The Peripheral Anionic Site of Acetylcholinesterase: Structure, Functions and Potential Role in Rational Drug Design

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Abstract: The peripheral anionic site of acetylcholinesterase lies at the entrance to the active site gorge. It is composed of five residues (Tyr 70, Asp 72, Tyr 121, Trp 279 and Tyr 334; Torpedo numbering); associated with it are a number of surface loops, conferring a high degree of conformational flexibility on the area. The site is involved in the allosteric modulation of catalysis at the active centre and is the target of various anti-cholinesterases. It is also implicated in a number of non-classical functions, in particular, amyloid deposition, cell adhesion and neurite outgrowth. A number of peptide and protein ligands for the site have been identified. In this review, the structure and multiple functions of the peripheral anionic site are discussed, together with its potential as a target in rational drug design for the development of novel and improved inhibitors and of therapeutics for the treatment of neural cancers, nerve regeneration and neurodegenerative disorders such as Alzheimer's disease.

Key Words: Acetylcholinesterase, peripheral anionic site, allosterism, inhibitors, non-cholinergic, rational drug design.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7; AChE) belongs to the α/β hydrolase fold protein superfamily, a group defined by common structural homology and including the cholinesterases, carboxylesterases and lipases [1]. AChE is most closely related to butyrylcholinesterase (EC 3.1.1.8; BChE), and also shows significant sequence homology to a number of non-enzymatic proteins, many of which are involved in cell adhesion and signalling.These include neurotactin [2], glutactin [3], gliotactin [4], neuroligin [5] and the *Dictyostelium* crystal protein [6], as well as thyroglobulin [7].

AChE itself is found in all vertebrates and in all invertebrate groups in which its presence has been investigated. There are reports of AChE-like proteins in algae [8], *Paramecium* [9] and the slime mould, *Dictyostelium*, where it promotes aggregation [10]. Its principal physiological function is the rapid hydrolysis of acetylcholine in the synapse and neuromuscular junction, resulting in the termination of the nerve impulse. Evolutionary pressure to perfect this crucial role has undoubtedly driven AChE to become one of the most powerful and efficient enzymes known, with a turnover number of 10^4 s^{-1} [11]. AChE's significance has resulted in its being targeted by a variety of anti-cholinesterases, ranging from snake venoms to pesticides and the nerve gases used in chemical warfare. In medicine, AChE inhibitors are used in the treatment of dementias.

The peripheral anionic site (PAS) lies essentially on the surface of AChE, approximately 20A distant from the active site itself [12]. It binds acetylcholine as the first step in the catalytic pathway [13, 14] and allosterically modulates catalysis [15] as well as binding specific inhibitory compounds. The PAS has also been identified as a site promoting non-cholinergic functions: cell adhesion and neurite outgrowth in developing and transformed neural cells [14-17] and amyloidosis through an interaction with the amyloid β-peptide in Alzheimer's disease [18, 19]. The site has also been shown to interact with an omega loop on an adjacent AChE subunit [20], and with the extracellular matrix molecules laminin-1 and collagen IV [21].

The structure of the PAS and the number of functions that have been assigned to it suggest that it is an area of extraordinary versatility. How one site can fulfill multiple functions, interacting with a variety of ligands, is a fascinating question, and offers perhaps unique possibilities for rational drug design (RDD). Modelling studies, based on structural information of AChE and its complexes with inhibitors and other ligands, can be used to gain insight both into the molecule's interactions and into the mechanisms of catalysis and inhibition, leading to the design of novel and more effective inhibitors and reactivators, as well as possible therapeutics for the treatment of cancer and amyloidoses.

OVERVIEW OF ACHE STRUCTURE

Alternative splicing of the *ACHE* gene results in a number of splice variants [22], and this, together with the association of AChE catalytic subunits with additional domains and proteins, results in an array of oligomeric forms, broadly grouped into globular (including monomers, dimers and tetramers) and asymmetric (with a C-terminal-associated collagen-like domain) forms [23].

