Effective Charge on Acetylcholinesterase Active Sites Determined from the Ionic Strength Dependence of Association Rate Constants with Cationic Ligands†

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ABSTRACT: The reaction of the specific fluorescent cationic ligand *N*-methylacridinium with the active site of 11S acetylcholinesterase from electric eel was monitored by temperature-jump relaxation kinetics at a variety of ionic strengths. The ionic strength dependence of the bimolecular association rate constant is analyzed with a Brønsted–Debye–Hückel expression and leads to estimates of the association rate constant at zero ionic strength of $k_{12}^0 = 1.1 \times 10^{10}$ M^{-1} s^{-1} at 25 °C and the net charge number of the enzyme active site of $Z_E = -6.3$. The ionic strength dependence of the second-order hydrolysis rate constant k_{cat}/K_{app} for acetylthiocholine under steady-state conditions is also very pronounced and indicates a value of $Z_E = -9$. Thus, a large effective negative charge on the enzyme active site appears to be a general characteristic of its interaction with cationic ligands. The ionic strength dependence of k_{cat}/K_{app} is identical with that of sodium chloride, sodium phosphate, and sodium citrate, thus ruling out any possibility that the phenomena arise from a specific, partially competitive binding of Na^+ to the enzyme active site. Substitution of the calculated electrostatic parameters into theoretical equations indicates that the most significant effect of these Z_E values is a 2–3 order of magnitude reduction in the rate constant for dissociation of the initial ligand–enzyme encounter complex; this decrease renders the bimolecular reaction diffusion controlled. The high value of k_{12}^0 and the space requirements of six to nine charged groups suggest that regions of the enzyme surface area larger than the catalytic sites themselves are effective in trapping cationic ligands.

Acetylcholinesterase is an extremely efficient catalyst of acetylcholine hydrolysis at neuromuscular junctions [see, for example, Neumann et al. (1978) and Rosenberry (1979)]. This efficiency reflects both a very high first-order hydrolysis rate constant of $k_{cat} = 1.6 \times 10^4$ s^{-1} at high substrate concentrations and a very high second-order constant of $k_{cat}/K_{app} = 2 \times 10^8$ M^{-1} s^{-1} at lower substrate concentrations or under conditions of excess enzyme [Rosenberry, 1975a; values at 25 °C, ionic strength (I_c) of 0.1 M]. Recently, we investigated the basis of this high second-order rate constant by measuring the bimolecular association rate constant k_{12} for the reaction of the fluorescent ligand *N*-methylacridinium with acetylcholinesterase from the electric eel *Electrophorus electricus* (Rosenberry & Neumann, 1977). This cationic ligand binds with high specificity to each of the four independent sites in the 11S enzyme tetramer (Mooser & Sigman, 1974). Temperature-jump relaxation kinetics showed that $k_{12} = 1.2 \times 10^9$ M^{-1} s^{-1} (23 °C, pH 8, $I_c = 0.13$ M), an unusually high value for an enzyme–ligand interaction.

In this study we have pursued the physical basis for this high k_{12} value by examining its ionic strength dependence. Previous studies have demonstrated the dramatic effect of ionic strength on cationic ligand affinities for acetylcholinesterase from *Electrophorus* (Mooser & Sigman, 1974), from the torpedo *Torpedo marmorata* (Changeux, 1966), and from erythrocytes (Pyttel & Robinson, 1974). Our report confirms this effect on equilibrium affinities and extends the analysis to individual association and dissociation rate constants to provide a mechanistic basis for the ionic strength dependence. A high net negative charge on the enzyme active site appears to be responsible for making the association reaction primarily diffusion controlled. The isoelectric point (p*I*) of the eel enzyme is 4.5 (Chen et al., 1974). Thus, at pH 7 anionic groups dominate the surface charge and several of these groups appear to influence the association reaction at each catalytic site. Similar electrostatic features govern the interaction of both *N*-methylacridinium and the acetylcholine analogue acetylthiocholine with the enzyme active site.

Experimental Section

Materials. Acetylcholinesterase from the electric organs of the eel *E. electricus* was an 11S preparation. Its specific activity corresponded to ~55% of the maximal activity reported for 11S acetylcholinesterase (Rosenberry, 1975a), and its normality was determined from equilibrium and kinetic titrations with *N*-methylacridinium. The concentration of recrystallized *N*-methylacridinium perchlorate in solutions was determined from $\epsilon_{358} = 21\,300$ M^{-1} cm^{-1} (Rosenberry & Neumann, 1977).

Buffer solutions contained defined mixtures of sodium monobasic and dibasic phosphates, and other salts were omitted except where noted to avoid the quenching of *N*-methylacridinium fluorescence (Rosenberry & Neumann, 1977). The solution ionic strengths (I_c) were calculated by assuming complete dissociation of NaH_2PO_4 and partial dissociation of Na_2HPO_4 according to the equilibrium $[NaHPO_4^-]/([Na^+][HPO_4^{2-}]) = 4.0$ M^{-1} (Smith & Alberty, 1956). The ionic strength of sodium citrate was calculated by assuming $pK_{a3} = 6.39$ (Bates & Pinching, 1949).

Fluorescence Measurements. Fluorescence data were collected in a temperature-jump apparatus similar to that described previously (Rigler et al., 1974; Rosenberry & Neumann, 1977). The temperature increase was triggered by a 20- or 30-kV discharge from a 1×10^{-8}, 2×10^{-8}, or 5×10^{-8} F capacitor through the 0.8-mL sample compartment, but the final temperature in all cases was adjusted to 25.0 °C.

