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Prediction of the binding sites of huperzine A in acetylcholinesterase by docking studies

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SUMMARY

We have performed docking studies with the SYSDOC program on acetylcholinesterase (AChE) to predict the binding sites in AChE of huperzine A (HA), which is a potent and selective, reversible inhibitor of AChE. The unique aspects of our docking studies include the following: (i) Molecular flexibility of the guest and the host is taken into account, which permits both to change their conformations upon binding. (ii) The binding energy is evaluated by a sum of energies of steric, electrostatic and hydrogen bonding interactions. In the energy calculation no grid approximation is used, and all hydrogen atoms of the system are treated explicitly. (iii) The energy of cation–π interactions between the guest and the host, which is important in the binding of AChE, is included in the calculated binding energy. (iv) Docking is performed in all regions of the host's binding cavity. Based on our docking studies and the pharmacological results reported for HA and its analogs, we predict that HA binds to the bottom of the binding cavity of AChE (the gorge) with its ammonium group interacting with Trp^{84}, Phe^{330}, Glu^{199} and Asp^{72} (catalytic site) and to the opening of the gorge with its ammonium group partially interacting with Trp^{279} (peripheral site). At the catalytic site, three partially overlapping subsites of HA were identified which might provide a dynamic view of binding of HA to the catalytic site.

INTRODUCTION

Huperzine A (HA, Fig. 1), isolated from Chinese folk medicine, is one of the most potent and selective, reversible inhibitors of acetylcholinesterase (AChE). This compound might be used as a potential drug for the palliative treatment of mild or moderate forms of Alzheimer's disease [1]. To understand the mechanism of the interaction of HA and to enable rational design of HA

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analogs that are synthetically more accessible at a stage when the crystal structure of the HA–AChE complex is not yet available, we have engaged in determining the HA binding sites in AChE by means of a docking study, searching for specific conformations, positions and orientations of both the guest and the host in which they interact most tightly and specifically with each other by evaluating systematically the potential energies of the resulting complexes. These potential energies, which are termed 'binding energies', are relative to the potential energies of the guest and host in their free state, and are a function of these conformations, positions and orientations.

SYSDOC

As summarized recently by Stoddard and Koshland [2], all docking algorithms use one or more simplifications to reduce the computing time, so that the docking is feasible within a reasonable time. These simplifications include the assumptions that the guest and/or the host is rigid [3–5], that the guest binds to certain restricted areas in the host [3–6], and that steric or electrostatic fit is the determining factor in binding affinity [4–11], as well as the grid approximation used in molecular affinity analysis for the calculation of a sum of independent energies of steric, electrostatic and hydrogen-bonding interactions [12–15]. Such simplifications can save computing time, but trade-off accuracy and reliability of the results. Recently, the interaction between an ammonium group and aromatic groups (cation–π interaction) has been reported to play a role in binding [16–23], especially in the binding of AChE, because the binding cavity of AChE (the gorge)

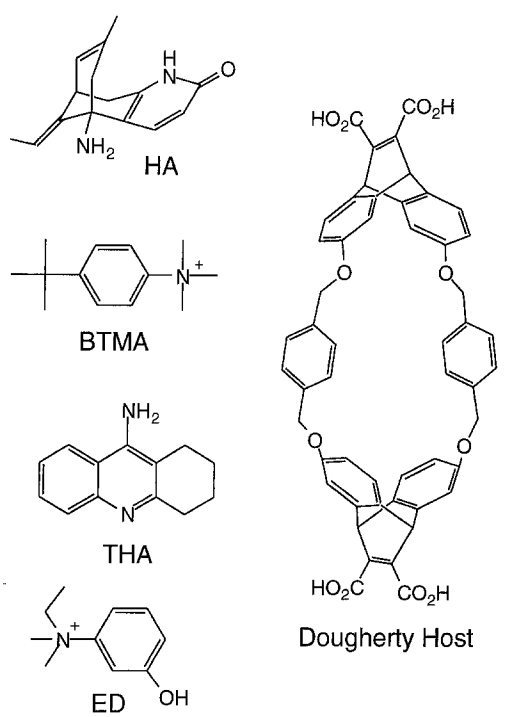


Fig. 1. Structures of HA, BTMA, the Dougherty host, ED and THA.

contains primarily aromatic groups, and all AChE substrates are positively charged [24]. However, this interaction has not been included in the reported docking algorithms.