The catalytic or acylation site of AChE (Ser 200 (203), His 440 (447) and Glu 327 (334))[Torpedo numbering is given first, followed by mammalian numbering in brackets] lies deep within the molecule at the base of a narrow 20A deep gorge, lined predominantly with aromatic residues (Fig. **1a**) [12]. A number of additional subsites, also important in the catalytic process, have been differentiated within the

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gorge [24, 25]. The "anionic" subsite (Trp 84 (86), Tyr 130 (133), Tyr 330 (337) and Phe 331 (338)) binds the quaternary trimethylammonium choline moiety of the substrate, largely through π-cation interactions [26], optimally positioning the ester at the acylation site. The acyl pocket, responsible for substrate selectivity by preventing access of the larger members of the choline ester series, is composed of Phe 288 (295) and Phe 290 (297). The oxyanion hole, Gly 118 (121), Gly 119 (122) and Ala 201 (204), provides hydrogen bond donors that stabilise the tetrahedral transition state of the substrate [27].

THE STRUCTURE OF THE PAS

The PAS consists of 5 residues (Tyr 70 [72], Asp 72 [74], Tyr 121 [124], Trp 279 [286] and Tyr 334 [341]) clustered around the entrance to the active site gorge [24, 28, 29] (Fig. **1b**). A number of surface loops are associated with the PAS, and incorporate several of its residues. The large omega loop Cys 69 – Cys 96 incorporates Tyr 70 (72) and Asp 72 (74). The latter section of this loop forms part of the outer wall of the gorge, and includes Trp 84 (86), the principal component of the anionic site [12]. This loop is structurally homologous to the lid loop that sequesters substrate in neutral lipases, and is a structural element conserved throughout the esterase/lipase family [30]. It has been proposed that it may be involved in the accessibility of small molecules to the active centre as well as allosterism [31-34]. The surface loop 275-305 lies on the opposite side of the gorge, and includes Trp 279 (286).

There are ten acidic residues in the area surrounding the PAS. This concentration of negative charge, the "annular electrostatic motif" [35], is shared by AChE and the homologous signalling molecules neurotactin, gliotactin and neuroligin, but not by BChE.

Species differences in PAS structure occur, seen in different responses to PAS-binding inhibitors. Hagfish AChE exhibits much weaker inhibition by both propidium and BW284c51 [36]. Fasciculin 2 shows relatively weak inhibition of avian, reptile and insect AChEs [37]. AChE from cobra venom apparently does not contain a PAS at all [38].

The aromatic PAS residues, with Trp 279 (286) at their core, appear to act synergistically, shown by the very large increases in inhibition constants induced by multiple mutants [39]. The aromatic rings of Tyr 70 (72) and Tyr 121 (124) flank the indole of Trp 279 (286) and together interact with charged groups of ligands. Crystal structures of AChE complexed with decamethonium [20] show a direct interaction of Tyr 70 (72) with the inhibitor. The indole ring of Trp 279 (286) displays a variety of interaction modes, including stacking, aromatic-aromatic and π-cation [39], depending upon the nature of the ligand. Significant movement of the indole is also observed in comparison of crystal structures of apo- and complexed AChE [40], indicating its importance in ligand binding. It is possible that the π electron system of the indole may be polarised by the adjacent Glu 285, and this may enhance the stabilising effect of all the interaction modes. An argument may be made for including Glu 285 in the PAS, as mutations of this residue have a pronounced influence on the inhibitory effects of PAS ligands [39]. Glu 285 plays an important role in the binding of fasciculin-2 in particular, interacting with His 27 of the peptide [40]. Asp 72 (74), like Trp 279 (286), is also able to use several different interaction modes with ligands: its carboxylate uses both charge-charge and hydrogen bonded interactions [39].

