Protein Science (1999), *8*:2553–2561. Cambridge University Press. Printed in the USA.

Acetylcholinesterase of *Schistosoma mansoni*—Functional correlates

Contributed in honor of Professor Hans Neurath's 90th birthday

RUTH ARNON,[1] ISRAEL SILMAN,[2] AND REBECA TARRAB-HAZDAI[1]

[1]Department of Immunology, The Weizmann Institute of Science, Rehovot, 76100 Israel
[2]Department of Neurobiology, The Weizmann Institute of Science, Rehovot, 76100 Israel

(RECEIVED September 9, 1999; ACCEPTED October 15, 1999)

Abstract

Acetylcholinesterase (AChE) is an enzyme broadly distributed in many species, including parasites. It occurs in multiple molecular forms that differ in their quaternary structure and mode of anchoring to the cell surface. This review summarizes biochemical and immunological investigations carried out in our laboratories on AChE of the helmint, *Schistosoma mansoni*. AChE appears in *S. mansoni* in two principal molecular forms, both globular, with sedimentation coefficients of ~6.5 and 8 S. On the basis of their substrate specificity and sensitivity to inhibitors, both are "true" acetylcholinesterases. Approximately half of the AChE activity of *S. mansoni* is located on the outer surface of the parasite, attached to the tegumental membrane via a covalently attached glycosylphosphatidylinositol anchor. The remainder is located within the parasite, mainly associated with muscle tissue. Whereas the internal enzyme is most likely involved in termination of neurotransmission at cholinergic synapses, the role of the surface enzyme remains to be established; there are, however, indications that it is involved in signal transduction. The two forms of AChE differ in their heparin-binding properties, only the internal 8 S form of the AChE being retained on a heparin column. The two forms differ also in their immunological specificity, since they are selectively recognized by different monoclonal antibodies. Polyclonal antibodies raised against *S. mansoni* AChE purified by affinity chromatography are specific for the parasite AChE, reacting with both molecular forms, but do not recognize AChE from other species. They interact with the surface-localized enzyme on the intact organism, and produce almost total complement-dependent killing of the parasite. *S. mansoni* AChE is thus demonstrated to be a functional protein, involved in multifaceted activities, which can serve as a suitable candidate for diagnostic purposes, vaccine development, and drug design.

Keywords: acetylcholinesterase; heparin-binding; immunological properties; molecular forms; parasites

This short review will briefly summarize and analyze extensive investigations performed in our laboratories on the enzyme acetylcholinesterase (acetylcholine hydrolase; EC 3.1.1.7; AChE) of the parasite *Schistosoma mansoni* (*Sm*AChE). One of the major and puzzling issues in the study of host-parasite relationships in general, and in schistosoma in particular, is the ability of the parasite to evade the immune defense mechanism of the host. A major strategy is thus the identification of functional proteins that might elicit protective immunity against infection. A protein that falls exactly within this category is AChE. The goals of this study were, therefore, to characterize *Sm*AChE and to investigate its function in the parasite.

Reprint requests to: Ruth Arnon, The Weizmann Institute of Science, Department of Immunology, Rehovot, 76100 Israel; e-mail: liarnon@weizmann.weizmann.ac.il.

The principal role of AChE is termination of transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter, acetylcholine (ACh) (Taylor, 1990). It is indeed concentrated at both central and peripheral cholinergic synapses. In keeping with its biological function, AChE is characterized by unusually high catalytic activity, especially for a hydrolase, displaying a turnover number of ~10,000/s, and thus operating close to the diffusion-controlled limit (Quinn, 1987).

Due to its crucial biological role, AChE is the target of a wide repertoire of drugs and poisons, both natural and synthetic (Koelle, 1963; Taylor, 1990). These include organophosphate (OP) nerve agents (Millard et al., 1999), both carbamate and OP insecticides (Casida & Quistad, 1998) and antihelmintics (Martin, 1997), which act as powerful covalent inhibitors of AChE (Aldridge & Reiner, 1972). AChE inhibitors are also employed as drugs to alleviate cholinergic insufficiency in such conditions as myasthenia gravis

and glaucoma (Taylor, 1990) and, most recently, anticholinesterases have become prominent as the first generation of anti-Alzheimer drugs (Fisher et al., 1998).

The three-dimensional (3D) structure of *Tc*AChE was solved in 1991 (Sussman et al., 1991), subsequent to solubilization, purification, and crystallization of the GPI-anchored dimer present in large amounts in *Torpedo* electric organ (Sussman et al., 1988). The 3D structure proved that, like other serine hydrolases, AChE contains a catalytic triad, albeit with a glutamate in place of the aspartate found in the serine proteases. However, the 3D structure displays a number of unexpected features. The active site is deeply buried, being located almost 20 Å from the surface of the catalytic subunit, at the bottom of a long and narrow cavity (Fig. 1). This cavity was named the active site gorge, or, since over 60% of its surface is lined by the rings of conserved aromatic residues (Sussman et al., 1991; Axelsen et al., 1994), the aromatic gorge. Despite the prediction that the "anionic" site would contain several negative charges (Nolte et al., 1980), in fact only one negative charge is close to the catalytic site, that of Glu199, adjacent to the active site serine, Ser200. Based both upon docking of ACh within the active site (Sussman et al., 1991), and upon affinity labeling (Weise et al., 1990), the quaternary group of ACh appears to be interacting, via a cation -π-electron interaction (Dougherty & Stauffer, 1990), with the indole ring of one of the conserved aromatic residues, Trp84. Another conserved aromatic residue, Phe330, is also involved in the interaction (Harel et al., 1993).

AChE displays structural polymorphism, being expressed as a repertoire of molecular forms (Massoulié et al., 1993) Most vertebrates contain a single gene coding for AChE, and alternative splicing gives rise to two principal catalytic subunits, H and T. H subunits form GPI-anchored dimers (Silman & Futerman, 1987), whereas T subunits occur as monomers, dimers, and tetramers, the latter being dimers of disulfide-linked dimers. T subunits also associate with structural subunits (P and Q) to form membrane-anchored tetramers and asymmetric forms, in which 1–3 tetramers are attached to a collagen like tail (Massoulié et al., 1998). Although the biological significance of this phenomenon is not well understood, different types of synapses display different repertoires of molecular forms. This, in turn, may reflect strict requirements for spatial distribution and/or orientation of AChE at a given synapse.

Both the temporal and the spatial appearance of AChE, and of its companion enzyme, BChE, have raised the possibility that they may play biological roles other than termination of synaptic transmission by hydrolysis of ACh (Layer, 1983; Greenfield, 1984; Layer et al., 1988). It has been suggested that they may play trophic roles during synaptogenesis, whether catalytic or noncatalytic, and that catalysis may involve substrates other than ACh (George & Balasubramanian, 1981). In recent years, it has been noticed that a number of adhesion proteins display significant sequence homology with the ChEs. These include three such adhesion proteins from *Drosophila*, neurotactin (Barthalay et al., 1990), glutactin (Olson et al., 1990), gliotactin (Auld et al., 1995), and the mammalian protein, neuroligin (Ichtchenko et al., 1995). This prompted the suggestion that the ChEs may serve as adhesion proteins (Krejci et al., 1991), and indeed experimental evidence has been offered to support this possibility (Darboux et al., 1996). Solution of the 3D structure of *Tc*AChE, and the concomitant recognition of the α/β hydrolase fold family of proteins (Ollis et al., 1992), revealed that these adhesion proteins were all mem-