For these reasons, we have developed an automated docking program, SYSDOC, whose major features are the reduction of computing time by use of the fast affine transformation and improvements in computer programming, which make the above-mentioned simplifications unnecessary. SYSDOC still neglects, like most other docking programs, the direct interaction of the guest with solvent molecules or metal cations. Affine transformation comprises three fundamental forms of coordinate system change that include translation of origin, change of scale (this transformation is not used in docking) and rotation of axes; the fast affine transformation uses different mathematical methods to perform the transformation, which gives the same results as the conventional affine transformation and requires less computing time (Pang, Y.-P., unpublished work; see below for a comparison of fast affine transformation with the conventional method). Consequently, SYSDOC provides the following unique features in comparison with other docking programs: (i) given a reasonable time constraint and the current computer speed, molecular flexibility of the guest and the host is taken into account, which permits the guest and the host to change their conformations upon binding, rather than assuming the guest and/or the host to be rigid; (ii) the total binding energy is calculated as the sum of the energies of steric, electrostatic and hydrogen-bonding interactions, rather than applying the grid approximation or assuming that either steric or electrostatic interaction is the determining factor for binding affinity; (iii) cation–π interaction between the guest and the host, which was recently reported to play a role in binding, is taken into account (vide infra), rather than ignoring this type of interaction; (iv) docking is performed in all regions of the binding cavity of the host, rather than assuming that the guest binds to certain restricted areas.

The SYSDOC algorithm identifies the positions and orientations of the guest for each conformation of host and guest that corresponds to a binding energy for the complex below a user-defined threshold. These complexes are generated by a systematic combination of translation of a guest along the x, y and z axes and rotation of a guest around the x, y and z axes with user-defined increments (normally 0.2 to 1.0 Å and 5 to 20° of arc) in a user-defined box that encloses the assumed binding cavity of the host. Users must test the docking results with different translational and rotational increments to make sure that use of the largest increments for one particular system provides the same docking result as when using smaller increments. The resulting complexes are not energy minimized, but the structures of the guest and host are subjected to energy minimization prior to docking. The calculated binding energy is composed of the Lennard-Jones energy, the Coulombic energy and the energy of hydrogen-bonding interactions. In this energy calculation (referred to as 'explicit energy calculation'), no grid approximation is used, and all hydrogen atoms of the guest and the host are treated explicitly. The all-atom force fields TRIPOS, CHARMm and AMBER are implemented in SYSDOC [25–27]. The general TRIPOS force field is set as the default, and the uses of other force fields are noted accordingly. When using the force fields TRIPOS or CHARMm, the explicit hydrogen-bond energy term is not included, but hydrogen atoms attached to atom types designated as hydrogen-bond donors are given a radius of zero in the 6–12 nonbonded term for their interactions with atom types designated as hydrogen-bond acceptors [28,29]. A cutoff criterion (R_c = 8 Å) is applied to the energy calculation for the nonbonded interaction [30]. A distance-dependent dielectric constant ($\varepsilon_r = r$) is used in the Coulomb term [31]. Molecular flexibility is taken into account by generating

complexes with different conformers of the guest and host. The different conformers of the host can be obtained from molecular dynamics simulation using CHARMm [26] or other dynamics simulation programs, while the different conformers of the guest can be derived from a systematic conformational search employing the SysSearch program [32] or other conformational search programs. Provided an adequate number of different conformers of the host is available, which are generated from an extended molecular dynamics simulation of the crystal structure of the host in either the bound or unbound form, no special starting geometries of the guest and the host are required for docking. However, it requires less simulation time to use a host structure that is first derived from the guest–host complex and then filled with water molecules in the binding cavity as the starting structure in the dynamics simulation for collecting an ensemble of different conformations of the host. The water molecules serve as a surrogate of the guest to prevent shrinkage or collapse of the binding site during the dynamics simulation. Given a fixed number of conformers of the guest and the host and the upper limits of translational and rotational increments, the docking results were independent of the initial docking conditions (such as orientation and position of the guest relative to the host and initial conformers of the guest and host). Importantly, SYSDOC performs explicit energy calculations on as many as 140 000 000 HA–AChE complexes consuming only 8 h of CPU time on a Silicon Graphics R4000 computer (50 MHz processor). In contrast, the conventional affine transformation, applied with the same programming algorithm as used in the fast affine transformation, requires at least 22 h of CPU time to complete the calculation with the same result. In addition, a fine-tuning module has been implemented in SYSDOC. This module automatically performs the docking at 0.2 Å and 5° of arc increments in user-defined ranges (usually in a range of 30° of arc for rotation and 1.2 Å for translation) at the positions and orientations identified at larger increments. SYSDOC has been fully vectorized (110 MFlops vector length) on a Cray YMP-C90 supercomputer, massively paralleled on a CM2 supercomputer for use with peptide or protein guests, and optimized on an IBM RS/6000 workstation with a better benchmark than on the SGI R4000.

