Simulation of Charge-Mutant Acetylcholinesterases†

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ABSTRACT: A recent experimental study of human acetylcholinesterase has shown that the mutation of surface acidic residues has little effect on the rate constant for hydrolysis of acetylthiocholine. It was concluded, on this basis, that the reaction is not diffusion controlled and that electrostatic steering plays only a minor role in determining the rate. Here we examine this issue through Brownian dynamics simulations on *Torpedo californica* acetylcholinesterase in which the surface acidic residues homologous with those mutated in the human enzyme are artifically neutralized. The computed effects of the mutations on the rate constants reproduce quite well the modest effects of the mutations upon the measured encounter rates. Nonetheless, the electrostatic field of the enzyme is found to increase the rate constants by about an order of magnitude in both the wild type and the mutants. We therefore conclude that the mutation experiments do not disprove that electrostatic steering substantially affects the catalytic rate of acetylcholinesterase.

AChE[1] is a critical enzyme in many organisms, including humans. It primarily serves to clear neuronal and neuromuscular synapses of the cationic neurotransmitter acetylcholine, through hydrolysis of its ester bond. AChE is a very efficient enzyme believed to operate at, or near, the diffusion control limit (Quinn, 1987; Bazelyansky et al., 1986). Furthermore, visual inspection of computed electrostatic fields around the newly solved structure of TcAChE led to the suggestion that the electrostatic field generated by negatively charged amino acid side chains accelerates the rate of encounter of cationic substrates with the active site (Ripoll et al., 1993). More recently, BD simulations of the diffusion of substrate in the vicinity of AChE have supported this notion (Tan et al., 1993; Antosiewicz et al., 1994a, 1995), which also fits with the experimental observation that the apparent bimolecular rate constants for the hydrolysis of cationic substrates by ACh drop with increasing ionic strength.

Shafferman and co-workers have recently described an elegant study in which negatively charged amino acids on the surface of human AChE were substituted by neutral amino acids, and rate constants for the hydrolyses of a cationic and a neutral substrate were measured at two ionic strengths (Shafferman et al., 1994). The mutations were designed to diminish the negative electrostatic potential thought to guide cationic substrates into the active site, without perturbing the active site itself. Up to seven amino acids were changed, for a net change of +7e in the charge of the enzyme. The mutated amino acids were all located in the general vicinity of the active site entry, where they might be expected to have a maximal effect. Nonetheless, the mutant enzymes displayed modest, if any, reductions in the apparent bimolecular rate constant (k_{app}) for hydrolysis of a cationic substrate (Shafferman et al., 1994). These results led to the suggestion that the electrostatic field of AChE does not, in fact, guide cationic substrates into the active site.

In interpreting these results, it may be instructive to refer to the case of SOD, whose k_{app} is believed to be increased substantially by the electrostatic guidance of its anionic substrate, the superoxide radical. Recent experiments of Getzoff and co-workers (Getzoff et al., 1992) demonstrate that mutations that significantly alter the net charge of the enzyme can have little effect upon k_{app}, and BD simulations of SOD have given the same basic result (Allison et al., 1985). Therefore, that the charge mutations in human AChE have only small effects upon k_{app} may not be inconsistent with the existence of an electrostatic steering effect of the enzyme's field upon a cationic substrate.

This issue is amenable to further exploration by the BD method, which we have used in simulating wild-type TcAChE. In particular, we are interested in whether the

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[1] Abbreviations: AChE, acetylcholinesterase; ACh, acetylcholine; TcAChE, *Torpedo californica* acetylcholinesterase; BD, Brownian dynamics; SOD, Cu,Zn-superoxide dismutase; ATC, acetylthiocholine; TB, 3,3-dimethylbutyl thioacetate.

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results of our previous BD simulations, which suggest that electrostatic steering increases the hydrolysis rate by increasing the rate of encounter of cationic substrates with the active site, can be reconciled with the experimental study of Shafferman et al., which suggests the opposite. Here we describe simulations of the encounter of substrate with TcAChE, with acidic residues homologous to those mutated in the human enzyme modeled as electrically neutral. The use of simulations of TcAChE to interpret experiments on human AChE is justified by the high degree of sequence homology between the two enzymes (Cygler et al., 1993). We find, in agreement with the experimental data, that the mutations have only modest effects upon the encounter rates. Nonetheless, a change in the charge of the ligand from +1 to 0 substantially reduces the computed encounter rates. This demonstrates that the modest effects of the mutations upon rate are not inconsistent with significant rate effects due to the electrostatic steering of cationic substrates. Further implications are discussed in the context of the enzymatic reaction mechanism.

MATERIALS AND METHODS

The methodology used here is as described previously (Antosiewicz et al., 1994a, 1995). Briefly, the bimolecular rate constant, k, for the first diffusional encounter between an enzyme and its ligand can be represented as

$$k = k(b)\beta$$

where $k(b)$ is the rate constant for the encounter of the ligand with a spherical surface of radius b centered on the enzyme, and β is, in effect, the probability that the ligand, having encountered this "b-surface", will proceed to encounter the active site, rather than wandering away to infinity (Madura et al., 1994).

The electrostatic interaction between a charged ligand, such as acetylcholine, and the charged atoms of the enzyme results in a potential of mean force, U, which affects the diffusion of the ligand in the vicinity of the enzyme. When the ligand is far from the enzyme, U becomes centrosymmetric to a good approximation. The radius b is chosen so that U is highly centrosymmetric at the b-surface. This permits $k(b)$ to be obtained analytically as a solution of the one-dimensional diffusion equation, yielding

$$\frac{1}{k(b)} = \int_b^\infty \frac{e^{U(r)/k_B T}}{4\pi D r^2}\, dr$$

where k_B is Boltzmann's constant, T is absolute temperature, and D is the relative diffusion coefficient for the two species.