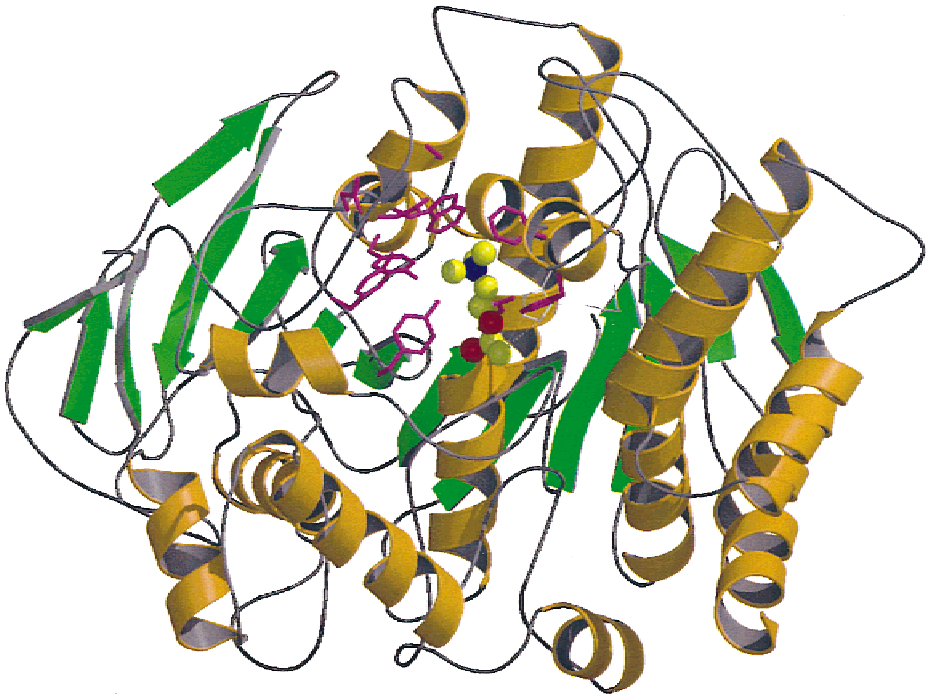

Fig. 1. Ribbon diagram of the 3D structure of TcAChE. Green arrows represent β-strands, and brown coils, α-helices. The side chains of the catalytic triad and of key aromatic residues in the active site gorge are indicated as purple stick figures. ACh, manually docked in the active site, is represented as a space-filling model, with carbons shown in yellow, oxygens in red, and the nitrogen in blue. Its quaternary group faces the indole of Trp84.

bers of the family, even though most likely devoid of hydrolase activity due to the absence of one or more of the residues of the catalytic triad. Recently, Botti et al. (1998) have shown that an electrostatic motif, at the mouth of the active-site gorge of AChE, is also present in the noncatalytic adhesion proteins. They suggested that this motif contributes to long-range electrostatic interactions associated with their adhesive function and have suggested that they constitute a novel class of adhesion proteins, which they have named the electrotactins.

AChE in parasites

AChE is very broadly distributed in many species including parasites. It is present in all helmintic invertebrates, including trematodes and nematodes (Chalfie & White, 1988; Pax et al., 1996). In trematodes, as will be discussed below, AChE is associated with both the surface and the internal subcellular structures. In nematodes, a substantial fraction of the AChE is secreted, e.g., as G_1 and G_4 forms in *Nippostrongylus brasiliensis* (Hussein et al., 1999) and as a G_2 dimer in *Parascaris equorum* (Talesa et al., 1997). As to its localization in helmints, AChE may be associated with neuronal cell bodies or with the musculature, presumably at cholinergic sites, or with the external surface membrane where, presumably, it fulfills one of the noncholinergic functions which have been suggested for it (Camacho & Agnew, 1995; Camacho et al., 1996). This issue is discussed later in relation to the possible functions of *Sm*AChE in the parasite.

It is important to understand the biological role of AChE in helmints in the context of development of antihelmintic drugs. Various AChE inhibitors were shown to significantly decrease the amplitude of muscle contraction in various parasites, such as *Faciola hepatica* (Sukhdeo et al., 1986) and *Schistosoma mansoni* (Pax & Bennett, 1991), and have found use as antihelmintic drugs (Marshall, 1987; Martin, 1997). The effect on secretion of AChE was one of the parameters used in the evaluation of the effectiveness of antihelmintics on *N. brasiliensis* (Rapson et al., 1986). Furthermore, levels of parasitic AChE in the serum, as assayed after immunoprecipitation, were used to monitor circulating filaria parasites both in humans and experimental animals (Rathaur et al., 1992).

In *S. mansoni*, AChE was first demonstrated in adult worms by Bueding (1952) and was partially characterized both histochemically and biochemically (Fripp, 1967). Early studies also demonstrated that anticholinesterases produce paralysis of the worms, suggesting involvement of AChE in the motor activity of *S. mansoni* (Barker et al., 1966). More recently, it was shown that eserine, an AChE inhibitor, produces significant relaxation of the longitudinal muscle of the parasite (Pax et al., 1981). It is, therefore, apparent that the cholinergic mechanisms are associated with neuromuscular function (Pax et al., 1996). Indeed, schistosomal AChE is the target for several antiparasite drugs, including hycanthon, lucanthone, metrifonate, and phosphonium compounds (Bueding et al., 1972; Hillman et al., 1978; Nordgren et al., 1981; Levi-Schaffer et al., 1984a; Marshall, 1987).

In an early study in our laboratory, during the isolation of the tegumental membrane of schistosomula and analysis of its major components, we observed that AChE activity was highly enriched in the isolated external membrane, ca. 350-fold as compared to the entire worm (Levi-Schaffer et al., 1984b). It is thus apparent that *Sm*AChE is associated with the outer membrane and is probably exposed on the surface of the parasite. The presence of AChE on the outer surface of the parasite raised the possibility of a role other than termination of synaptic transmission and also suggested that it might serve as an effective immunological target.

The simplest way to address the latter issue was to investigate whether antibodies raised against AChE would have an effect on the parasite. In our earlier studies, in the absence of specific antibodies against *Sm*AChE, we employed antibodies elicited against AChE of the electric eel, *Electrophorus electricus* (Anglister et al., 1979). We observed strong cross-reactivity, both by radioimmunoassay and by immunofluorescence, at all stages of the life cycle (Tarrab-Hazdai et al., 1984a). Furthermore, interaction with these antibodies led to marked complement-dependent cytotoxicity in intact schistosomula. These results prompted a more detailed investigation of *Sm*AChE.

Purification and biochemical characterization of S. mansoni AChE

To permit more detailed analysis of *Sm*AChE, we purified it from *S. mansoni* extracts, using affinity chromatography techniques similar to those used previously for purification of AChE from other species (Dudai & Silman, 1974). The affinity matrices used for purification of enzymes are usually constructed by covalently linking an appropriate competitive inhibitor to Sepharose via a spacer arm. The affinity ligand *m*-[(ϵ-aminocaproyl-ϵ-amino-caproyl)-*m*-aminophenyl] trimethyl ammonium bromide (mTA) is one of the inhibitors that has been used for purification (Viratelle & Bernhard, 1980), as has the corresponding *para* isomer (pTA) (Dudai & Silman, 1974). Other inhibitors, such as *N*-methylacridinium (MAC) (Dudai & Silman, 1974) and *N*-methyl-3-aminopyridinium (Arnon et al., 1987), have also been employed. The AChE was usually released by elution with a competitive inhibitor such as decamethonium or tensilon.