According to Meot-Ner and Perutz, the cation–π interaction is primarily a hydrogen-bonding interaction that arises from the van der Waals and electrostatic interactions between atoms [16,18], and can be evaluated by Lennard-Jones and Coulombic energy terms in a system where polar and nonpolar atoms are treated explicitly [18]. Therefore, in principle the explicit energy calculation in SYSDOC is expected to include the cation–π interaction, although the empirical force field parameters used in the energy calculation may cause a deviation from theory. To confirm this assumption, we performed docking studies of the binding sites of the (*p-tert*-butylphenyl)trimethylammonium (BTMA) cation in the Dougherty host (Fig. 1) by systematically docking BTMA at 0.4 Å and 20° increments into a box (47.5 × 23.5 × 22.7 Å) encompassing the Dougherty host and large enough to allow BTMA to interact also with the exterior carboxyl anions of the host. The SysSearch program generated 120 low-potential energy conformers of the Dougherty host. One conformer, containing an open aromatic cavity, was used in the docking; others were not used because the smaller aromatic cavity cannot accommodate the bulky trimethylammonium or *t*-butyl groups of BTMA. Dougherty's NMR binding studies established that the ammonium group of BTMA binds to the cavity, revealing the importance of the cation–π interaction in this case [19]. Four different binding sites were identified by SYSDOC (Fig. 2; the coordinates of the SYSDOC-generated complexes will be deposited in the Brookhaven Protein Data Bank (PDB) [33]). The calculated relative affinities ($K_{I\ reference} / K_{I\ individual}$) of BTMA

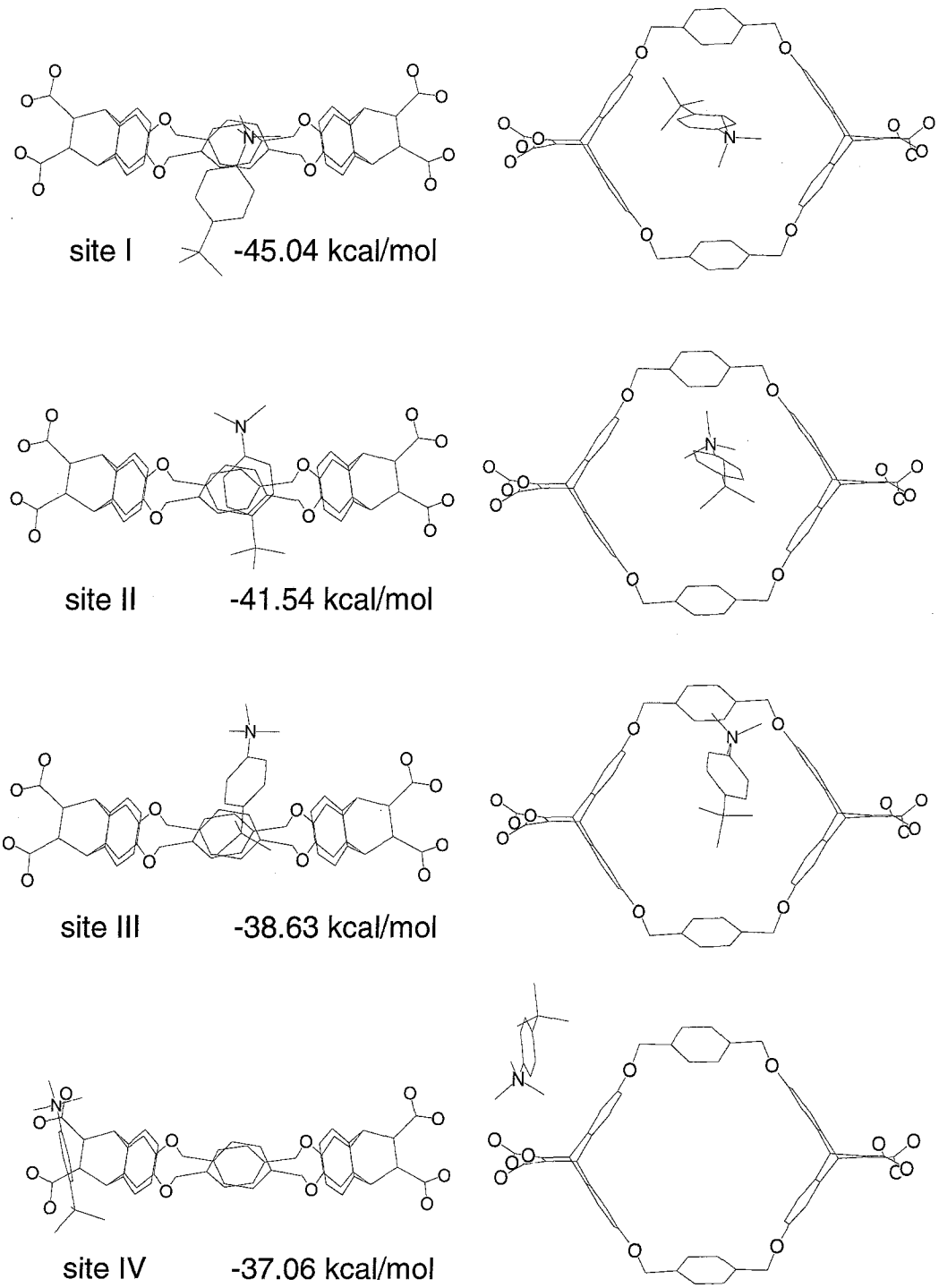


Fig. 2. SYSDOC-generated Dougherty complexes with the corresponding calculated relative binding energy. Left: front view; right: top view.