The probability β is estimated by initiating many diffusional BD trajectories of the ligand at random points on the b-surface and continuing them until they either satisfy some reaction criterion (see the following) by reaching the active site or wander away to a "quit-surface" (q-surface) beyond the b-surface. A correction term accounts for the possibility that a trajectory that terminates at the q-surface may wander back to the b-surface again. Each BD step is biased by the local electrostatic field generated by the enzyme. The fraction of trajectories that end with encounter is β (McCammon et al., 1986; Davis et al., 1991).

The calculations are based on the TcAChE crystal structure [Protein Data Bank (Bernstein et al., 1977) accession code 1ACE] solved by Sussman and co-workers (Sussman et al., 1991), to which unresolved atoms are added as described previously (Antosiewicz et al., 1994a, 1995). Calculations of the ionization state of the enzyme, and the electrostatic potential field it generates, are based upon finite difference solutions of the linearized Poisson–Boltzmann equation (McQuarrie, 1973), as previously described (Antosiewicz et al., 1994a,b, 1995). In all calculations, the ionic strength is set to 150 mM and the temperature to 300 K to match the experimental conditions. The pH is set to 7.0 in these calculations. This differs somewhat from the experimental pH of 8.0; however, none of the mutant amino acids are expected to titrate between pH 7.0 and 8.0. The choice of pH is somewhat arbitrary in any case, because the isoelectric point of human AChE is likely to differ from that of TcAChE. A more detailed comparison of simulation results with the data of Shafferman et al. will be possible when the structure of human AChE becomes available. "Mutations" are created computationally by artificially adjusting the pK_a's of the appropriate acidic side chains to a high value, which forces them to remain neutral. The hydrodynamic models of the enzyme and the single-bead substrate are as described previously (Antosiewicz et al., 1994a). Steric and electrostatic interactions between substrate and enzyme are modeled by treating the substrate as a sphere, which may possess a central unit charge and which cannot enter the van der Waals envelope of the enzyme (see the following paragraph). The program UHBD (Davis et al., 1991) is used for all electrostatic and BD calculations. The multiple-site titration problem is treated by either the cluster method of Gilson (1993) or the Monte Carlo method of Antosiewicz and Porschke (1989).

As noted earlier, an enzyme–substrate encounter is said to occur when the diffusing substrate satisfies a reaction criterion. This typically means arriving within a specified distance of a point in the active site gorge. The precise definition of the reaction criterion is somewhat arbitrary; ultimately, BD simulations must be interfaced with more detailed simulations of the dynamics of the substrate within the active site, using methods such as that set out by Luty and co-workers (Luty et al., 1993). The case of AChE is particularly difficult to model with the BD method because, as previously noted (Sussman et al., 1991; Gilson et al., 1994), its active site gorge is somewhat too narrow to admit the substrate. Presumably, conformational fluctuations of the gorge allow the substrate to reach the catalytic residues at the base. We have argued that computation of accurate rate constants will require that these conformational fluctuations be accounted for (Antosiewicz et al., 1995). In this study, we attempt to account for them rather crudely by artifically reducing the steric radius of the substrate. This approach has been used successfully in BD simulations of SOD (Allison et al., 1988). We have previously assigned the substrate a radius of 3 Å. This is roughly the radius of the choline moiety of the substrate. Here we consider radii of 3.0, 2.5, and 2.0 Å. Because the smaller substrate radii permit deeper penetration of the active site gorge than was possible with the 3.0 Å radius, we are able to use deeper reaction criteria, which require the substrate to travel some distance into the gorge.

The reaction criteria considered here are diagrammed in Figure 1. As before (Antosiewicz et al., 1994a), we define the center of the active site entry to be the geometric center

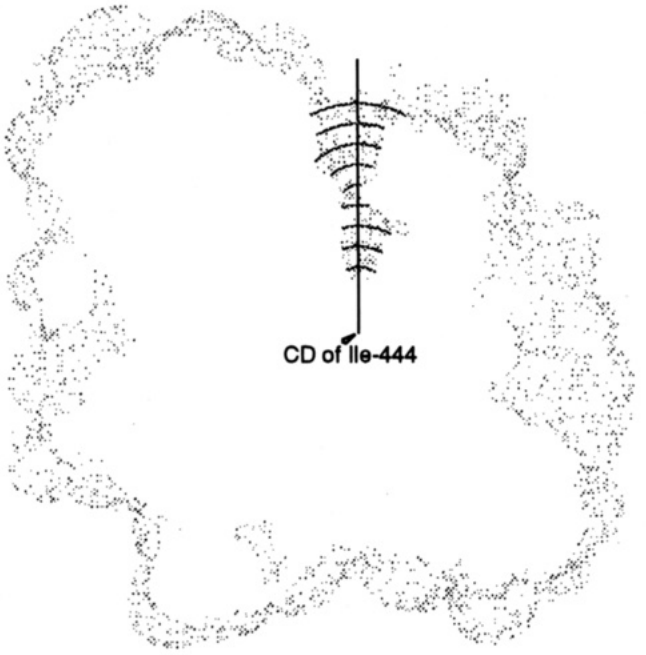

FIGURE 1: Cross section of a dot representation of the surface of TcAChE accessible to the center of a 2.0 Å spherical probe, showing the active site gorge, the atom defining the base of the gorge (Ile-444 CD), the gorge axis, and arcs corresponding to the various reaction criteria.

Table 1: Acidic Residues in TcAChE Homologous to Those Mutated in Human AChE and Their Distances from Points Defining the Entry and Base of the Active Site Gorge (See Text)

residue in HuAChE	residue in TcAChE	distance from gorge entry (Å)	distance from gorge base (Å)
Glu-84	Glu-82	17	17
Glu-292	Asp-285	9.4	28
Glu-285	Glu-278	12	20
Asp-349	Asp-342	18	23
Glu-358	Asp-351	16	29
Glu-389	Asp-380	31	34
Asp-390	Asp-381	29	36

of the following atoms: CA of Glu-73, CB of Asn-280, CG of Asp-285, and O of Leu-333. The base of the active site gorge is taken to be CD of Ile-444, and the axis of the gorge connects the base and the entry. We define two reaction centers, both on the gorge axis: the first lies 9.0 Å and the second 18 Å deeper than the point defining the active site entry. We consider five different reaction criteria for the first reaction center; these are satisfied when the substrate arrives within 10, 8, 6, 4, and 2 Å of this reaction center. The first two of these are similar to the 3 and 1 Å reaction criteria we have used previously (Antosiewicz et al., 1994a, 1995). We consider four reaction criteria for the second reaction center; these are satisfied by arrival within 9, 7, 5, and 3 Å of the second reaction center. The two sets of reaction criteria cover distances from ~7 to ~23 Å from the base of the active site (see Figure 1 and Table 3).