In our studies, Sepharose conjugates of three inhibitors were evaluated for purification of *S. mansoni* AChE. These include mTA, pTA, and MAC. Only the mTA-Sepharose conjugate served as a suitable affinity column for purification of *Sm*AChE (Goldlust et al., 1986). The enzyme eluted from this column by decamethonium bromide was purified 300-fold and had a specific activity of 11,000 units per mg protein. It migrated as a single band of 500 kDa on nondenaturing PAGE, and a major polypeptide band of ~80 kDa was seen on SDS-PAGE under reducing conditions (Fig. 2, insert), as well as light band at ~110 kDa. In our early studies, an additional band of 30 kDa was observed (Goldlust et al., 1986); subsequently, we were able to eliminate it by improving the antiprotease cocktail used for extraction of the enzyme.

The purified enzyme was found to be "true" AChE, since it hydrolyzed acetylcholine (ACh) at a sevenfold higher rate than butyrylcholine (Fig. 2). Furthermore, it was inhibited both by eserine and by the specific AChE inhibitor, BW284C51, but was almost unaffected by the specific butyrylcholinesterase inhibitor, iso-OMPA.

Sucrose gradient centrifugation of crude extracts of cercaria, and of the purified enzyme, revealed activity with a sedimentation coefficient of ~7 S (Goldlust et al., 1986). Under certain conditions, heavier species, ~10 and 32 S, were also observed (Tarrab-Hazdai et al., 1984b). These data suggest the existence of multiple molecular forms of *Sm*AChE, which differ in their solubility characteristics and quaternary structure. This phenomenon, which has been observed in AChE of many other species (Massoulié & Bon, 1982), was more pronounced in the extracts of adult worms than in

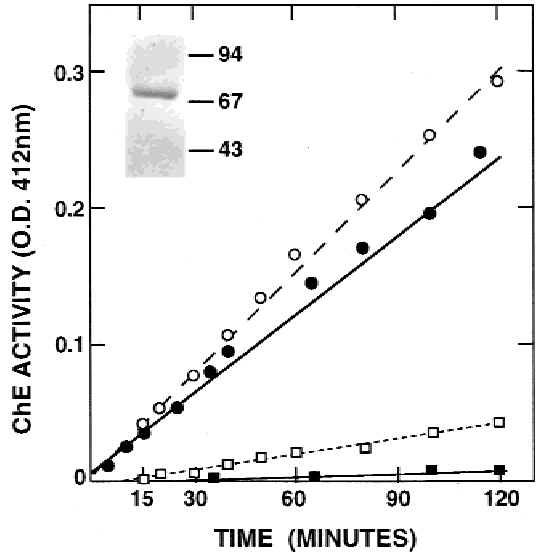

Fig. 2. Substrate specificity of crude and purified *S. mansoni* AChE. Cholinesterase activity was assayed by the colorimetric assay with acetylthiocholine or butyrylthiocholine as substrates. ○, crude enzyme + acetylthiocholine; ●, purified enzyme + acetylthiocholine; □, crude enzyme+ butyrylthiocholine; ■, purified enzyme + butyrylthiocholine. Insert: Coomassie Blue staining of the purified *Sm*AChE enzyme on SDS-PAGE under reducing conditions.

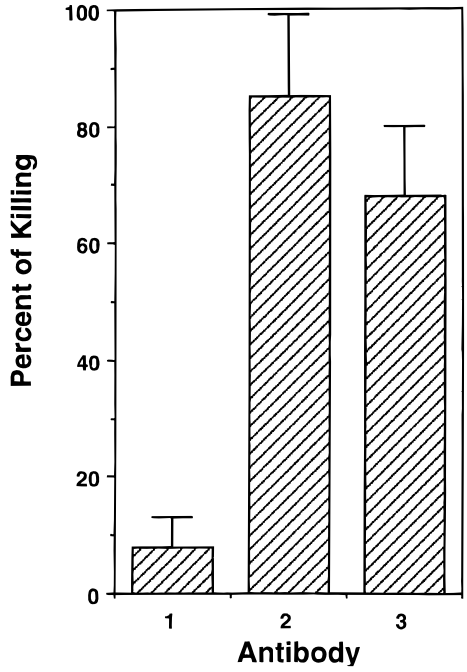

Fig. 3. Cytotoxic effect of anti-AChE antibody. Mechanically transformed schistosomula (200) were exposed to normal rabbit serum (1), anti-*Sm*AChE serum (2), or to rabbit antiserum raised against a total parasite extract (3), in the presence of guinea pig complement. The extent of killing was determined after 18 h incubation at 37 °C in 10% CO_2 by counting under the microscope.

extracts of cercariae or schistosomulae (Tarrab-Hazdai et al., 1984b; Camacho et al., 1994; Camacho et al., 1996). As will be shown below, further fractionation, based on the heparin-binding characteristics, permitted a more detailed analysis of the molecular forms of *Sm*AChE.

Immunological characterization

The availability of pure enzyme permitted the preparation of specific antibodies against *Sm*AChE, which were capable of binding to the surfaces of both 3 h schistosomula and adult worms. These antibodies were shown to be AChE-specific, since they recognized a single 8 S fraction in the total extract of the parasite, which coincided with the fraction containing AChE activity (Espinoza et al., 1991b). Furthermore, they precipitated AChE activity following immunoprecipitation of the schistosomal extracts, revealing major bands of 76 and 110 kDa. Most significantly, these antibodies were highly cytotoxic to the parasite in the presence of guinea pig complement (Arnon et al., 1987; Arnon, 1991) (Fig. 3).

The availability of specific antibodies permitted use of immunohistochemical procedures for localization of AChE in the parasite (Espinoza et al., 1991b). Immunogold labeling demonstrated that the AChE is located partly within the parasite, mainly associated with muscle, and partly on the outer face of the tegument, indeed providing a possible target for immune attack (Fig. 4). Staining of the longitudinal muscle is concentrated in patches that presumably correspond to muscle endplates.

In view of the above results, *Sm*AChE could be a potential candidate for vaccine development. However, considering the ubiquity of AChE and its vital role in neurotransmission in the host, absence of cross-reactivity with human AChE is a prerequisite for its being proposed as a suitable candidate antigen. This is an important point, especially in view of our previous observations that antibodies against electric eel AChE do cross-react with the schistosomal enzyme (Tarrab-Hazdai et al., 1984a). To study this issue we employed purified human erythrocyte AChE, together with polyclonal antibodies raised against it. No cross reaction was observed, either between human AChE and polyclonal antibodies against *Sm*AChE, or between the *S. mansoni* enzyme and the antibodies raised against the human AChE (Espinoza et al., 1991b). These results, which were demonstrated by radioimmunoassay and corroborated by immunoblotting and immunoprecipitation, are indeed encouraging and call for further exploration of *Sm*AChE as a potential vaccine.

