Acetylcholinesterase: Molecular Modeling with the Whole Toolkit

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Abstract: Molecular modeling efforts aimed at probing the structure, function and inhibition of the acetylcholinesterase enzyme have abounded in the last decade, largely because of the system's importance to medical conditions such as myasthenia gravis, Alzheimer's disease and Parkinson's disease, and well as its famous toxicological susceptibility to nerve agents. The complexity inherent in such a system with multiple complementary binding sites, critical dynamic effects and intricate mechanisms for enzymatic function and covalent inhibition, has led to an impressively diverse selection of simulation techniques being applied to the system, including quantum chemical mechanistic studies, molecular docking prediction of noncovalent complexes and their associated binding free energies, molecular dynamics conformational analysis and transport kinetics prediction, and quantitative structure activity relationship modeling to tie salient details together into a coherent predictive tool. Effective drug and prophylaxis design strategies for a complex target like this requires some understanding and appreciation for all of the above methods, thus it makes an excellent case study for multi-tiered pharmaceutical modeling. This paper reviews a sample of the more important studies on acetylcholinesterase and helps to elucidate their interdependencies. Potential future directions are introduced based on the special methodological needs of the acetylcholinesterase system and on emerging trends in molecular modeling.

Keywords: Acetylcholinesterase, Alzheimer's disease, organophosphorus nerve agents, molecular docking, molecular dynamics, quantum chemistry, QSAR, COMBINE.

I. INTRODUCTION

One of the most catalytically efficient enzymes in nature is acetylcholinesterase (AChE) [1], a serine hydrolase found in the neuromuscular junctions of all known animals. AChE is responsible for deacylating excess quantities (post neural discharge) of the neurotransmitter acetylcholine (ACh) in the synaptic cleft. As such, normal AChE function is critical to a wide range of biological processes, most notably including vital central nervous system, cardiac and respiratory functions.

AChE has garnered substantial interest in the medical community over the past several decades for a variety of reasons. AChE has been targeted for reversible inhibition in pursuit of treatments for myasthenia gravis (MG) [2] and Alzheimer's disease (AD) [3]. In the former case, the primary symptom of MG is muscle weakness caused by autoimmune attack on the nicotinic receptors found in voluntary muscles, leading to diminished capacity to receive ACh-mediated instructions. Selective inhibition of a sizeable percentage of available AChE receptor sites leads to increased ACh-persistence in the cleft, thus enhancing the opportunity for successful ACh transmission. For the latter, a precise etiological characterization of AD causality remains elusive, however one key symptom is degradation in synaptic efficiency that appears to be somewhat alleviated by the similar mechanism of AChE-inhibiting / ACh-bolstering therapy applied in MG treatment [4]. Early hypotheses holding that the synaptic inefficiency might actually be a cause of the disease rather than just an effect [5] have proven inadequate in explaining many observations, however, increasing evidence suggests that although AChE-inhibition therapy tends to provide temporary cognitive improvements in AD patients, the tangible benefits toward reversing or even slowing the progression of the disease remain under debate [6,7].

Diminished AChE activity is also a dysfunction that has a variety of health impacts. Onset of Parkinson's disease (PD), for example, correlates with reduced AChE function in muscarinic receptors in the temporal cortex [8]. A much more obvious issue, however, is the direct link between organophosphorus (OP) and carbamate toxicity and AChE inhibition, providing a biochemical basis for many pesticide formulations as well as most of the nerve agent class of chemical weapons. Not surprisingly, recent studies have suggested an important possible link between OP exposure and elevated risk of PD [9].

Whether the medical point of interest has to do with targeting AChE for inhibition, in the case of MG or AD treatment, or trying to prevent this from occurring (i.e., PD and nerve therapy and prophylaxis), there is a strong motivation for wanting to understand aspects of the AChE activity and deactivation in atomic-level detail. Given the complexity of the system that will be elaborated upon in greater detail in the next section, there is little wonder that molecular modeling methods and other chemical informatics techniques have been applied extensively to the study of this enzyme; in trying to derive as much insight into a variety of different aspects of the system as possible, numerous different computational methods have been applied. This review aims to examine a reasonable selection of these different methods and their intended rhetorical value. Dynamic aspects of the enzyme system itself, and of ligand-

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enzyme conjugates have been studied extensively *via* molecular dynamics (MD) simulations, of which a representative sample are discussed in Section III. Optimization of non-covalent inhibition strategies, pursued extensively in the search for non-toxic treatments for MG and AD, has made substantial contributions *via* molecular docking simulations, some of which are discussed in Section IV. Many aspects of covalent inhibition cannot be modeled *via* classical simulations and thus require quantum chemical (QC) treatment, which has also been applied toward understanding the underlying enzymatic and inhibitive reaction mechanisms, as well as other electronic structural effects governing enzyme activity. This is reviewed in Section V. Finally, given the multifaceted nature of the AChE inhibition problem and the fact that the potency of a given ligand may depend on any combination of (or all of) the aforementioned considerations, it is proving necessary and valuable to develop statistical methods for assessing the relative importance of given properties and phenomena. Various quantitative structure activity relationship (QSAR) models applied to AChE inhibition are thus described in Section VI. Some speculations regarding the impact of emerging computational methodologies are discussed in Section VII.

II. ACETYLCHOLINESTERASE STRUCTURE, FUNCTION AND INHIBITION

The amenability of AChE to detailed studies of structure-function relationships has been greatly augmented by the publication of numerous well-resolved crystal structures of the enzyme in the apo form and in various states of inhibition. The two most widely available crystal structures are those of the *Torpedo californica* (tcAChE) [10] and Mus Musculus (mAChE) [11], although in recent years the human form (huAChE) has also been published [12] and is depicted in Fig. (**1**). AChE tends to be very well conserved across species, with huAChE and mAChE sequences exhibiting 88% identity and 97% homology, while even the great evolutionary divergence between humans and *Torpedo californica* affords 57% identity and 86% homology over the aligned portion of their sequences. Therefore, while we will focus primarily on huAChE in this review as the system of primary pharmacological interest (residue numbering will be according to huAChE except as noted), the vast majority of assertions derived from any one of the above three structural models should hold reasonably well for the other two.

AChE monomer units such as the one depicted in Fig. (**1a**), are classifed according to the Cluster-Architecture-Topology-Homologous Superfamily scheme as αβα-sandwiches [13]. The two α-rich masses (one of which is on the left side of Fig. (**1a**), while the other is near the bottom-center) provide sites for the cysteine-cysteine disulphide bridging that leads to dimerization and tetramerization of AChE, thereby optimizing the enzyme's hydrophilicity and catalytic efficacy [14]. Within a coil-rich mass between the two α-rich associative lobes lies the main enzymatic functionality of AChE, whose main features are shown in greater detail in Fig. (**1b**). The view in Fig. (**1b**) is oriented obliquely above a gorge whose mouth is demarcated by a peripheral anionic site (PAS), coloured dark blue, comprised mostly of hydrophobic residues (Tyr72, Tyr124, Trp286, Tyr341) but also an anionic Asp74. The hydrophobic contact surface plus a single anionic point appears to be an ideal match for attracting the cholinic end of ACh which combines a hydrophobic moiety with a quaternary (i.e., electrophilic) amine.

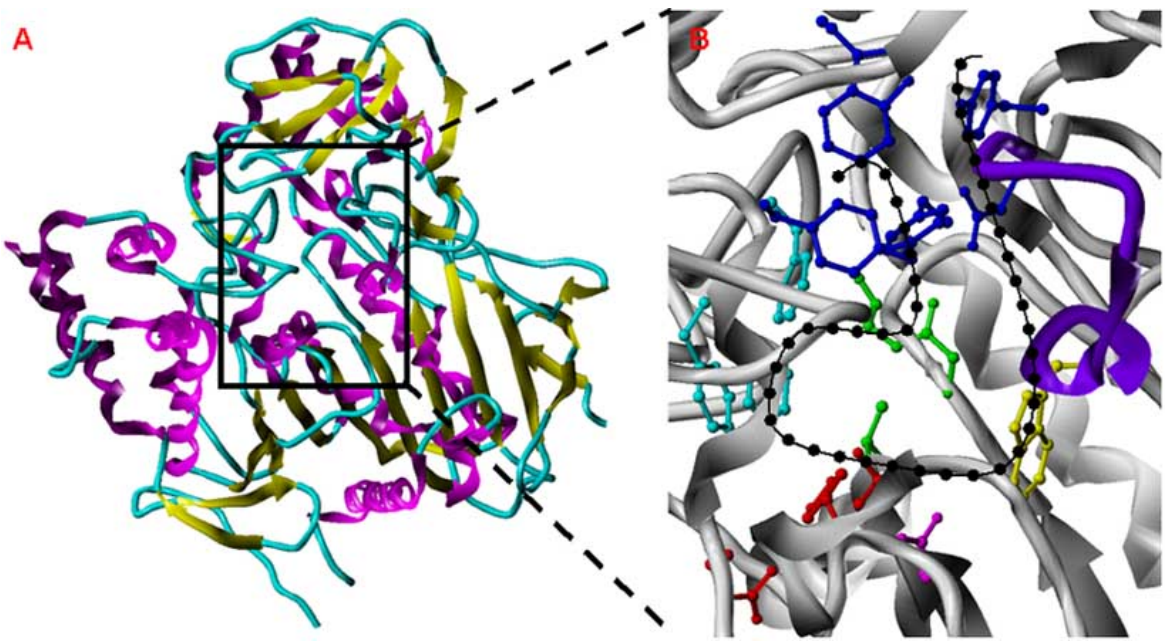

Fig. (1). Crystal structure of human AChE showing A) secondary structure, and B) active site features. Secondary structural features in A) are colored in standard form (yellow = β-sheets, magenta = α-helices, cyan = coils). In B), the catalytic triad residues are colored red, the oxyanion hole is green, acyl pocket is cyan, the peripheral active site is blue, Glu202 is magenta, Trp86 is yellow and the mobile loop is purple. The approximate extent of the gorge is depicted in B) as a brown dotted line.