In modeling the mutants, the similarity between human and *Torpedo* AChE permits identification of the surface acidic residues in TcAChE homologous to those mutated in the human enzyme (Cygler et al., 1993). The correspondences are listed in Table 1. The experimental study generated 20 single and multiple mutants. We present the results of electrostatic and titration calculations on all of these and the results of BD calculations on the wild type, two single mutants, two quadruple mutants, and the heptamutant. The pairs of single and quadruple mutants were selected to include one whose k_{app} equals that of wild type and one whose k_{app} deviates maximally from the wild type. The single and quadruple mutants are (human numbering (Shafferman et al., 1994)) E84Q, E292A, E84Q/E292A/D349N/E358Q, and D349N/E358Q/E389Q/D390N; for simplicity, we designate these mutants 1a, 1b, 4a, and 4b, respectively.

Figure 2 presents two views of the active site of TcAChE, showing the acidic residues that correspond to those mutated in human AChE. Table 1 gives the distances from the carboxyl carbon of each mutated group to the gorge entry and the base of the gorge, as defined earlier. Because the catalytic residues lie at the base of the gorge, and no mutated residue lies closer than 17 Å from the base, it seems unlikely that any rate changes result from direct effects upon the integrity of the catalytic site.

RESULTS

Electrostatic Properties of TcAChE and Its Mutants. Ripoll and co-workers pointed out that TcAChE possesses a large dipole moment, oriented along the active site gorge. They also used finite difference PB calculations to show that the entry to the active site of the enzyme is surrounded by a large region of negative electrostatic potential (Ripoll et al., 1993). On the basis of these observations, they proposed that the electrostatic field of TcAChE helps to guide cationic substrates into the active site of the enzyme. Shafferman and co-workers designed their mutants to weaken the dipole moment of the enzyme and used the PB method to show that the mutations significantly shrink the region of negative electrostatic potential surrounding the mouth of the active site (Shafferman et al., 1994). Here we examine the electrostatic properties of the wild-type and mutant enzymes in further detail.

The electrostatic properties described here are computed as outlined in the Materials and Methods section. Note that the charges and dipole moments are actually computed as averages over ensembles of ionization states at pH 7.0 (Antosiewicz et al., 1994b). The dipole moments are origin dependent because the enzyme has non-zero net charge. However, as long as a consistent origin is used, the dipole moments can be used to characterize the charge distributions in the various mutants. We used the center of mass of the enzyme as the origin of coordinates in computing all dipole moments.

Table 2 presents the computed charge of each mutant enzyme at pH 7.0. For two single, two double, two triple, two quadruple, two quintuple, and the hexa- and heptamutants, it also presents the magnitude of the dipole moment and the angle of the dipole moment with respect to the axis of the active site gorge (see above). The results are plotted in Figures 3 and 4. The net charges and the magnitudes of the dipole moments vary quite linearly with the number of mutations. Interestingly, although the net charge of the enzyme rises from about −7 to about 0 as all seven mutations are progressively added, the magnitude of the dipole moment does not go to zero. Instead, it falls only about 40%, from 1700 to 1100 D. Furthermore, its orientation with respect to the gorge axis does not change significantly (see Table 2). Thus, one of the key features identified by Ripoll and co-workers (Ripoll et al., 1993), the dipole moment oriented

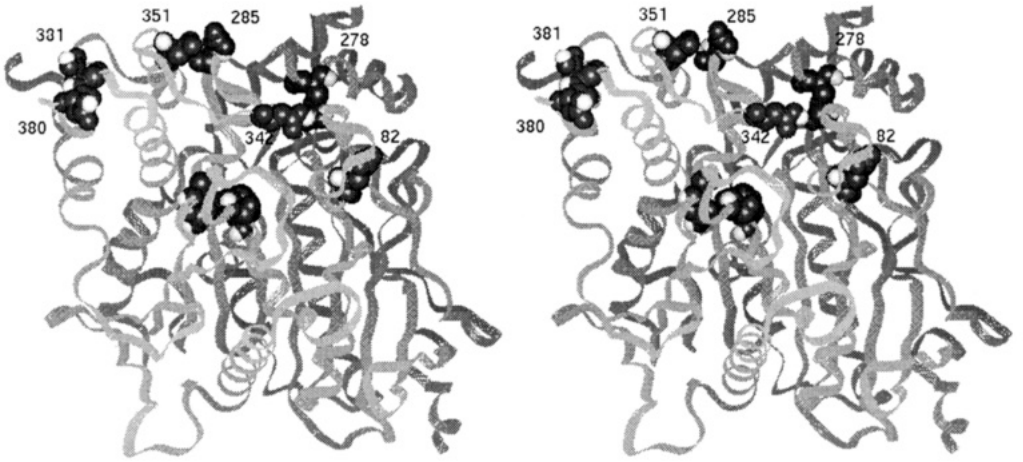

FIGURE 2: Stereoview of TcAChE and the negatively charged residues apparently homologous to those mutated in human AChE by Shafferman and co-workers (Shafferman et al., 1994). The ribbon represents the peptide backbone. The catalytic residues, Ser-200 and Glu-327, and His-440 are shown but not labeled.