In an extension of the above study, interesting results were achieved with a series of monoclonal antibodies (MAb) raised against *Sm*AChE (Espinoza et al., 1995). For their preparation, tests were developed in which the hybridomas were screened for MAbs capable of specifically recognizing catalytically active AChE. The various MAbs recognized different epitopes (either protein or carbohydrate) and could distinguish between the various life cycle stages of the parasite, as well as between the parasite AChE and those from other species. In contrast to the polyclonal antibodies against *Sm*AChE, one MAb showed cross reactivity with human AChE. Interestingly, the same MAb also cross-reacted with a dodecapeptide corresponding to the conserved sequence found in the active site of AChE from different species (Fig. 5). However, most of the schistosomal AChE epitopes are species-specific and are not shared with vertebrate AChE. Several MAbs displayed complement-dependent toxicity toward the parasite; hence, the epitopes that they recognize are suitable candidates for design of vaccines that

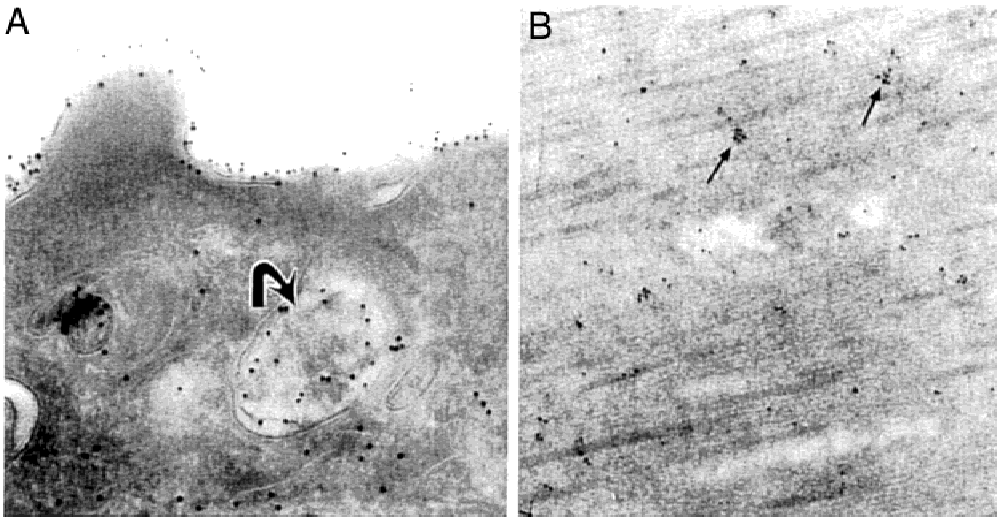

Fig. 4. Transmission electromicrograph of specific labeling of AChE in a frozen section of schistosomula. Schistosomula, 4 h after mechanical transformation, were incubated with specific anti-*Sm*AChE antibodies, followed by gold conjugated goat anti-rabbit IgG antibodies. Straight arrows and arrowheads show aggregates of immunogold particles in muscle. Curved arrow shows *Sm*AChE localization in a membranal body in the tegument; clear labeling is also seen on the outer surface membrane; (**A**) × 40,000; (**B**) × 30,000.

could provide protective immunity. Finally, as demonstrated immunohistochemically, some MAbs recognized the surface AChE, while others reacted only with the internal enzyme, indicative of possible differences in properties and function of the internal and external AChE.

Surface-localized AChE and its anchoring

A related issue concerns the mode of association of AChE with the surface membrane of the parasite. Taking advantage of the impermeability of the tegumental membrane to ACh, we were able to show that about 50% of the total parasite enzyme is present on the surface of intact schistosomula. This result was shown by inhibition studies using impermeable and permeable enzyme inhibitors (Camacho et al., 1994). As shown in Table 1, when intact schistosomula were exposed to either of the charged AChE inhibitors, BW284C51 and eserine, just under 50% of the AChE activity was inhibited, presumably corresponding to surface AChE. The permeable inhibitor, diisopropylfluorophosphate (DFP), produced almost total inhibition. However, inhibition with DFP in the presence of BW284C51 yielded 54% residual activity, presumably because the charged reversible inhibitor could protect the surface AChE, but not the internal enzyme, from inhibition by DFP. These results are in accord with the data mentioned above concerning detection of both internal and surface AChE by an immunohistochemical technique. It was, therefore, of interest to characterize the interaction of both the external and internal enzyme with their sites of attachment.

Over a decade ago, a novel mechanism for hydrophobic attachment of proteins to surface membranes was described, which is now known to be shared by a large number of surface proteins of diverse functions and origins (Low et al., 1986; Ferguson & Williams, 1988). The hydrophobic anchor is the 1,2-diacylglycerol moiety of a single phosphatidylinositol (PI) molecule, which is covalently attached to the carboxyl terminus of the polypeptide chain via an intervening oligoglycan. The initial evidence for this mode of attachment came from the observation that certain ectoenzymes, including AChE, could be released from the surface membranes to which they were attached by a PI-specific phospholipase C (PIPL-C) of bacterial origin (Low, 1987). We subsequently showed that AChE is susceptible to release from the *S. mansoni* tegumental membrane vesicles by PIPL-C of either *Staphylococcus aureus* or *Bacillus thuringiensis*, suggesting that AChE of this parasite, as in higher organisms, is anchored to the membrane via a covalently attached glycophosphatidylinositol (GPI) anchor. The enzyme so released was shown to be true AChE on the basis of its substrate specificity and sensitivity to selective inhibitors. Sucrose gradient centrifugation revealed a sedimentation coefficient of ~7.5 S. Thus, this form is most probably a G_2 dimer, like the GPI-anchored form of AChE from *Torpedo* (Espinoza et al., 1988; Tarrab-Hazdai et al., 1999).

An interesting phenomenon is the effect of PIPL-C on intact living schistosomula. We were able to demonstrate that PIPL-C removed large amounts of AChE from the surface of schistosomula in culture, without impairing the viability of the parasite (Espinoza et al., 1988). The amount of AChE released by PIPL-C increased with time of culture of the parasite, reaching a maximum for 24 h old schistosomula (Fig. 6), and paralleled an increase in overall levels of AChE in the parasite. Whereas release of AChE to the supernatant from membrane vesicles was paralleled by concomitant depletion of the enzyme from the membrane pellet, in the case of the intact parasite, the total amount of AChE within and on the surface of the parasite was not diminished; on the contrary, a significant increase was observed (insert of Fig. 6), despite the release of enzyme to the medium. Since PIPL-C is known to cleave only inositol phosphate groups present on the external surface, it must be assumed that such release of the AChE triggers immediate replenishment of the surface enzyme. This phenomenon is not observed for another GPI-anchored protein, alkaline phosphatase, that is also present on the parasite tegument.

The mechanism of this up-regulation has not been fully elucidated, but definitely involves de novo protein synthesis, since it is prevented by the protein synthesis inhibitor, cycloheximide (Espinoza et al., 1991a). Furthermore, as indicated above, cleavage of the GPI anchor by PIPL-C releases free 1,2-diacylglycerol (DAG).