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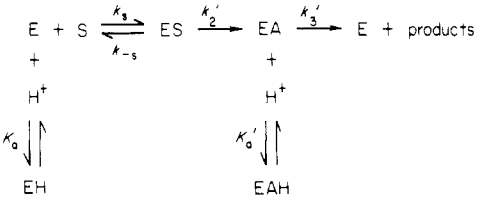
Scheme I

$$E + L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EL$$

Scheme II



The fluorescence relaxation trace was stored on a Bruker transient recorder, and relaxation times and amplitudes were obtained by superpositioning the stored trace upon a calibrated multiple exponential function [see Rosenberry & Neumann (1977)] with a dual-beam oscilloscope. Usually three to four temperature-jump measurements per titration step were sufficient to assure good reproducibility and each titration involved 8 to 13 C_L^{tot} points.

Steady-State Acetylcholinesterase Assay. Enzyme activity toward acetylthiocholine was measured with a modification of the spectrophotometric assay of Ellman et al. (1961) at 25 °C. The concentration of 5,5′-dithiobis(2-nitrobenzoic acid) was fixed at 0.33 mM, but the phosphate buffer I_c was varied between 2.2 and 250 mM, the acetylthiocholine concentration ranged from 1.6 to 25 μM, and the pH was adjusted between 5.9 and 8.2. Small corrections (<10%) were applied to rates measured at the lowest pH values to compensate for slight decreases in the absorbance of reduced 5,5′-dithiobis(2-nitrobenzoate). Typical rates with 3.3 μM acetylthiocholine were measured at 412 nm on the 0.05-A full-scale setting of a Perkin-Elmer 572 spectrophotometer. Final rates were triplicate averages randomized with respect to the independent variable, usually ionic strength.

Ligand Interaction Schemes. The binding of the fluorescent ligand *N*-methylacridinium to acetylcholinesterase active sites was analyzed according to the simple, one-step process indicated in Scheme I. The association rate constant k_{12} and the dissociation rate constant k_{21} define the equilibrium dissociation constant $K_D \equiv k_{21}/k_{12}$. Inclusion of an independent additional binding site for the ligand in enzyme preparations [Scheme II′ in Rosenberry & Neumann (1977)] was considered during analysis of some experiments but was rejected due to insufficient evidence (see Results).

The steady-state hydrolysis of acetylthiocholine by acetylcholinesterase was interpreted according to the mechanism in Scheme II. In Scheme II the substrate S combines with the free enzyme E and the complex ES is converted to an intermediate acetylenzyme EA [see Rosenberry (1975a)]. A proton with dissociation constant K_a competes with *cationic* substrates for the binding site in E, and a proton with dissociation constant K_a' blocks deacetylation of EA (Krupka, 1966; Rosenberry, 1975b). The rate equation involving the hydrolysis rate v that corresponds to Scheme II is given by

$$\frac{1}{v} = \frac{1}{k_{cat}C_E^{tot}}\left[1 + \frac{k_{cat}C_{H^+}}{k_3'K_a'} + \frac{K_{app}}{C_S}\left(1 + \frac{C_{H^+}}{K_a}\right)\right] \quad (1)$$

where C_X is the concentration of species X, C_E^{tot} is the total enzyme concentration, $k_{cat} = k_2'k_3'/(k_2' + k_3')$, and $K_{app} = k_{cat}(k_{-s} + k_2')/(k_sk_2')$ [see Rosenberry (1975a)].

Relaxation Data. The expression relating the observed relaxation time τ to the intrinsic rate constants in Scheme I (Eigen & DeMaeyer, 1963) is given by

$$\tau^{-1} = k_{12}(\bar{C}_E + \bar{C}_L) + k_{21} \quad (2)$$

where $\bar{C}_E$ and $\bar{C}_L$ are the respective *equilibrium concentrations* of enzyme active sites and of ligand. An alternative expression (Rosenberry & Neumann, 1977) that involves only *total concentrations* C_E^{tot} and C_L^{tot} (i.e., in Scheme I $C_E^{tot} = \bar{C}_E + \bar{C}_{EL}$) is given by eq 3 and is used here.

$$\tau^{-2} = k_{12}^2(K_D + C_E^{tot})^2 + 2k_{12}^2(K_D - C_E^{tot})C_L^{tot} + k_{12}^2(C_L^{tot})^2 \quad (3)$$

When C_E^{tot} is constant during a progressive titration with the ligand, eq 3 is a second-order polynomial in C_L^{tot}; determination of the coefficients of the polynomial by regression analysis allows simultaneous evaluation of k_{12}, C_E^{tot}, and K_D. Slight corrections for enzyme dilution during the titration can be introduced (Rosenberry & Neumann, 1977) that permit calculation of the initial value of $C_E^{tot} \equiv C_E^{init}$. Enzyme dilution arises from the progressive addition of stock ligand solution but is partially compensated for by evaporation accompanying deaeration of the sample after each ligand addition. An average dilution factor was calculated from the known volume of ligand solution added (generally 0.6–2.5% of the total sample volume) and the average incremental volume loss due to evaporation as measured by the increased conductivity of the sample (generally 0.3–1.3%).

In experiments where two observed relaxation steps suggested the presence of a second independent binding site for ligand, an analysis of relaxation amplitudes was conducted to provide quantitative estimates of the coupling factor θ between the two relaxation steps (Rosenberry & Neumann, 1977). If coupling is negligible, θ increases to a value of 1. Values of θ less than 1 can also be detected by analysis of relaxation times according to eq 2. When coupling is significant, the slower relaxation time is given by an expression analogous to eq 2 in which $\bar{C}_E$ is replaced by $\bar{C}_E\theta$ (Rosenberry & Neumann, 1977). Thus, deviation of τ^{-1} values at low $\bar{C}_L$ from the linear relationship predicted by eq 2 is an indication of a possible coupling factor θ.

Analysis of equilibrium fluorescence data was carried out as previously described (Rosenberry & Neumann, 1977).