for these sites are 1.0 for site I, 9.0×10^{-8} for site II, 1.0×10^{-6} for site III and 1.5×10^{-12} for site IV. The relative affinity can be calculated according to the equation $K_{I\ reference} / K_{I\ individual} = \exp[(\Delta H_{reference} - \Delta H_{individual}) / RT]$, assuming the entropy change to be the same for all sites, where K_I is the dissociation constant and the enthalpy change (ΔH) is the sum of the interaction energy calculated by SYSDOC and the solvation energy change of the complexes. The solvation energy change is calculated by subtracting the sum of the minimized potential energy of the separate systems of the host and the guest immersed in a box of water molecules generated by Monte Carlo simulations [34] from the minimized potential energy of the system with the complex immersed in the water box. The calculated relative binding energies of four binding sites of BTMA, as well as the fact that the highest relative affinity found by SYSDOC for site I with the ammonium group of BTMA enmeshed in the cavity agrees with the NMR binding studies, confirm that the energy of the cation–π interaction is implicitly included in the calculated binding energy of SYSDOC. In addition, the second lowest binding energy calculated by SYSDOC at site II, with the phenyl group of BTMA buried in the aromatic cavity, implies that the π–π interaction [35,36] is also implicitly included in the calculated binding energy.

As a check of the force field parameters implemented in SYSDOC, calculations were carried out to reproduce the binding sites for THA and edrophonium (ED, Fig. 1) in *Torpedo* AChE, that were identified from the crystal structures of the THA–AChE and ED–AChE complexes [20]. The calculation protocols are the same as described below for HA, except that the guest and the host structures were taken directly from the crystal structures and molecular flexibility was not taken into account. In both cases, the calculated binding site with the lowest binding energy agrees well with that determined by crystallography. The rms deviations of the guest coordinates, which were obtained by first overlapping the AChE (host) portion in the crystal complex and in the SYSDOC-generated complex and then calculating the rms deviation of all non-hydrogen atoms of the guest portion in the two complexes, are 0.48 Å for THA and 0.77 Å for ED (Fig. 3). The conformation of AChE was taken from the crystal complex to reproduce the THA or ED complexes only for the purpose of checking the force field parameters. It was our intention to validate SYSDOC later by comparing the SYSDOC-generated huperzine A and E2020 complexes [37] with the corresponding crystal complexes that are not yet available, instead of by using the examples of THA or ED complexes whose crystal complexes were

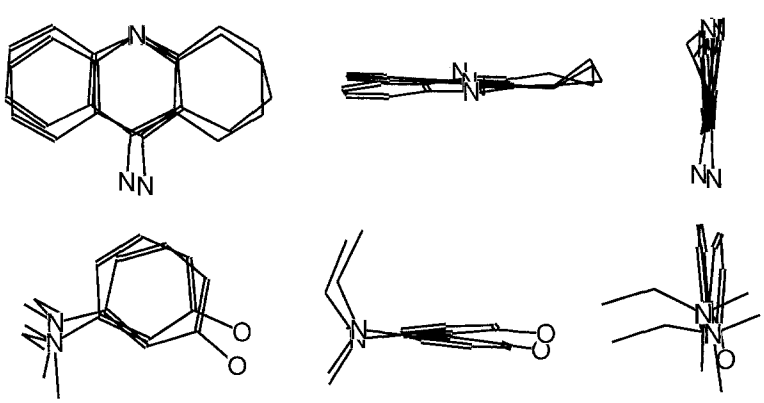


Fig. 3. Representation of rms deviations between the calculated structure and the crystal structure for THA (top) and ED (bottom). Left: front view; middle: top view; right: side view.

available in advance. Interestingly, although there are at present no crystal structures of AChE with ED or THA bound to Trp^{279} in the opening of the gorge (the peripheral domain), docking studies of ED and THA in the static *Torpedo* AChE also reveal binding sites for ED and THA in the peripheral domain. This result is consistent with the experimental finding of a peripheral site in AChE for ED and THA [38,39]. At the peripheral site of ED, its ammonium group interacts with the aromatic groups of Trp^{279}, Tyr^{334}, Phe^{331} and Phe^{290}, and its phenyl ring interacts with the aromatic groups of Phe^{331}, Phe^{290} and Trp^{279} (Fig. 5e; the coordinates of the SYSDOC-generated complexes in Fig. 5 will be deposited in the Brookhaven PDB). At the peripheral site of THA, its positively charged nitrogen atom interacts with the indole ring of Trp^{279} and the phenyl group of Tyr^{70}; its aromatic moiety interacts with the indole ring of Trp^{279} and the phenyl group of Phe^{290} (Fig. 5f).

