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Understanding the Enzyme-Ligand Complex: Insights from All-Atom Simulations of Butyrylcholinesterase Inhibition

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Abstract

All-atom molecular dynamics simulations of butyrylcholinesterase (BChE) *sans* inhibitor and in complex with each of fifteen dialkyl phenyl phosphate derivatives were conducted to characterize inhibitor binding modes and strengths. Each system was sampled on the 250 ns timescale in explicit ionic solvent, for a total of over 4 μ s of simulation time. A K-means algorithm was used to cluster the resulting structures into distinct binding modes, which were further characterized based on atomic-level contacts between inhibitor chemical groups and active site residues. Comparison of experimentally observed inhibition constants (K_i) with the resulting contact tables provides structural explanations for relative binding coefficients and highlights several notable interaction motifs. These include ubiquitous contact between glycines in the oxyanion hole and the inhibitor phosphate group; a sterically-driven binding preference for positional isomers that extend aromaticity; a stereochemical binding preference for choline-containing inhibitors, which mimic natural BChE substrates; and the mechanically-induced opening of the omega loop region to fully expose the active site gorge in the presence of choline-containing inhibitors. Taken together, these observations can greatly inform future design of BChE inhibitors, and the approach reported herein is generalizable to other enzyme-inhibitor systems and similar complexes that depend on non-covalent molecular recognition.

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Keywords

organophosphate; reversible inhibition; contact table; omega loop; docking

I. INTRODUCTION

Alzheimer's Disease, Parkinson's Disease, and preeclampsia are all associated with overactivity of butyrylcholinesterase, or BChE (Darvesh, Hopkins, & Geula, 2003; Dong et al., 2017; Giacobini, 2001; Greig et al., 2005; Mesulam et al., 2002; Rahimi et al., 2013), a non-specific enzyme that hydrolyzes the neurotransmitter acetylcholine and other choline-based esters (Allderdice et al., 1991). Because these health problems continue to afflict the population, the development of selective and potent BChE inhibitors continues to be a valuable area of research (Akincio lu et al., 2017; Cavallaro, Moglie, Murray, & Radivoy, 2018; Giacobini, 2004; Kamal et al., 2008; Senol et al., 2017; Yu et al., 2017). Organophosphorous compounds constitute one class of inhibitors that have been explored thus far (Fukuto & Metcalf, 1956; Hong & Raushel, 1999; Moretto, 1998). Structurally, they are related to metrifonate, an irreversible inhibitor of acetylcholinesterase, or AChE, shown to increase cognitive ability in patients suffering from Alzheimer's Disease (Farlow & Cyrus, 2000).

We previously explored the inhibitory potential of dialkyl phenyl phosphates (DAPs) and, based on the success of that initial study (Law et al., 2007), we opted to systematically synthesize, assay, and model derivatives of the dibutyl phenyl phosphate species (Nakayama et al., 2017). This report builds on that previous effort by exploring the binding mechanics of DAP derivatives through all-atom, explicit solvent, molecular dynamics (MD) simulations. Indeed, this proof-of-concept effort presents an approach to understanding and exploring the binding mode space of molecular complexes via rigorous MD simulations and a novel binding mode analysis based on high-dimensionality clustering and visualization of interactions between chemical groups in each inhibitor and the residues that compose the BChE active site gorge.

This gorge, or pocket, is approximately 690 Å³ in volume (Pezzeменти, Nachon, & Chatonnet, 2011) and, as represented in Figure 1, contains a number of subsites that play various roles during catalysis. Substrates approaching BChE first interact with residues in the peripheral anionic site, or PAS (Masson, Froment, Bartels, & Lockridge, 1996; Masson et al., 1997), which passes the substrate further into the gorge (Chatonnet & Lockridge, 1989; Masson et al., 1996; Masson et al., 1997; Nicolet, Lockridge, Patrick Masson, Fontecilla-Camps, & Nachon, 2003), where the acyl binding site, or ABS (Dighe et al., 2016; Pezzementi et al., 2011; Wandhammer et al., 2011), and the choline binding site, or CBS (Biberoglu, Tacal, & Akbulut, 2011; Dighe et al., 2016; Pezzementi et al., 2011), assist in the positioning of the substrate for catalysis. At the bottom of the gorge lie the catalytic triad, or CAT (Chiou, Huang, Hwang, & Lin, 2009; Zhan & Gao, 2005), and the oxyanion hole, or OAH (Warshel, Naray-Szabo, Sussman, & Hwang, 1989; Zhan & Gao, 2005). The CAT of BChE operates similarly to the Ser-His-Asp motif seen in many serine proteases, with the aspartic acid residue replaced by glutamic acid (Warshel et al., 1989). During

catalysis, the OAH stabilizes high-energy anionic intermediates and transition states via hydrogen bonding. Additionally, the omega-loop, or OML (Fetrow, 1995; Masson, Xie, Froment, & Lockridge, 2001), resides near the binding pocket entrance and can, depending on the interactions of the inhibitor with active site gorge residues, seal off the gorge via a capping mechanism or, as we explore later, shift to fully expose the pocket in the presence of certain choline-containing inhibitors that were initially proposed in an effort to mimic natural substrates.

II. METHODS

A model of human BChE was prepared by removing all water molecules, ions, and ligands from the crystal structure (PDB ID: 1P0I), inserting missing atoms and sidechains (none of which were near to, or part of, the enzyme active site gorge), and performing geometry optimization on these regions using Accelrys Discovery Studio (BIOVIA, 2007). The resulting structure was then energy minimized, including sidechain rotamer relaxation, using the SwissPDB software (Guex & Peitsch, 1997). Simulated inhibitor molecules were modeled using the General AMBER Force Field (Wang, Wolf, Caldwell, Kollman, & Case, 2004), which was designed in tandem with partial charge calculation via the semi-empirical (AM1) method with bond charge correction (BCC) to match the molecular electrostatic potential computed at the Hartree-Fock 6-31G* theory level (A. Jakalian, Bush, Jack, & Bayly, 2000; Araz Jakalian, Jack, & Bayly, 2002). Partial charges were calculated using the Quacpac Tool Kit from OpenEye Scientific (Ellingson et al., 2014).