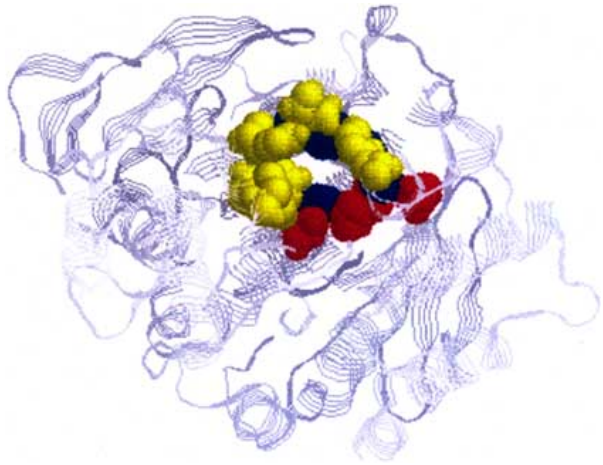


Fig. (1a). Structure of Torpedo AChE showing the active site (in red), the anionic site (in blue) and the peripheral anionic site (in yellow).

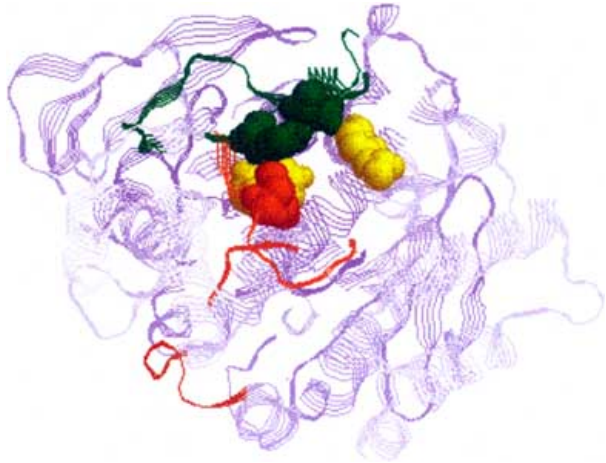


Fig. (1b). Structure of Torpedo AChE showing the PAS-associated omega loops. PAS residues are shown in spacefilling mode. Omega loop 69-96 is shown in green, including the two PAS residues Tyr 70 and Asp 72 (also green). Omega loop 274-308 is shown in orange, including the PAS residue Trp 279. The remaining PAS residues (Tyr 121 and Tyr 334) are shown in yellow.

Asp 72 (74), like Trp 279 (286) is also able to utilise several different interaction modes, operating by charge-charge or hydrogen bond interactions mediated by its carboxylate moiety [39]. The residue also appears to act as a trap for charged substrate [13, 14], thus contributing to catalytic efficiency. Similar transient binding also occurs with cationic organophosphates [14]. In the binding of PAS ligands, Asp 72 (74) appears to be unique in its ability to affect both the active site and the PAS [29].