It should be noted that the purpose of SYSDOC is to provide *likely* binding sites, because the program does not count solvent and ion effects. These likely binding sites serve as candidates to judge which are the actual binding sites according to the scientist's intuition and structure–activity relationship studies, or as starting geometries for free energy perturbation studies to quantitatively analyze the most likely binding sites. The cutoff of the SYSDOC-calculated binding energy for eliminating unreasonable binding sites is usually set to values that are 5 to 20 kcal/mol higher than the lowest binding energy of the complex identified by SYSDOC. Consequently, the binding sites identified by SYSDOC in vacuo through energy minimization of the separated guests and hosts and followed by translating and rotating the guests relative to the hosts to achieve the energetically favorable sites, cover the binding sites generated from energy minimization of the guest–host complexes, so that the induced-fit effect is taken into account, or from dynamics simulation of the complexes in solution, so that the solvent effect is counted. However, the preference in binding energies of the binding sites generated in vacuo will not be the same as those generated from the energy-minimized complexes or from the complexes simulated in solution.

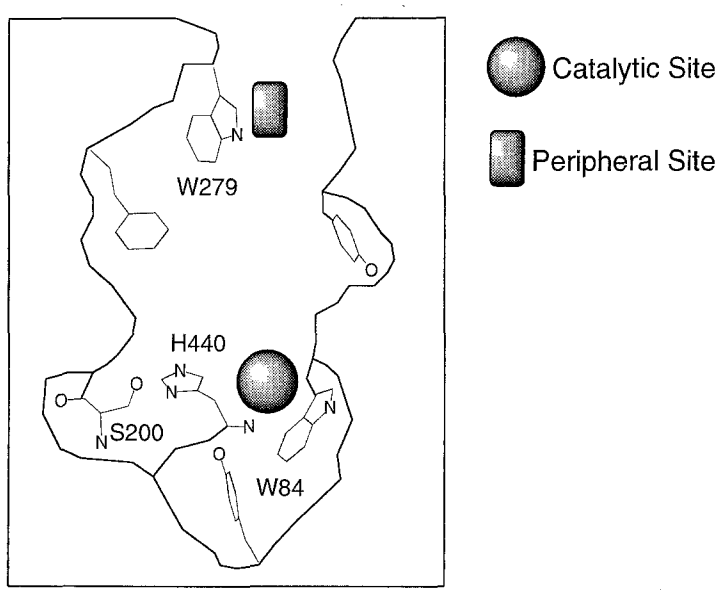


Fig. 4. A representation of the gorge and the catalytic and peripheral sites of HA.