All-atom molecular dynamics simulations of native BChE *sans* inhibitor, and of the protein in complex with each inhibitor described below, were performed using GROMACS 4.5.3 software suite (Pronk et al., 2013). The protein and counterions were modeled using the AMBER03 force field (Duan et al., 2003) ported to the GROMACS suite (Sorin & Pande, 2005) and solvated with the TIP3P explicit water model (Mahoney & Jorgensen, 2000). To optimize simulation time a periodic octahedral box was used, yielding a total system size of approximately 72,350 atoms. All simulations were performed in the NPT ensemble at 1.0 atm and 300 K using the Berendsen and modified-Berendsen barostat and thermostat, respectively (Berendsen, Postma, van Gunsteren, DiNola, & Haak, 1984; Bussi, Donadio, & Parrinello, 2007), with a 2.0 fs timestep using the LINCS algorithm (Hess, Bekker, Berendsen, & Fraaije, 1997) to constrain bonds involving hydrogen atoms. A switching function from 7 Å to 9 Å and a standard long-range correction term were applied to van der Waals interactions, and electrostatic interactions beyond 9 Å employed a reaction-field treatment with a dielectric coefficient of 80.

The ICM Pro computational suite (Abagyan, Totrov, & Kuznetsov, 1994; J., M., & R., 2005) was used to perform 10,000 molecular docking trials of each inhibitor within the active site gorge of BChE, where the best scoring docked structure was taken as the MD starting conformation for each inhibitor. Five simulations per BChE-inhibitor complex were then collected, with an average simulation time of approximately 60 ns, thereby yielding 4.23 μs of total sampling with structures stored every 100 ps with the initial 10.0 ns equilibration period of each simulation excluded from the analysis reported below. While this is an appreciable timescale on which to simulate systems of this size in atomistic detail, it is

important to emphasize that the limited sampling per complex reported herein is not expected to have reached, or to provide information regarding, conformational equilibrium or statistically relevant populations of the observed binding modes. For this reason, the characterization below focuses on the qualitative nature of protein-inhibitor binding.

Following 3D alignment of each resulting complex structure to a reference protein structure, each conformation was characterized by the position of the inhibitor relative to the protein center-of-mass and vectors internal to the inhibitor representing the orientation of the three constituents bonded to the phosphate group, as well as a vector normal to the aromatic group to capture rotational position. These vector components were then used to cluster inhibitor conformations into thermodynamic microstates, or binding poses. To address the primary limitation present in most K-means algorithms, the need to know *a priori* how many data clusters are present in a given data set (Shao, Tanner, Thompson, & Cheatham, 2007), a modified K-means algorithm that initially overestimates the number of clusters present in the data and then slowly eliminates empty clusters (Sorin & Pande, 2005) was employed. The most statistically dominant binding poses were then characterized in terms of specific interactions between chemical groups within the inhibitor and the binding pocket residues of the enzyme. Atomic interactions for each binding mode were identified as protein atoms within 5.0 Å of inhibitor phosphate or aromatic group atoms, or within 3.0 Å of alkyl or choline group atoms, that occurred with a frequency of 0.25 or higher.

III. RESULTS AND DISCUSSION

A. Reference Inhibitor and Contact Tables

As the largest of the dialkyl phenyl phosphates (DAPs) that did not encounter solubility issues during assay in our recent collaborative study (Nakayama et al., 2017), dibutyl phenyl phosphate (DAP4) serves as the reference structure to which the inhibitors studied herein are compared. This moiety has a measured K_i value of $94.5(\pm 9.0)$ μM and an optimal ICM Pro docking score of -82.6 . A total MD simulation time of 268.9 ns was collected over five simulations for DAP4. To assess the stability of our simulated complexes, the all-atom root-mean-square deviation (RMSD) of the protein was monitored in tandem with the distance between the center-of-mass of the catalytic triad and the inhibitor center-of-mass, which are shown in Figure 2a for the BChE-DAP4 reference complex. While the inhibitor is quite dynamic with respect to the catalytic triad location, the protein RMSD remains below 3.25 Å in all but one simulation, during which a maximum protein RMSD of 4.04 Å was observed.

The clustering of structures from our DAP4 simulations yielded nine statistically relevant binding poses. To assess the quality of this clustering result, we defined the similarity score for any two structures as the root-mean-squared difference between the min-max normalized clustering vectors for those structures, where highly-similar inhibitor structures would thus yield low numeric scores. Figure 2b shows the resulting similarity matrix for our DAP4 clustering. As expected, intra-cluster similarities along the diagonal are generally very good and off-diagonal comparisons demonstrate far lower inter-cluster similarity. Given our focus on small molecules in non-covalent complex with a large protein, it is not unexpected to see some similarity between a limited number of cluster pairs, as multiple clusters can include a significant number of shared protein-inhibitor interactions. This does not imply, however,

that the employed clustering method or resulting binding modes have been optimized, and we are currently evaluating approaches to best describe the protein-inhibitor complex in a cluster-friendly vector form that will fully overcome the heuristic nature of the K-means algorithm.

Identical structural and clustering assessments were applied to all BChE-inhibitor complexes discussed below, with each complex demonstrating similar protein stability as observed for BChE-DAP4 and each clustering result demonstrating comparable cluster similarities as observed for BChE-DAP4. For two of the simulated systems, BChE in complex with dibutyl 4-methyl-phenyl phosphate (MET4) and 3,4,5-trimethylphenyl phosphate (TRIM), a single inhibitor ejection event was observed, and data stored after these dissociation events was thus not included in clustering or the analysis of protein-inhibitor interactions.

For DAP4 and all other inhibitors studied herein, *contact tables* were generated. This tabular format, shown in Table 1 below for DAP4, identifies contacts between BChE binding pocket residues and specific chemical substituents of each inhibitor, from which the type of intermolecular interaction is inferred.

Significantly populated binding modes are listed vertically in Table 1 from the most populated (Mode 0) to the least populated, with relevant binding pocket amino acids listed horizontally, grouped as summarized in the introduction and colored to match the scheme shown in Figure 1. These groups include the peripheral anionic site (PAS, red), the catalytic triad (CAT, yellow), the oxyanion hole (OAH, orange), the choline binding site (CBS, green), the acyl binding site (ABS, blue), and the omega loop (OML, charcoal), with additional protein residues of interest also shown (APR, gray). Per the key provided at the bottom of Table 1, pastel colors are used to denote electrostatic interactions (green), hydrogen bonding (pink), cation- π interactions (yellow), π -stacking (cyan), van der Waals interactions (violet), and non-polar interactions (light blue), where the latter two differ only in title to distinguish non-polar/non-polar interactions from other possible combinations. Interactions between the inhibitor and the protein backbone are uncolored. The graphical motif presented in Table 1 will be used throughout the remainder of this article to characterize and compare interactions inherent to BChE in complex with the inhibitors studied herein.