At the base of the gorge lies a classic serine-protease catalytic triad comprised of three residues: Ser203, Glu334 and His447 (shown in red) that collectively serves to deacylate ACh *via* the mechanism illustrated in Fig. (**2**). The charge buffering role played by His477 is enhanced by prior extraction of the H_δ proton on the imidazole ring by Glu334. Reaction irreversibility is believed to be conferred by the nearby Glu202 which may serve the function of attracting the imidazole's H_ε (corresponding to the protonated N_ε in Fig. (**2**), step 4) during the deacylation (Fig. (**2**), step 5). The role of Glu202 will be discussed in more detail later.

Proximal to Ser 203, one finds two glycines (121, 122) and an alanine (204) whose backbone amido protons co-orient in such a fashion as to provide a strong H-bond donating site amenable to spatially fixing a small nucleophile, such as an ester carbonylic oxygen. In the specific case of ACh, these three residues, collectively referred to as the oxyanion hole, assist in deacylation by selectively orienting esters so as to render its central carbon favorable to nucleophilic attack by Ser 203 (Fig. (**2**); step 1), and also favorable for subsequent removal of choline (Fig. (**2**); step 2). Across the base of the gorge from the catalytic triad one notes the presence of a hydrophobic Trp 86 residue (rendered yellow in Fig. (**1b**)). The presence of the aromatic Trp side chain appears to be well suited to accommodating the hydrophobic but positively charged $(CH_3)_3NCH_2$ ¾ tail of ACh during the reactive process.

Also of relevance, is the acyl pocket shown as cyan residues (Phe 295, Phe 297, Phe 338) in Fig. (**1b**), whose primary role appears to be in housing the acyl methyl group in the activated ACH-AChE complex. Given the fact that this region comprises one of the few major differences in the of AChE receptor structure versus that of the otherwise closely related butyrylcholinesterase (BChE) enzyme (Phe295 → Leu 295; Phe297 → Ile297), it has been postulated as a primary source of AChE and BChE enzymatic selectivity [15]. Specifically, the smaller hydrophobic groups in the BChE acyl pocket would be

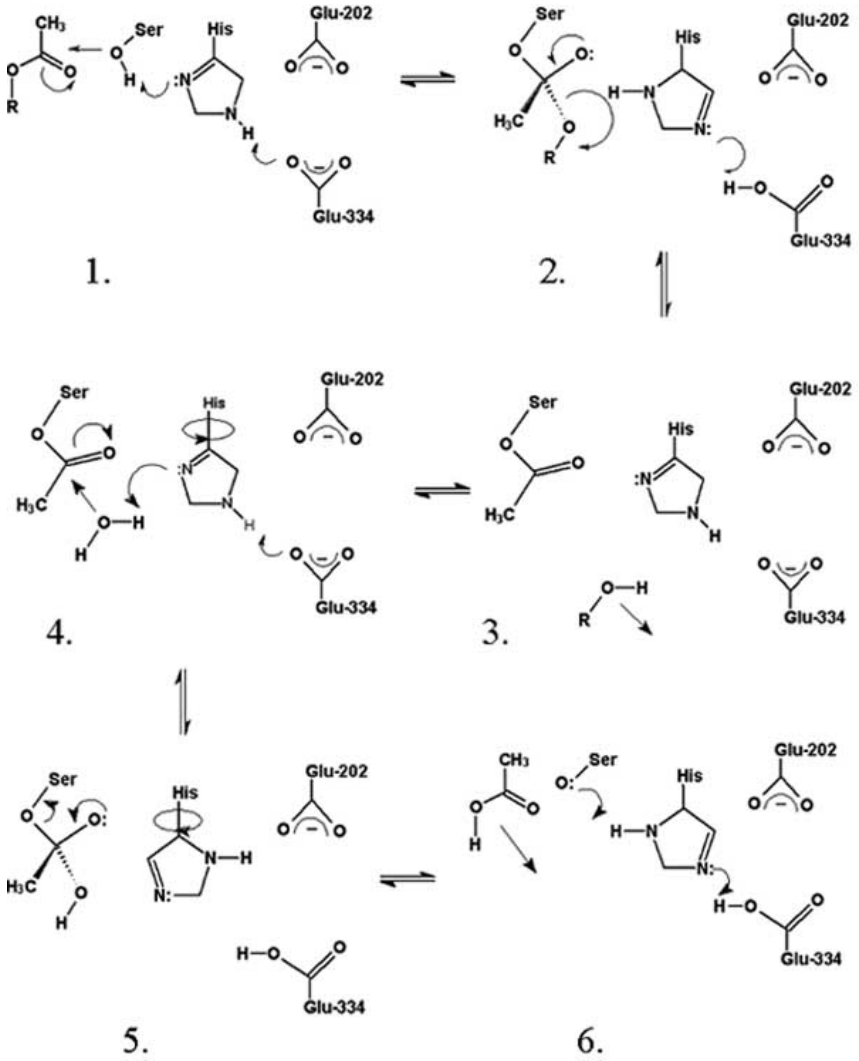

Fig. (2). Reaction diagram illustrating the putative six step deacylation of ACh by AChE. **R** corresponds to ACh's $(CH_3)_3NCH_2$ moiety, **Ser, His** and **Glu** indicate serine (203), histidine (447) and glutamine (202,334) AChE receptor residues.

expected to yield a larger chamber capable of processing the larger substrate molecules (e.g., butyrylcholine and benzoylcholine) that cannot be admited into the AChE receptor.

AChE's impressive enzymatic efficiency suggests that the various structural features described above likely produce an optimal mechanism for attracting, trapping and decomposing the ACh substrate, as well as expelling the choline and acetic acid products. It is broadly assumed that much of the efficiency is conferred by an ideal spatial relationship between the different features, whereby **1)** the PAS and Trp86 respectively attract and guide the $(CH_3)_3NCH_2$ moiety into place, **2)** the oxyanion hole and acyl pocket collectively anchor the ester into a position suitable for nucleophilic substitution, **3)** proton shuttling within the triad induces highly effective nucleophilic attack onto the ester center, **4)** Glu202 effects a conformational rearrangement of His447 to prevent premature back-sliding of the shuttled proton (although the exact role of Glu202 remains a matter of debate, as will be discussed later) , and **5)** the spacious nature of the gorge permits the presence of ample water molecules, of which one is required to effect deacylation. It has also been speculated that a number of electrostatic and dynamic effects may enhance catalytic efficiency. Chief among them is the presence of a highly mobile Cys69-Cys96 loop that is believed to engage in gorge gating selectivity mechanisms [11]. The fact that Trp 86 seems to be both a structural keystone of this mobile loop [16] as well as a key lipophilic component of the active site suggests a possible role as a gating trigger that signals the loop to occlude the gorge exit thus preventing the departure of unreacted ACh. Another so-called *back-door* hypothesis exists, claiming that a concerted post-complexation rearrangement (centered in the Trp86, Val132 and Gly448 region) facilitates departure of the bulky choline leaving group from gorge by an alternate route [17], thus reducing the likelihood that the choline will linger in the region of Trp86 or the PAS and hinder admission of subsequent ACh substrate. Originally postulated as a result of molecular modeling studies, the concept has not been incontrovertibly established. Subsequent mutagenesis studies argue against the possible mechanism [18], but later crystallographic evidence [19] and kinetic data [20] provide support for it.

Researchers have also postulated the existence of an efficient mechanism to guide the substrate down the extensive gorge to the active site[21]. Detailed studies of the electrostatics of several forms of AChE and BChE [22-24] have demonstrated the conservation of key residues lining the gorge. These residues are postulated to produce a marked electrostatic profile which facilitates guidance of the substrate down the gorge and subsequent ejection of the leaving group.

The very features on which AChE relies for its highly efficient ACh enzymatic processing can be very effectively turned against the system by choice of inhibitors capable of exploiting the various binding sites and subsites in the AChE receptor. Given the physicochemical diversity of these sites and subsites, it is not surprising that an extensive variety of different chemicals exhibit some tendency toward AChE inhibition. Inhibition data within a single online repository exists for compounds from each of the following families: organophosphorus (OP) compounds, carbamates, alkaloids, haloketones, coumarins, ketomalonates, acridines, piperidines, xanthins, benzyl/phenyl amine/amide derivatives, tryptamines, bipyridines, alkylpyridnium oligomers, bis-(and other) quaternary amines, polyethylene ester oligomers, quinolines, quinazolines, triazolines, phenothiazines, curariniums, diazepines, steroids, opioids, fluorides, numerous peptides, and even some quaternary platinum species [25].