Steady-State Data. Measurements of K_a were made at low acetylthiocholine concentrations ($C_S < K_{app}$) by determining the ratio R for v_1 at low pH_1 to v_2 at high pH_2 at a fixed I_c. Calculated values of K_a were obtained from R according to

$$K_a = \frac{R10^{-pH_1} - 10^{-pH_2}}{1 - R} \quad (4)$$

Equation 4 assumes that $K_a \simeq k_3'K_a'/k_{cat}$. Second-order hydrolysis rate constants k_{cat}/K_{app} were calculated from pseudo-first-order rate constants $k_{cat}C_E^{tot}/K_{app}$ by assuming $k_{cat}/K_{app} = 2.5 \times 10^8$ M^{-1} s^{-1} at $I_c = 0.10$ M and pH 7.5 (Hillman & Mautner, 1970; Rosenberry, 1975a). Estimates of $k_{cat}C_E^{tot}/K_{app}$ were obtained at $C_S < K_{app}$ by rearrangement of eq 1 according to

$$\frac{k_{cat}C_E^{tot}}{K_{app}} \simeq \frac{v}{C_S}\left(1 + \frac{C_S}{K_{app}}\right)\left(1 + \frac{C_{H^+}}{K_a}\right) \quad (5)$$

Equation 5 also assumes that $K_a \simeq k_3'K_a'/k_{cat}$. This assumption is equivalent to considering the "apparent K_{app}" (i.e., the negative abscissa intercept of a plot of v^{-1} vs. C_S^{-1} according to eq 1) to be independent of pH. For acetylcholine hydrolysis at $I_c = 0.1$ M, the assumption appears valid: $pK_a = 6.3$, $k_3'/k_{cat} \simeq 1$, and $pK_a' = 6.5$ (Rosenberry, 1975a,b); furthermore, the apparent K_{app} appears to be independent of pH

at various I_c values (H. R. Smissaert, unpublished results). Thus, the assumption appears reasonable for acetylthiocholine hydrolysis. Moreover, eq 4 and 5 were utilized here only at low C_S (except for the lowest I_c investigated, $C_S/K_{app} < 0.25$), a condition under which K_a and $k_{cat}C_E^{tot}/K_{app}$ estimates are little affected even by moderate differences between K_a and $k_3'K_a'/k_{cat}$.

Theoretical Treatment of Ionic Strength Dependence. Analysis of the dependence of observed rate constants on ionic strength was based on concepts introduced by Brønsted and Bjerrum as developed by Scatchard (1932). The association rate constant k_{12} in Scheme I thus is given by

$$k_{12} = k_{12}^0 f_E f_L / f^* \quad (6)$$

where f_E and f_L are activity coefficients of E and L, f^* is the activity coefficient for the activated complex formed on the reaction pathway, and k_{12}^0 is the association rate constant at zero ionic strength where all f terms equal 1. Application of the Debye–Hückel theory to the calculation of the activity coefficients [e.g., see Kortüm (1972)] leads to

$$\log k_{12} = \log k_{12}^0 + \frac{2AZ_E Z_L I_c^{1/2}}{1 + B\bar{a}I_c^{1/2}} \quad (7)$$

In eq 7, A and B are constants (for 25 °C and the dielectric constant of water $\epsilon = 79$, $A = 0.509$ $M^{-1/2}$ and $B = 3.29$ $M^{-1/2}$ nm^{-1}); the ionic strength $I_c = 0.5\sum_i Z_i^2 C_i$, where Z_i and C_i are the charge number and concentration of species i; $\bar{a}$ is the mean distance of closest approach between E and counterions. In this study, $Z_L = +1$ for all ligands.

The application of eq 6 and 7 to a diffusion-controlled reaction involves certain approximations. For example, the equilibrium condition for the ionic atmospheres appears to be unfulfilled for ionic diffusional encounter complexes (Eigen, 1954; Eigen et al., 1964). It must be assumed that the relatively high concentrations of inert strong electrolytes in these experiments provide sufficient electrostatic screening to permit estimation of the activity coefficients for the nonequilibrium states during the relaxation in the same formal way as for equilibrated ionic atmospheres. A second approximation involves the solution of the Poisson–Boltzmann equation used in the derivation of eq 7. The solution generally utilizes the assumption that $Z_L e_0 \bar{\psi}_E(r) \ll kT$, where $\bar{\psi}_E(r)$ is the mean electrostatic potential at a distance r from the enzyme active site (see the Appendix), but this assumption does not appear justified, particularly with the large Z_E estimates obtained here. An alternative solution which avoids this assumption has been developed by Pierce [see Rice & Nagasawa (1961)]. The solution to the electrostatic potential is expressed as a power series, the first term of which is equivalent to the corresponding expression in the Debye–Hückel approximation. When this method is applied to our case with the parameter values collected in Table III, it is shown that the Debye–Hückel term is indeed large compared to the other terms which describe the deviations from the Debye–Hückel approximation. The method can be used to estimate the accuracy of the parameter values, in particular the charge number Z_E, as determined by eq 7 and will be given in detail elsewhere (H.-J. Nolte and E. Neumann, unpublished results).

Data Analysis. Relaxation times were analyzed according to eq 3 with a second-order polynomial regression computer program introduced by Rosenberry & Neumann (1977). Ionic strength dependencies were quantified according to eq 7 with a minimization function program (subroutine VAO4A, Harwell Subroutine Library) obtained from Dr. D. Lancet (Lancet & Pecht, 1976). Error factors for parameters estimated from eq 7 were obtained from a weighted least-squares analysis of the following linear form of eq 7:

$$\left[\frac{\Delta I_c^{1/2}}{\Delta(\log k_{12})}\right]^{1/2} = \frac{1 + B\bar{a}\bar{I}_c^{1/2}}{(2AZ_+Z_-)^{1/2}} \quad (8)$$

where each $\bar{I}_c$ is the mean of I_c values associated with each ΔI_c.