There are several common contacts seen in the DAP4 binding modes presented in Table 1, the most notable of which are (i) the phosphate group hydrogen-bonding with HIS438 in the CAT; (ii) the phosphate group consistently hydrogen bonding with GLY116 and GLY117 in the oxyanion hole; (iii) significant van der Waals/non-polar interactions between inhibitor alkyl chains and residues SER198 (CAT), ASN397 (APR), and the five residues that compose the acyl binding site. As can be seen in Figure 3, while one alkyl group is surrounded by ABS residues in the deepest end of the active site gorge, the other engages with residues from several subgroups within the pocket, as described above. Across all observed binding modes, the phenyl group, pointing out of the plane of the page in Figure 3, forms van der Waals contact with GLN119 (PAS) and THR120 (APR), as well as π -stacking with TRP82 (CBS), PHE73 (OML), and TYR332 (PAS). These latter interactions are

assumed to stabilize small molecule binding in the absence of the cationic choline group that is present in natural substrates.

B. Methylphenyl Derivatives

The initial round of chemical modifications to the DAP4 reference species explored in our collaborative work was the methylation of the phenyl ring at the ortho, meta, and para positions (Nakayama et al., 2017). Table 2 shows the structures, total simulation times, optimal docking scores (with standard deviations), and experimentally measured K_I values for these three species, which will be referred to below as MET2 (ortho), MET3 (meta), and MET4 (para). The addition of a single methyl group allowed the methylphenyl series to outperform the reference species, DAP4, by as much as an order of magnitude (MET4).

As shown below in Table 3, the three methylphenyl derivatives exhibit many of the same contacts observed for the DAP4 reference structure, with all four inhibitors demonstrating extensive non-polar interactions between their alkyl chains and the acyl binding site, as well as interactions between the inhibitor phosphate group and the oxyanion hole. In addition, all four of these inhibitors exhibit phenyl group π -stacking with PAS residue TYR332; van der Waals contact between inhibitor butyl groups and CAT residue SER198; and further van der Waals contact between the phenyl ring and THR120, as well as between a butyl group and ASN397.

Whereas DAP4 binding modes show consistent van der Waals contact between the inhibitor phenyl group and both GLN119 in the peripheral anionic site and TRP82 in the choline binding site, these two residues are also largely in contact with butyl chains for each of the three MET species, signaling a much more diverse set of binding modes upon addition of only a single methyl group on the phenyl ring. Indeed, the methylphenyl groups in the three MET species favor π -stacking interactions with PHE76 and TYR332 in the neighboring omega loop and peripheral anionic sites, respectively, as was also observed for DAP4.

In contrast, MET2 was observed to strongly favor a single binding mode that is visualized in Figure 4a. While the tendency to occupy a single binding mode could result from a lack of sampling on sufficient timescales, the presence of multiple observed binding modes suggests that this species has a single energetically-favorable conformation available. Here, that favored mode includes π -stacking and van der Waals interactions between the phenyl group and the entirety of the choline binding site, as well as π -stacking interactions between the phenyl group and residues PHE73 and PHE76 in the OML. Figure 4a demonstrates how the phenyl group of MET2 is situated between TYR332 of the PAS, TRP82 of the CBS, and neighboring OML residues.

For the more effective of the methylphenyl inhibitors (MET3 and MET4), but not MET2, we observe a new electrostatic interaction between the phenyl group and residue MET437, as well as hydrogen bonding between the inhibitor phosphate group and residue THR120, neither of which are observed for DAP4. The most effective of these species, MET4 (visualized in Figure 4b), exhibits an additional π -stacking interaction between the phenyl group and residue TRP430, and the rotation of the inhibitor within the active site that affords these contacts also facilitates new van der Waals contact between an alkyl chain and

SER287, as well as added non-polar interactions between an alkyl group and numerous hydrophobic sidechains in the omega loop region.

C. Di- and Trimethylphenyl Derivatives

Additional methylations of the phenyl ring led to the di- and trimethylphenyl derivatives of MET3 and MET4 assayed recently (Nakayama et al., 2017) and shown in Table 4. While the addition of a second and third methyl group to the meta positions of MET4 had no appreciable impact on inhibitor strength, the di-*m*-methyl species (DIM5) shown in Table 4 improved the measured K_I value by an additional order of magnitude.

As illustrated in Table 5, this group of inhibitors maintained significant alkyl chain contact with acyl binding site and catalytic triad residues, as well as phosphate and alkyl chain contacts with residues in the oxyanion hole, and π -stacking interactions between the phenyl group and TRP82 in the CBS, all of which were observed for DAP4 and the methylphenyl series above. This clear symmetry between binding modes for these multi-methylated species and the previously discussed inhibitors justifies the relative constancy of measured K_I values for both DIM4 and TRIM shown in Table 4. In contrast to DAP4 and the MET series, the methylated phenyl group of both DIM4 and TRIM also showed significant π -stacking with TRP430, electrostatic interaction with MET437, and van der Waals contact with TYR440, all of which are shown in the APR section of Table 5.

The most potent inhibitor examined in this study, DIM5, exhibits an improved K_I value that is an order of magnitude lower than the MET4, DIM4, and TRIM analogs. This is largely due to the positioning of the DIM5 phosphate group, which forms hydrogen bonds with catalytic triad residues SER198 and HIS438, something that is seen only sparingly for the previously discussed inhibitors above. It is notable that this PO_4 -CAT hydrogen bonding is analogous to the first step in the BChE catalytic mechanism of natural substrate hydrolysis (Sirin & Zhang, 2014) and stabilizes the deep-pocket positioning of DIM5. In addition, the DIM5 phosphate group interacts not only with the GLY residues in the oxyanion hole, GLY116 and GLY117, but also with the neighboring GLY115, thereby strengthening the anchoring of the phosphate group to the protein backbone in the CAT region.

It is also noteworthy that, of the three di- and trimethylphenyl species, only DIM5 consistently maintains interaction between the inhibitor aromatic group and both GLN119 (PAS) and THR120 (APR), which were observed for DAP4 and MET series inhibitors. The capacity of GLN119 and THR120 to interact with all three types of functional groups present in the studied inhibitors reflects the amphipathic nature of these amino acids, which allows them to adopt hydrophobic and hydrophilic roles. That the versatility of these residues allows them to interact with chemically disparate functional groups suggests that these interactions are sterically driven and will persist when inhibitor size allows them to take positions adjacent to nearly any substituted functional group.

