA as X, $\log(\rho) = 0.11$, and with MTSET as X, $\log(\rho) = 0.06$. Taking MS and TNB as T and V and Y as MMTS, we calculate, with MTSES as X, $\log(\rho) = 0.03$, with MTSEA as X, $\log(\rho) = -0.06$, and with MTSET as X, $\log(\rho) = 0.46$. Except for the last value, all of the $\log(\rho)$ values are close to zero, as required. The deviation of the last value from zero reflects the unusually high reactivity of TNB with MTSET (Table 1), perhaps due to the interaction of the aromatic ring of TNB with the quaternary ammonium group of TNB. The assumption of eq 14 and the other assumptions we made in deriving eq 16 appear to be reasonably justified.

Electrostatic Potential of the ACh Binding Site. One complication of our analysis is that the structure of the receptor could change with ionic strength, so that the number and arrangement of charges could be different at physiological ionic strengths and at zero ionic strength. Since the dependence of the rate constants on $(u)^{1/2}$ was linear, with relatively small slopes, there was at least no large, discontinuous change in structure or charge distribution as the ionic strength was lowered.

Another complication is that we have analyzed the ACh binding site with the binding-site disulfide reduced. Reduction of this disulfide changes the functional properties of the receptor moderately: the K_{app} for agonists increases by 3–10-fold (Karlin & Bartels, 1966; Walker et al., 1981). Reduction is unlikely to increase markedly the electrostatic potential of the binding site since reduction lowers the affinity for agonists, so that at worst we may be underestimating the potential. (At pH 7, the cysteine thiols are not likely to contribute much charge to the binding sites; in any case, the electrostatic potential due to the reacting $-S^-$ was subtracted from the calculated electrostatic potentials.)

Additional complications are that there are two ACh binding sites per receptor, that these sites react at very different rates with MBTA, and that there are two cysteines in each ACh binding site. At the concentrations of MBTA used here, however, MBTA reacts with just one of the two ACh binding sites per receptor and with one of the two cysteines in the binding site (Damle & Karlin, 1978). Since the binding-site cysteines are adjacent, it is likely that the reaction of either MTSX or MBTA at one of the two cysteines not only blocks further reaction at that cysteine but retards reaction at the other cysteine. We analyze the competition between MBTA and MTSX as if it were for a single thiol, assuming that any errors introduced thereby are small compared to the observed effects of reactant charge on the rates of reaction.

The assumed constancy of the ratio of intrinsic reactivities of the MTSX (eq 14) appears to be a good approximation for the simple thiols (see above). For MTSEA and MTSES, the constancy of the ratio of intrinsic reactivities, and the validity

of eq 16, appear to extend to the ACh binding-site thiols. This is indicated by the similar values of the binding-site electrostatic potential *minus* the simple thiol electrostatic potential we obtained with MTSEA and MTSES, each compared with MMTS, and with the receptor compared to each of the simple thiols (Table 3). The potential differences obtained with MTSET, however, were in all cases greater in magnitude than those obtained with MTSEA and MTSES. This is most probably due to the binding of the quaternary ammonium MTSET to the ACh binding site. MTSET acted as a weak agonist of the mouse ACh receptor expressed in *Xenopus* oocytes (Akabas et al., 1992) and competed with ACh binding to unreduced *Torpedo* receptor (data not shown). (MTSEA at concentrations up to 2.5 mM had no detectable activity as an agonist or as an antagonist of the unreduced receptor and thus has very low affinity for the binding site.) Although MTSET and MTSEA both have a single positive charge, MTSET reacted about 6 times more rapidly than MTSEA with the binding-site thiolates. By contrast, MTSET reacted only about 2 times more rapidly than MTSEA with all of the simple thiols except MS (Table 1). The extra increase in reactivity of MTSET with the binding-site thiolates and the greater electrostatic potential differences reported by MTSET are likely due to the moderate affinity of MTSET for the binding site; i.e., the short-range interactions of MTSET with the ACh binding site are stronger than those with the simple thiols. Nevertheless, the values for the potential differences reported by MTSET are not more than 50% different from those reported by MTSEA and MTSES. Furthermore, the means of the binding-site electrostatic potentials were similar over all of the simple thiols (Table 3, last column). These results support the approximate validity of eqs 14 and 16 as applied to the binding-site thiols.

The estimate that the effective charge at the binding site is −2.6 (Table 3) is a plausible result, consistent with the identification of two negatively charged residues in or close to the ACh binding site (Czajkowski et al., 1993). Nevertheless, even with the simple thiols, the values of the charges estimated by eq 16 were scattered, and the geometry and the ionic screening of charge are certainly more complicated for the ACh binding site than for the simple thiols. The calculation of the electrostatic potential from the extrapolated value of $\log(\rho)$ at zero ionic strength, however, is free of the assumptions of the Debye–Hückel theory involved in the calculation of the charges of the reactants from the slopes.

Electrostatic Contribution to the Binding of ACh by the Receptor. An electrostatic potential of −80 mV and the equivalent of two to three negative charges at the binding site are consistent with a long-range electrostatic contribution to the free energy of binding of ACh. At any ionic strength, the effective electrostatic potential at the ACh binding-site thiol can be estimated as $-(1/(z_X - z_Y))(RT/F)\ln(\rho)$, where ρ is formed with the receptor as T and ME as V, and X and Y are two MTSX. At the highest ionic strength tested (u = 135 mM), taking MTSEA as X and MTSES as Y, ρ = 8, and taking MTSET as X, ρ = 12; i.e., after normalization by the ratio of intrinsic reactivities, the positively charged MTSEA reacted with the binding-site thiol 8 times more rapidly, and MTSET reacted 12 times more rapidly, than did the negatively charged MTSES. These ratios imply that the effective electrostatic potential at the binding-site thiols at u = 135 mM is about −30 mV; i.e., there is a considerable negative potential at the binding-site thiols even when the electric field is screened by mobile ions. The binding-site thiols are approximately 1 nm away from the negative subsite of the ACh binding site (Karlin, 1969), and the electrostatic potential closer to the aspartyl and glutamyl residues that are likely to contribute to the negative subsite (Czajkowski et al., 1993) could be considerably greater in magnitude than that at the thiols.

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