Table I: Kinetic Parameters[a] for the Interaction of *N*-Methylacridinium with Acetylcholinesterase at Various Ionic Strengths

expt no.	ionic strength (mM)	k_{12} (nM^{-1} s^{-1})	K_D (μM)	k_{21} (s^{-1})
1	1.37	6.38 ± 0.21	0.012	77
2	4.54	4.52 ± 0.07	0.025	114
3	13.5	3.40 ± 0.07	0.046	157
4	13.5	3.57 ± 0.07	0.039	140
5	26.9	2.10 ± 0.04	0.073	153
6	39.7	1.58 ± 0.02	0.087	137
7	75.9	1.11 ± 0.02	0.131	145
8	121	0.89 ± 0.02	0.187	166
9	121	0.80 ± 0.03	0.260	209

[a] Determined at 25 °C, pH 7.1 ± 0.2, according to eq 3 and corrected for dilution and evaporation as outlined under Experimental Section. Values of C_E^{init} calculated from eq 3 were 0.09 μM (expt 1), 0.17 μM (expt 2), 0.61 μM (expt 3–8), and 6.28 μM (expt 9). Error estimates for k_{12} were calculated by computer analysis, but error estimates for K_D and k_{21} could not yet be calculated by the program [see Rosenberry & Neumann (1977)].

Results

Ionic Strength Dependence of Relaxation Spectra Associated with N-Methylacridinium and Acetylcholinesterase. Relaxation measurements were conducted at pH 7 during the course of a titration of a fixed amount of enzyme with increasing amounts of the fluorescent ligand in the temperature-jump cell. Analysis of the dependence of the single observed slow relaxation time τ on the total ligand concentration C_L^{tot} gave simultaneous estimates of the total initial enzyme normality C_E^{init}, the equilibrium dissociation constant K_D, and the bimolecular association rate constant k_{12}. Values of these estimates in experiments at various ionic strengths are given in Table I. In addition, the equilibrium fluorescence titration data also provided independent estimates of C_E^{init} and K_D. An indication of the agreement between the kinetic and equilibrium estimates is given by the average C_E^{init} values calculated from experiments 3–8 in Table I for which the input enzyme quantities were the same: the relaxation time analysis gave 0.61 ± 0.02 μM, while the equilibrium analysis yielded 0.64 ± 0.05 μM.

Estimates of k_{12} and K_D in Table I indicate that these parameters are highly dependent on ionic strength. This dependence of k_{12} is shown graphically in Figure 1, where a plot based on eq 7 is presented. According to Scheme I, the dissociation rate constant k_{21} is given by the product of k_{12} and K_D. Values of k_{21} are also listed in Table I, and these values show much less dependence on ionic strength than k_{12} values do. A slight tendency toward lower k_{21} values is apparent at lower ionic strengths, but the statistical significance of this trend is questionable. The mean value of k_{21} for experiments 2–9 is 153 ± 10 s^{-1}. Thus, most of the ionic strength dependence of K_D is contributed by k_{12}.

Relaxation measurements were conducted at pH 7 in contrast to the pH 8 measurements reported earlier (Rosenberry & Neumann, 1977) for two reasons: (1) the greater buffering capacity of pH 7 phosphate better maintains the pH in the

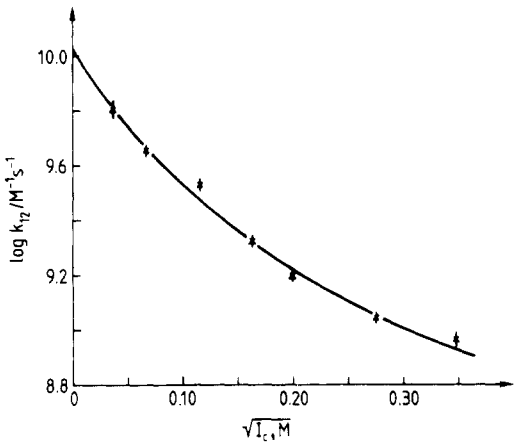


FIGURE 1: Dependence of the bimolecular association rate constant k_{12} for *N*-methylacridinium and acetylcholinesterase on ionic strength at 25 °C. Values of k_{12} are taken from Table I and plotted according to eq 7. The best fit line was calculated as noted under Experimental Section. The vertical bar through each point indicates twice the standard error.

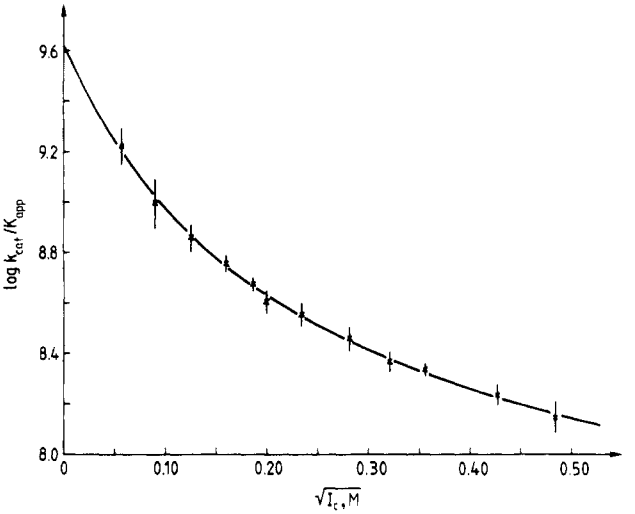


FIGURE 2: Dependence of the second-order hydrolysis rate constant k_{cat}/K_{app} for acetylthiocholine and acetylcholinesterase on ionic strength at 25 °C. Values of k_{cat}/K_{app} were determined from eq 5 as outlined under Experimental Section at pH 6.8–7.1 and C_S = 3.3 μM. The calculated line was determined as in Figure 1.