THE ROLE OF THE PAS IN CATALYSIS

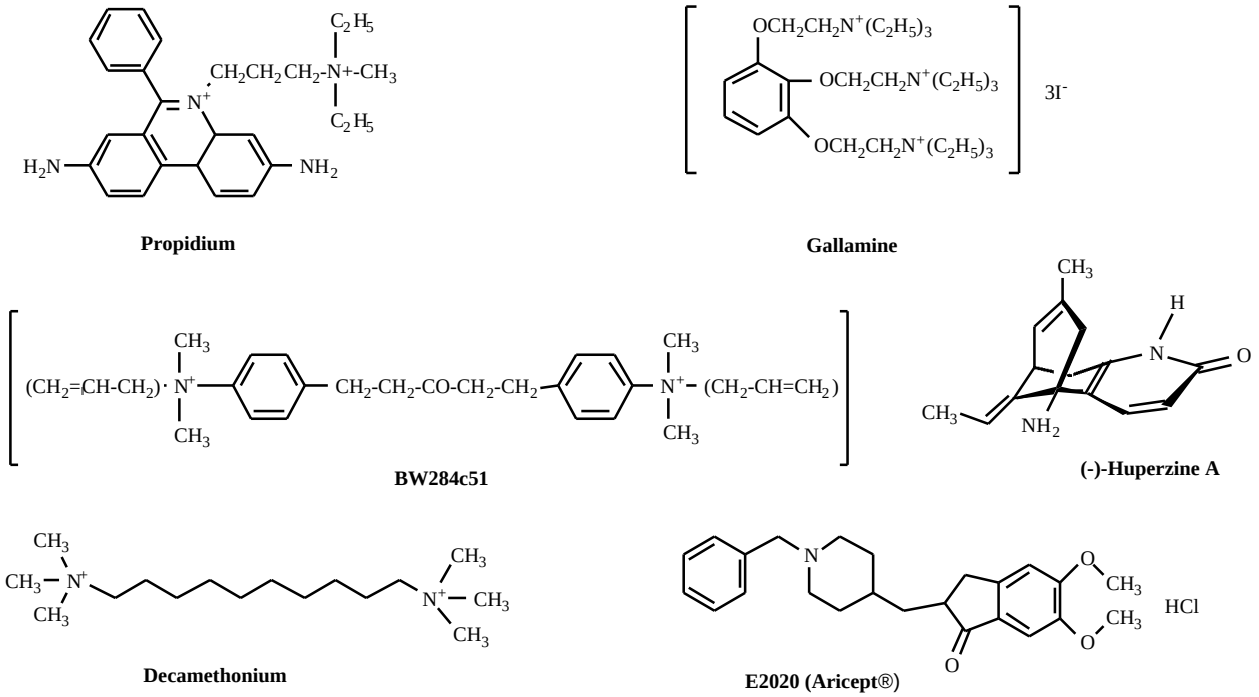
The PAS binds substrate transiently as the first step in the catalyic pathway [13, 14, 41], enhancing catalytic efficiency by trapping substrate on its way to the active site. At high substrate concentrations, a reduction in turnover i.e. substrate inhibition, is seen; it has been recognised for some time that this is a consequence of substrate binding at the PAS [15, 42]. Theoretically, this could occur for a number of reasons: physical blocking of the gorge entrance preventing access of additional substrate molecules, charge repulsion between the molecules, or an allosteric interaction between the active and peripheral sites involving conformational changes in the protein molecule [43].

It has long been known that binding of ligands at the peripheral site induces changes within the gorge: binding at the PAS was observed to accelerate carbamoylation at the active site [44], and polarometric and NMR data indicated extensive conformational changes on binding [45, 46]. This supported the hypothesis of Changeux [47] attributing allosteric regulation to a site physically removed from the active site. This hypothesis has been substantiated by numerous studies [e.g. 48, 49]. It would appear that changes in the anionic site are crucial [31, 48]: mutation of Glu199Asp eliminated substrate inhibition [50], and Shafferman *et al.* [29] proposed that substrate inhibition operates through a sequence of changes leading to movement of Tyr 337 as result of substrate binding at the PAS. Phe 297 of the acyl pocket is also involved. It appears that a transition of Trp 84 (86) to a conformation in which it shifts to occlude the active centre is crucial [31]. It was suggested [31, 48] that this conformational mobility was controlled by movement of the omega loop at residues 69-96. Part of this loop forms a section of the outer wall of the gorge, and includes Trp 84 (86). Grubic [51] observed that on the binding of propidium or monoclonal antibodies to the PAS, the freedom of motion of the gorge became more restricted, suggesting closure of the gorge. Recent evidence [32, 52-53] has shown that the loop is highly flexible, responding to conformational changes induced by both PAS and active site inhibitors, and is thus able to induce conformational fluctuations in the gorge itself, allowing transient opening and closing to alter substrate accessibility.

PAS-BINDING INHIBITORS

A large number of compounds inhibit AChE. Many of these, such as the organophosphates and carbamates, bind directly and competitively to the active site serine. Others, such as edrophonium, bind to the anionic site. Yet others bind noncompetitively to the PAS. The sites controlling substrate inhibition and inhibitor binding overlap, as shown by competition of substrate for binding with ligands specific for the PAS [15, 54], as well as by site-directed mutagenesis studies [15, 29].