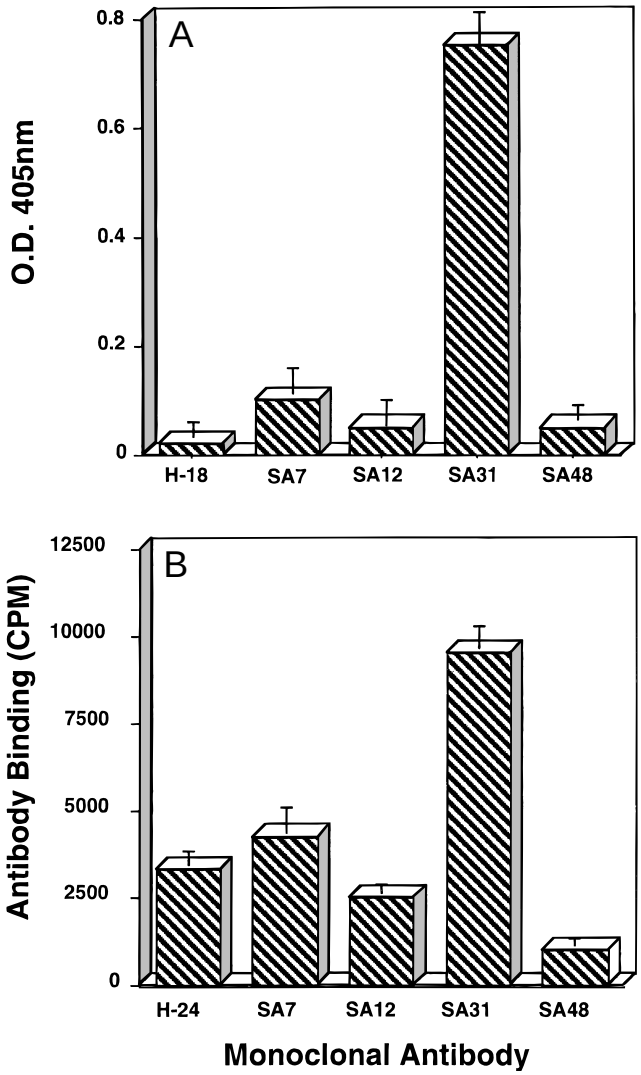

Fig. 5. **A:** Binding of MAbs to human erythrocyte AChE. **B:** Binding of MAbs to the conserved region of the AChE catalytic site. Semipurified human erythrocyte AChE (5 μg/100 μL/well), or the synthetic 12 amino acid peptide, were adsorbed to microplates. After 2 h incubation at 25 °C, the wells were washed and the indicated MAbs Ig were added (25 μg/50 μL/well) for an additional 18 h incubation at 4 °C. Following washes, MAb binding was determined in (**A**) by ELISA, using a goat anti-mouse Ig conjugated to β-galactosidase and ONPG as substrate, and in (**B**) by RIA using ^{125}I-labeled goat anti-mouse Ig. The monoclonal antibodies SA7, SA12, SA31, and SA48 were anti-*Sm*AChE, while H-18 and H-24 were negative controls.

We hypothesize that the de novo synthesis of AChE is triggered by second messenger activity of the released DAG. Indeed, we have demonstrated that three different DAGs, namely dioctanoyl-*sn*-glycerol (C_8), 1-oleoyl-2-acetyl-*sn*-glycerol (C_{18}), and dimyristin (C_{14}), could also elicit an increase in AChE activity similar to that caused by PIPL-C. As these compounds are known to be activators of protein kinase C, it is possible that the increase in levels of AChE as a consequence of PIPL-C cleavage is actually a protein kinase C-mediated process. In any event, regardless of the exact mechanism, the mere fact that the release of the AChE from the parasite membrane triggers an immediate synthesis and replenishment of the enzyme on the surface of the schistosomula is a further indication of a vital function for the tegumental AChE in *S. mansoni*.

Table 1. *Qualification of surface vs. internal SmAChE*

Inhibitor[a]	CPM[b] (residual activity %)	Surface-associated activity (%)
Total activity	33,078 (100)	
BW 284C51	18,157 (55)	45
Eserine	19,153 (58)	42
DFP	571 (2)	0
DFP +BW 284C51	17,930 (54)	54

[a]Quantification of surface-associated and internal *Sm*AChE: 24 h after transformation, schistosomula were incubated with BW284C51 (10^{-3} M), eserine (10^{-4} M), BW284C51+DFP (10^{-4} M), or in the absence of inhibitor, for 10 min; soluble extracts were then made prior to (in the case of BW) or after washing of the parasites.
[b]AChE was quantified radioenzymatically/3000 schistosomula.

Interaction of globular species of AChE with heparin

The existence of two forms of AChE in *S. mansoni*, external and internal, received new impetus in our recent study on the interaction of *Sm*AChE with heparin. As mentioned above, AChE occurs both in vertebrates and invertebrates in a repertoire of molecular forms (Massoulié et al., 1993; Talesa et al., 1995). These include the globular (G) forms, containing 1, 2, or 4 catalytic subunits (G_1,

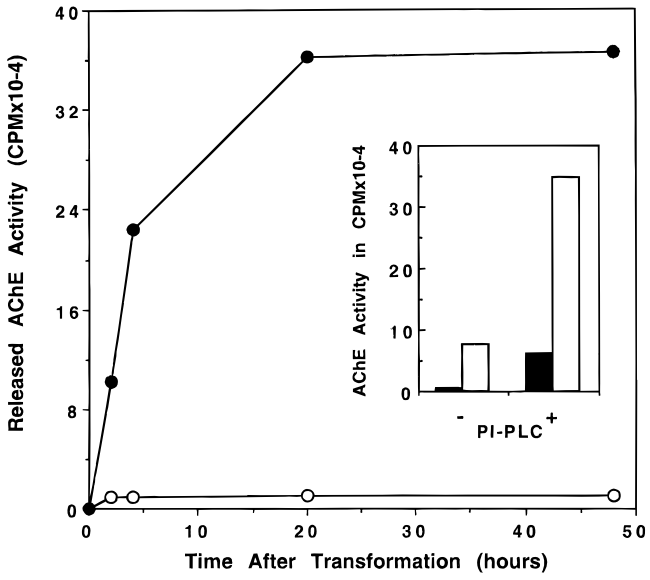

Fig. 6. Effect of age of schistosomula on the release of *Sm*AChE by PIPL-C. Schistosomula in culture (10,000), obtained at different times after transformation, were washed and incubated with 8 μg/mL PIPL-C for 60 min. Released *Sm*AChE activity was assayed in the supernatants of these cultures (●), as well as in the controls without PIPL-C (○). Insert shows overall levels of *Sm*AChE in the cultured schistosomula, before and after treatment with PIPL-C. Full bars represent *Sm*AChE activity in the supernatant, and empty bars the activity in the pellet. Results are expressed as total cpm of [^{3}H]ACh hydrolyzed.