Visualizations of the primary binding modes of DIM4 and DIM5 shown in Figures 5a and 5b, respectively, demonstrate the distinctly different orientations of these species within the active site gorge in tandem with relatively similar gorge structures. In Figure 5a, the DIM4 aromatic group occupies the upper left quadrant of the pocket (from the perspective shown)

and the phosphate P=O oxygen points away from the active site toward the mouth of the gorge. In contrast, the orientation of the DIM5 phosphate group deep inside the active site, and the hydrogen bonding between phosphate group and CAT residues that results, fosters the interactions between the aromatic group and the numerous residues noted above.

D. Naphthyl-Containing Derivatives

Other than MET2, all mono-, di-, and tri-methylations of the DAP4 phenyl group discussed above showed significant impact on inhibitor binding strength. With this in mind, and the dominant role that the phenyl group aromaticity clearly plays in stabilizing inhibitor binding, a natural next step in our study was to replace the phenyl ring with a naphthyl group. As shown in Table 6, this can be done at either of two naphthyl positions. This extension of the aromatic group significantly improved inhibition, with NAP1 showing a 5-fold decrease in K_I relative to the reference DAP4 structure and NAP2 improving upon that reference K_I by approximately two orders of magnitude.

As one might expect, the contact table for these two naphthyl-containing species, Table 7, largely mimicked that of the reference DAP4 inhibitor. These interactions common to NAP1, NAP2, and DAP4 include (i) extensive naphthyl contact with GLN119 (van der Waals) and TYR332 (π -stacking) in the peripheral anionic site; (ii) significant van der Waals contact of inhibitor alkyl chains with SER198 and/or HIS438, both within the catalytic triad, as well as hydrogen bonding between the phosphate group and residue HIS438; (iii) ubiquitous interactions of the phosphate group and alkyl chains with GLY and ALA residues in the oxyanion hole; (iv) ubiquitous π -stacking of the naphthyl group with TRP82, along with electrostatic interaction between the phosphate group and PHE329, in the choline binding site; (v) dominant non-polar interactions between inhibitor alkyl and naphthyl groups with all residues in the acyl binding site; and (vi) consistent van der Waals contact of the naphthyl group with THR120.

Such similar binding profiles in comparison to DAP4 immediately raise the question *what leads to the much-improved K_I values for these two naphthyl-containing species?* Indeed, NAP2 ranks a very close second to the most potent inhibitor studied, DIM5. With the same aromatic chemistry in play, the difference here is expected to result from steric limitations inherent to the larger naphthyl group, which should then lead to the slight repositioning of other inhibitor functional groups. Indeed, visual comparison of observed binding modes of NAP2 and DIM5, which have nearly identical K_I values, shows that these species sample nearly identical interactions with the active site. Unlike NAP2, however, DIM5 is able to form an additional phosphate-to-GLY115 hydrogen bond, which effectively extends the oxyanion hole to the neighboring GLY115 residue.

As shown in Table 7, both NAP1 and NAP2 exhibit added interaction between inhibitor alkyl chains and the backbone of SER287 in the PAS, as well as consistent contact between the inhibitor phosphate group and GLU197, neither of which are significant for the DAP4 reference inhibitor. The differences between additional contacts observed in NAP1 and NAP2 should then explain their relative potencies. To begin with, NAP1 exhibits some naphthyl π -stacking with omega loop residues PHE73 and PHE76, as was also observed for DAP4, and the NAP1 naphthyl group also shows prominent van der Waals contact with

GLN71 in the omega loop, which is not seen for DAP4. The sterically less-extended covalent connection of the naphthyl group to the phosphate in NAP1 thus favors these interactions between the NAP1 naphthyl group and omega loop residues, which are only minimally present in our NAP2 simulations. Indeed, an examination of omega loop positioning and motion after the initial equilibration period within our simulations confirms that NAP1 interactions with the OML hold that structure relatively in place, whereas the lack of NAP2 interactions with the OML allows for significantly larger deviation from the initial docked structure and more flexibility overall.

The visualizations of NAP1 and NAP2 in Figures 6a and 6b, respectively, demonstrate this distinct difference in their omega loop positioning. While the NAP1 naphthyl appears to protrude farther toward the active site entrance, in relatively close proximity to omega loop residues, the analogous NAP2 naphthyl group favors a more internal orientation, yielding greater contact areas with active site residues in the PAS and CBS, and leaving the OML residues largely without tether. The more extended structure resulting from the covalent linkage between the NAP2 naphthyl group and the phosphate also promotes consistent van der Waals contact between inhibitor alkyl groups and ASN397, which was ubiquitous in our BChE-DAP4 simulations yet went unobserved in our BChE-NAP1 sampling. Overall, the seemingly minimal difference in positioning of the naphthyl group within the inhibitor has large scale consequences in three dimensional space that result in dramatically different enzyme active site structure and dynamics, with seemingly minor differences in enzyme-inhibitor interactions, yielding a 10-fold difference in binding affinity.

E. Choline-Containing Derivatives

As a final deviation from the basic DAP structure, a choline group, $-\text{CH}_2\text{CH}_2\text{N}^+(\text{CH}_3)_3$, was substituted in place of one of the two alkyl chains in an effort to mimic the chemistry of natural BChE substrates such as acetyl- and butyrylcholine. As this substitution results in stereoisomerism, the (*R*) and (*S*) forms were examined separately *in silico* alongside minor changes to the phenyl and alkyl substituents, as shown in Table 8. Although we have not yet published the experimental components of this study, racemic mixtures of several choline-containing DAP analogs were synthesized and assayed as done in our previous report (Nakayama et al., 2017), leading to the K_i values presented in Table 8, and we are currently in the process of investigating the potential impact of the absolute stereochemistry on inhibitor binding strength.

As predicted by the docking scores for the CP4 and CB4 species in Table 8, the choline-containing inhibitors exhibit binding modes that depend directly on stereochemistry. The binding modes of (*S*)-enantiomers, shown in Table 9, largely reflects those of the reference DAP4: the OAH binds predominantly with the phosphate group, all residues in the ABS form non-polar van der Waals interactions with the alkyl group, the phenyl group forms π -stacking interactions with TYR332 in the PAS, and both the phosphate and phenyl groups interact with PHE339 in the CBS. The most prominent difference observed between our simulations of (*S*)-enantiomers and those of DAP4 was an increase in binding between the choline and phenyl groups with numerous amino acids in the surrounding APR, particularly residues GLU197, TRP430, MET437, and TYR440. The additional electrostatic and π -

stacking interactions seen for (*S*)-enantiomers of the larger choline-containing inhibitors is thus expected to enhance the inhibition of their dialkyl analogs.