lowest ionic strength experiments and (2) the progressive loss of ligand fluorescence during the titration, presumably due to the adsorption of the ligand on the quartz cell walls, is decreased. At equivalent 0.12 M ionic strengths, the progressive loss of fluorescence at each C_L^{tot} point in this study averaged 1% or about half that observed previously at pH 8. However, the progressive loss at lower ionic strengths at pH 7 was greater and approached 3% per point in some experiments. Complete compensation for such progressive losses would alter k_{12} and k_{21} estimates by at most 10–15% [see Rosenberry & Neumann (1977)]. Furthermore, the variability of the progressive loss called such compensation into question; for example, experiment 3 averaged a 1.0% progressive loss while duplicate experiment 4 averaged a 2.5% progressive loss, yet the kinetic parameter estimates are similar. No compensation for progressive loss was made for estimates in Table I.

The experimental data in Table I were obtained at relatively low C_L^{tot} values to maximize the accuracy of the kinetic parameter estimates involving the enzyme catalytic site. No consistent evidence of a second peripheral enzyme site for *N*-methylacridinium was observed in either the equilibrium or the relaxation data. Tentative evidence consistent with such a site had been demonstrated with somewhat higher C_L^{tot} points at pH 8 in our earlier study. Examination of the slow relaxation amplitudes in this study revealed a linear dependence on $\bar{C}_E\bar{C}_L/(\bar{C}_E + \bar{C}_L + K_D)$ as in the previous study, but the corresponding ΔH derived from this dependence was 18.0 ± 1.7 kJ/mol (4.3 ± 0.4 kcal/mol; average of six experiments) or only one-half that reported previously. The discrepancy is probably due to an incorrectly labeled capacitor in the study of Rosenberry & Neumann (1977). Very fast ligand relaxation step(s) associated with the presence of enzyme were also observed here in addition to the generally predominant slow relaxation. The time course of these fast step(s) (<5 μs) is beyond the resolution capacity of the temperature-jump method. The dependence of these fast relaxation amplitude(s), ΔF_{fast}, on $\bar{C}_L$ differed from that expected for ligand binding to a low-affinity peripheral site in that $\Delta F_{fast}/\bar{C}_L$ decreased over about the same $\bar{C}_L$ range as the corresponding ratio $\Delta F_{slow}/\bar{C}_L$ for the slow relaxation. Thus, calculation of any coupling factor θ was precluded, and the possible correction of the slow relaxation times for ligand coupling to fast relaxations was resolved, as outlined under Experimental Section, by plotting τ^{-1} according to eq 2. These plots were linear with $\theta = 1$, and therefore coupling effects on the slow relaxation times were assumed to be negligible.

Table II: Kinetic Parameters for the Interaction of Acetylthiocholine with Acetylcholinesterase at Various Ionic Strengths

ionic strength (mM)	K_{app}[a] (μM)	k_{cat}[b] ($s^{-1} \times 10^{-4}$)	pK_a
3.2	4.2 ± 0.5	0.69 ± 0.09	6.95 ± 0.10
8.2	13.1 ± 5.7	1.29 ± 0.58	6.91 ± 0.08
15.9	18.2 ± 2.4	1.30 ± 0.19	6.92 ± 0.11
25.6	30.3 ± 2.3	1.73 ± 0.14	6.79 ± 0.07
39.6	33.6 ± 3.2	1.34 ± 0.13	6.81 ± 0.04
79.5	40.4 ± 6.6	1.16 ± 0.20	6.62 ± 0.15
126.7	48.9 ± 3.1	1.06 ± 0.07	6.43 ± 0.08

[a] Estimates were obtained from weighted reciprocal plots (Rosenberry & Bernhard, 1971) according to eq 1 at pH 7.0 ± 0.1 with assumption that $K_a \simeq k_3'K_a'/k_{cat}$; 25 °C. [b] Calculated as the product of the corresponding K_{app} in this table and k_{cat}/K_{app} in Figure 2.

Ionic Strength Dependence of k_{cat}/K_{app} for Acetylthiocholine Hydrolysis by Acetylcholinesterase. The second-order hydrolysis rate constant k_{cat}/K_{app} was determined under steady-state conditions at several ionic strengths. A graphical presentation of these data is given in Figure 2. Values of k_{cat}/K_{app} increase at lower ionic strengths in a manner very similar to that shown earlier for k_{12} with *N*-methylacridinium in Figure 1.

Estimates of k_{cat}/K_{app} required small to moderate corrections for the ionic strength dependencies of K_{app} and for K_a, the equilibrium dissociation constant for a proton which inhibits the hydrolysis of positively charged ester substrates (see Scheme II and eq 1 and 5). As shown in Table II, the dependence of K_{app} closely parallels the ionic strength dependence of K_D in Table I. In particular, k_{cat}, the product of k_{cat}/K_{app} and K_{app}, shows evidence of only a slight ionic strength dependence except at the lowest ionic strength investigated. Thus, the ionic strength dependence of K_{app} for acetylthiocholine, like that of K_D for *N*-methylacridinium, is contributed almost exclusively by the dependence of the bimolecular association rate constant for the interaction of the cationic ligand with the enzyme active site. The proton species associated with