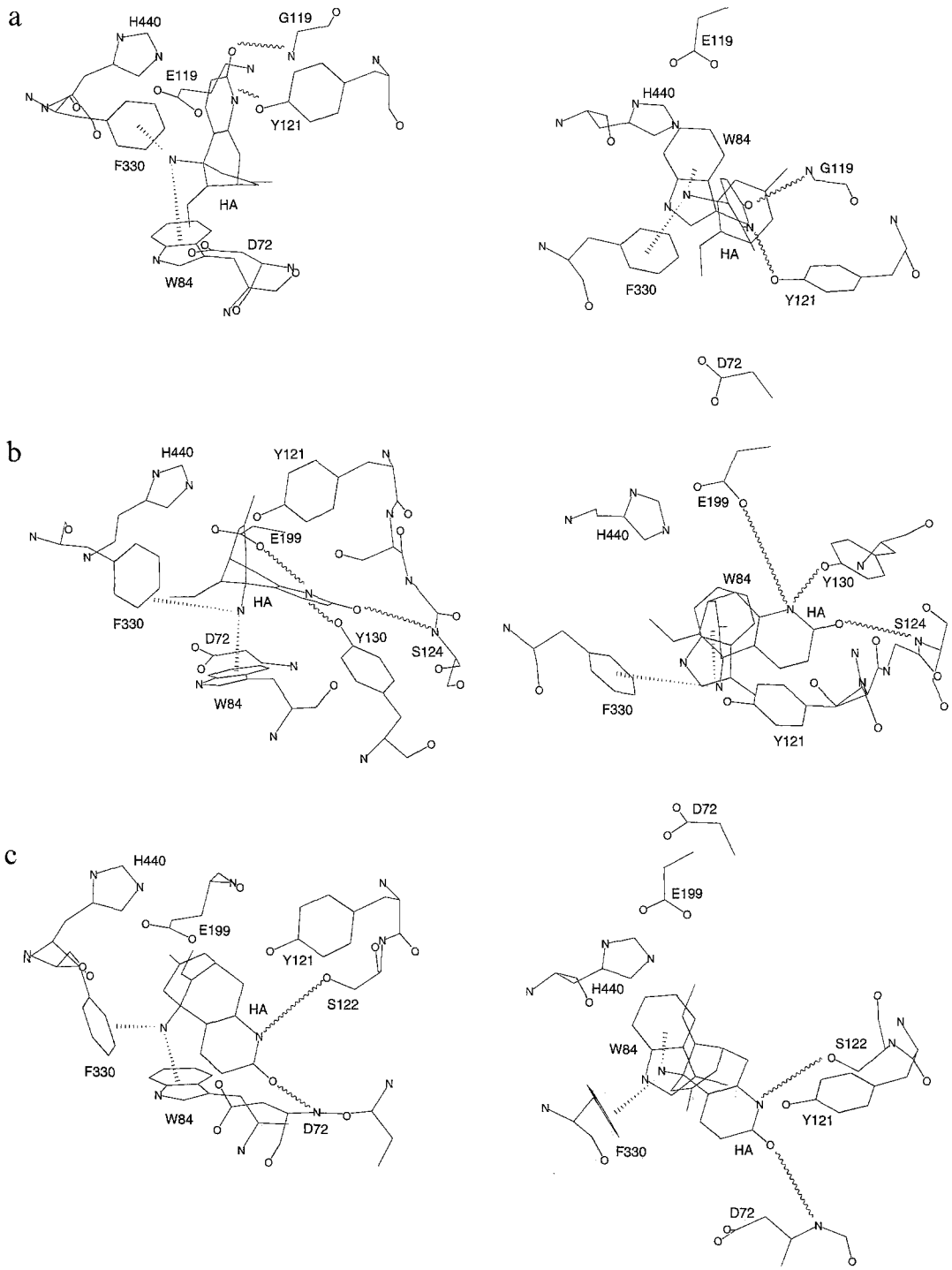


Fig. 5. Topography of the catalytic site of HA ((a) subsite 1; (b) subsite 2; (c) subsite 3; left: front view; right: top view) and the peripheral sites of (d) HA; (e) ED and (f) THA from the perspective of looking down into the gorge. ∿∿∿: hydrogen bond; ····· : cation–π interaction; ||||| : offset π–π stacking interaction.

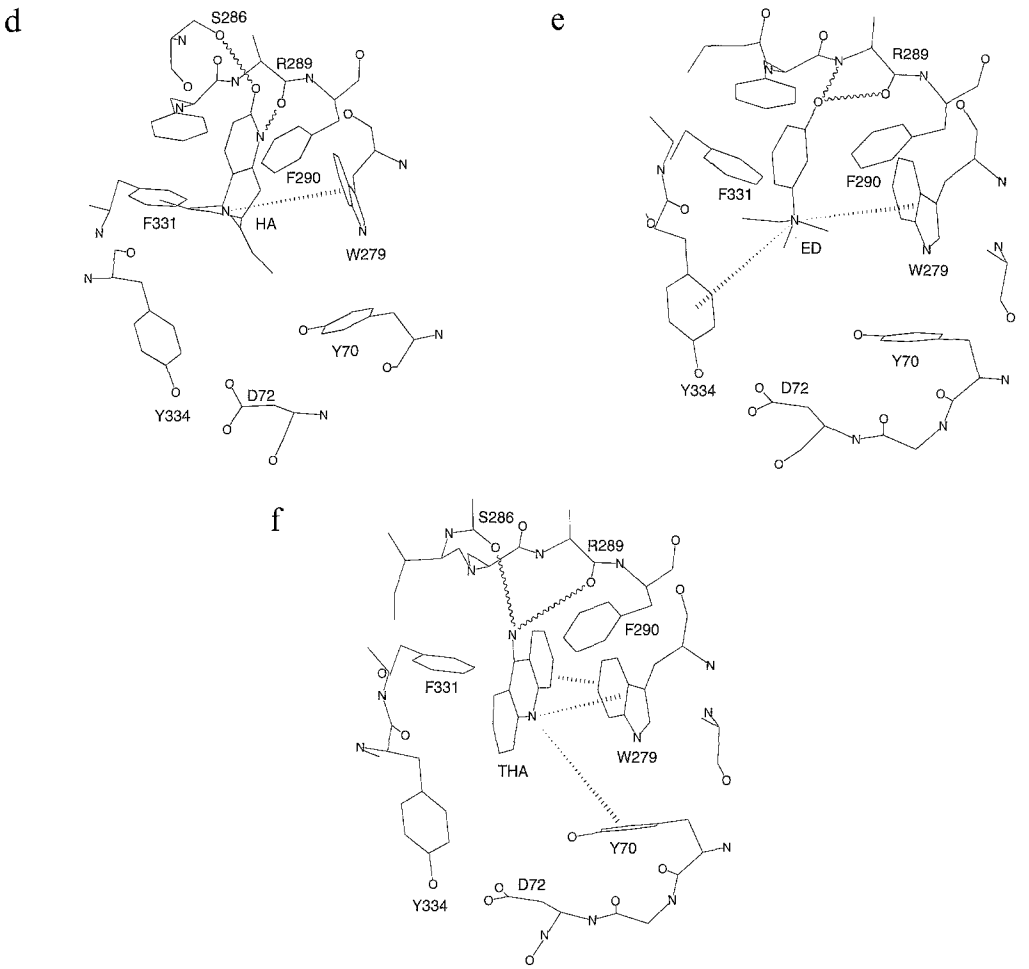


Fig. 5. (continued).