In contrast, (*R*)-enantiomers engage in qualitatively different binding behavior than that of DAP4, with contacts characterized by specific phosphate, phenyl, and choline interactions within the active site gorge and the alkyl group solvated. The choline group supplants the previously seen phenyl ring position to interact with PAS and CBS residues, and also engages in electrostatic interactions with GLU197 in the APR and both MET81 and GLN71 in the OML. This repositioning of the inhibitor relative to DAP4 binding allows (*R*)-enantiomers to maintain interactions between the phosphate group and GLY116 that were observed for DAP4, and replaces the similar phosphate-GLY117 interactions seen in DAP4 with phenyl-GLY117 contacts. The ubiquity of this contact between the phosphate (and now, phenyl) group with GLY116 and GLY117 emphasizes the importance of the oxyanion hole and the role it can play in stabilizing electron-dense regions of strong inhibitors.

In comparing the (*S*)- and the (*R*)-enantiomers, there is thus a discernible absence of choline and alkyl contacts, respectively: that is, choline substitution of the pro-*S* butyl group with the choline moiety leaves interactions with the binding site largely unchanged, while choline substitution of the pro-*R* butyl group effectively replaces contacts between the alkyl group and the active site gorge with choline-gorge contacts. That electrostatics is a dominant intermolecular force cannot be underemphasized here, and the effective replacement of non-polar interactions with electrostatic interactions undoubtedly contributes significantly to the improved K_I value observed for the racemic CP4 mixture in comparison to the reference DAP4. It is therefore natural to accept the predictions of both docking calculations and MD simulations that predict the (*R*) form of CP4 and other choline-containing DAP analogs as the more inhibitory stereoisomers, which largely determine the degree to which these choline-containing species outperform their DAP analogs. Initial synthetic and assay efforts to evaluate CP4r and CP4s inhibition constants suggest that the (*R*) stereoisomer is indeed the stronger inhibitor (to be published).

F. The Omega Loop & TRP82

It is well established that the omega loop of AChE undergoes conformational changes in the presence of substrate and some reversible inhibitors (Shi, Radi, & Taylor, 2002), and also exhibits a gating behavior that imparts acetylcholine specificity by physically blocking substrate access to the active site for larger substrates (Stank, Kokh, Fuller, & Wade, 2016; Zhou, Wlodek, & McCammon, 1998). In contrast, the OML region of BChE in our simulations does not exhibit this gating behavior in the absence or presence of inhibitors. Rather, we observe conformational changes including (i) an “open” state in which the OML is fully solvent-exposed and allows access of small molecules to the active site gorge (Figure 1a), alongside (ii) a structural preference of the active site gorge for “closed” configurations via two possible mechanisms. The first involves the closing of the OML into the mouth of the active site gorge, resulting in “capping” of the gorge (Figure 8a), in which hydrophobic residues within the OML and the ABS are in contact. In the second, the peripheral anionic site and acyl binding site come together to “pinch off” access to the gorge (Figure 4a, with

MET2 bound), and the link between the PAS and the OML results in the OML being pulled into a partially closed position.

These two closed conformations (capped and pinched) were observed frequently for non-choline-containing inhibitors, some choline-containing (*S*)-enantiomers, and BChE *sans* inhibitor. That these conformations are observed in the absence of inhibitors establishes that they are natural states of the protein and, more specifically, the active site. While the limited sampling reported herein does not allow us to approximate the statistical weights of these closed states or the open state represented by Figure 1a, that these closed conformations appear to dominate our sampling suggests that OML position and dynamics plays an important role in the inhibition process, and one might then surmise that inhibitors of proper size, geometry, and/or chemistry might be more omega loop compatible, or complementary, than other inhibitors.

Indeed, the choline-containing (*R*)-enantiomers appear to achieve such complementarity with the OML and the adjacent choline binding site. The binding of (*R*)-enantiomers, as discussed above, includes consistent choline cation- π interactions with TRP82. The result of this interaction and the binding orientation that fosters it, particularly for the more strongly binding CP4r and CB4r species, is the observation of a fourth omega loop state in which the OML is mechanically leveraged into a wide open and rigid conformation (Figure 8b) that exposes the active site gorge to a greater extent than the natural “open” state seen in Figure 1a. A plot of the change in root-mean-squared fluctuation per residue (RMSF) is shown in Figure 8c. As shown there, the flexibility of omega loop residues is greatly reduced when CP4r or CB4r are bound. This, of course, raises the question: *what is the purpose of such mechanical leveraging of the omega loop into an open position that fully exposes the active site gorge?*

We speculate that this mechanism acts to counter the observation that closed conformations of the gorge and omega loop are thermodynamically preferred for BChE. That is, trapping of the products of catalysis by closed-gorge conformations, via capping or pinching, would not provide those products to the local area in which they are needed, and would instead temporarily lock the enzyme in a non-functional state. However, if natural substrates such as butyrylcholine exhibit this mechanical leveraging of the OML into an open position, thus exposing the active site gorge, increased rates of product emission and substrate arrival would be expected, thereby contributing to BChE efficiency and providing product species to the local environment.

IV. CONCLUSION

The clustering of structures from all-atom MD simulations allowed for characterization of distinct BChE-inhibitor binding modes, which are intricate and fluid three dimensional engagements that are concisely summarized via contact tables. Examination of these contact tables revealed several motifs of particular interest. First, the enlargement of a specific functional group by substitution of a chemically-similar species, such as the replacement of a phenyl ring with a naphthyl group, allows the derivative species to maintain many of the originally observed contacts while potentially increasing the area of contact between that

group and the active site gorge. Second, contacts formed by oxyanion hole residues GLY116 and GLY117 are ubiquitous, appearing in nearly every binding mode for every inhibitor studied, and most often involve the inhibitor phosphate group, which replaces the anionic transition state that is inherent to catalysis. Future design of cholinesterase inhibitors might then begin by assuming significant phosphate-OAH contact and constructing outwardly from there. Third, and most surprising, is the clear and significant dependence of binding strength on stereochemistry: (*S*)-enantiomers studied herein maintained the binding behavior of the base structure, DAP4, whereas (*R*)-enantiomers consistently adopted different binding poses that employed electrostatic interactions to strengthen BChE-inhibitor contact. Lastly, splaying of the active site gorge upon formation of choline-CBS contact effectively counteracts the thermodynamic favoring of closed-gorge BChE conformations, which we attribute to molecular evolution toward more efficient catalysis.