Table 2: Electrostatic Properties of Wild-Type and Hypothetical Charge Mutants of TcAChE[a]

neutralized residues (ID)	q (e)	μ (D)	α (deg)	k_{app} (10^7 M^{-1} s^{-1})
none (wt)	−6.51	1697	31	4.8
E82 (1a)	−5.68	1627	30	2.7
D285	−5.60			4.3
E278	−5.73			2.2
D342	−5.59			4.3
D351	−5.60			4.2
D380	−5.57			4.8
D381 (1b)	−5.56	1597	32	4.8
E82/D285	−4.74	1529	31	2.0
D380/D381	−4.61	1519	32	4.7
D342/D351	−4.67			5.0
E82/D342/D351	−3.82	1397	28	2.0
D285/D380/D381	−3.70	1427	34	5.8
D285/D342/D351	−3.75			4.2
E82/D285/D342/D351 (4a)	−2.89	1294	29	2.1
D342/D351/D380/D381 (4b)	−2.76	1307	32	5.5
E82/E278/D285/D342/D351	−2.07	1244	31	1.8
D285/D342/D351/D380/D381	−1.84	1219	35	4.5
E82/D342/D351/D380/D381	−1.91			2.2
E82/D285/D342/D351/D380/D381	−0.98	1140	32	2.1
E82/E278/D285/D342/D351/D380/D381 (7)	−0.16	1095	33	2.1

[a] Residues listed are homologous to those mutated in human AChE by Shafferman et al. (1994). The six forms for which BD calculations were done are marked with the identifying symbols used in the text (ID). q, average net charge. μ, magnitude of average dipole moment. α, angle of dipole moment of relative to gorge axis. k_{app}, measured apparent bimolecular rate constant for the corresponding human form of AChE, at 150 mM ionic strength (Shafferman et al., 1994).

along the gorge axis, is largely preserved, even in the heptamutant. On the other hand, there is no clear correlation between the computed dipole moments of the various enzyme forms and the measured apparent bimolecular rate constants for the mutants of human AChE, which are also listed in Table 2.

In previous work, we have noted that the electrostatic steering of a cationic substrate becomes substantial only rather close to the active site gorge (Antosiewicz et al., 1994a, 1995). This conclusion was based upon the observation that the encounter rate constants for cationic substrates exceed those for neutral substrates by large factors only when the reaction criteria are at least a short distance inside the active site gorge. This implies that the degree to which the field increases the encounter rate is likely to correlate best with the fields in the immediate vicinity of the gorge. Similar results have been found in calculations on SOD (Allison et al., 1988). We therefore examine the electrostatic potentials along the gorge axis for the wild type and mutants 1a, 1b,

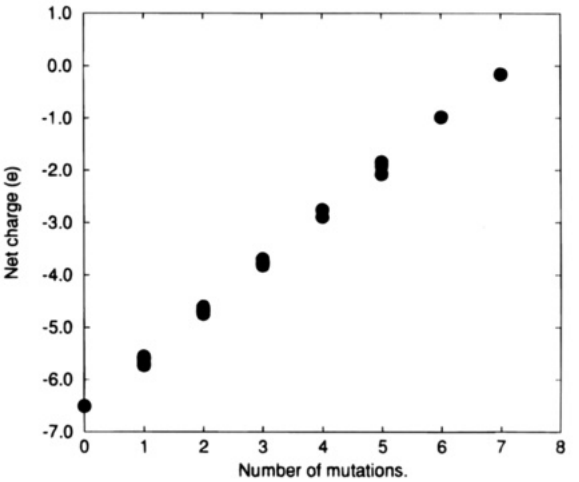

FIGURE 3: Mean total charge (e) of monomer TcAChE, at pH 7, 300 K, and 150 mM ionic strength, as a function of the number of mutations of acidic residues to neutral ones. See Figure 1 and Table 1 for the identity of mutated residues.

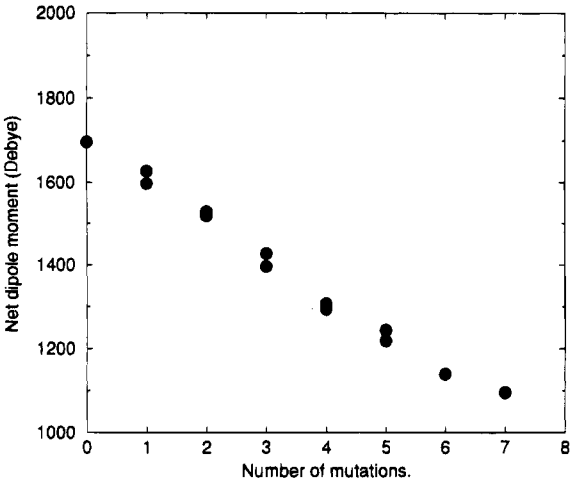

FIGURE 4: Mean total dipole moment (D) of monomer TcAChE at pH 7, 300 K, and 150 mM ionic strength, as a function of the number of mutations. See text for details.

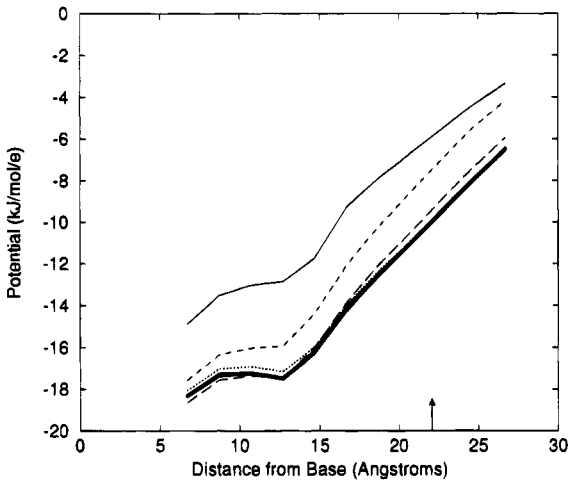

FIGURE 5: Potentials (kJ/mol/e) along the gorge axis at points intersecting the reaction surfaces. Distances are from Ile-444 CD: heavy solid line, wild type; dots, mutants 1a and 1b; long dashes, mutant 4b; short dashes, mutant 4a; thin solid line, mutant 7. The arrow indicates the position of gorge entry, as defined in the text.