HA BINDING SITES

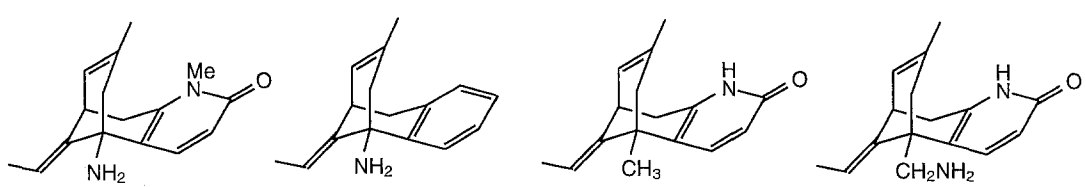
Calculation of the HA binding sites was carried out by docking HA at 0.4 Å and 10° increments into a box (18.6 × 19.1 × 20.3 Å) encompassing the space confined by the 14 aromatic residues on the surface of the gorge of AChE [20,24], and the results were fine-tuned at 5° of arc increment (in the range of 30°) and at 0.2 Å increment (in the range of 1.2 Å). The amino group of HA was treated as an ammonium cation. A single low-potential-energy conformer of HA was generated by the SysSearch program. Two conformers of HA were judged different if one of the torsion angles differed by more than 30°. SYSDOC selected 69 different conformers of AChE from an ensemble of conformers generated in a 40 ps dynamics simulation in vacuo of the AChE structure with water molecules occupying the gorge at 300 K. The entire protein was allowed to move in the simulation. It was found that simulations with substrate-free AChE at 600 or 300 K for more than 100 ps led to collapse or shrinkage of the gorge. Two conformers of AChE were

judged different if the rms deviation of selected atoms was greater than 0.8 Å. The selected atoms are those belonging to residues on the surface of the gorge. The initial structure of AChE for the simulation was taken from the crystal structure of the decamethonium-bound *Torpedo* AChE complex [20] and was subjected to structural modifications that include addition of the missing side chains of the residues on the surface of AChE, all polar hydrogen atoms and the CHARMm template charges (a net charge of eight electrons was assigned to the AChE structure consisting of the segments of residues 4–484 and 490–534) as well as energy minimization. The selected conformers used for docking studies were subjected to further modifications, entailing removal of the water molecules in the binding site, energy minimization, addition of all nonpolar hydrogen atoms and the CHARMm template charges, and energy minimization of the hydrogen atoms.

SYSDOC reveals that HA binds to the bottom of the gorge in *Torpedo* AChE above Trp^{84} (catalytic domain, Fig. 4) and to the opening of the gorge near Trp^{279} (peripheral domain, Fig. 4). The catalytic domain contains three partially overlapping subsites (catalytic site), differing in the hydrogen bonding motif (i.e., the lactam hydrogen atom of HA forms a hydrogen bond with the hydroxyl oxygen atoms of Tyr^{121} in subsite 1, Tyr^{130} in subsite 2 and Ser^{122} in subsite 3; the pyridone oxygen atom of HA forms a hydrogen bond with the peptide hydrogen atoms of Gly^{119} in subsite 1, Ser^{124} in subsite 2 and Asp^{72} in subsite 3) but sharing the cation–π interactions of the ammonium group of HA with the aromatic groups of Trp^{84} and Phe^{330}, as well as the ionic interactions of the ammonium group with the carboxyl groups of Asp^{72} and Glu^{199} (Figs. 5a–c). In the peripheral domain, there is only one site (peripheral site) where the ammonium group of HA partially interacts with the indole ring of Trp^{279} and is exposed to the solvent, possibly forming hydrogen bonds with solvent molecules. At this site the amide hydrogen atom of HA forms a hydrogen bond with the carbonyl oxygen atom of Arg^{289} and the pyridone oxygen atom of HA forms a hydrogen bond with the hydroxyl hydrogen atom of Ser^{286} (Fig. 5d). Taking into account the reported experimental information described below on HA and its analogs, as well as the facts that the 14 aromatic residues of the gorge are conserved in different species of AChE and that the primary structures of AChE in different species exhibit strong similarity [24], we predict that both the catalytic site and the peripheral site calculated by SYSDOC are the binding sites for HA in AChE.