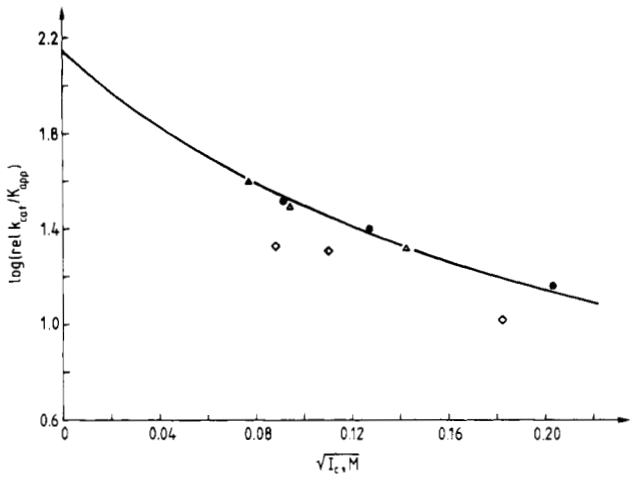


FIGURE 3: Comparison of dependence of relative k_{cat}/K_{app} on ionic strengths, I_c, generated by three sodium salts and one calcium salt. Relative k_{cat}/K_{app} is defined as $k_{cat}C_E^{tot}C_S/K_{app}$ according to eq 5, where C_S = 3.3 μM acetylthiocholine and C_E^{tot} was constant for all data points. Corrections for K_{app} and K_a were carried out as in Figure 2 according to eq 5. The calculated line from Figure 2 was normalized to these data by means of a single control point in the assay solvent plus sodium phosphate, pH 6.93, at I_c = 3.2 mM. This sodium phosphate concentration was retained as a buffer for the other data points. These points and the respective *additional* I_c contributed by added salts were as follows: (●) sodium chloride, 5.0 (pH 6.84), 13.0 (pH 6.81), and 38.0 mM (pH 6.76); (△) sodium citrate, 2.74 (pH 6.96), 5.55 (pH 7.05), and 16.9 mM (pH 7.15); (◇) calcium chloride, 4.5 (pH 6.87), 9.0 (pH 6.76), and 30.0 mM (pH 6.58).

K_a also appears to interact with the enzyme active site in an ionic strength dependent manner, as indicated by the pK_a estimates in Table II. However, the 3.3-fold difference in K_a between the highest and lowest ionic strengths is less than the 10-fold difference shown by K_{app}; when pK_a values are plotted against $I_c^{1/2}$, the dependence is linear within experimental error in contrast to the increase in slope observed for k_{cat}/K_{app} in Figure 2 with increasing $I_c^{1/2}$. The difference is further considered under Discussion. Presumably the ionic strength dependence of K_a arises largely from the bimolecular association rate constant, although the rate constants comprising K_a have not yet been separated.

Relationship of Specific Ion Binding to the Ionic Strength Dependence. Despite the agreement between eq 7 and the experimental data in Figures 1 and 2, it could be argued that the apparent ionic strength dependence instead arises from specific Na^+ ion binding to the active site that *partially competes* with cationic ligands like *N*-methylacridinium, acetylthiocholine, and acetylcholine (H. R. Smissaert, unpublished results). This alternative mechanism was examined in Figure 3 by comparing the ionic strength dependence of relative k_{cat}/K_{app} values in the presence of sodium chloride and sodium citrate with the calculated dependence from Figure 2 in the presence of sodium phosphate. The largely trivalent citrate ion contributes most of the ionic strength in sodium citrate solutions, and thus at a given ionic strength the Na^+ concentration in a sodium citrate solution is only about half that in a sodium chloride solution. It is clear from Figure 3 that k_{cat}/K_{app} follows the same ionic strength dependence for all three sodium salts and therefore that a specific Na^+ ion binding to the active site is not involved.

The ionic strength dependence of relative k_{cat}/K_{app} in the presence of calcium chloride is also shown in Figure 3 and does not follow the dependence of the sodium salts. Values of k_{cat}/K_{app} are some 30% lower at an equivalent ionic strength in calcium chloride. This suggests that a specific Ca^{2+} binding may interfere modestly with cationic ligand binding to the active site, particularly at low ionic strengths.

Table III: Effective Enzyme Charge Number Z_E and the Mean Approach Distance $\bar{a}$ for the Interaction of Cationic Ligands with Acetylcholinesterase[a]

ligand	Z_E	$\bar{a}$ (nm)
N-methylacridinium	−6.3	0.91
acetylthiocholine	−9.0	1.30
	−8.5 ± 2.2[b]	1.16 ± 0.29[b]
proton	−1.7 ± 0.3	(0)

[a] Values of Z_E and $\bar{a}$ were calculated from eq 7 with program VA04A (see Experimental Section) with the data for $k_{1,2}$ in Figure 1 for *N*-methylacridinium and for k_{cat}/K_{app} in Figure 2 for acetylthiocholine. Values associated with the proton utilized pK_a data in Table II plus three additional points (not shown) and result from a linear analysis according to eq 7 in which $\bar{a}$ is assumed equal to zero. [b] Calculated with the same acetylthiocholine data as in footnote *a* from eq 8. Ten roughly equal $\Delta[\log (k_{cat}/K_{app})]$ values were generated from the twelve experimental points by using no experimental point more than twice.

Discussion

High Bimolecular Association Rate Constants. Extrapolation to zero ionic strength provides estimates of $k_{12}^0 = 1.1 \times 10^{10}$ M^{-1} s^{-1} for *N*-methylacridinium in Figure 1 and of $(k_{cat}/K_{app})^0 = 4.2 \times 10^9$ M^{-1} s^{-1} for acetylthiocholine in Figure 2. This k_{12}^0 value is the highest reported for the interaction of a small ligand with a specific protein binding site [see Hammes & Schimmel (1971)], and in the following sections the electrostatic features that account for this high rate constant are examined from experimental and theoretical standpoints. These features also apply to acetylthiocholine (and presumably to acetylcholine) because $(k_{cat}/K_{app})^0$ from the steady-state measurements appears closely analogous if not identical with a bimolecular association rate constant: $k_{cat}/K_{app} \equiv k_s k_2'/(k_{-s} + k_2')$ (Scheme II). Also, previous evidence (Rosenberry, 1975a,b) has suggested that $k_2' > k_{-s}$ and thus that k_{cat}/K_{app} is a close minimum estimate of the biomolecular association rate constant k_s.