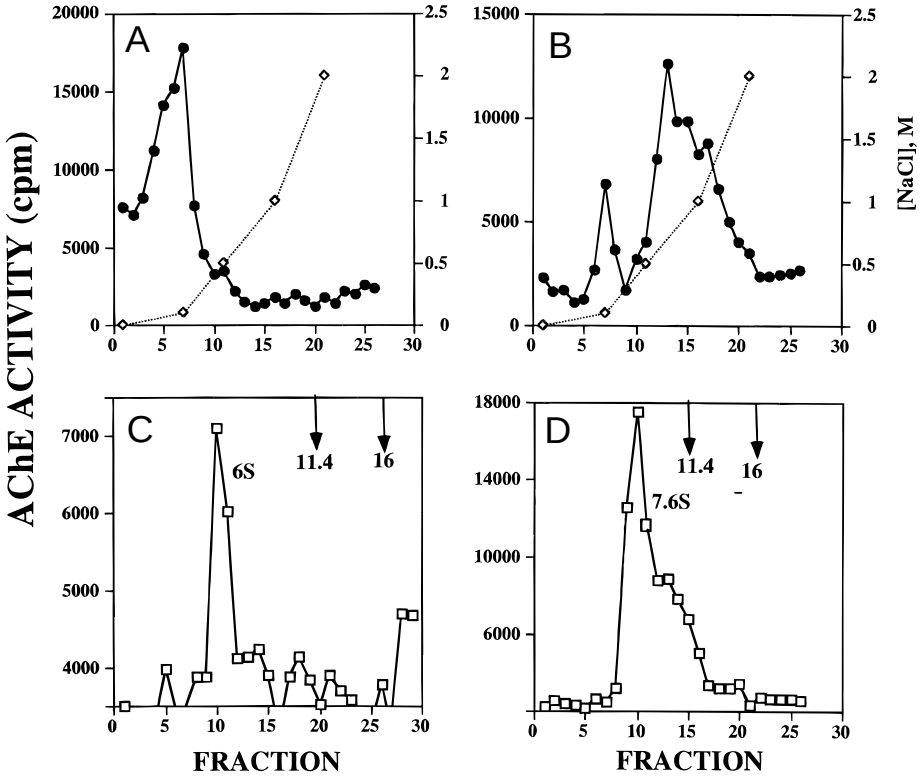

Fig. 7. Heparin-binding capacity and characterization by sucrose gradient centrifugation of AChE from schistosomula. Schistosomula obtained after 24 h in culture were incubated in fresh medium with PIPL-C (8 μg/mL) for 2 h at 37 °C. The parasites were separated from the incubation medium by centrifugation (80 × *g*, 1 min). The supernatant, representing the PIPL-C-solubilized fraction, was retained. The parasite pellet, containing residual AChE activity, was solubilized with 1% Triton X-100 in 50mM Tris-HCl, pH 7.5. Both the PIPL-C (**A**) supernatant and the (**B**) extract were subjected to affinity chromatography on heparin-Sepharose. The AChE activity passing through the heparin-Sepharose column in the case of the PIPL-C supernatant was analyzed by sucrose gradient centrifugation on a 5–20% sucrose gradient in 0.1% Triton X-100/50 mM Tris-HCl, pH 7.5 (**C**), and the material eluted by NaCl from the heparin-Sepharose column to which the extract of the pellet had been applied was analyzed similarly (**D**).

G_2, and G_4, respectively), and the asymmetric (A) forms, containing 4, 8, or 12 subunits (A_4, A_8, and A_{12}, respectively), attached to a collagenous tail (Massoulié et al., 1993). The G forms are either water-soluble or membrane-bound, the latter being anchored to the surface membrane by hydrophobic moieties that are sometimes attached post-translationally. The A forms are believed to be attached to the basal lamina within the synaptic cleft by interaction with heparan sulfate (Casanueva et al., 1998). The reason for this elaborate polymorphism is, as yet, unknown, but has been ascribed to the functional requirements of different types of synapses (Magazanik et al., 1984; Silman & Futerman, 1987; Massoulié et al., 1993). It appears to arise, at least in part, from differential targeting, to either the presynaptic or postsynaptic surface membranes, or to secretion (Rosenberry, 1985). Thus, for example, the A forms are localized to the endplate with cholinergic synapses in the central nervous system (Inestrosa & Perelman, 1990). As already mentioned, the A forms interact specifically with heparin or heparin-like glycosaminoglycans, and motifs on the collagen tail have been identified that are involved in this interaction (Deprez & Inestrosa, 1995). The G forms of AChE lack the collagen tail and are usually considered to lack the capacity to interact with heparin. However, it has been shown that the G_4 form from chick muscle binds to heparin with low affinity (Ramirez et al., 1990), and that an amphiphilic G_2 form of butyrylcholinesterase extracted from mucosal cells of rat intestine can also interact with heparin (Sine et al., 1994).

As mentioned above, it was shown earlier (Camacho et al., 1994, 1996) that in adult *S. mansoni* AChE activity could be partially resolved into two peaks of ~6 and 8 S, both, presumably, representing G forms of the enzyme. The AChE released from the parasite surface by PIPL-C is GPI-anchored and is probably homologous to the GPI-anchored G_2 form of AChE present in *Torpedo* electric organ (Silman & Futerman, 1987; Mehlert et al., 1993). It was, therefore, of interest to find that *Sm*AChE could be fractionated into two fractions on the basis of their heparin-binding properties, and that the two fractions, which correspond to the internal and external AChE pools, both represent G forms.

Upon passing an extract of *S. mansoni* with the nonionic detergent Triton X-100 over a heparin-Sepharose column, only the 8S form of AChE was retained on the column (Tarrab-Hazdai et al., 1999). The bound AChE could be progressively eluted by increasing the salt concentration, complete elution being achieved at 0.5–0.6 M NaCl. To correlate the heparin-binding capacity of *Sm*AChE and its localization, we studied separately the properties of the surface and the internal enzyme. For this purpose, intact schistosomula, cultured for 24 h, were exposed to PIPL-C, and both the released AChE,and the residual enzyme extracted from the whole organism were fractionated on a heparin column. The results (Fig. 7A) clearly demonstrate that the surface, namely PIPL-C-released, AChE does not bind to the column and is almost completely recovered in the effluent. Sucrose gradient centrifugation of

this fraction reveals a major peak of 6.5 S. In contrast, most of the AChE activity in the extract of the residual organism was bound to the heparin column and was eluted at 0.6 M NaCl; upon sucrose gradient centrifugation it displayed a major peak at ~8 S (Fig. 7B,D). These data were corroborated by selective inhibition experiments on live parasites in which the covalent AChE inhibitor echothiophate (phospholine), which does not penetrate the tegument, selectively inhibited the 6.5 S form, but not the 8 S form, suggesting an internal localization for the latter. Furthermore, the monoclonal antibody, SA7, which was previously shown to inhibit the surface AChE, bound to the 6.5 S form selectively, while the SA57 MAb preferentially recognized the heparin-bound 8 S form, which is internally located.

Conclusion

Functional role of schistosomal AChE

Efforts of recent years led to the identification and characterization of several functional components in parasites that might be involved in their drug sensitivity or resistance, as well as in their susceptibility to immune attack. In the case of schistosomes such a functional protein is the enzyme AChE.

Our findings clearly demonstrate the presence of two forms of AChE in *S. mansoni*. One is internal, associated with the musculature and involved in cholinergic processes. Indeed, immunoelectron microscopy reveals association of AChE with the musculature of schistosoma, and pharmacological evidence supports its functional role at the motor endplates of the parasite. The other form is external, associated with the tegument, and presumably involved in noncholinergic processes, perhaps in signal transduction (Espinoza et al., 1991a). Another possibility is that the AChE serves as an adhesion protein, as has been suggested for other species (Botti et al., 1998). It is worth noting that in addition to the above, a role for tegumental AChE in modulation of ACh-induced glucose import in *Schistosoma hematobium* was proposed by Camacho and Agnew (1995).

Since the GPI-anchored AChE is on the external surface of the tegument, it is not surprising that exposure of schistosomula to specific monoclonal antibodies does not affect the motor activity in the parasite, even though it inhibits the AChE activity. On the other hand, polyclonal anti-AChE are cytotoxic and lead to almost total complement-dependent killing of the parasite, indicating its susceptibility as an immunological target.

Only the internal form interacts with heparin; hence, it is the heparin-binding fraction that is the functional form in the muscle. In the extensive literature on molecular forms of AChE in skeletal muscle, both A and G forms are described (Massoulié et al., 1993). However, it is generally assumed that the A forms, associated with the basal lamina at the motor endplate, are the functional forms, at least in fast and slow twitch muscles (Barnard et al., 1984). We have demonstrated that in striated muscles of *S. mansoni*, just as is the case for the frog synaptic basal lamina (Anglister et al., 1994), the functional forms of AChE are G forms, and that they are associated with the extracellular matrix.

AChE is thus demonstrated to be a functional antigen, involved in multifaceted activities, and can hence serve as a suitable candidate for both diagnostic purposes, vaccine development, and drug design.

Acknowledgments

We are grateful to J.L. Sussman for providing Figure 1.

References

Aldridge WM, Reiner E. 1972. *Enzyme inhibitors as substrates. Interaction of esterases with esters of organophosphorus and carbamic acids*. North Holland, Amsterdam.

Anglister L, Haesaert B, McMahan UJ. 1994. Globular and asymmetric acetylcholinesterase in the synaptic basal laminal of skeletal muscle. *J Cell Biol 125*:183–196.