Many of the above compounds, especially those with substantial hydrophobic or electrophilic groups, noncovalently inhibit the PAS and/or the Trp86 choline-binding site. However, the strongest AChE inhibitors appear to be species that covalently bind to the esteratic Ser203, especially organophosphorus (OP) compounds or carbamates. The so-called 'nerve agents', of deadly inhibitive efficacy are OP species with the general formula shown in Fig. (**3**). The most powerful OP nerve agents effectively mimic the ACh ester structure that is so conducive to binding within the AChE

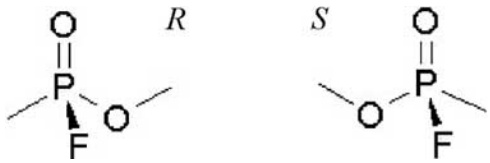

Fig. (3). general structure of *S*- and *R*-stereoisomers for OP nerve agents. **R** and **R'** are usually alkyl groups. **X** is generally an electronegative group (often a fluoride). Note that the sp3-hybridized ester O may be substituted with S in some formulations (i.e., thioesters).

esteratic subsite. Inhibitive OP species possess a phosphonyl group with an electrostatic and steric profile similar to that of an ester (thus being well-accommodated by the oxyanion hole), and an electrophilic phosphorus apparently even more amenable to Ser203 nucleophilic attack than the native ACh substrate. Effective inhibitors typically possess a small aliphatic group (***R***) that fits the contours provided by the acyl pocket and a larger hydrophobic group (***R'***) suitable for interacting with the Trp86 choline-binding site. From Fig. (**4**), however, we see that the final disposition of OP ligands differs from the catalytic ACh decomposition process in that water is not a sufficiently strong nucleophile as to effect a process analogous to the deacylation in steps 4-6 of Fig. (**2**). Despite its relatively high electronegativity, the ***X*** group in OP nerve agents does not appear to detract from the ligand's efficacy for initial complexation and, as is seen in steps 2,3 of Fig. (**4**), subverts the normal AChE catalysis by serving as the preferred leaving group instead of the OR group (as would be analogous to ACh). This produces a phosphorylated active site that is inherently resistant to the normal hydrolysis reaction (steps 4-6 in Fig. (**2**)) that would otherwise permit enzymatic regeneration of the AChE enzyme. Although the phosphorylation is still technically reversible at this stage, the AChE receptor has been rendered largely inaccessible to ACh, thus providing the principle source of OP toxicity. Some OP agents are capable of undergoing a further dealkylation reaction, illustrated in steps 4,5 of Fig. (**5**). This reaction, generally referred to as 'aging', creates a resonance stabilized inhibition state that can be considered irreversible for most practical purposes

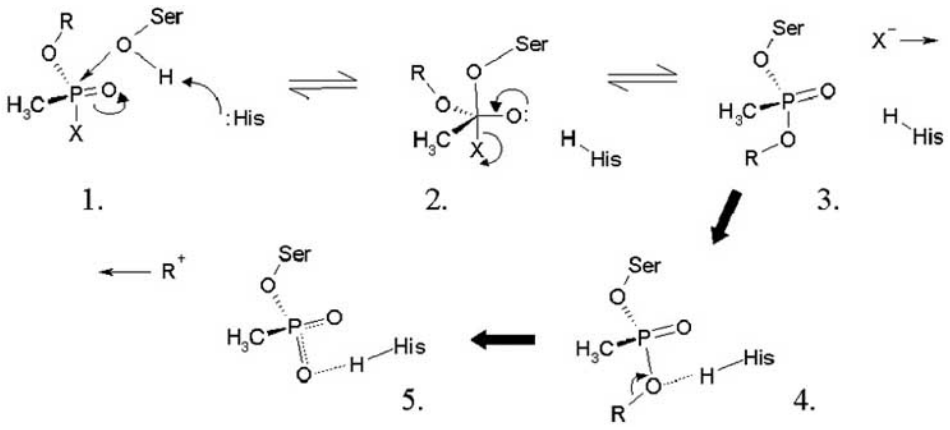

Fig. (4). Reaction diagram illustrating the putative phosphorylation and aging reactions for organophosphorus reactions with the AChE active site. **Ser** and **His** refer to the serine and histidine residues in the AChE catalytic triad (numbered Ser 203 and His 447, according to huAChE sequence).

[26]. While therapeutic species (primarily oximes) have been developed that can dephosphorylate OP-poisoned AChE enzymes from the point described by step 3 in Fig. (**5**), most aged nerve agents aged states remain resistant to chemical remediation.

III. DYNAMIC PROPERTIES AND EFFECTS

The most prolific body of work corresponding to molecular dynamics simulations of AChE corresponds to that produced by the McCammon group at the University of California at San Diego. In addition to numerous contributed articles on AChE dynamics, of which some will be discussed shortly, the McCammon group has produced a recent review article [27] that establishes an important premise for the importance of MD simulations to the understanding of AChE function. Namely, they point out that the structure of the gorge is so deep and narrow that the rigid models corresponding to most available crystal structures do not afford sufficient channel width to admit the known substrate and active site inhibitors. The facts that AChE actually does a) allow both substrate and inhibitors into the furthest depths of the cavity and b) the enzyme is viewed as one of the most catalytically efficient systems in the known proteome [1] clearly suggests that dynamic effects play a major role in diffusional efficacy. Unraveling the precise nature of this dynamic facilitation has been a major source of research interest over the past decade.

To the best of our knowledge, the first published simulations focusing on AChE structural dynamics include the following three papers dating from 1994: 1) an important but relatively uncontroversial comparison of a static AChE crystal structure and its dynamic virtual analog [28], 2) the aforementioned (see previous section) ground breaking study in which prediction of the presence of a back door exit channel adjacent to the known gorge was first made [17], and 3) the first detailed assessment of the role of the mobile Cys69-Cys96 loop [29]. The first of these papers provided enlightening insight into the degree of enzyme-wide structural rearrangement that is possible upon transition from an apo state to inhibition, while the second and third raised the possibility of specific dynamic features, such as transient channels for the evacuation of product molecules and trafficking of solvent, that might not have been readily intuited from the crystal structure alone.

While the acceptance of a back door hypothesis within the experimental community has been a gradual process [18], further MD studies within the McCammon group [30-32], and by other researchers [29], were fairly quick to confirm the feature and have since carefully characterized it. Later studies also postulated the presence of a side door (around Thr75, Leu76, and Thr83) [33], which has been subsequently validated by the McCammon group [34]. In the apo form of the enzyme, it appears that the back door is relatively occluded, only spending about 1% of its existence open broadly enough to admit or emit a water molecule (~1.4 Å radius), while the side door remains shut effectively 100% of the time [32]. When ligated or inhibited, however, the doors are predicted to be much more accessible. With fasciculin bound across the PAS, for example, the back door was found to remain open (a radius of at least 1.4 Å) upwards of 18% of the time, and the side door 13% of the time. It has been hypothesized that these alternate doors not only serve to expedite solvent and product exchange, but possibly also act as an adaptive biological defense mechanism that may permit some degree of ACh processing to occur even when the gorge entry is fully occluded [32]. This may offer a reasonable explanation for continued deacylation activity in the face of extensive fasciculin-AChE inhibition [35,36]. Some evidence exists for the possible existence of as many as two other transient portals to the active site cavity [32], suggesting an inherently very porous gorge. It must be additionally noted these dynamics studies address not only the question of ingress and egress of ACh and inhibitors into the gorge, but also applied to the critical question of solvation within the gorge and diffusion of waters to the active site [22-24]

Detailed analysis of MD simulations suggest that the process of opening and closing of the back and side doors is not merely a localized phenomenon, but entails significant concerted motion across much of the AChE monomeric unit [32]. Interestingly, while Trp86 is generally considered to be a critical residue for the back door, essentially forming part

of the mouth of the open portal [17], it is also strongly correlated to side door motion, being one of the residue side chains that moves the most upon side door opening / closing [32]. There does not, however, appear to be a simple correlation between to the relative states (i.e., open *vs.* closed) of the side and back doors. During a MD simulation with PAS-bound fasciculin, they coexisted in an open (*OO*) state 8% of the time, were both closed (*CC*) approximately 77% of time and in the remaining 15% of the observed time steps one was found to be open and the other closed (*OC* or *CO*) [32]. Despite this apparent independence, it is still possible that both aperture states depend at least in part on the nature of ligand interactions with Trp86, with different contact conformations favoring each of the four different (*OO*, *OC*, *CO* or *CC*) states. Since Trp86 has also been identified as possibly performing a gorge gate-switching role through action on the mobile Cys69-Cys96 loop [11], evidence appears to point to this hydrophobic / basic residue as serving (and perhaps helping to coordinate) multiple important enzyme regulatory functions. If so, it may prove to be a very attractive inhibition or mutagenesis target for enzyme modulation.