4a, 4b, and 7. Figure 5, plots these computed potentials as a function of distance from the base of the active site gorge (CD of Ile-444). The largest distance of about 27 Å from the base corresponds to a position 5 Å beyond the point defining the gorge entry (Figure 1). Not surprisingly, the potentials in the gorge become progressively less negative as more acidic residues are neutralized. However, even with all seven mutations, the potentials along the axis retain a strongly negative potential. Perhaps more importantly, the sharp inward gradient for the wild type is minimally reduced in the mutants. This is significant, because it is the gradient that biases the motion of the substrate. That the potentials are weakened more than the field presumably results from the fact that the field generated by a charge falls off more sharply with distance than does the potential.

It is interesting to examine not only the general trends but also the potentials generated by the specific mutants. The two single mutants, 1a and 1b, yield potentials very similar to those of the wild type. This means that the potentials will not explain the experimental observation that the human mutant 1a has a lower rate constant than the wild type and mutant 1b. However, the potentials for mutants 4a and 7

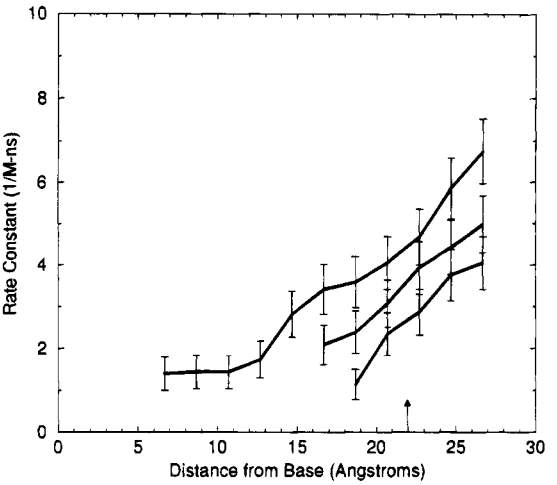

FIGURE 6: Computed rate constants for diffusional encounter (M^{-1} ns^{-1}) for substrate exclusion radii of 3.0 (lowest curve), 2.5 (middle curve), and 2.0 Å (highest curve), as a function of the distance of the reaction criterion from the base of the active site gorge, Ile-444 CD. The arrow indicates the position of gorge entry, as defined in the text.

are weaker than those for 4b, consistent with the lower measured rates for the corresponding human mutants.

Absolute Rate Constants. As described in Materials and Methods, we attempt to account for enzyme flexibility in a crude fashion by reducing the steric radius of the substrate. We consider radii of 2.0, 2.5, and 3.0 Å. Because the smaller radii permit the substrate to move fairly close to the bottom of the active site, we also examine reaction criteria located as little as 6.7 Å from the base. Results for the wild type are summarized in Figure 6, which, for each substrate radius, plots computed encounter rates for all of the reaction criteria diagrammed in Figure 1. These calculations use 1000 trajectories for each substrate radius. The deeper reaction criteria yield lower rates because they are harder to reach. The smaller substrate radii yield higher rates because they make it easier for the substrate to enter the active site. (We use the same diffusion constant for all three steric radii). In fact, while the 2.0 Å substrate reaches a point about 7 Å from the base of the gorge, the 2.5 Å substrate never satisfies any reaction criterion closer than about 17 Å to the base, and the 3.0 Å substrate never comes closer than about 19 Å.

The plot of reaction rate constant versus depth for the 2.0 Å substrate radius shows an interesting feature. With increasing depth, the rate constant declines gradually, shows a sudden drop at about 14 Å, and then remains constant. It would appear that once the substrate passes a "choke point" at about 14 Å, it is unlikely to escape and goes on to satisfy all the deeper reaction criteria.

As an initial assessment of the quality of the present simulations, we wish to compare the computed encounter rate constants for the wild-type enzyme with measured rate constants. For an enzyme that is truly diffusion limited, the appropriate experimental rate constant for comparison is $k_{app} = k_{cat}/K_m$, the apparent bimolecular rate constant. The experimental value of k_{app} for the hydrolysis of acetylthiocholine by wild-type human AChE is 4.8×10^7 M^{-1} s^{-1} at 150 mM ionic strength and pH 8.0 (Shafferman et al., 1994). For the *Torpedo* enzyme under similar conditions, Berman and co-workers (Berman et al., 1991) and Radić and co-workers (Radić et al., 1992) have reported values of 5.3 ×

Table 3: Computed Encounter Rates (M^{-1} ns^{-1}) of Cationic Substrate (ATC) for Wild-Type (wt) TcAChE and Mutants of TcAChE Corresponding to Mutants of Human AChE, as a Function of Assumed Distance of Reaction Criterion from Base of Active Site Gorge[a]

reaction distance from base (Å)	mutant reacting with ATC							
	wt	1a	1b	4a	4b	7	wt TB	wt ATC/TB
6.7	1.34/0.23	1.04/0.20	1.03/0.20	0.50/0.14	1.02/0.20	0.37/0.12	nr	
8.7	1.36/0.23	1.05/0.20	1.50/0.20	0.50/0.14	1.02/0.20	0.37/0.12	nr	
10.7	1.37/0.23	1.08/0.20	1.11/0.21	0.50/0.14	1.05/0.20	0.37/0.12	nr	
12.7	1.59/0.24	1.23/0.22	1.27/0.22	0.56/0.15	1.20/0.21	0.38/0.12	0.02/0.02	83.1
14.7	2.55/0.30	1.98/0.27	2.25/0.29	1.13/0.21	1.92/0.27	0.72/0.17	0.06/0.04	44.5
16.7	3.14/0.33	2.64/0.31	2.72/0.31	1.60/0.24	2.69/0.31	1.01/0.20	0.11/0.05	29.8
18.7	3.35/0.34	2.97/0.32	2.98/0.32	1.81/0.26	2.91/0.32	1.31/0.22	0.16/0.06	20.6
20.7	3.80/0.36	3.32/0.34	3.26/0.34	2.11/0.28	3.26/0.34	1.56/0.24	0.30/0.09	12.8
22.7	4.29/0.38	3.70/0.36	3.79/0.36	2.79/0.32	4.02/0.37	2.32/0.29	0.64/0.13	6.7
expt	0.048/0.007	0.027/0.044	0.048/0.007	0.021/0.003	0.055/0.008	0.021/0.003	0.003/0.0005	16

[a] Also included are computed encounter rates for a neutral substrate (TB) with the active site, the ratio of the ATC to TB encounter rates, and experimental values of k_{app} (Shafferman et al., 1994) for the wild-type and mutant enzymes and for reaction of TB with wild-type enzyme. The values after the slashes are 90% confidence limits of BD simulations for the computed values and published error estimates for experimental values.