Anglister L, Tarrab-Hazdai R, Fuchs S, Silman I. 1979. Immunological cross-reactivity between electric eel acetylcholinesterase and rat tail-tendon collagen. *Eur J Biochem 94*:25–29.

Arnon R. 1991. Immuno-parasitological parameters in schistosomiasis—A perspective view of a vaccine-oriented immunochemist. *Vaccine 9*:379–394.

Arnon R, Espinoza-Ortega B, Tarrab-Hazdai R. 1987. Acetylcholinesterase of *Schistosoma mansoni*—An antigen of functional implications. *Mem Inst Oswaldo Cruz 82*:163–170.

Auld VJ, Fetter RD, Broadie K, Goodman CS. 1995. Gliotactin, a novel transmembrane protein on peripheral glia, is required to form the blood-nerve barrier in *Drosophila*. *Cell 81*:757–767.

Axelsen PH, Harel M, Silman I, Sussman JL. 1994. Structure and dynamics of the active site gorge of acetylcholinesterase: Synergistic use of molecular dynamics simulation and X-ray crystallography. *Protein Sci 3*:188–197.

Barker LR, Bueding E, Timms AR. 1966. The possible role of acetylcholine in *Schistosoma mansoni*. *Brit J Pharmacol 26*:656–665.

Barnard EA, Barnard PJ, Jarvis J, Jedrzejczyk J, Lai J, Pizzey JA, Randall WR, Silman I. 1984. Multiple molecular forms of acetylcholinesterase and their relationship to muscle function. In: Brzin M, Barnard EA, Sket D, eds. *Cholinesterases: Fundamental and applied aspects*. Berlin: Walter de Gruyter. pp 49–71.

Barthalay Y, Hipeau-Jacquotte R, de la Escalera S, Jimenez F, Piovant M. 1990. *Drosophila* neurotactin mediates heterophilic cell adhesion. *EMBO J 9*: 3603–3609.

Botti SA, Felder CE, Sussman JL, Silman I. 1998. Electrotactins: A class of adhesion proteins with conserved electrostatic and structural motifs. *Protein Eng 11*:415–420.

Bueding E. 1952. Acetylcholinesterase activity of *Schistosoma mansoni*. *Br J Pharmacol 7*:563–566.

Bueding E, Liun CL, Rogers SH. 1972. Inhibition by metrifonate and diclorovos of cholinesterases in schistosomiasis. *Brit J Pharmacol 46*:480–487.

Camacho M, Agnew A. 1995. Schistosoma: Rate of glucose import is altered by acetylcholine interaction with tegumental acetylcholine receptors and acetylcholinesterase. *Exp Parasitol 81*:584–591.

Camacho M, Alsford S, Agnew A. 1996. Molecular forms of tegumental and muscle acetylcholinesterase of Schistosoma. *Parasitology 112*:199–204.

Camacho M, Tarrab-Hazdai R, Ezpinoza B, Arnon R, Agnew A. 1994. The amount of acetylcholinesterase on the parasite surface reflects the differential sensitivity of schistosome species to metrifonate. *Parasitology 108*:153–160.

Casanueva OI, Garcia-Huidobro T, Campos EO, Aldunate R, Garrido J, Inestrosa NC. 1998. A major portion of synaptic basal lamina acetylcholinesterase is detached by high salt- and heparin-containing buffers from rat diaphragm muscle and *Torpedo* electric organ. *J Biol Chem 273*:4258–4265.

Casida JE, Quistad GB. 1998. Golden age of insecticide research: Past, present, or future? *Annu Rev Entomol 43*:14–16.

Chalfie M, White J. 1988. The nervous system. In: Wood WB, ed. *The nematode Caenorhabditis elegans*. New York: Cold Spring Harbor. pp 337–391.

Darboux I, Barthalay Y, Piovant M, Hipeau-Jacquotte R. 1996. The structure-function relationships in *Drosophila* neurotactin show that cholinesterasic domains may have adhesive properties. *EMBO J 15*:4835–4843.

Deprez PN, Inestrosa NC. 1995. Two heparin-binding domains are present on the collagenic tail of asymmetric acetylcholinesterase. *J Biol Chem 270*: 11043–11046.

Dougherty DA, Stauffer DA. 1990. Acetylcholine binding by a synthetic receptor: Implications for biological recognition. *Science 250*:1558–1560.

Dudai Y, Silman I. 1974. Acetylcholinesterase. *Methods Enzymol 34*:571–579.

Espinoza B, Parizade M, Ortega E, Tarrab-Hazdai R, Zilberg D, Arnon R. 1995. Monoclonal antibodies against acetylcholinesterase of *Schistosoma mansoni*: Production and characterization. *Hybridoma 14*:577–586.

Espinoza B, Silman I, Arnon R, Tarrab-Hazdai R. 1991a. Phosphatidylinositol-specific phospholipase C induces biosynthesis of acetylcholinesterase via diacylglycerol in *Schistosoma mansoni. Eur J Biochem 195*:863–870.

Espinoza B, Tarrab-Hazdai R, Himmeloch S, Arnon R. 1991b. Acetylcholinesterase from *Schistosoma mansoni*: Immunological characterization. *Immunol Lett 28*:167–174.

Espinoza B, Tarrab-Hazdai R, Silman I, Arnon R. 1988. Acetylcholinesterase in *Schistosoma mansoni* is anchored to the membrane via covalently attached phosphatidylinositol. *Mol Biochem Parasitol 29*:171–179.

Ferguson MAJ, Williams AF. 1988. Cell-surface anchoring of proteins via glycosyl-phosphatidylinositol. *Ann Rev Biochem 57*:285–320.

Fisher A, Brandeis R, Chapman S, Pittel Z, Michaelson DM. 1998. M1 muscarinic agonist treatment reverses cognitive and cholinergic impairments of apolipoprotein E-deficient mice. *J Neurochem 70*:1991–1997.

Fripp P. 1967. Histochemical localization of esterase activity in schistosomes. *Exp Parasitol 21*:380–390.

George ST, Balasubramanian AS. 1981. The aryl acylamidases and their relationship to cholinesterases in human serum, erythrocyte and liver. *Eur J Biochem 121*:177–186.

Goldlust A, Arnon R, Silman I, Tarrab-Hazdai R. 1986. Acetylcholinesterase of *Schistosoma mansoni*: Purification and characterization. *J Neurosci Res 15*:569–581.

Greenfield S. 1984. Acetylcholinesterase may have novel functions in the brain. *TINS 7*:364–368.

Harel M, Schalk I, Ehret-Sabatier L, Bouet F, Goeldner M, Hirth C, Axelsen PH, Silman I, Sussman JL. 1993. Quaternary ligand binding to aromatic residues in the active-site gorge of acetylcholinesterase. *Proc Natl Acad Sci USA 90*:9031–9035.

Hillman GR, Senf AW, Gibleur WB. 1978. The mode of action of hycanthone revisited. *J Parasitol 64*:754–756.

Hussein AS, Grigg ME, Selkirk ME. 1999. *Nippostrongylus brasiliensis*: Characterisation of a somatic amphiphilic acetylcholinesterase with properties distinct from the secreted enzymes. *Exp Parasitol 91*:144–150.

Ichtchenko K, Hata Y, Nguyen T, Ullrich B, Missler M, Moomaw C, Sudhof TC. 1995. Neuroligin 1: A splice site-specific ligand for beta-neurexins. *Cell 81*:435–443.

Inestrosa NC, Perelman A. 1990. Association of acetylcholinesterase with the cell surface. *J Membrane Biol 118*:1–9.