The structures of PAS-binding inhibitors are shown in Fig. **2**. Quaternary inhibitors (e.g. gallamine) have structures resembling the quaternary ammonium group of acetylcho-



TMCYSHTTTSRAILTNCGENSCYRKSRRHPPKMVLGRGCGCPPGDDNLEVKCCTSPDKCNY

Fasciculin-2

Fig. (2). Structures of PAS-binding inhibitors.

line, and bind in the same manner. Bisquaternary inhibitors, such as BW284c51, decamethonium and E2020 (Aricept), are long thin molecules with quaternary ammonium groups on each end, and span both the anionic site and the PAS, where Tyr 70 (72), Tyr 121 (124) and Trp 279 (286) are involved in the binding [55-57]. Propidium, a small cationic inhibitor, binds in a pocket formed by the aromatic PAS residues centred around Trp 279 (286). In the case of (-)-huperzine A, various parts of the molecule bind to the anionic site, Phe 330 and the PAS [58]. Although steric blockade accounts for much of the effects of PAS-binding inhibitors [59, 60], there is also evidence of allosteric effects, for instance, mutation of Trp 84 (86) reduces the ability of propidium to inhibit AChE [24], suggesting coupling between this residue and the PAS.

Fasciculin-2, a 61 amino acid peptide from mamba venom, is a member of the 3 loop toxin family that includes α-bungaratoxin and the cardiotoxins, and has evolved from this basic structure to have a binding site highly specific for the PAS [61]. One of the loops inserts into the gorge entrance, essentially blocking substrate access [62], while another loop binds in a complementary fashion to the 69-96 omega loop [40]. There is also contact with residues of the 275-305 loop on the opposite side of the gorge. Van der Waals contacts are made with the PAS tyrosine residues (Tyr 20 (72), Tyr 121 (124) and Tyr 334 (341)) by loop II of fasciculin, which also packs against Trp 279 (286). Approximately 200A^2 of AChE's surface is obscured by fasciculin binding [40].

ELECTROSTATICS

AChE has a marked asymmetrical distribution of surface charge, with a large "dipole moment" of approximately 1500 debye orientated roughly along the axis of the active site gorge [12]. The negatively charged "pole" is located in the vicinity of the gorge entrance. The acidic residues responsible for this charge concentration include eight on the surface (Asp 72, Glu 82, Glu 282, Glu 285, Glu 342, Asp 351, Asp 380, and Asp 381) and two near the base of the gorge (Glu 199 and Glu 443) (all residues Torpedo numbering) [35, 64]. All these residues are highly conserved in sequence alignments [64].

This anionic surface charge appears to play a significant role in the electrostatic attraction of cationic substrates and inhibitors towards the active site gorge [65]. Mutations of any of the acidic residues involved significantly reduce enzyme-substrate or enzyme-inhibitor interactions [66]. Changes in ionic strength and pH [67, 68] also affect the binding of ligands at the PAS, indicating electrostatic interactions.

Very similar electrostatic motifs have been noted between AChE and cholinesterase-homologous proteins (neurotactin, glutactin and neuroligin) that are involved in cell adhesion and signalling, all having comparable concentrations of negative charge [35]. BChE, which does not promote cell adhesion, lacks this motif. The authors coined the term "electrotactins" to describe these molecules that appear to mediate adhesion by electrostatic means. The finding that AChE is able to bind to the extracellular matrix molecules, laminin-1 and collagen IV, by an electrostatic mechanism at the PAS [21] supports the hypothesis.