Apparent Charge on the Enzyme Active Site and the Ligand Approach Distance. The correspondence of the ionic strength dependence of the bimolecular association rate constants in Figures 1 and 2 to the Debye–Hückel relationship in eq 7 permits estimates of the net enzyme active site charge $Z_E e_0$ and of the counterion approach distance $\bar{a}$.[1] These estimates, given in Table III, must be interpreted with care. The apparent active-site charge may well include a substantial contribution from the net charge on the overall enzyme [see Hammes & Alberty (1959)]; the low acetylcholinesterase isoelectric point of 4.5 (Chen et al., 1974) suggests a considerable net negative charge at pH 7. Furthermore, the possibility that the enzyme active site may be partially buried in a hydrophobic area of low dielectric constant [see Rosenberry (1975a)] could lead to an overestimate of $Z_E e_0$, because both *A* and *B* in eq 7 are inversely proportional to the dielectric constant at the active-site surface. Thus, in our discussion of Z_E and $\bar{a}$ it should be understood that these are empirical parameters that reflect the overall interactions involving cationic ligands and the enzyme active site.

The most striking feature in Table III is the high effective charge on the enzyme active site with *N*-methylacridinium and

[1] Because for acetylcholinesterase there is no evidence for polyvalent charged groups, $|Z_E|$ is the effective number of monovalent anionic groups involved in the association of cationic ligands to the active site.

Scheme III

$$E + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EL$$

acetylthiocholine. Large values of $|Z_E|$ and $\bar{a}$ contribute substantially to the high k_{12}^0 estimate as noted below. It is unclear whether the difference in the Z_E or $\bar{a}$ estimates arising from these two ligands is statistically significant, because the VAO4A computational program unfortunately does not provide error estimates. Error estimates included in Table III for the alternative differential analysis (eq 8) may be somewhat higher than those expected from VAO4A because of the inherent scatter in a differential analysis. In addition to possible intrinsic differences in active-site characteristics toward the two ligands, differences might also arise from the nature of the rate constants measured. As noted in the previous paragraph, k_{cat}/K_{app} appears to be closely related to but may not be identical with the association rate constant k_s. However, neither the measured dissociation rate constant k_{21} (Table I) nor the unimolecular catalytic steps in k_{cat} that involve the enzyme–substrate complex (Table II) showed large ionic strength dependencies, so it is likely that the dependence of k_{cat}/K_{app} accurately reflects the dependence of k_s. Another experimental difference between the k_{12} and the k_{cat}/K_{app} analyses is that no corrections for K_a were applied to the k_{12} data, although such corrections do not appear to account for the differences in Z_E and $\bar{a}$ estimates.[2]

Table III indicates that Z_E for the enzyme active site is less toward the proton which acts as an active-site inhibitor than toward the other two cationic ligands. The precision of the K_a data was not sufficient to estimate an $\bar{a}$ value according to eq 7. Since pH changes alter the Z_E value of the enzyme active sites by neutralization of anionic groups, it is difficult to get complete K_a data [this effect may account for the nonlinear inhibition by H^+ below pH 6 noted by Rosenberry (1975b)]. Above pH 6, this effect was not major; estimates of five of the seven pK_a values in Table II were averages of separate determinations in which pH_I (eq 4) was ~6 on the one hand and ~7 on the other, and the separate determinations agreed within their standard errors. Equilibrium K_a values in other protein systems have ionic strength dependencies which suggest $\bar{a}$ values of ~0.3 nm [Shire et al., 1974; but see Beetlestone & Irvine (1968)]. If $B\bar{a} = 1$ is inserted into the expression for pK_a corresponding to eq 7, a Z_E value of -2.4 ± 0.2 can be calculated from the same data utilized in Table III. It is not surprising that values of Z_E and $\bar{a}$ for the proton interaction are quite different from those for other cationic ligands. Although protons and cationic ligands compete for the active site, the protonation site is thought to be a histidine residue adjacent to the anionic group(s) in the active site (Krupka, 1966).

Binding of N-Methylacridinium to the Active Site Appears To Be Diffusion Controlled. For assessment of the question of diffusion control, the simple overall reaction in Scheme I must be expanded to Scheme III (Eigen, 1954). Scheme III differs from Scheme I by inclusion of an intermediate, an initial diffusional encounter complex E·L (in which the original hydration shells of the individual reaction partners E and L may still be intact), prior to the chemical transformation to EL that results in fluorescence quenching. Our relaxation studies with *N*-methylacridinium show no evidence of a second relaxation with a characteristic dependence of time constant and amplitude on ligand concentration, and thus E·L must be present only in very low steady-state concentrations. In this case, the rate constants in Schemes I and III are related according to (Eigen, 1954)

$$k_{12} = k_1 k_2/(k_{-1} + k_2) \quad (9)$$

$$k_{21} = k_{-1} k_{-2}/(k_{-1} + k_2) \quad (10)$$

For the overall reaction to be diffusion controlled, the condition $k_2 > k_{-1}$ must be met. In the Appendix, theoretical values for k_1^0 and k_{-1}^0, the zero ionic strength values of k_1 and k_{-1}, are calculated with the assumption of the Z_E and $\bar{a}$ estimates for *N*-methylacridinium in Table III. The calculations yield $k_1^0 = 1.7 \times 10^{10}$ M^{-1} s^{-1} and $k_{-1}^0 = 7 \times 10^7$ s^{-1}. From Figure 1 and Table II, the respective experimental estimates of k_{12}^0 and k_{21}^0 are 1.1×10^{10} M^{-1} s^{-1} and 1.5×10^2 s^{-1}. Insertion of these four rate constant values in eq 9 and 10 indicates that $k_2^0 \geq 1 \times 10^8$ s^{-1} and $k_{-2}^0 \geq 1 \times 10^2$ s^{-1}. Thus, $k_2^0 \geq k_{-1}^0$, and the overall bimolecular reaction in Scheme I is at least partially and perhaps completely under diffusion control.