10^7 and 5.8×10^7 M^{-1} s^{-1}, respectively. More recently, Quinn and co-workers have reported a k_{app} value of 5.6×10^8 M^{-1} s^{-1} for the acylation of ATC by TcAChE at 120 mM ionic strength and pH 7.3 (Quinn et al., 1995). We do not know why the experimental values of k_{app} vary so widely. The computed encounter rate constants (Figure 6) are tabulated in the first column of Table 3. [The modest differences from previous calculations on this system (Antosiewicz et al., 1994a,b) result primarily from the use of somewhat different reaction criteria.] The computed rate constants range from 1.3×10^9 to 4.3×10^9 M^{-1} s^{-1}, depending upon the choice of the reaction criterion. Although these exceed the measured values of k_{app}, the discrepancy of the lower computed values from the recent data of Quinn and co-workers (Quinn et al., 1995) is not great.

It is important to note that, if AChE is not entirely diffusion limited, the computed encounter rate constants are expected to exceed measured values of k_{app}. The recent experimental study of Quinn and co-workers suggests that the degree to which TcAChE is diffusion limited depends upon ionic strength. The study also provides estimates of the actual rate constants for enzyme–substrate encounter. These should be comparable with our computed encounter rate constants, and in fact, the reported values of 2×10^9 and 1.6×10^8 s^{-1} for 0 and ~200 mM ionic strength, respectively, bracket the values we calculate at 150 mM ionic strength for our deeper reaction criteria.

Effect of Substrate Charge upon Rate Constants. The results discussed so far apply to a cationic substrate. Shafferman and co-workers have also measured apparent bimolecular rate constants for the hydrolysis of a neutral isostere of ATC, 3,3-dimethylbutyl thioacetate (TB) (Shafferman et al., 1994). For the wild-type enzyme, this rate constant is 16-fold lower than that for the cationic substrate ATC at 150 mM ionic strength. We simulate the effect of replacing cationic ATC with neutral TB by replacing our cationic substrate model with a neutral one that is otherwise identical. We assign a steric radius of 2.0 Å to the substrate (see the preceding section). BD simulations (4000 trajectories) with this neutral isostere yield rates of encounter with the active site that are considerably lower than those for the 2.0 Å cationic substrate at 150 mM ionic strength, as shown in the last column of Table 3. Although the 90% error intervals are rather broad for the deeper reaction criteria, the

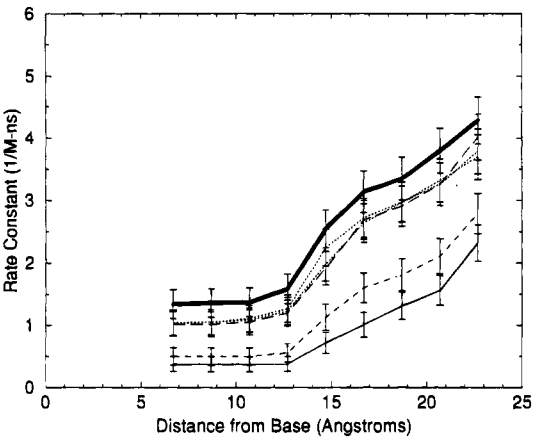

FIGURE 7: Computed rate constants for diffusional encounter of 2.0 Å substrate (M^{-1} ns^{-1}) for wild-type TcAChE and mutants, as a function of the distance of the reaction criterion from the base of the active site gorge: heavy solid line, wild type; dots, mutants 1a and 1b; long dashes, mutant 4b; short dashes, mutant 4a; thin solid line, mutant 7.

simulations clearly demonstrate a substantial electrostatic steering effect. As observed in previous simulations (Antosiewicz et al., 1994a, 1995), the ratio of rates for the charged to the neutral substrate—a measure of the influence of electrostatics—increases with increasing stringency of the reaction criterion. The experimental ratio of 16 (Shafferman et al., 1994) corresponds to a reaction criterion located about 19 Å from the base of the active site. However, it is important to note that the differences between the kinetics of ATC and TB probably do not only result from differences in their diffusive encounter, as discussed in some detail in the following. Therefore, a direct comparison between the computed and experimental ratios of k_{app} for these two substrates probably is not meaningful.

Effects of Mutations upon Rate Constants. Here we describe the results of simulations of charge mutants of TcAChE. The mutated residues correspond to those neutralized experimentally in the human enzyme (Shafferman et al., 1994). We use the 2.0 Å steric substrate radius in these calculations. Because of uncertainty about the best model, we examine the dependence of the results upon the choice of reaction criterion. Table 3 and Figure 7 present computed rate constants for the encounter of a cationic substrate with wild-type TcAChE and with the hypothetical mutants 1a,

1b, 4a, 4b, and 7. The calculations use 3000 BD trajectories for each mutant.

The simulations of mutants 1a, 1b, and 4b yield encounter rate constants slightly below those of the wild type. The simulation of mutant 4a yields rate constants significantly below those of the wild type, and the simulation of mutant 7 yields rate constants somewhat below those of mutant 4a. That the mutations in general produce modest effects upon the rate constants agrees with the measured results for k_{app} in the human enzyme. That mutants 4a and 7 show larger reductions in rate constant than mutants 1b and 4b also agrees with experiment. On the other hand, the experiments show a larger rate drop for mutant 1a than that found in the simulations. Also, the computed rate constants for mutants 1b, 4b, and 7 fall, relative to the wild type, somewhat more than do the experimental rate constants, at least for the deeper reaction criteria. Given the approximate nature of the simulation model, and the fact that the simulations are based upon *Torpedo* rather than human AChE, the agreement between simulated and measured values is rather good.