Dynamic modeling has played an important role in the original identification of the mobile loop [28], and characterizing its motion and function [16,29,37]. Named because of its apparent status as the most conformationally mobile portion of the AChE molecule, the flexibility of the Cys69-Cys96 loop was first noted from MD analysis, and was thus postulated to serve a role analogous to that observed in lipases [38,39] as a transitory cap on the active site [28]. Kovach *et al.* used this loop's mobility as rationale for the existence of the various alternate product channels (e.g., back door, etc.), suggesting that loop motion uncovered transient apertures between the three main permanent channels in the enzyme: the primary gorge, a side channel capable of accommodating cationic products such as choline, and a cavity admitting nucleophiles such as the deacylation leaving group [29]. Subsequent MD studies have supported this assessment, suggesting that the binding of a lipophilic cation (e.g., choline or the phosphorylation leaving group) to Trp86, induces partial unwinding of the loop up into the gorge, opening an aperture suitable for cation evacuation [16]. The importance of the mobile loop has been effectively validated by enzyme kinetic studies as a key participant in allosteric modulation of AChE catalytic activity [40].

Motion of additional residues had also been postulated as being of key importance to binding [41]. Carlacci [42] and Olsen *et al.* [43] have studied the motion of the acyl pocket loop (residues TcAChE 287-290) through a variety of techniques, including MD and Monte Carlo (MC) simulation. This work analyzed alternative conformations of the acyl pocket loop residues and their influence on substrate binding. Residue Arg289 in particular was singled out as playing a critical role in stabilizing these alternative conformations. Additionally, the role of the solvent was recognized as being integral to this phenomenon. The MD work in particular provided a critical analysis of results using different solvation models. Interestingly, movement of the acyl pocket loop was also detected upon phosphorylation by Zeev-Ben-Mordecai *et al.* [44] in a non-traditional approach that used a morphing procedure to visualize and detect conformational changes in acetylcholinesterase crystal structures.

Another major contribution to our net understanding of AChE function and inhibition that MD studies have made is in the area of dynamic AChE-ligand interaction effects. In addition to the aforementioned study of PAS-binding fasciculin-AChE complexes [34] and more recent work on this system by the same group [45], there has been research on the dynamics of the non-covalent active-site binding huperzine [30], precomplexation studies on ACh and various of its analogs [46], and examinations of PAS-binding inhibitors such as the Alzheimers drug propidium [47], tacrine analogs [48,49], and of the novel tacrine-huperzine hydrid huprine-X [50,51]. As discussed in previous paragraphs in this section, the research on fasciculin binding was primarily geared toward elucidation of connections between ligand binding and the formation of the back and side door conduits [46]. Among the other enzyme-inhibitor dynamics studies, the ACh analog and propidium simulations were primarily focused on using MD methods to supplement static molecular docking evaluation of noncovalent bonding interactions between ligand and enzyme [46], which is a topic to be dealt with primarily in the next section. However it is worth noting that, in the study of ACh analogs, analysis of the different receptor conformers suggests that the presence of the ligand in the active site of AChE not only stabilizes the H-bonding interactions within the catalytic triad but also spatially tightens both subsites, thus collectively enhancing the binding environment [46]. The tacrine analog and huprine-X studies were also primarily geared toward elucidation of noncovalent bonding modes, but yielded important insight from their MD simulations that merits some elaboration here. In the tacrine studies, simulations on increasingly hydrophobic analogs suggest that such hydrophobicity produces important changes in the binding mode away from the gorge binding location of tacrine itself, exhibiting simultaneous lipophilic stacking with Trp84 and Phe330 (according to tcAChE numbering; huAChE = Trp86, Tyr337) and yielding an inhibitive mechanism arising more from PAS-binding interactions, thus raising the prospect of tailoring inhibitors toward simultaneous complexation with both subsites [48,49]. Conversely, when modeling huprine-X it was found that the amalgamation of chemical features from the noncovalent esteratic active site inhibitor huperzine and the gorge binding tacrine led to an extremely strongly binding noncovalent inhibitor spanning both the nucleophilic active site and the lipophilic gorge [50,51]. It was also noted that huprine-X was found to bind significantly more strongly to tcAChE than to huAChE, thus highlighting the importance of the Phe to Tyr mutation (tcAChE: Phe 330 / huAChE: Tyr337) distinguishing the two structures [50,51]. Both the tacrine analog and the huprine-X studies demonstrated the analytical power of MD methods for effective pharmacophore mapping within a complex, multifunctional receptor such as that of AChE.

MD simulations have also been used to probe precomplexation interaction between AChE and known covalent inhibitors such as soman [52,53]. These studies were quite effective in rationalizing the specific receptor-ligand interactions that stabilize and facilitate nerve agent

reactions in the active site. In comparing Figures (**2**) and (**4**), one notes that the standard ACh processing involves the usual series of proton transfer reactions and yields neutral products (Fig. (**2**)), however the OP covalent inhibition reaction involves the successive release of two ions (Fig. (**4**)) and, in this respect, might be expected to be less energetically favorable than an exchange of neutral species. To address this dilemma, a MD simulation of a fully solvated tetracoordinate phosphonate adduct (based on Soman inhibition of AChE) provided a trajectory whose ligand-enzyme interactions suggested a scenario whereby His447 might remain positively charged (rather than neutralizing the Glu334 anion) and thus effect a charge stabilization on the anionic leaving group (Fig. (**4**), step 3) [52,53]. In a commensurate fashion, Trp86, Phe338 and Glu201 were estimated to collectively attract and stabilize the lipophilic and electrostatic complement within a prospective carbenium leaving group and thus facilitate the aging portion of the covalent inhibition (Fig. (**4**), step 5) [52,53]. Although these assertions have not yet been definitively validated through spectroscopic or quantum chemical means, they remain generally accepted tenets of AChE inhibition theory.

IV. NON-COVALENT INHIBITION

One of the most active areas of AChE biochemical research has been in the pursuit and design of strongly binding but reversible inhibitors [54], with primary applications being improved AD and MG treatment, as well as development of new prophylaxis schemes for mitigating nerve agent toxicity. The spatial structure of the AChE peripheral and catalytic binding sites, well understood from extensive X-ray crystallographic analysis (see Section II), imposes substantial specificity in ligand conformation and the orientation upon binding, thus making AChE a tempting target for structure-based design and optimization of potential inhibitors. Substantial effort has thus been expended in search of novel AChE inhibitors that are both potent and sufficiently selective as to minimize collateral impact on other serine hydrolase enzymes. A strong desire exists for robust high throughput virtual screening techniques capable of sifting through the broad range of chemical space potentially suitable for AChE inhibition. This has placed a pronounced focus on development of docking algorithms capable of exploiting the wealth of available structural data for the purpose of rapidly and reliably identifying strong non-covalent inhibitors.

Early docking work often placed a primary emphasis on obtaining correct conformational and orientational predictions for the bound ligands. Work done by Pang *et al.* [55,56], for example, was mainly focused on predicting binding modes for a series of benzylamino AChE inhibitors. They successfully identified that the Trp84, located near the bottom of the binding pocket in *Torpedo californica* AChE, is the quaternary ammonium binding site for acetylcholine, and Trp279, in the peripheral hydrophobic site, is the binding site for other aromatic rings of the ligands (Trp86 and Trp286 respectively in huAChE numbering).

Structural predictions alone, however, do not provide sufficient insight for informed structure-based design. Given a capacity for correct conformational predictions, the next logical focus lies in accurate assessment of ligand binding affinities. *In silico*, this is generally attempted by devising a scoring function capable of inferring a free energy of interaction from the structure of the ligand-receptor complex. Due to the complexity of the interaction between ligands and AChE, the classical docking score appears to exhibit little or no significant correlation with experiment. Test calculations on a homogeneous set of non-covalent AChE inhibitors *via* a selection of the most commonly used generic docking score functions (including Chem-Score [57], Drug-Score [58], FlexX-Score [59], G-score [60], and PMF-score [61]) demonstrated a broad and fairly complete failure in predictivity, with no single method yielding a correlation better than $R^2 = 0.13$, and a specially trained linear combination of the five only producing $R^2 = 0.26$ [62].

The main shortcoming of generic docking score functions is typically an inability to reproduce entropic effects in systems where receptor flexibility plays an important role in driving the interaction [63]. Traditionally, the most popular scheme for incorporating aspects of receptor flexibility into the model has been to accumulate multiple enzyme conformers corresponding to different snapshots in a MD simulation and to dock ligands into this manifold of static conformers. To the best of our knowledge, the first studies on AChE inhibitors employing this paradigm were those of Pang and Kozikowski, who performed docking simulations for huperzine A and E2020 (aricept) into a series of 69 AChE conformers chosen from MD studies so as to approximate the receptor flexibility space available to an inhibiting ligand [55,56]. In the case of E2020, ligand flexibility was also incorporated in an ad hoc fashion by docking 1320 distinct ligand conformers into the receptor manifold. They found that E2020 spans the entire binding cavity of AChE, with the ammonium group interacting mainly with Trp84, Phe330 and Asp72 (tcAChE numbering; corresponding huAChE residues are Trp86, Tyr337, Asp74), the phenyl group interacting mainly with Trp84 and Phe330, and the indanone moiety interacting mainly with Tyr70 and Trp279 (tcAChE numbering; huAChE = Tyr72, Trp286). Huperzine A was found to have a similar binding mode to E2020. These structural predictions compare well with definitive results obtained from subsequent X-ray crystallographic studies [10,64], however no conclusions were reported in terms of resulting inhibition scores of these two systems relative to known ligand affinities across any control sets.