More generally, this work presents a method to ascertain and graphically display the observed binding modes of a simulated protein-inhibitor complex. As raw computational power, algorithmic sophistication, and large-scale resources now enable massive sampling (Borhani & Shaw, 2012; De Vivo, Masetti, Bottegoni, & Cavalli, 2016; Sorin et al., 2017), it is important to regularly assess both our computational models and the absolute predictions they make. With that in mind, and given the success of this initial proof-of-concept effort that employed limited computational sampling, we are currently simulating BChE-inhibitor complexes on a much larger scale with the aim of accumulating statistically relevant (and, therefore, thermodynamically relevant) sampling of binding modes and omega loop positions available to these complexes and to the protein *sans* inhibitor. We are also exploring the use of more sophisticated descriptors of inhibitor-active site contacts, with the goal of systematically identifying and discriminating between similar but non-identical binding modes in a fully reproducible and non-heuristic manner. It is expected that by significantly enhancing both the per-complex sampling and the quality of the clustering vector that describes protein-inhibitor interactions will allow for the most accurate predictions of statistical weights and relative free energies of all sampled binding modes for a given complex.

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Figure 1. Visualization of BChE in grayscale ribbon mode with active site residues shown as semi-transparent van der Waals surfaces and colored according to the inset color key, as described in the text, (a) facing into the active site gorge and (b) rotated 90° about the vertical axis.

Figure 2.

(a) All-atom RMSD of BChE (bottom) and absolute distance between the catalytic triad center-of-mass and inhibitor center-of-mass (top) for the first 60.0 ns of DAP4 simulations. (b) Similarity matrix for DAP4, which shows the root-mean-squared difference between descriptor vectors between and within each cluster of the DAP4 sampling, where lighter and darker shades represent higher and lower similarities, respectively. The matrix is sorted from most populated cluster 0 to least populated cluster 8.

Figure 3. Magnified view of the BChE active site with DAP4 bound following the graphical conventions described in Figure 1. The structure shown is the average structure from the most stable binding mode observed.

Figure 4.

Magnified view of the BChE active site with (a) MET2 and (b) MET4 bound following the graphical conventions described in Figure 1. The structures shown are the average structures from the most stable binding modes observed.

Figure 5. Magnified view of the BChE active site with (a) DIM4 and (b) DIM5 bound following the graphical conventions described in Figure 1. The structures shown are the average structures from the most stable binding modes observed.

Figure 6. Magnified view of the BChE active site with (a) NAP1 and (b) NAP2 bound following the graphical conventions described in Figure 1. The structures shown are the average structures from the most stable binding modes observed.

Figure 7:
Magnified view of the BChE active site with (a) CP4r and (b) CP4s bound following the graphical conventions described in Figure 1. The structures shown are the average structures from the most stable binding modes observed.

Figure 8.

Magnified view of the BChE active site, following the graphical conventions described in Figure 1, with (a) DAP4 bound inside a capped gorge structure; and (b) CP2r bound inside a wide open gorge structure. (c) Change in atomic root-mean-squared fluctuations of residues in and around the omega loop region of BChE, as compared to the enzyme *sans* inhibitor, are shown for the enzyme in complex with the reference inhibitor DAP4 (black), CP2r (green), CP4r (blue), and CB4r (red). Omega loop residues not assigned to adjacent active site regions are denoted by vertical lines.

Table 1.
Contacts observed in the most populated binding modes of the dibutyl phenyl phosphate reference inhibitor

Inh. & Mode	ASN8	ASP70	GLN119	ALA277	SER287	TYR332	SER198	GLU325	HIS438	GLY116	GLY117	ALA159	TRP82	ALA328	PHE329	TRP231	PRO285	LEU286	VAL288	PHE388	ILE69	GLN71	PHE73	PRO74	GLY75	PHE76	MET81	ASN63	SER79	TYR114	GLY115	PHE118	THR120	TYR128	GLU197	ASN397	TRP430	MET437	GLY439	TYR440	ILE442					
	DAP4		PAS				CAT			OAH			CBS			ABS					OML										Additional Protein Residues (APR)															
0			Ph				Alk		PO ₄	PO ₄	PO ₄		Ph		PO ₄	Alk	Ph	Alk	Alk	Alk			Ph										Ph			Alk										
1			Ph				Ph	Alk		PO ₄	PO ₄		Ph		PO ₄	Alk	Ph	Alk	Alk	Alk	Ph		Ph										Ph			Alk										
2	Ph		Ph					Alk		PO ₄	PO ₄				PO ₄	Alk	Alk	Alk	Alk	Alk		Ph				Ph								Ph			Alk									
3			Ph				Ph	Alk		PO ₄	PO ₄	Alk	Ph		PO ₄	Alk	Alk	Alk	Alk	Alk												PO ₄			Ph			Alk								
4	Ph		Ph		Alk	Alk	Alk		Alk	PO ₄	PO ₄	Alk	Ph		Alk	Alk	Ph	Alk	Alk	Alk													Ph			Alk										
		Electrostatic		Hydrogen Bonding		Cation- π		π -stacking		van der Waals		Non-polar		Backbone																																

Table 2.

Sampling, docking, and assay results for the methylphenyl inhibitors

Code	Nomenclature	Structure	Total Time (ns)	Docking Score	K_I (μ M)
MET2	dibutyl 2-methyl-phenyl phosphate		198.0	-94.88 (± 0.01)	50 (± 16)
MET3	dibutyl 3-methyl-phenyl phosphate		297.3	-95.14 (± 0.03)	16 (± 2)
MET4	dibutyl 4-methyl-phenyl phosphate		311.3	-95.87 (± 0.14)	8 (± 1)

Table 3:

Contacts observed in the most populated binding modes of the methylphenyl inhibitors

Inh. & Mode	ASN68	ASP70	GLN119	ALA277	SER287	TYR332	SER198	GLU325	HIS438	GLY116	GLY117	ALA199	TRP92	ALA328	PHE329	TRP231	PRO285	LEU286	VAL288	PHE398	ILE69	GLN71	PHE73	PRO74	GLY75	PHE76	MET81	ASN83	SER79	TYR114	GLY115	PHE118	THR120	TYR128	GLU197	ASN397	TRP430	MET437	GLY439	TYR440	ILE442																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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3			Alk		Alk	Alk	Alk		Alk	Alk	Alk		Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk	Alk			Alk	Alk			Alk	Ph				Alk			Alk					Ph																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
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Electrostatic

Hydrogen Bonding

Cation- π π -stacking

van der Waals

Non-polar

Backbone

Table 4.