DISCUSSION

We now discuss the conclusions that may be drawn from this work. One is that we remain uncertain about the optimal choice of reaction criterion and steric radius for the substrate. As we have suggested previously, a more detailed treatment of events in the active site is probably needed if full agreement with experimental rate constants is to be obtained (Antosiewicz et al., 1994a). An additional source of uncertainty in the present study is that, of necessity, we compare calculated results for the *Torpedo* enzyme—the only AChE whose three-dimensional structure has been solved—with experimental results for the human enzyme. This comparison is justified by the high degree of sequence homology between the two enzymes (Cygler et al., 1993), but it may also be the source of some discrepancies, such as the inability of the calculations to reproduce the drop in k_{app} for mutant 1a. Such issues presumably will be resolved once the structure of the human enzyme is solved. In any case, we find that the simulations of TcAChE yield qualitative results that are quite useful in interpreting the experimental data, as we now discuss in detail.

Mutants. Shafferman and co-workers reason that if the reaction of human AChE with ATC were diffusion limited, and if the encounter rate constant were enhanced by electrostatic guidance, then the charge mutants would operate at substantially slower rates than the wild-type enzyme (Shafferman et al., 1994). That the mutations in fact cause little change in the rate constants is interpreted as implying that the reaction is not diffusion limited and that electrostatic guidance is unimportant. The present study shows that the small effect of the mutations is actually consistent with diffusion limitation and a substantial electrostatic steering effect: our computations assume diffusion limitation, predict electrostatic steering, and yet yield rate constants that are not affected much by the mutations. This result is robust with respect to the choice of reaction criterion. It implies that most of the steering results from charges other than those that have been mutated to neutrality. Note, however, that the present calculations do not prove that diffusion is rate limiting and that electrostatic steering operates. They only show that the experimental data are consistent with this hypothesis.

If the mutations really do reduce the rate constants by diminishing electrostatic steering, then they should not reduce the rate constants for a neutral substrate for which steering cannot occur. This prediction is borne out by the experimental data, which show that the charge mutations have no detectable effect upon the rate constants of TB (Shafferman et al., 1994). In addition, the mutations should influence the apparent bimolecular rate constants ($k_{app} = k_{cat}/K_m$) for ATC through K_m only; if they have their effect through steering, they should not influence k_{cat}, which depends upon chemical steps. This, too, fits with the experimental data, which show that k_{cat} is completely unperturbed by the mutations.

Shafferman et al. also note that the small effects of the mutations upon the measured rate constants militate against the suggestion that the asymmetric charge distribution of AChE leads to faster catalysis through electrostatic guidance (Ripoll et al., 1993; Tan et al., 1993). The present calculations support the same conclusion, for although we assume a perfectly absorbing boundary in the enzyme active site, and thus perfect diffusion limitation, we find that reducing the asymmetry by eliminating up to seven negatively charged groups near the active site does not have a dramatic effect upon the encounter rate constants. The reason appears to be that the electrostatic field in the immediate vicinity of the active site is not drastically weakened by the mutations. On the other hand, we find that the mutations do not eliminate the overall dipole moment of the enzyme or its approximate alignment with the gorge. We estimate that even the heptamutant retains a dipole moment 60% of that of the wild type. Thus, the mutations do not actually abolish the electrical asymmetry of the enzyme, and it could be that greater reductions in the dipole moment would ultimately lower the rate constants.

Ionic Strength. Another question is whether the measured fall in k_{app} with increasing ionic strength for the reaction of ATC with AChE results from weakened electrostatic steering. This proposal seems to be supported in the case of the *Torpedo* enzyme by the work of Quinn and co-workers (Quinn et al., 1995). It is also consistent with the BD calculations of this and previous studies (Antosiewicz et al., 1994a,b). On the other hand, Shafferman and co-workers have argued that, because the ionic strength dependence of the charge mutants is quite similar to that of the wild type (Shafferman et al., 1994), it cannot result from the influence of dissolved ions upon electrostatic steering. However, the present simulations show that the mutants have little effect upon electrostatic steering to begin with. Therefore, the ionic strength dependence of their rate constants is, in fact, expected to be similar to that of the wild type, and the hypothesis of electrostatic steering to a diffusion-limited enzyme is not invalidated by these data. It should also be pointed out that k_{app} for neutral TB is independent of ionic strength, as one would predict from the hypothesis of electrostatic steering.

Comparison of Enzyme Kinetics for ATC and TB. At 150 mM ionic strength, k_{app} for TB is 16-fold lower than k_{app} for ATC. It is tempting to argue that the measured values of k_{app} for TB are lower because electrostatic steering is inoperative for this neutral substrate. However, if this were the only explanation, $k_{app} = k_{cat}/K_m$ should fall only because of an increase in K_m and not because of a decrease in k_{cat}, which involves chemical steps rather than encounter. In fact,

at 150 mM ionic strength, k_{cat}' is 8 times smaller than k_{cat}. (We will use primed symbols for TB, and unprimed for ATC.) That the kinetics of the chemical steps differs for these two compounds is not too surprising, and it implies that a comparison of the kinetics of these two substrates will not be as simple as a comparison of the kinetics of the various mutants or analysis of the influence of ionic strength.

These data also pose a problem: if electrostatic steering is important for AChE, then the encounter rate of neutral TB should be considerably lower than that for cationic ATC, and one might expect K_m' to be a good deal larger than K_m as a consequence. In fact, K_m' is only twice K_m at 150 mM ionic strength. Is this inconsistent with the hypothesis that electrostatic steering is important and that AChE is at least partly diffusion limited? It turns out that the kinetic scheme of AChE can accommodate the decrease in k_{cat} and a reduced encounter rate constant upon going from ATC to TB.