NON-CLASSICAL FUNCTIONS OF ACHE

The question as to whether AChE may have non-classical functions (those unrelated to acetylcholine hydrolysis in the synapse and neuromuscular junction) has intrigued investigators for many years. AChE is expressed transiently in early embryonic development, prior to synaptogenesis [69], and, in the adult, is found in areas of the brain that lack cholinergic innervation [70]. The discovery of sequence homologies between AChE and cell adhesion molecules suggested that AChE may function as a cell adhesion molecule. There is a considerable body of *in vitro* evidence indicating that AChE does indeed have non-classical functions promoting cell adhesion and neuritogenesis [e.g. 71-76], synaptogenesis [77], amyloidosis [18, 19], dopamine neuron activation [78], regulation of apoptosis [79, 80], nerve regeneration [81] and haemotopoiesis [82] and lymphocyte activation [83]. With the exception of synpatogenesis and lymphocyte activation, which show mixed cholinergic and non-cholinergic aspects, these functions do not appear to be catalytic in nature and are therefore presumed to be mediated by structural aspects of the AChE molecule. The PAS has been identified as the site of a number of these activities, particularly, cell adhesion/neurite outgrowth and amyloidosis, located to the adjacent surface loops 37-53 and 69-96 (cell adhesion/neurite outgrowth) [84] and 275-308 (amyloidosis) [85].

However, seemingly contrary to the almost overwhelming evidence of AChE's importance, the AChE knockout is not lethal, and the knockout mouse survived for three weeks postpartum [86]. AChE's classical function appears to have been taken over by BChE. This questions the significance of the *in vitro* evidence of non-cholinergic non-classical functions. Particularly difficult to reconcile are studies using non-inhibitory anti-AChE antibodies [76, 87], which resulted in loss of cell adhesion and induction of apoptosis *in vitro*, suggesting that a structural site on AChE is essential for neural cell survival and development. We hypothesise that AChE in its structural aspect is neither essential nor necessary for neural development, but that the site or sites identified on its surface, and recognised by the antibodies, indeed are; in other words, that these sites are functionally redundant with others on other molecules. Functional redundancy is not uncommon in signalling molecules, and has in fact been described between AChE and neuroligin in the mammalian CNS [88]. While this may reduce the import of the AChE molecule in these non-classical functions, it does offer a very interesting puzzle for the combinatorial chemist.

HETEROLOGOUS PEPTIDE AND PROTEIN ASSOCIATION AT THE PAS

The binding of the mamba venom peptide fasciculin to the PAS has been described above.

AChE binds specifically to the amyloid β-peptide and actively promotes the formation of the amyloid fibrils characteristic of Alzheimer's disease [18, 19]. The site of this interaction is the surface loop between residues 275-305; binding occurs by a hydrophobic mechanism [85].

Bourne *et al.* [20] observed an association of the PAS, in particular the 275-308 loop, with a small omega loop between residues 252-272 on an adjacent catalytic subunit in

the mouse tetramer. The function of this interaction would appear to be a stabilisation of the tetrameric assembly. What is interesting is that this short loop bears a significant structural resemblance to both the amyloid β-peptide and the prion proteins, suggesting common mechanisms of aggregation.

A site mediating cell adhesion and neurite outgrowth and binding laminin-1 and collagen IV *in vitro* has been identified with several of the PAS-associated surface loops, including the omega loop Cys69-Cys96 [84]. Binding was found to be sensitive to changes in ionic strength and pH, suggesting an electrostatic mechanism [21], which agrees with the acidic character of this region. The physiological relevance of AChE's binding to these extracellular matrix molecules is not known; it is possible that the interaction forms part of the AChE-mediated adhesion function, and may compensate for AChE's lack of cytoplasmic signalling capabilities. It is also possible that AChE, especially as it may show functional redundancy with other molecules, may be mimicking the action of these molecules. PAS-associated loops also appear to be responsible for the development of ester-hydrolysing catalytic behaviour in certain anti-AChE antibodies, although the mechanism by which this occurs is not yet clear (Johnson and Moore, unpublished results).