EXPERIMENTAL SUPPORT

The cation–π interaction of the ammonium group of HA with the aromatic group of residue 330 of AChE in the predicted HA catalytic site is supported by thermodynamic studies of different cholinesterases [40] which show a decrease of the dissociation constant on replacing the



1 1.5×10^{-5} **2** $> 10^{-4}$ **3** 1.7×10^{-4} **4** 9.5×10^{-4}

Fig. 6. Structures of HA analogs with IC_{50} values (M) of AChE inhibition. The IC_{50} of (±)-HA is 0.73×10^{-7} M.

phenyl group of Phe^{330} in *Torpedo* AChE (K_I = 215 nM) by the more π-electron-rich *p*-hydroxyphenyl group in human AChE (K_I = 40.5 nM), and an increase of K_I when this phenyl group is replaced by a methyl group (possessing no π electrons) in human butyrylcholinesterase (BChE; K_I = 42 000 nM). Similarly, the order of magnitude in the change of K_I for human BChE (in which, additionally, Trp^{279} is replaced by alanine) indicates the loss of an additional cation–π interaction of the ammonium group with Trp^{279} of AChE in the peripheral site of HA. The hydrogen bonds present in HA's catalytic and peripheral sites are supported by the observed weak inhibition of mouse AChE by both **1** (Fig. 6, Kozikowski, A.P., Yamada, F. and McKinney, M., unpublished work), whose amide hydrogen has been replaced by a methyl group, thus disabling one hydrogen bond, and **2** [41] (Fig. 6), whose pyridone ring has been surrogated by a benzene ring, thus eliminating all hydrogen bonds. The cation–π interactions between the ammonium group of HA and the aromatic groups of residues 84, 330 and 279 in the catalytic and peripheral sites of HA are also supported by the weak inhibitory potencies found for **3** (Kozikowski, A.P., Yamada, F. and McKinney, M., unpublished work) and **4** [41] (Fig. 6) towards mouse AChE. While a positive charge is missing in **3** altogether, it has been moved away from the aromatic groups in **4**.

DISCUSSION

The presence of a binding site in the peripheral domain of AChE (i.e., the peripheral 'anionic' site) for positively charged substrates has long been proposed and supported by experimental findings [24,42]. Therefore, it is reasonable to predict that the site near Trp^{279}, as calculated by SYSDOC, is the peripheral binding site of HA. This prediction is consistent with the mixed competitive and noncompetitive inhibition of AChE shown by HA, which experimentally reveals that HA possesses a binding site distinct from the catalytic site [42,43]. As is apparent from Figs. 5d–f, the peripheral sites of HA, THA and ED have in common the feature that binding of the inhibitors does not block the entrance of the gorge. Importantly, these peripheral sites do not consist of anionic residues, which suggests that the peripheral 'anionic' site might be a historical misnomer.

The preference of a guest for binding to one specific site of the host depends at least on the binding energy at that site and the number of different conformers of the host possessing that site. We can predict the preference for one of the two sites when one site has both a lower binding energy and a larger number of suitable conformers relative to the other, but not when one site has a lower binding energy and the other has a larger number of suitable conformers. As to the preference for either the catalytic or the peripheral site of HA, based on our docking studies we suggest that the catalytic site is more favorable than the peripheral site. The binding energies of the peripheral and catalytic sites are –41.22 and –59.61 kcal/mol, respectively, while the ratio of conformer numbers of AChE for the peripheral versus the catalytic site is 2 : 7. These binding energies were calculated, using the CHARMm program with its all-atom force field, distance-dependent dielectric constant and one conformer of AChE for comparison, by subtracting the sum of the potential energies of the separated AChE and HA compounds from the potential energy of their complex (the differences of these energies are identical to those calculated by SYSDOC with the TRIPOS or all-atom CHARMm force fields). The low-affinity peripheral site of HA in AChE may function to build up a higher concentration of HA at the

opening of the gorge, which facilitates HA crossing the 'valve' of the gorge to reach the catalytic site (Pang, Y.-P. and Sussman, J.L., unpublished work). The 'valve' of the gorge is the narrow space confined by the side chains of Phe^{331}, Phe^{330} and Tyr^{121} that is proposed to regulate the entrance of substrates to the catalytic site of AChE.

As to the preference among the catalytic subsites, we cannot specify which subsite is preferred due to the inconsistency between the binding energy of one particular subsite and the number of conformers of AChE possessing that subsite. Relevant to our determination of the subsites of HA is the report demonstrating the presence of two partially overlapping subsites for sulisobenzone in thymidylate synthase. These subsites were identified from two different crystal structures of the sulisobenzone–thymidylate synthase (STS) complexes obtained by crystallization from different buffers [44]. The subsites in the STS complex are similar to those for HA in the manner in which they overlap. In view of the above complications, it is conceivable that HA may bind in the catalytic domain to only one of its three subsites, or to one subsite under certain conditions and to another under other conditions, or it may switch among the three subsites. It should be noted that, from one perspective, the existence of three subsites could be an artifact resulting from the fact that the direct interaction of the guest with solvent molecules is ignored in the docking studies. However, from another perspective, the three subsites could also provide us with a dynamic view of binding of HA to the catalytic site of AChE. Further experimental investigations into the dynamics of binding of HA to AChE will be required to sort out these possibilities. A comparison of the predicted binding sites of HA with the X-ray results as a static view of binding of HA to AChE will be reported when the crystal structure of the HA–AChE complex becomes available (Sussman, J.L., personal communication).

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