A more thorough study bridging the capacities of MD and docking simulations, already introduced in Section III, was that of Kua and coworkers who combined multiple docking simulations with extensive MD calculations in the study of AChE interacting with its natural substrate ACh, assorted ACh analogs and choline [46]. In this case, they made no attempt to focus on unique characteristic receptor conformers, but rather docked the ligands *via* AutoDock [65] into a collection of 1998 receptor models corresponding to the enzyme conformer observed at 1 ps intervals of 1 ns MD simulations of a) the apo-AChE structure, and b) the AChE - ACh complex. From the resulting set of docking simulations, they retained the top scoring pose from each run (for each ligand) and discarded those conformers that placed neither the ligand's head nor tail in the its correct binding pocket.

Averaged docking free energies and inhibition constants were obtained for each ligand from the resulting manifold. These average inhibition constants correlate well with experimental k(cat)/K(M) values, as well as with experimental AChE binding affinities for a related series of trimethylammonio trifluoroacetophenone (TMTFA) inhibitors. As discussed earlier, a bound ACh appears to have a stabilizing effect on the both the catalytic triad as well as on coupling between the active and peripheral sites, leading to a free energy gain of 0.7 kcal/mol for ACh relative to that obtained for the static structure [46]. Binding of the substrate tail group to the anionic subsite appears to depend on both the size and the presence of a positive charge within the tail group. The removal of the positive charge leads to a loss of 1 kcal/mol in binding affinity. Substituting a hydrogen for the tail's methyl group results in both an incremental loss in docking energy as well as a decrease in the percentage of structures found to bind in an orientation suitable for catalysis.

While the combination of MD and docking analysis yields both reasonably accurate binding affinity predictions and substantial physicochemical insight into the factors affecting binding, a major drawback of the method is heavy computational cost that renders the strategy inappropriate for virtual screening, and cumbersome for the more speculative (i.e., trial and error) schemes of structure-based design. However, given the wealth of reliable experimental inhibition data across a fairly broad selection of AChE inhibitors, another powerful tool is available. Namely, the assumption that similar compounds should have similar binding conformations paves the way for the Comparative Molecular Field Analysis (CoMFA) method to be used for estimating activity.

The earliest application of CoMFA methodology to the AChE inhibition problem, to the best of our knowledge, appears to be the work of Cho *et al.* who employed templates obtained from crystal structures of AChE inhibited by edrophonium (EDR) and 9-amino-1,2,3,4-tetrahydroacridine (THA) to align a set of 60 structurally diverse known inhibitors [66]. These aligned structures were then used as the basis for generating a region-focused CoMFA model. Strong correlation was obtained relative to AChE assay IC50 data, with a leave-one-out (LOO) cross validated correlation Q^2=0.73.

A more recent study by Bernard and coworkers used a hybrid method to estimate the binding affinity of 82 N-benzylpiperidine derivatives by flexibly docking them into the mouse AChE active site and establishing a 3D QSAR model based on this ensemble *via* CoMFA analysis [67]. This methodology established an important contrast to conventional CoMFA studies wherein compounds are typically fitted to the conformation established by a single reference; the docked structures conformationally respond to the known (albeit static) receptor structure, thus providing a more reasonable rendition of the spatial and electrostatic effects that define the pharmacophore. The resulting CoMFA model, which attained a cross validated LOO correlation of Q^2=0.75 relative to an *in vitro* mouse AChE assay, was subsequently applied to analysis of another series of 29 N-benzylpiperidine derivatives whose inhibitory activity data corresponded to experimental conditions differing from those present for the training set (human AChE; different incubation times and pH). Reasonable agreement between predicted and experimental activity data was achieved (R=0.90), despite these important differences, demonstrating an inherent extensibility in the model relative to environmental effects, as well as similarity in AChE function / response across species.

In a study of 42 aminopyridazine compounds, Sippl *et al.* also used a docking strategy for aligning ligand structures as a precursor to the model generation [68]. The aligned conformations were then taken as the basis for a three dimensional (3D) QSAR analysis applying the GRID/GOLPE method [69,70]. A model of high quality was obtained using the GRID water probe, as confirmed by the cross-validation method (Q^2(LOO) = 0.937, Q^2(leave-50%-out) = 0.910). The validated model, together with the information obtained from the predicted AChE-inhibitor complexes, was applied as a paradigm for designing novel inhibitors. Seven of the prospective inhibitors were subsequently synthesized and tested, and were found to be highly active.

While CoMFA methods have a proven track record for generating models capable of reliable inhibition predictions, they are not an ideal basis for conceptual structure-based design strategies in that they produce a fairly abstract pharmacophore map that provides only vague notions of how to refine ligands in order to improve affinity. Of more intuitive value is a model that highlights specific atomic interactions between the receptor and ligand, and permits a ranking of those effects according to importance. In our group, a receptor-specific scoring function was developed for the purpose of predicting binding affinities for human AChE (huAChE) inhibitors and unraveling the underlying interactions [62]. This method is conceptually similar to CoMFA models such as those of Bernard *et al.* [67] with ligand conformations derived from explicit docking into the receptor target. It is more properly characterized as a Comparative Binding Energy (COMBINE) type approach [71], however, in that it replaces CoMFA's standard grid-based molecular field corresponding to a probe atom with an estimate of binding affinity in terms of 3D QSAR, using enthalpic interaction terms between the ligand and the AChE residues as primary descriptors. This resulting model thus entails a statistically trained weighted sum of electrostatic and van der Waals (VDW) interactions between ligands and the receptor residues. Within a 53 ligand training set composed of piperidinium, pyridinium, benzisoxazole and E2020 derivatives, our model yielded a strong correlation (R^2 = 0.89) between computed and experimental inhibition constants. Leave-one-out cross-validation also indicated high internal predictive power (Q^2 = 0.72), and analysis of a separate 16-compound test set of similar chemical constituency as the training set also produced very good correlation with experiment (R^2 = 0.69). The residue-specific scoring function is highly amenable to deconvolution, thus permitting the identification and characterization of important ligand-receptor interactions. The resulting list of residues making the most important electrostatic and VDW contributions within the main active site, gorge area, acyl binding pocket, and peripheral site is largely consistent with

available X-ray crystallographic and site-directed mutagenesis data and also provides new insight, supporting the concept of a gating role for Tyr337 in huAChE analogous to that of Phe330 in tcAChE as predicted in prior MD calculations [72]. Interestingly, Tyr337 registers within our model not as one of the dominant hydrophobic interactions (as would clearly be expected for Phe330 in tcAChE) but rather as an electrostatic contributor. This subtle difference among residues serving an otherwise analogous role between tcAChE and huAChE may prove to be key to devising inhibitors capable of differentiating between AChE from mammalian and other sources.

Another recent study employing COMBINE methodology was carried out by Martin-Santamaria and coworkers, exploring ligand binding modes within the tcAChE catalytic active site to identify the key residues that modulate the inhibitive potential of a collection of tacrine-, huprine-, and dihydroquinazoline-based acetylcholinesterase inhibitors [72]. The resulting set of interaction enthalpic descriptors, optimized *via* partial least-squares fitting across a unique training set containing the full set of compounds, produced a model exhibiting both strong correlation and predictivity (R^2 = 0.91 and Q^2 = 0.76, using 4 principal components) across the entire set of ligands. Greater internal predictivity (Q^2 = 0.81 and standard deviation of errors in prediction (SDEP) = 0.25 for 3 principal components) was achieved when restricting the training (and corresponding testing) set to just the collection of huprines. The authors uncovered a pronounced correlation between the affinity trends among the inhibitors and their corresponding receptor desolvation term. This may prove to be a simple and powerful descriptor for intuiting new and improved AChE inhibitors.

Such COMBINE methods, with their valued attributes of computational efficiency, accuracy, and mechanistic insight, may prove to be vital tools in the pursuit of high throughput virtual screening of irreversible non-covalent AChE inhibitors. Results from past virtual screening studies on AChE have not been overwhelmingly successful. A recent study by Mizutani and Itai [73] screened a virtual library of about 160 000 commercially available compounds against the tcAChE X-ray crystallographic structure. A total of 1551 potential inhibitors were identified. Among the 114 virtual hits that could be purchased and assayed, 35 molecules spanning a reasonable selection of chemical space showed inhibitory activities with IC(50) values less than 100 μM. Thirteen compounds had IC(50) values between 0.5 and 10 μM, most of which displayed substantial chemical uniqueness relative to known inhibitors. While the fact that this exhaustive study uncovered potential new scaffolds for inhibitor discovery is in itself an important contribution, none of the identified and validated inhibitors remotely approach the known subnanomolar efficacy of various non-covalent inhibitors identified through purely *in vitro* screens [74]. Furthermore, the relatively high false positive ratio (69%) suggests relatively poor scoring, and may also imply a sizeable false negative rate that is leaving potentially viable chemical space untapped. It would be interesting to apply a more accurate scoring technique such as a COMBINE-based assessment to a collection of this size.