In order to discuss this, we write the following reaction scheme (Quinn, 1987; Quinn et al., 1995):

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} EA + P_1 \xrightarrow{k_5} E + P_1 + P_2 \qquad (1)$$

where E is the free enzyme, S is the substrate, ES is the enzyme−substrate complex, EA is the acylated enzyme, and P_1 and P_2 are the two products. For ATC, the products are thiocholine and acetic acid. Experimentally, reaction velocity is measured as the rate of production of thiocholine. With the steady-state approximation for ES and EA, one obtains

$$k_{cat} = \frac{k_3 k_5}{k_3 + k_5} \qquad (2)$$

$$K_m = \frac{k_5}{k_1}\frac{k_2 + k_3}{k_3 + k_5} \qquad (3)$$

We wish to know whether it is possible to satisfy the experimental results at 150 mM ionic strength,

$$K_m' = 2K_m \qquad (4)$$

$$k_{cat}' = {}^1/_8 k_{cat} \qquad (5)$$

subject to the hypotheses that the encounter rate constant for TB is less than that for ATC and that the kinetics is at least partly diffusion limited for the cationic substrate. We also require that the dissociation rate constant for TB is greater than that for ATC, as seems appropriate if the encounter rate constant is smaller. That is, we require

$$k_1' < k_1 \qquad (6)$$

$$k_3 \approx k_2 \qquad (7)$$

$$k_2' > k_2 \qquad (8)$$

If we can find sets of kinetic constants for ATC and TB that satisfy the preceding requirements, then we will have shown that the relative values of K_m and k_{cat} for ATC and TB are in fact consistent with a marked reduction in the encounter rate, k_1, of TB relative to ATC.

In fact, such sets of kinetic constants do exist. We present two different examples. Set I assumes that the encounter rate constant of TB with the active site is 10 times lower than that of ATC, and set II assumes a 20-fold drop. For both sets, we use the value of the commitment, $k_3/k_2 = 1.7$, obtained by Quinn and co-workers for TcAChE (Quinn et al., 1995), in order to stay as close as possible to existing experimental data. Purely for the sake of mathematical simplicity, we also assume $k_3 \gg k_5$. Thus, we begin with the following:

I	II
$k_1' = {}^1/_{10} k_1$	$k_1' = {}^1/_{20} k_1$
$k_3/k_2 = 1.7$	$k_3/k_2 = 1.7$
$k_3 \gg k_5$	$k_3 \gg k_5$

(9)

The various requirements and assumptions given earlier may then be satisfied by

$k_3' = k_3$	$k_3' = 4.4k_3$
$k_2' = 2.7k_2$	$k_2' = 2.0k_2$
$k_5' = {}^1/_8 k_5$	$k_5' = {}^1/_8 k_5$

(10)

We emphasize that these sets are meant only to be illustrative, as we have made seveal arbitrary assumptions in constructing them. However, they serve to show that the experimental data on the hydrolysis of ATC and TB are not inconsistent with the reduced encounter rate constant for TB compared with that for ATC.

Although they are hypothetical, the two sets of kinetic constants do deserve further discussion. Both sets postulate an 8-fold drop in k_5 upon going from ATC to TB. Because k_5 corresponds to the deacylation step, and because the acyl groups of TB and ATC are the same, our hypothesis that $k_5' < k_5$ requires justification. We suggest that the choline product of ATC hydrolysis might persist in the active site long enough to accelerate the deacylation step for this substrate. This would be consistent with the experimental observation that choline and other cations accelerate deacylation (Kitz et al., 1969; Krupka, 1966a,b). We have hypothesized that the mechanism for this acceleration involves competition between cation binding near Trp-84 and proton binding by the catalytic histidine, His-440 (numbering of TcAChE) (Wlodek et al., 1995).

Set II differs from set I in proposing that the acylation step is about 4 times faster for TB than for ATC; that is, $k_3' = 4.4k_3$. This appears to be inconsistent with the proposition that TB is intrinsically less reactive than ATC, an idea that is based upon the observation that the hydroxide-catalyzed hydrolysis of neohexyl acetate, the ester relative of the thioester TB, is slower than that of acetylcholine, the ester relative of ATC (Hasan et al., 1980). It is worth noting, however, that part of the reason neohexyl acetate is hydrolyzed more slowly by hydroxide may be that the local concentration of hydroxide is somewhat lower in the vicinity of this neutral molecule than in the vicinity of cationic acetylcholine. This would mean that the reactivity difference seen in solution might not be obtained in the active site of AChE. Still, the idea that $k_3' > k_3$ is purely hypothetical. Note that it is possible to set up alternative sets of kinetic parameters that also meet the requirements set out here. The essential point is that the observations that $k_{cat}' = {}^1/_8 k_{cat}$ and

that $K_m' = 2K_m$ do not necessarily imply that the encounter rate for TB is similar to that for ATC, i.e., that $k_1' \approx k_1$.

CONCLUSIONS

The present study shows that a model that assumes diffusion limitation and electrostatic steering can account for the experimental results on charge-mutant human AChE. Even if we assume complete diffusion limitation by equating k_{app} with our computed encounter rate constants, we find that the charge mutations have only modest effects on k_{app}, in accord with experiment. Nonetheless, we find a significant electrostatic steering effect: the encounter rate constants for a cationic substrate are substantially larger than those for a neutral substrate.

On the other hand, this work does not provide proof that human AChE is diffusion limited. In fact, even though *Torpedo* AChE is faster than human AChE, its commitment (k_3/k_2) is not extremely large (Quinn et al., 1995). It is worth noting that if the commitment of human AChE is similar to that of TcAChE, then the fact that k_{app} is lower for the human enzyme would require that the encounter rate for the human is proportionally lower. Interestingly, the recent study of Quinn and co-workers suggests that the degree to which TcAChE is diffusion limited actually increases with ionic strength. This is presumably because diminishing electrostatic steering due to increasing ionic strength reduces the rate constants for diffusional encounter.

The acetylcholinesterases continue to present intriguing experimental and theoretical challenges. Further development of methods to account for kinetics that are diffusion influenced but not necessarily diffusion limited, along with continued comparison with experiment, should in time yield a comprehensive picture of the mechanisms of this family of enzymes.

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