Sampling, docking, and assay results for the di- and trimethylphenyl inhibitors

Code	Nomenclature	Structure	Total Time (ns)	Docking Score	K_I (μ M)
DIM4	3,4-dimethyl phenyl phosphate		298.4	-98.69 (± 0.56)	9 (± 1.2)
DIM5	3,5-dimethylphenyl phosphate		303.4	-100.24 (± 0.79)	1 (± 0.4)
TRIM	3,4,5-trimethylphenyl phosphate		309.7	-100.69 (± 0.86)	11 (± 1)

Table 5.
Contacts observed in most populated binding modes of the di- and trimethylphenyl inhibitors

Inh. & Mode	ASN68	ASP70	GLN119	ALA277	SER287	TYR332	SER198	GLU325	HIS438	GLY116	GLY117	ALA199	TRP82	ALA328	PHE329	TRP231	PRO285	LEU286	VAL288	PHE398	ILE69	GLN71	PHE73	PRO74	GLY75	PHE76	MET81	ASN83	SER79	TYR114	GLY115	PHE118	THR120	TYR128	GLU197	ASN397	TRP430	MET437	GLY439	TYR440	ILE442		
DIM4	PAS						CAT			OAH			CBS			ABS					OML						Additional Protein Residues (APR)																
0						Ph	Alk		Ph	PO ₄	PO ₄		Ph	Ph	Ph	Alk	Alk	Alk	Alk	Alk										PO ₄		PO ₄				Ph		Ph	Ph				
1							Alk		Ph	PO ₄	Alk		PO ₄	Ph	Ph	Alk	Alk	Alk	Alk	Alk								Ph					PO ₄		PO ₄			Ph		Ph	Ph		
2						Ph	Alk		Alk	PO ₄	Alk		Ph		PO ₄	Alk	Alk	Alk	Alk	Alk		Ph		Ph										PO ₄				Ph		Ph	Ph		
3		Ph	PO ₄				Ph	Alk	Alk	PO ₄	Alk		Ph		PO ₄	Alk	Alk	Alk	Alk	Alk														PO ₄				Alk					
4							PO ₄	Alk		Ph	Alk	Alk		Ph	Alk	Alk	Alk	Alk	Alk	Alk		PO ₄								Alk	PO ₄						Alk	Ph	Ph		Ph		
DIM5	PAS						CAT			OAH			CBS			ABS					OML						Additional Protein Residues (APR)																
0			Ph				Ph	PO ₄		PO ₄	PO ₄	PO ₄	Alk		Ph		PO ₄	Alk	Alk	Alk	Alk	Alk	Ph									PO ₄		Ph		PO ₄							
1			Ph				Ph	Alk		Alk	PO ₄	PO ₄	Alk				Alk	Alk	Alk	Alk	Alk	Ph	Ph										PO ₄		Ph			Alk					
2						Alk	Ph	PO ₄		Alk	PO ₄	PO ₄	Alk	Ph		PO ₄	Alk	Alk	Alk	Alk					Ph								PO ₄		Ph		Alk						
3			Ph				Alk	Ph	PO ₄		PO ₄	PO ₄	Alk	Ph		PO ₄	Alk	Ph	Alk	Alk				Ph									PO ₄		Ph		PO ₄	Alk					
4	Ph		Ph			Alk		Alk		Alk	PO ₄	PO ₄	Alk	Ph		PO ₄	Alk	Alk	Alk	Alk		Ph		Ph											Ph		Alk						
TRIM	PAS						CAT			OAH			CBS			ABS					OML						Additional Protein Residues (APR)																
0						Alk		Ph	Alk		PO ₄	PO ₄	Alk	Alk	Ph	Ph	Ph	Alk	Alk	Alk		Ph	Ph		Ph									Alk				Ph					
1			Alk	Alk			Ph	Alk		Alk	Alk	Alk	Alk	PO ₄	Ph	Alk	Alk	Alk	Alk	Alk														Alk			Alk	Ph	Ph		Ph	Ph	
2		Alk	Alk	PO ₄			Alk	Alk		Ph	Alk	PO ₄	Alk	Alk	Ph	Ph	Alk	Alk	Alk	Alk		Alk	Alk		Alk		Alk			Alk				Alk			Alk						

ElectrostaticHydrogen BondingCation-ππ-stackingvan der WaalsNon-polarBackbone

Electrostatic   Hydrogen Bonding   Cation- π π -stacking   van der Waals   Non-polar   Backbone

Table 6.

Sampling, docking, and assay results for the naphthyl-containing inhibitors

Code	Nomenclature	Structure	Total Time (ns)	Docking Score	K_I (μ M)
NAP1	dibutyl 1-naphthyl phosphate		290.9	-100.76 (± 0.87)	22 (± 2)
NAP2	dibutyl 2-naphthyl phosphate		479.7	-101.75 (± 1.02)	1.9 (± 0.4)

Table 7:
Contacts observed in most populated binding modes of the naphthyl inhibitors

Inh. & Mode	ASN88	ASP70	GLN119	ALA277	SER287	TYR332	SER198	GLU325	HIS438	GLY116	GLY117	ALA189	TRP82	ALA328	PHE329	TRP231	PRO285	LEU286	VAL288	PHE388	ILE89	GLN71	PHE73	PRO74	GLY75	PHE76	MET81	ASN83	SER79	TYR114	GLY115	PHE118	THR120	TYR128	GLU197	ASN387	TRP430	MET437	GLY439	TYR440	ILE442			
NAP1	PAS					CAT			OAH			CBS			ABS					OML										Additional Protein Residues (APR)														
0	Nap	Nap			Alk		Alk		PO ₄	Nap	PO ₄		Nap		PO ₄	Alk	Nap	Alk	Alk	Alk		Nap	Nap										Nap											
1		Nap					Alk		PO ₄	PO ₄	PO ₄		Nap		PO ₄	Alk	Nap	Alk	Alk	Alk		Nap	Nap										Nap			PO ₄								
2			Nap		Alk	Nap		PO ₄		PO ₄	Nap	PO ₄		Nap		PO ₄	Alk	Nap	Alk	Alk		Nap	Nap										Nap			PO ₄								
3			Nap		Alk	Nap	Alk		Alk	PO ₄	PO ₄	Alk	Nap		PO ₄	Alk	Nap	Alk	Alk	Alk													Nap			PO ₄								
4	PO ₄		PO ₄		Alk			Alk		Alk	Alk	Alk	PO ₄		Alk	Alk	Nap	Alk	Alk	Alk	Alk	Nap	Nap			Nap							PO ₄											
NAP2	PAS					CAT			OAH			CBS			ABS					OML										Additional Protein Residues (APR)														
0			Alk		Alk	Nap	PO ₄		PO ₄	PO ₄	PO ₄	PO ₄	Nap	Nap	Nap	Alk	Nap	Alk	Alk	Alk											PO ₄		Nap			Alk								
1	Nap		Nap		Alk		Alk		Alk	PO ₄	PO ₄	Alk	Nap		PO ₄	Alk	Nap	Alk	Alk	Alk		Nap	Nap										Nap				Alk							
2			Nap		Alk	Nap		PO ₄		PO ₄	Nap	PO ₄	Alk	Nap		PO ₄	Alk	Nap	Alk	Alk													Nap			PO ₄	Alk							
3	Nap		Nap		Alk	Nap	Alk		Alk	PO ₄	PO ₄	Alk	Nap		PO ₄	Alk	Nap	Alk	Alk	Alk													Nap			PO ₄	Alk							
4			Nap		Alk	Nap	Alk		Alk	PO ₄	PO ₄	Alk	Nap		PO ₄	Alk	Nap	Alk	Alk	Alk	Nap												Nap			PO ₄	Alk							
		Electrostatic		Hydrogen Bonding		Cation- π		π -stacking		van der Waals		Non-polar		Backbone																														

Table 8.