V. COVALENT BONDING EFFECTS

The scientific literature abounds with examples of computational studies of enzyme catalysts, amply demonstrating that some level of quantum theory must be used to properly model reactive chemistry occurring at the enzyme active site. This is especially vital for AChE, where multiple reaction mechanisms are of immediate interest. Although we have discussed in detail the function of cholinesterase inhibitors that involve non-covalent interaction, a number of systems (both benign and deleterious) exist which function through binding at the active site Serine 203. Among these covalent binders, OP compounds appear to be unique in being posited to undergo an additional side chain dealkylation (a reaction commonly known as 'aging') which greatly exacerbates their toxicity. Finally, numerous compounds of potential therapeutic value have been proposed to facilitate the OP-AChE dephosphorylation reaction, thus restoring enzyme functionality. A thorough understanding of the mechanisms of these processes would be of immense value in the design of improved antidotal therapeutics.

The use of quantum models to elucidate such reaction mechanisms evokes problematic issues of model truncation, the effect of missing residues, solvation, and lack of the enzymatic environment *in toto.* In the absence of quantum mechanical methods capable of handling thousands of atoms in a biological system in an efficient, reliable fashion, mixed quantum mechanical /classical mechanical (QM/MM) models are often employed as a palliative. These QM/MM algorithms have been an area of rapid development over the last decade or so, and many of the major quantum software packages have implemented some form of QM/MM in an attempt to facilitate mainstream study of biomolecular systems.

AChE was recognized early in the development process as a candidate enzyme likely to benefit from use of an enlarged model. Vasilyev published a very effective QM/MM study [75] (utilizing semiempirical PM3 theory and the OPLS forcefield), demonstrating the strong influence of hydrogen bonding within the catalytic triad on the configuration of the bare enzyme, as well as on the acylation and phosphorylation reactions. This work demonstrated the stabilization by the enzymatic environment of the tetrahedral intermediate (TI) formed during acylation. Particular attention was paid to the role of the oxyanion hole residues, although these residues were not included in the quantum layer of the model system.

Several truncated but fully quantum mechanical models for the acylation of the AChE active site were used in conjunction with Brownian dynamics studies by Wlodek *et al.* [76]. Four models of the active site were utilized to further investigate the role of Glu202 and the oxyanion hole. These models consisted of:

1. WI (model I Wild Type Enzyme): the substrate, the sidechains of the active site triad, and Glu202 sidechain
2. MI (model I mutant): WI model with no Glu202
3. WII: (model II Wild type) WI plus ammonia groups representing backbone amide of Gly121, Gly122, Ala204

4. MII: (model II mutant) WII with Glu202 sidechain replaced by Gln

As is often the case with extremely truncated models, it was necessary to impose constraints by fixing a selection of backbone atoms, including the amide nitrogens of each oxyanion hole residue and the C_α for all other residues. Energy profiles were generated for formation of the tetrahedral intermediate (TI) at the HF/3-21G level for all 4 model systems. Although there were definite questions raised by the truncation of the active site and the level of the quantum treatment, these results confirmed the critical role of the oxyanion hole in stabilization of the TI. It was additionally suggested that the Glu202 stabilizes the transition state of the acylation *via* electrostatic interactions with the His447 imidazole ring.

Fuxreiter and Warshel studied AChE acylation through calculation of activation free energies using the empirical valence bond (EVB) and an all-atom free energy perturbation (FEP) approach [77], in conjunction with the PDLD/S-LRA method. This work treated the acylation as a two-step process consisting of an initial proton transfer from the active site Ser203 to His447 and a subsequent nucleophilic attack by acetylcholine on the serine. The importance of the ionization state of both nearby glutamates Glu202 and Glu334 was demonstrated, and the significance of the oxyanion hole residues were again confirmed. The effect of the enzymatic environment was determined to be beneficial to both steps of the mechanism. The overall decrease on the activation barrier was determined to be ~10-15 kcal/mol relative to an aqueous reference reaction, ostensibly in line with the catalytic effect observed experimentally.

Vagedes *et al.* [78] followed this work with an EVB simulation of the deacylation reaction. This group studied two different mechanisms for the reaction: a two-step process (proton transfer from water to the active site His447 followed by nucleophilic attack of the remaining hydroxide ion on the acetylated Ser203 to produce the tetrahedral intermediate TI of the deacylation step) and a concerted (simultaneous proton transfer and nucleophilic attack) mechanism were studied. Preliminary to the EVB analysis, the investigators performed Monte Carlo simulations to study the protonation states of all 145 titratable groups in the AChE, and reached the conclusion that Glu202 is protonated in both the free and acylated enzyme. The EVB work determined an energy barrier lowering of 11-12 kcal/mol in AChE relative to an aqueous reference reaction, and again demonstrated a sizable effect from Glu202 and the oxyanion hole residues. Both the concerted and stepwise mechanisms were accelerated in a similar fashion by the enzyme. The investigators recommended the concerted mechanism through comparison to experimental kinetic data.

More explicit quantum detail on the acylation reaction was provided by Zhang *et al.* [79], who used a pseudobond ab initio QM/MM approach with HF/3-21G for the quantum layer and the AMBER95 forcefield for the remainder of the enzyme (Additional single point calculations were carried out at MP2/6-31+G* and B3LYP/6-31+G* for points of interest along the reaction profile). Two different models were used for the quantum layer, a smaller model consisting of the ACh and the active site serine and histidine sidechains, as well as a larger model that also included the active site Glu334. A concerted mechanism was proposed (simultaneous acylation of active site Ser203 and proton transfer from Ser203 to His447), and a transition state obtained which resulted in a 10.5 kcal/mol potential energy barrier for the large quantum model at MP2/6-31+G* (12.6 kcal/mol for the smaller system). This result was determined to be consistent with experimental data. As there were minimal energy differences between the small and large quantum models, it was proposed that the Glu334, while providing a vital electrostatic interaction as well as forming a short hydrogen bond with the His447, did not interact *via* a charge-relay mechanism. The role of the oxyanion hole was again confirmed, suggesting continued existence of two hydrogen bonds, between the ACh and the Gly121 and Gly122 backbones, throughout the course of the reaction. Formation of a weak hydrogen bond between ACh and Ala204 occurred as the reaction proceeded. The role of the notorious Glu202, while important, was less clear and left to future work.

The use of QM/MM was expanded past the acylation reaction to predict binding free energies for *O,O'*-1,4-xylene bispilocarpic acid diester C6 as well as the inhibitors bambuterol and TMTFA. This study included both AChE *and* BChE, and undertook to understand the differences in the active site regions of the two enzymes [80]. This work, done at the AM1/Dreiding FF level, used a large quantum layer consisting of all residues within 5 Å of the active site Ser oxygen (13 residues for AChE, including the active site, oxyanion hole, Glu199 (tcAChE numbering), as well as additional residues). The thrust of the analysis was aimed at differentiating between BChE and AChE. The role of conformational flexibility of the Phe330 residue (tcAChE numbering) was discussed, and the direct role of solvent in the active site proposed.

Additional quantum work has been performed on reactions within the active site related to enzyme phosphorylation as well, although in some cases the role of the quantum simulation was primarily to provide input (refined adduct structure and charges) to further classical simulations. Bencsura *et al.* [52,81] utilized this technique to optimize the side chains of the active site serine (and neighboring two residues), as well as assorted adducts mimicking soman, aged soman, and ACh. This work utilized the MNDO method to generate refined structures and charges, which were then employed in molecular mechanics simulations. Evidence was provided again for stabilization provided by the oxyanion hole and catalytic His, and proposed the role of Glu202 as a driving force in the aging reaction, by "pushing" the alkoxy group. This work also provided an analysis of the role of stereochemistry (P_R *vs* P_S soman) in active site inhibition, and was expanded further later. A similar approach [82] has been applied to VX, albeit using a very truncated active site model and a numerical basis.

Further quantum mechanical and QM/MM exploration of the phosphorylation reaction was performed by Hurley *et al.* [83] The mixed quantum/classical work explored the dependence of the catalytic triad configuration on model details such as force field choice, model size, and quantum theory level. Multiple quantum models were used in the fully

QM work, which studied the binding of the simulant methyl methyl phosphonofluoridate to the active site. Small models were used which consisted of the substrate and the active site sidechains. The expanded quantum models treated the entirety of the oxyanion hole residues in an *ab initio* fashion, some of the first work to do so. Analysis of the transition state for the small model performed at the B3LYP/6-31G* level demonstrated several points. The transition state barrier was calculated to be ~15 kcal/mol, which was approximately 15 kcal/mol lower than the concomitant aqueous reaction, fully in line with previously cited work on the related deacylation reaction [83]. The transition state itself corresponded to a concerted mechanism (expanded past that of previous work), demonstrating agent binding simultaneous with *dual* proton transfer across the active site (Ser -> His and His-> Glu), an argument in favor of including the full active site in the quantum model. It must be noted that the product state in this work was a pentacoordinate phosphorus adduct (later work demonstrated the role of solvent in removal of the leaving group on OP binding [84]). Additionally, results from the expanded quantum model demonstrated not just hydrogen bond formation, but appreciable charge transfer upon binding between the OP compound and the oxyanion hole residues, a telling argument for inclusion of the oxyanion hole in the quantum model. Short hydrogen bonds within the active site were found in both the reactant and product states, and calculated distances were in good agreement with experimental results.