Closer examination of eq 1A and 2A (see Appendix) reveals the contributions of the large negative Z_E to the observed rate constants k_{12} and k_{21}. The major effect is to reduce k_{-1} by the electrostatic potential factor ϕ_{-1} and thus to make the overall reaction diffusion controlled. The factor ϕ_{-1} is 1.0 if E and L have no charge, ~0.7 if $Z_E = -1$ and $Z_L = +1$, but 0.037 if $Z_E = -6.3$ and $Z_L = +1$. Thus, the large negative Z_E reduces k_{-1} 30-fold to a value of $k_{-1} \leq k_2$ *independent of ionic strength*. According to eq 9 and 10, this reduction in k_{-1} results in a significant increase in k_{12} and a somewhat smaller decrease in k_{21}. The lack of ionic strength effects on k_{-1} is supported experimentally by the small ionic strength dependence of k_{21} (Table I). The large negative Z_E also contributes directly to an increase in k_1 and hence to k_{12}, but this effect is only 4.9-fold at zero ionic strength and, being ionic strength dependent, decreases to rather small values at physiological ionic strengths.

The comparatively large values of k_{12} observed with *N*-methylacridinium led to our previous suggestion (Rosenberry & Neumann, 1977) that an enzyme surface area larger than the ligand binding site itself was effective in "trapping" a cationic ligand in the encounter complex E·L (Scheme III). This larger surface area might include peripheral anionic sites from which ligand would move to the active site by surface diffusion. The high negative Z_E (as well as the comparatively large $\bar{a}$) values in Table III support this concept, because six to nine negatively charged enzyme groups would be expected to be dispersed over an enzyme surface area greater than the immediate catalytic site. Furthermore, the differences in Z_E and $\bar{a}$ among the cationic ligands in Table III suggest that ligands may differ in their relative rates of surface diffusion and thus in the area of surface over which trapping is effective. Further studies are required to extend and clarify differences in apparent electrostatic parameters among ligands.

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[2] It is difficult to analyze the coupled system $H^+ + EL \rightleftharpoons E + L^+ + H^+ \rightleftharpoons EH + L^+$ involving *N*-methylacridinium, because multiple relaxations are observed at low pH (Rosenberry & Neumann, 1977; H.-J. Nolte and T. L. Rosenberry, unpublished results) that may preclude correction of k_{12} by a simple factor of $1 + C_H/K_a$. The effects of this K_a correction on the acetylthiocholine data were assessed by analyzing the ionic strength dependence of k_{cat}/K_{app} prior to correction of C_H/K_a in eq 5. The K_a correction had little effect on Z_E, decreased $\bar{a}$ by ~30%, and increased $(k_{cat}/K_{app})^0$ by 60%. Thus, this correction would not reconcile the differences between Z_E and $\bar{a}$ for *N*-methylacridinium and acetylthiocholine in Table III.

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Appendix

The theoretical expression for k_1 in Scheme III (Eigen, 1954, 1974; Eigen et al., 1964; Debye, 1942) may be written as

$$k_1 = \frac{\Omega N}{1000}(D_E + D_L)d_{E\cdot L}\phi_1 \qquad (1A)$$

In eq 1A, $\Omega = 2\pi$ is the physically plausible value for the solid angle of diffusional approach, N is the Loschmidt–Avogadro number 6.02×10^{23} mol^{-1}, $D_E + D_L \simeq D_L \simeq 10^{-5}$ cm^2 s^{-1} is the estimate for the sum of the diffusion constants of the enzyme and the ligand, $d_{E\cdot L}$ is the mean distance between the centers of the ligand and the active site in the encounter complex; the electrostatic potential factor $\phi_1 = \phi_1{}^0 f_E f_L / f^*$ (cf. eq 6), and at zero ionic strength $\phi_1 = \phi_1{}^0 \equiv \varphi[\exp(\varphi) - 1]^{-1}$, where $\varphi \equiv Z_E Z_L e_0{}^2/(4\pi\epsilon_0\epsilon kT d_{E\cdot L})$. The term φ, the ratio between the electrostatic and thermal energy, includes the elementary charge e_0 (1.60×10^{-19} C), the permittivity constant ϵ_0 (8.85×10^{-12} C^2 J^{-1} m^{-1}), the dielectric constant of water ϵ (79), and the thermal energy kT (4.12×10^{-21} J at 25 °C). Substitution of $Z_E = -6.3$ and $d_{E\cdot L} = \bar{a} = 0.91$ nm from Table III gives $\varphi = -4.9$, $\phi_1{}^0 = 4.9$, and $k_1{}^0 = 1.7 \times 10^{10}$ M^{-1} s^{-1}.

Derived from a similar approach, the theoretical expression for k_{-1} (Eigen, 1954) is

$$k_{-1} = \frac{1.5}{d_{E\cdot L}{}^2}(D_E + D_L)\phi_{-1} \qquad (2A)$$

In eq 2A the electrostatic potential factor $\phi_{-1} \equiv \phi_{-1}{}^0 f_{E\cdot L}/f^*$; but since both the intermediate encounter complex and the activated complex are ion pairs, $f_{E\cdot L} \simeq f^*$ and $\phi_{-1} \simeq \phi_{-1}{}^0$ at all ionic strengths [but see Eigen et al. (1964)], where $\phi_{-1}{}^0 \equiv \varphi[1 - \exp(-\varphi)]^{-1}$. With $\varphi = -4.9$, $\phi_{-1}{}^0 = 0.037$ and $k_{-1}{}^0 = 7 \times 10^7$ s^{-1}.

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