THE PAS AS A MATRIX OF BINDING SITES

It is apparent that the PAS is a site of association for numerous ligands, ranging from small cationic substrates and inhibitors to peptides (fasciculin and the amyloid β-peptide) and large proteins (AChE itself, laminin-1 and collagen IV) (Fig. **3**). Functional redundancy has been demonstrated between neuroligin and AChE for binding to the cell surface

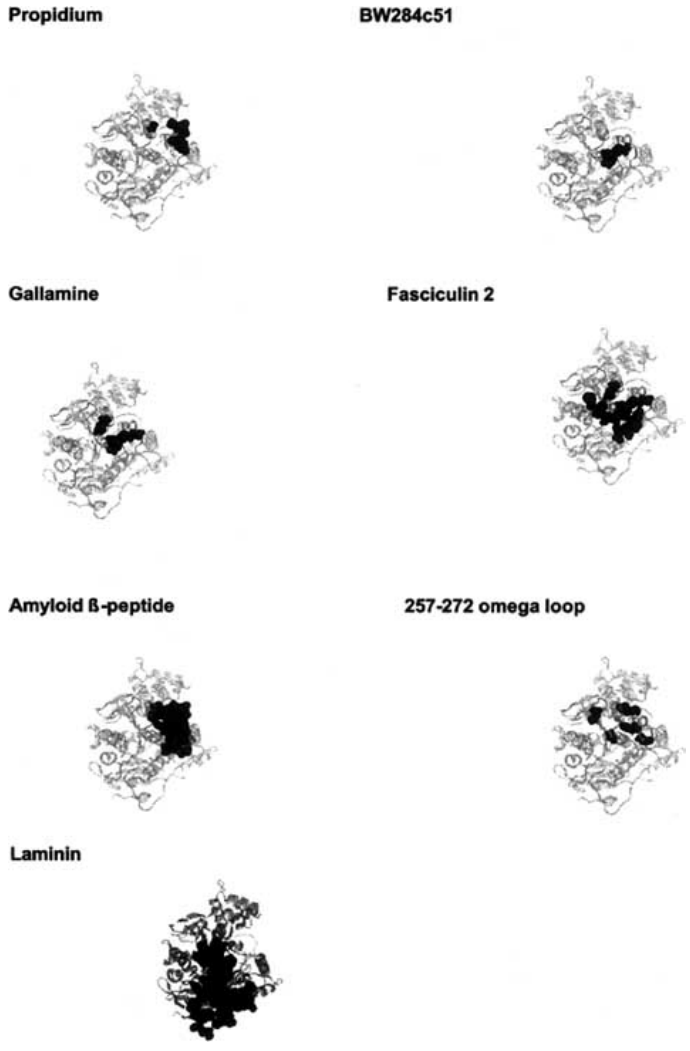


Fig. (3). Residues involved in the binding of various PAS ligands.

Note that all structures shown are Torpedo AChE, and where the structures determined belong to another species (e.g. mouse), the equivalent Torpedo residues are shown. The binding sites of propidium, BW284c51 and gallamine were determined by crystallography [86], as were the sites of fasciculin [37] and the 257-272 omega loop interaction [19]. The binding site of the amyloid β-peptide was determined by molecular docking and binding to synthetic peptides [84]. The binding site of laminin was determined by binding to synthetic peptides (Johnson and Moore 2004).

receptors, the β-neurexins [88]. As the β-neurexins and laminin contain similar homologous domains, it is possible that certain β-neurexins may also be PAS ligands. Differences in the positions of binding of various PAS ligands have been demonstrated directly by crystallographic studies [26, 40, 56, 57, 89] and indirectly by the different effects observed by ligands on catalysis [50]. It has been shown that the sites of the amyloid β-peptide and laminin and collagen binding are different [84, 85]. The concept of the PAS as a matrix of overlapping binding sites has been proposed [37, 89]. It is likely that at least part of this promiscuity is due to the conformational flexibility of the region, a function of the surface loops. Comparison of the loop homologous to the 69-96 loop in lipases [30] and dynamic studies on the loop in AChE itself [53, 90] show its extraordinary flexibility. It has been observed that omega loops are often found clustered on one surface of a protein, frequently in areas involved in substrate, ligand binding or active sites [91]. This flexibility, coupled with the electrostatic motif and the versatility of individual PAS residues, in particular, Trp 279 (286) and Asp 72 (74), as described above, make the PAS an ideal site for ligand interaction.