The role of Glu199 has been a recurring theme throughout this work, yet it remains a question that has not been definitively answered by simulation or experiment. This mobile glutamate has been nominated for everything from a somewhat passive role in electrostatic stabilization to active participation ("pushing") in reactions including both binding and aging. Additionally, X-ray crystallographic work [85] demonstrated mobility in the active site His440, revealing that it could move from a position binding to the active site Glu327 to the Glu199, or alternatively reorient the other side of the imidazole ring from binding to the active site Ser200 to the organophosphorus sidearm (all tcAChE numbering; huAChE = His447, Glu334, Glu202, Ser203). This was furthered by subsequent proton NMR data [86]. Later quantum work [87,88] made an initial attempt to address some of these issues. The model used was expanded from earlier work to include the full catalytic triad residues (rather than sidechain models), the full oxyanion hole residues, Glu202, and an aged DEFP (organophosphorus compound). This work, performed at the B3LYP/6-31G** level, studied relative energetics on variation of the binding pattern of the active site histidine, and demonstrated the feasibility of the mobile His447 scheme. An additional insight was provided upon analysis of the calculated proton NMR signal and hydrogen bond distances within the active site of the above models. Experimental NMR data and previous molecular dynamics work [52,81] had suggested a shortening of the hydrogen bonding within the active site upon binding. It was demonstrated in the work of Hurley *et al.* that this in fact will only occur in quantum models containing the Glu202 residue [87,88]. It must be noted that this is the case even in configurations where His447-Glu334 is the primary hydrogen bond (rather than His447-Glu202). It is apparent from this, that the effect of Glu202 on His447 has it's subtle influences as well as it's overt ones. This result merits further exploration, and more detailed analysis is underway.

VI. UNIFYING THE MODELS

The application of computational models to reproduce experimentally determined conformational details of the ligand-receptor complex has traditionally proven quite straightforward, both for noncovalent inhibitors (as in Section IV) and covalent species (Section V). Translating the results of computational studies into accurate prediction of *in vitro* and *in vivo* efficacy has proven to be significantly more challenging for classes of covalent inhibitors than for non-covalent, however.

In addition to the successful structure-based methods discussed in Section IV, quantitative structure-activity relationship (QSAR) analysis has proven reasonably effective in assessing and predicting non-covalent AChE inhibition. A number of early QSAR studies explored the function of various ligand substituent groups *via* simple charge- and shape-based descriptors, achieving reasonable reproduction of experimental *in vitro* AChE inhibition results [89,90]. Tong *et al.* developed a CoMFA model for a series of 1-benzyl-4[2-(N-benzoylamino)ethyl]piperidine derivatives and N-benzylpiperidine benzisoxazoles, finding a strong correlation between the inhibitory activity of those N-benzylpiperidines and both steric and electronic aspects of the ligand set [91]. Hasegawa and coworkers applied a genetic algorithm-based region selection scheme to refine the CoMFA model, obtaining a model with improved predictivity [92]. Sulea *et al.* pursued the topic in a somewhat different manner [93], deriving a structure-activity relationship as a function of an ensemble of different ligand conformers, each optimized at the AM1 level [94] *via* the adjusted multiconformational minimal steric / topology difference (MTD-ADJ) method. The resulting topological pharmacophore maps corresponded well with the spatial constraints apparent in crystallographic receptor structures.

Prediction of toxicity trends among covalent AChE inhibitors such as organophosphorus (OP) compounds has proven much more difficult, largely because of other complex, target-nonspecific interactions that tend to occur *in vivo*. Eldred and Jurs evaluated an array of different descriptors that might be appropriate for encoding the topological, electronic and geometrical features for a set of 54 OPs with acute oral acute oral mammalian toxicity [95]. Given serious limitations in the body of experimental information regarding the important structural features driving interactions between OP compounds and mammalian AChE, they used a genetic algorithm scheme to find subsets of descriptors that would support a high quality computational neural network (CNN) model capable of linking the structural features to activity trends. A non-linear CNN model was determined that yielded strong correlation relative to experiment. A principle drawback of such a nonlinear model, however, is that is does not provide clear insight into the mechanism underlying the OP toxicity, especially since some of the descriptors used in their study have at best an abstract relationship with the actual

mechanism and are not readily connected with precise physicochemical processes.

Other QSAR studies on covalent / toxic inhibition have focused more on concrete physicochemical parameters with tangible relationships to the OP - AChE inhibitive process. Yazal *et al.* used a pharmacophore model to describe the inhibition arising from a small set of 8 specific OP compounds, ultimately determining that OP inhibitive potential seems to hinge on the availability of one ligand hydrogen bond acceptor (corresponding to the phosphonyl group to be anchored within the oxyanion hole) and 2-3 ligand hydrophobic sites [96]. More recently, Zhao *et al.* used CoMFA to study the acute housefly toxicity for a set of 35 OP compounds and determined that the length of ligand's alkyl group, and the electronegativity of the various substituent groups on the P center have significant effects on the AChE activity, but the overall hydrophobicity of the OP molecule appears to have minimal influence [97]. This is in keeping with wide-spread consensus that steric and electronic properties of the OP species play a dominant role in the covalent inhibition of AChE. Bernard and coworkers also explored the interaction between OP compounds and AChE by using CoMFA for a set of 35 compounds with conformations obtained from docking calculations [98]. Somewhat surprisingly given the non-covalent (Michaelis complex) basis for the ligand conformational ensemble, a reasonable correlation (Q^2 = 0.70) was found relative to *in vitro* AChE assay data arising from a variety of different experimental sources.

While such QSAR studies based on correlations with *in vitro* data do have demonstrable value in deriving insight into conditions responsible for effective AChE inhibition, true OP toxicity assessment is only really accessible from *in vivo* studies. Frequently, experimental *in vitro* data has been found to exhibit poor and even minimal correlation with *in vivo* toxicity studies, primarily because of contributing effect of metabolic biotransformation present in the latter [99]. To complicate matters, it has been found that OP metabolites may produce a greater toxic response than their parent compounds. Furthermore, even within the AChE receptor cavity itself, the binding energy of the Michaelis OP-AChE complex is only one of several tangible factors affecting the OP inhibition, since phosphorylation and aging reactions are critical aspects of the inhibition process and may be expected to manifest some differential properties across even a relatively similar family of inhibitors. In the recent work, we have been applied information from a number of different computational methodologies and sources in pursuit of a simple model that both improves the basic mechanistic understanding of OP toxicity and yields reasonable toxicity predictions [100]. Within our training set of 30 known covalent AChE inhibitors (all OPs), 21 were known to act primarily through well characterized metabolites, whereas the other nine are believed to act directly on AChE in their parent form. For the former subset, cytochrome P450 metabolic chemical reactions were quantified approximately as enthalpy-driven processes by including the quantum chemical (AM1) electronic energies of the parent compound and metabolite in the QSAR. Additional absorption, distribution, and excretion effects were accounted for *via* simple physicochemical parameters. The Michaelis complex binding affinity was incorporated *via* CoMFA calculations. Finally, OP phosphorylation and aging reactions were approximated enthalpically, again *via* inclusion of reactant and product energy terms in the QSAR expression. This simple yielded a strong correlation relative to experimental acute toxicity data (R^2=0.90), and provided valuable information about the relative weights of these empirical terms (and their corresponding mechanistic processes) in the net toxic process. Further refinement of the model is envisioned through consideration of factors such as ligand solubility, receptor desolvation, processes stabilizing the phosphorylation and aging leaving groups and so forth.

VII. FUTURE DIRECTIONS

At the present time (ca. 2005) an *ideal* theoretical model of an enzymatic system might be envisioned as an atomic level representation that accounts for all relevant external environment effects, evolves dynamically over biologically meaningful time periods as a function of plausible biological temperature variations, permits substrate or inhibitors (or both, in a competitive fashion) to enter the model under biologically reasonable transport conditions, supports dynamic chemical reactivity in a realistic time frame and permits products to leave the system at similarly reasonable rates. For AchE, such a model would probably entail a fully solvated active form (i.e., tetrameric) AChE, thus amounting to a coupled quantum-classical molecular dynamics simulation of around 500,000 atoms (of which perhaps 200-300 would be treated at quantum mechanical levels) running over a period of at least μ-seconds (the time to process an ACh substrate molecule) and perhaps extending up to the seconds (aging half life for soman) time frame. Needless to say, such a simulation is computationally far in excess of current capabilities and is unlikely to become viable soon, even under the most optimistic interpretations of Moore's Law. In the foreseeable future, therefore, human ingenuity will continue to be our most valuable resource as we uncover increasingly sophisticated methods for approximating such complex and lengthy mesoscopic phenomena. A vision of computational simulation of acetylcholinesterase-related biological phenomena on the grand scale is given in a recent publication of Tai [101].