Sampling, docking, and assay results for the choline-containing inhibitors

Code	Nomenclature	Structure	Total Time (ns)	Docking Score	K_I (μ M)
CP2r	ethyl choline phenyl phosphate		259.8	-80.04 (± 2.22)	30.0 (± 1)
CP2s			259.4	-79.25 (± 2.34)	
CP4r	butyl choline phenyl phosphate		220.3	-91.72 (± 0.48)	10.5 (± 0.27)
CP4s			214.1	-89.99 (± 0.74)	
CB4r	butyl choline benzyl phosphate		259.4	-103.34 (± 1.51)	N/A
CB4s			259.8	-100.92 (± 1.10)	

Table 9:

Contacts observed in the most populated binding modes of the choline-containing inhibitors

Inh. & Mode	ASN68	ASP70	GLN119	ALA277	SER287	TYR332	SER198	GLU325	HIS438	GLY116	GLY117	ALA189	TRP82	ALA328	PHE329	TRP231	PRO285	LEU286	VAL288	PHE388	ILE69	GLN71	PHE73	PRO74	GLY75	PHE76	MET81	ASN83	SER79	TYR114	GLY115	PHE118	THR120	TYR128	GLU197	ASN387	TRP430	MET437	GLY439	TYR440	ILE442				
CP2r	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0							Ch	Ch	PO ₄	Ph	Ch	Ch	Ch	Ph	Ph	Ph	Ph	Ph	Ph	Ph							Ch					Ch	Ch		Ch					Ch					
1							Ch	Ch	PO ₄	Ph	PO ₄	Ph	PO ₄	Ch		Ph	Ph	Ph	Ph	Ph												PO ₄	Ch		Ch					Ch	Ch		Ch	Ch	
2			PO ₄		Ph	Ch	Ch	Ch	Ch	PO ₄	Ph	Ch	Ch	Ch	PO ₄	Ph	Ph	Ph	Ph	Ph	Ph	Ch						Ch				Ch	Ch	PO ₄	Ch					Ch	PO ₄	Ch			
CP2s	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0						Ph	PO ₄	PO ₄	PO ₄	PO ₄	Ph	PO ₄			Ph	Ph	PO ₄	Alk		Alk																					Ch				
1						Ph	PO ₄	PO ₄	PO ₄	Ph	PO ₄	PO ₄			PO ₄		Ph	Alk	Alk	Alk															Ph	PO ₄					Ch				
2			Ph				Alk	PO ₄	Ph	PO ₄	Ph				PO ₄	Alk	Ph	Alk	Ph	Alk														Ph								Ch			
CP4r	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0							Ch	Ch	PO ₄	Ph	Ph	Ch	Ch	Ch	Ph	Ph	Ph	Ph	Ph	Ph												Ch	PO ₄	Ch	Ch					Ch	Ch				
1	Ch	Ch	PO ₄				Ch	Ph		Ch	PO ₄	Ph		Ch	Alk	Ph	Ph	Ph	Ph	Ph	Ch												Ch	PO ₄	Ch	Ch			Ch	Ch					
2	Ch	Ch	Ch				Ch	Ch	Ch	Ch	PO ₄	Ph	Ch	Ch	Ph	Ph	Ph	Ph	Ph	Ch								Ch					Ch	Ch	Ch	Ch		Ch	Ch		Ch	Ch			
CP4s	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0						Ph	Alk		Ph	PO ₄	PO ₄		Ph	Ph	Ph	Alk	PO ₄	Alk	Alk	Alk																PO ₄						Ph			
1						Ph	Alk		Ph	PO ₄	PO ₄	PO ₄		Ph	Ph	Ph	Alk	PO ₄	Alk	Alk	Alk															PO ₄	Alk					Ph			
2			Alk			Ph	Alk		Ph	PO ₄	PO ₄	Alk	Alk	Ph	Ph	Alk	Alk	Alk	Alk	Alk															Ch	Alk	Ph	Ph	Ch	Ph					
CB4r	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0			PO ₄				Ph	Ph	PO ₄	Ph		Ch			Ph	Ph	Ph	Ph	Ph	Ph		Ch										Ch	PO ₄	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch			
1			PO ₄			Ch	Ph		Ch	PO ₄	Ph		Ch		Ph	Ph	Ph	Ph	Ph	Ph	Ch	Ch						Ch				Ch	PO ₄	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch			
2			PO ₄			Ch	Ph		Ch	PO ₄	Ph		Ch	Ch	Ph	Ph	Ph	Ph	Ph	Ph	Ch	Ch						Ch				Ch	PO ₄	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch			
CB4s	PAS							CAT		OAH				CBS			ABS																Additional Protein Residues (APR)												
0						Ph	Alk		PO ₄	PO ₄	Alk	Alk	Ph	Ph	PO ₄	Alk	Alk	Alk	Alk	Alk											Ph				Ch		Ph			Ch	Ph				
1						Ph	Alk		Ph	PO ₄	Alk	Alk	Ph	Ph	PO ₄	Alk	Alk	Alk	Alk	Alk														Alk	PO ₄	Ch	Ch	Ch	Ch	Ch	Ch	Ch			
2			Ph			Ph	PO ₄		Alk	PO ₄	PO ₄	PO ₄	Ph		PO ₄	Alk	Alk	Alk	Alk	Alk		Ph											PO ₄	Alk	Ph	PO ₄				Ch					

Electrostatic

Hydrogen Bonding

Cation-π

π-stacking

van der Waals

Non-polar

Backbone

Electrostatic

Hydrogen Bonding

Cation- π π -stacking

van der Waals

Non-polar

Backbone