PHARMACOLOGICAL PERSPECTIVES: THE PROSPECTS FOR RATIONAL DRUG DESIGN

The combination of our knowledge of the three-dimensional structure of AChE and structures of AChE-binding ligands, together with molecular modelling, offers a rational foundation for drug design. The PAS, with its documented structure and multiplicity of functions and ligands, provides such an opportunity. Rational design has already proved successful in the development of AChE inhibitors of increased potency [92, 93].

Neurological Disorders and Alzheimer's Disease

It has been estimated [94] that approximately 4.5% of the population over the age of 70 years suffers from Alzheimer's disease, and with increased life expectancy and an ageing population, this figure is likely to increase, placing an enormous burden on health services. Inhibitors of AChE are the only drugs thus far approved for the treatment of Alzheimer's, and are also used in the management of other neurological disorders and dementias. Research into improved treatments and the design of novel inhibitors are thus important [92, 95, 96]. Inhibitor treatment is, nevertheless, merely palliative, and is associated with a variable clinical response giving rise to questions as to whether it has reached its "therapeutic ceiling" [97]. The ultimate goal would be either to prevent the formation of amyloid deposits or to break them down once formed. RDD based on the information of the interaction of the PAS and the amyloid β-peptide could provide a means of interfering with fibrillogenesis.

Organophosphate Poisoning

Apart from binding inhibitors, the PAS is also involved in the reactivation of organophosphate-inactivated AChE by oximes. The dimeric oxime, 2-PAM, binds with one oxime end at the catalytic site and the other at the PAS [98]. Design of improved reactivators of AChE is important, in view of the prevalence of organophosphate pesticides and chemical weapons. The role of the PAS could be further explored in this regard.

Cancer

Less toxic, more effective and more selective anticancer therapies are constantly being sought and rational drug design plays a crucial role in the development of drugs targeting specific cancer-related processes, especially angiogenesis, cell cycle and apoptosis regulators [99]. Retinoids have potent differentiation-inducing effects, but have significant disadvantages, in particular, toxicity, the development of resistance and their ineffectiveness against most solid tumours [100]. The use of retinoids in combination with other, novel, differentiation-inducing therapies, or the use of such therapeutics alone, is a viable alternative [101].

AChE promotes differentiation of both neural and hematopoietic tumour cells *in vitro*. The site promoting neural cell adhesion and differentiation is known. Furthermore, this site also appears to be the epitope recognised by catalytic anti-AChE antibodies, which are potent apoptosis-inducing agents [87]. Structural characterisation of both the AChE and the complementary antibody sites, together with rational drug design, suggests the possibility of a novel therapy, promoting either differentiation or apoptosis.

Autoimmune Disease

Anti-AChE autoantibodies have been observed in a number of neurological and myasthenic syndromes [102-104], although it remains unknown as to what role they may play in the pathology. The YT blood group antigen has been found to be AChE in the red blood cell membrane [105]. Catalytic antibodies, raised either as idiotypic [87] or anti-idiotypic [106] antibodies against AChE, develop with relative ease and frequency, and induce apoptosis in neuroblastoma cells [87]. Although the presence and frequency of occurrence of such antibodies in autoimmune disorders is not known, their existence would appear to be a distinct possibility, and suggests a role for RDD in the treatment of these disorders.

CONCLUSIONS

It is apparent that the PAS is a complex of binding sites, mediating catalysis modulation, ligand binding, nucleation, initiation of signalling pathways and the development of catalytic behaviour in antibodies. It would appear that much of this versatility resides in the conformational flexibility of the region. Although many of the mechanisms of molecular interactions are presently unclear, the elucidation of these through structural studies, combinatorial chemistry, modelling and docking techniques has the potential for opening the door to RDD for the treatment of a variety of conditions and disorders.

ABBREVIATIONS

AChE = Acetylcholinesterase

BChE = Butyrylcholinesterase

PAS = Peripheral anionic site

RDD = Rational drug design

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