For the time being, more practical methods such as QSAR will undoubtedly remain important computational tools for enzyme inhibition research. In the previous section, some near-term plans were discussed for the development and refinement of predictively robust AChE toxicity QSAR models. Attaining reliable and physically intuitive descriptors, in this case, is proposed *via* detailed analysis of the system through higher level modeling techniques such as quantum chemistry and MD. The basic assumption behind this approach is that many important phenomena governing the enzymatic process (e.g., chemical reaction mechanisms, intra-receptor diffusion, etc.) occur in a fundamentally similar fashion across a broad range of potential inhibitors, and that the differences in behavior from one instance to another can be distilled down to simple differences in the structure and properties of the ligands themselves as elucidated from detailed atomic simulations that are localized in space (i.e., only covering a single AChE unit for

MD, or even just a fragment of a unit for quantum) and, if dynamic, occurring over a relative short (ns) time scale.

A similar strategy of integrating results from detailed atomic level simulations may also prove useful in further refining our COMBINE representation of non-covalent binding. One of the most important limitations in the current model is the approximate treatment of receptor-related dynamic effects, incorporated empirically within statistically trained weights factors applied to the relative enthalpic contribution of different receptor residues to the net ligand binding affinity. In reality, it is very difficult to extract all relevant relaxation-related entropic contributions from a system without some explicit account of the real torsional motions from whence they originate. One possible scheme for recovering a substantial portion of these dynamic effects in a computationally viable fashion is by isolating characteristic receptor conformers from the trajectory of a MD simulation and expressing each ligand-receptor complex not as a static single structure, but rather as a linear combination of multiple different complex conformers. As discussed in Section IV, prior work by Kua *et al.* [46] sought to implement such a multi-conformational receptor model, although the conformer selection used (fixed-period snapshots every 1 ps over the course of a 1 ns simulation) has the deficiencies of extensive redundancy (typically sampling numerous very similar conformers) and of not ensuring the inclusion of rare-event conformers that are often critical to biochemical processes [102]. As a more discriminating alternative, we plan instead to use an intelligent feature-selection based technique [103] to extract from MD trajectories a small targeted set of structurally unique receptor conformers that are chosen to optimize coverage of conformational space. Flexibly docking ligands into this more modest-sized but also more diverse ensemble of receptor conformers should enhance our ability to approximate a true flexible docking simulation and entail only a small fraction of the computational cost of a more arbitrary sampling.

While QSAR and COMBINE methods (and their hitherto unpublished future variants) will undoubtedly continue to provide valuable predictions for a wide range of inhibitive issues, they remain limited in their ability to predict phenonema or behavior too far beyond the breadth of their empirical programming. This is to say that a model parametrized under one set of conditions (e.g., type of chemical inhibitive mechanism or physical binding mode) may yield predictions of questionable accuracy when extrapolated to another disparate set of conditions (e.g., different mechanism or binding mode, mutated receptor, significant differences in ligand chemical functionality, etc.). In this respect, such methods can be viewed as somewhat *inflexible*. The desire to probe AChE modulation under diverse conditions of inhibition, mutation, environment, etc., has perpetuated a strong interest in more methodologically *flexible* models capable of reliably predicting response to variable or unusual stimuli. For this reason, MD and quantum chemical simulations (and combinations thereof) will almost certainly remain important tools of choice in that they are highly adaptive, and thus generate modes that effectively respond to situations that may not have been anticipated and empirically encoded into the original paradigm.

The drawback with both quantum mechanical methods and MD simulations is computational cost. Treating an entire enzyme quantum mechanically at an accuracy level sufficient for qualitative reproduction of even fairly basic phenomena is very challenging, and while computational capacity is gradually rising to the task it is our assumption that complex geometry searches (e.g., transition states, internal reaction coordinations, shallow local minima, etc.) and quantum MD studies of reasonable length (more than a few ps) will remain impractical for the foreseeable future and that scientific progress will continue to depend as much on new algorithmic tricks and judicious computational shortcuts as on brute-force large scale computations. Conversely, for classical MD simulations it is already very feasible to model entire proteins *via* fully atomic models, however the time scales in which to resolve many phenomena of interest (frequently μs for ligand diffusion, loop restructuring or receptor relaxation) remain far beyond our current capacity (tens of ns). One wonders, therefore, what sort of refinements and modifications will be made to these techniques in order to enhance their adequacy for simulating biologically meaningful systems, properties and phenomena?

The time scale issue is probably the single most challenging barrier to a biologically realistic treatment of deep / narrow gorge enzymes such as AChE. In such cases, the steric constraints make it increasingly probable that particle diffusion will be the primary rate determining step in such embedded receptor structures. The corresponding diffusion kinetics are frequently governed by rare event phenomena whose incidences are most readily ascertained through computationally demanding MD simulations. Conventional MD simulations of systems as large as proteins currently tend to be limited to the nanoseconds time scale, at which level they frequently produce inadequate sampling of all potentially relevant rare events. Accelerated MD methods may yield a reasonable solution to this dilemma, although the precise method of acceleration must be carefully validated against known physical behaviors before any degree of trust may be placed in the predictions for hitherto uncharacterized processes. Temperature accelerated molecular dynamics for ligand diffusion into an inherently flexible system (as is the case for many enzyme receptors including the AChE active site) may, in addition to speeding up physically reasonable processes, induce unphysical motion and thus compromise simulation accuracy. Other methods require advance specification of a subset of the accessible potential energy surface to be explored, ultimately prejudicing the system to intuitively straightforward conformers and thus falling into the methodological *inflexibility* trap that we seek to avoid. Other accelerated methods, however, may well prove suitable. One such method is that of Hamelberg *et al.* [102] wherein barriers are softened *via* bias potentials. Initial signs that this scheme reproduces known equilibrium structures and identifies plausible rare event conformers make it an encouraging prospective mode of analysis for intra-cavity ligand diffusion and prediction of complementary receptor motion [104].

On another front, the work of Song *et al.* has demonstrated a push to larger length scales through a novel application of continuum (i.e., non-atomistic) mechanics [105]. This work utilizes a finite element (FE) solution of the steady-state Smoluchowski equation to model diffusion through the gorge of both monomeric and tetrameric AChE and thereby estimate diffusion-controlled ligand binding rate constants [105,106]. This audacious approach has been tuned to the AChE system to yield calculated reaction rates in reasonable agreement with experimental data at far less computational cost than similar Brownian Dynamics (BD) simulations.

Incorporation of reactive processes entails another set of challenges. QM/MM formalisms provide a promising paradigm for taking simultaneous account of whole protein structural properties and localized reactive or polarizational phenomena. People have envisioned applying such techniques toward the treatment of concurrent dynamic and reactive phenomena so as to permit receptor dynamics to exert their due influence on reactive processes. Careful design and validation is likely still required before effective application is made to the AChE system, however, in that broadly available IMOMM-based methods have exhibited significant deficiencies in their ability to reproduce essential interactions in AChE-OP complexes [83]. It is likely that such problems can be largely mitigated through developement of carefully tuned polarization schemes for softening the quantum-classical boundary, and through careful analysis of forces across the boundary to ensure that the quantum and classical regimes are in an appropriate balance.

Even given a microscropically robust QM/MM formalism, however, there are major practical challenges to be addressed before dynamic reactive simulations are viable across a reasonable spread of biologically relevant time scales. The task of recomputing a wavefunction at every time step in a lengthy trajectory is currently impractical, even for relatively modest sized quantum shells. Integration of classical MD with a Carr Parrinello representation of the quantum region provides somewhat enhanced efficiency [107] by virtue of intelligently extrapolating an estimated wavefunction from step to step, however the simulation length is currently limited to the picosecond time domain and thus of only limited applicability. A possible solution to this dilemma may be achieved at some point by selectively switching on the localized quantum mechanical representation only at times when it is likely to have a tangible impact on the accuracy of the structural evolution. The main kinetic limitation for most chemical reactions tends to be a low probability of achieving conformations suitable to activated complex formation. Once the trajectory zeroes in on an activated complex, most chemical reactions occur relatively quickly if accorded sufficient energy along the reactive vibrational mode. To the best of our knowledge, such adaptive methods have not been published yet, however it is possible that they will ultimately provide a reliable basis for dynamic reactive simulations on biologically useful time scales.

The inherent complexity of the AChE enzyme system and its importance to a number of critical health related issues have colluded to yield a vibrant subdiscipline of computational enzymology that is driving the development of innovative, increasingly reliable and efficient simulation techniques that are transforming the computational chemistry field. The fact that a Google search (ca. April, 2005) on the terms "acetylcholinesterase" and "simulation" of "computational" or "modeling" yields approximately 22,900 hits is a testament to the current importance of the topic. While the breadth of computational research in the AChE modeling arena is too great to fully capture in a single review article, we hope to have provided a reasonable overview of some of the more important contributions and their potential implications for future therapeutics discovery.

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