Koelle GB, ed. 1963. *Cholinesterases and anticholinesterase agents. Handbuch der experimentellen pharmakologie*, Vol XV. Berlin: Springer Verlag.

Krejci E, Duval N, Chatonnet A, Vincens P, Massoulié J. 1991. Cholinesterase-like domains in enzymes and structural proteins: Functional and evolutionary relationships and identification of a catalytically essential aspartic acid. *Proc Natl Acad Sci USA 88*:6647–6651.

Layer PG. 1983. Comparative localization of acetylcholinesterase and pseudocholinesterase during morphogenesis of the chicken brain. *Proc Natl Acad Sci USA 80*:6413–6417.

Layer PG, Rommel S, Bulthoff H, Hengstenberg R. 1988. Independent spatial waves of biochemical differentiation along the surface of chicken brain as revealed by the sequential expression of acetylcholinesterase. *Cell Tissue Res 251*:587–595.

Levi-Schaffer F, Tarrab-Hazdai R, Meshulam H, Arnon R. 1984a. Effect of phosphonium salts and phosphoranes on the acetylcholinesterase activity and on the viability of *Schistosoma mansoni* parasites. *Int J Immunopharmac 6*:619–627.

Levi-Schaffer F, Tarrab-Hazdai R, Schryer MD, Arnon R, Smolarsky M. 1984b. Isolation and partial characterization of the tegumental outer membrane of schistosomula of *Schistosoma mansoni*. *Mol Biochem Parasitol 13*:283–300.

Low MG. 1987. Biochemistry of the glycosyl-phosphatidylinositol membrane protein anchors. *Biochem J 244*:1–13.

Low MG, Ferguson MAJ, Futerman AH, Silman I. 1986. Covalently attached phosphatidylinositol as a hydrophobic anchor for membrane proteins. *Trends Biochem Sci 11*:212–215

Magazanik LG, Fedorov VV, Giniatullin RA, Nikolsky EE, Snetkov VA. 1984. Functional role of cholinesterase in different types of neuromuscular junction. In: Brzin M, Barnard EA, Sket D, eds. *Cholinesterases: Fundamental and applied aspects*. Berlin: Walter de Gruyter. pp 229–242.

Marshall I. 1987. Experimental chemotherapy. In: Rollinson D, Simpson AJG, eds. *The biology of the schistosomes from genes to latrines*. London, New York: Academic Press. pp 399–423.

Martin RJ. 1997. Modes of action of antihelmintic drugs. *Vet J 154*:11–34

Massoulié J, Anselmet A, Bon S, Krejci E, Legay C, Morel N, Simon S. 1998. *J Physiol (Paris) 92*:183–190.

Massoulié J, Bon S. 1982. The molecular forms of cholinesterases in vertebrates. *Ann Rev Neurosci 5*:57–106.

Massoulié J, Pezzementi L, Bon S, Krejci E, Vallette FM. 1993. Molecular and cellular biology of cholinesterases. *Prog Neurobiol 41*:31–91.

Mehlert A, Varon L, Silman I, Homans SW, Ferguson MAJ. 1993. Structure of the glycosyl-phosphatidylinositol membrane anchor of acetylcholinesterase from the electric organ of the electric fish, *Torpedo californica*. *Biochem J 296*:863–870.

Millard CB, Kryger G, Ordentlich A, Greenblatt HM, Harel M, Raves ML, Segall Y, Barak D, Shafferman A, Silman I, et al. 1999. Crystal structures of aged phosphonylated acetylcholinesterase: Nerve agent reaction products at the atomic level. *Biochemistry 38*:7032–7039.

Nolte HJ, Rosenberry TL, Neumann E. 1980. Effective charge on acetylcholinesterase active sites determined from the ionic strength dependence of association rate constants with cationic ligands. *Biochemistry 19*:3705–3711.

Nordgren I, Bengtsson E, Holmstedt B, Pettersson BM. 1981. Levels of metrifonate and dichlorvos in plasma and erythrocytes during treatment of schistosomiasis with Bilarcil. *Acta Pharmacol Toxicol (Copenhagen) 49 Suppl 5*:79–86.

Ollis DL, Cheah E, Cygler M, Dijkstra B, Frolow F, Franken SM, Harel M, Remington SJ, Silman I, Schrag J, et al. 1992. The alpha/beta hydrolase fold. *Protein Eng 5*:197–211

Olson PF, Fessler LI, Nelson RE, Sterne RE, Campbell AG, Fessler, JH. 1990. Glutactin, a novel *Drosophila* basement membrane-related glycoprotein with sequence similarity to serine esterases. *EMBO J 9*:1219–1227.

Pax RA, Bennett JL. 1991. Neurobiology of parasitic platyhelminths: Possible solutions to the problems of correlating structure and function. *Parasitology 102*:S31–S39.

Pax RA, Day TA, Miller CL, Bennett JL. 1996. Neuromuscular physiology and pharmacology of parasitic flatworms. *Parasitology 113 Suppl*:S83–96

Pax RA, Siefker C, Hickox T, Bennett JL. 1981. *Schistosoma mansoni*: Neurotransmitters, longitudinal musculature and effects of electrical stimulation. *Exp Parasitol 52*:346–355.

Quinn DM. 1987. Acetylcholinesterase: Enzyme structure, reaction dynamics and virtual transition states. *Chem Rev 87*:955–979.

Ramirez G, Barat A, Fernandez HL. 1990. Interaction of asymmetric and globular acetylcholinesterase species with glycosaminoglycans. *J Neurochem 54*:1761–1768.

Rapson EB, Chilwan AS, Jenkins DC. 1986. Acetylcholinesterase secretion—A parameter for the interpretation of in vitro anthelmintic screens. *Parasitology 92*:425–430.

Rathaur S, Muller S, Maizels RM, Walter LD. 1992. Identification of circulating parasite acetylcholinesterase in human and rodent filariasis. *Parasitol Res 78*:671–676.

Rosenberry TL. 1985. Structural distinctions among acetylcholinesterase forms. In: Martonosi AN, ed. *The enzymes of biological membranes*, vol. 3. New York: Plenum Press. pp 403–429.

Silman I, Futerman AH. 1987. Modes of attachment of acetylcholinesterase to the surface membrane. *Eur J Biochem 170*:11–22.

Sine JP, Toutant JP, Colas B. 1994. Butyrylcholinesterase amphiphilic forms of the mucosal cells of rat intestine bind heparin. *Biochem Biophys Res Comm 201*:1376–1381.

Sukhdeo SC, Sangester NC, Mettrick DF. 1986. Effects of cholinergic drugs on longitudinal muscle contractions of *Fasciola hepatica*. *J Parasitol 72*:858–864.

Sussman JL, Harel M, Frolow F, Oefner C, Goldman A, Toker L, Silman I. 1991. Atomic structure of acetylcholinesterase from *Torpedo californica*: A prototypic acetylcholine-binding protein. *Science 253*:872–879.

Sussman JL, Harel M, Frolow F, Varon L, Toker L, Futerman AH, Silman I. 1988. Purification and crystallization of a dimeric form of acetylcholinesterase from *Torpedo californica* subsequent to solubilization with phosphatidylinositol-specific phospholipase C. *J Mol Biol 203*:821–823.

Talesa V, Grauso M, Giovannini E, Rosi G, Toutant JP. 1995. Acetylcholinesterase in tentacles of *Octopus vulgaris* (Chephalopoda). Histochemical localization and characterization of a specific high salt-soluble and heparin-soluble fraction of globular forms. *Neurochem Int 27*:201–207.

Talesa V, Romani R, Grauso M, Rosi G, Giovannini E. 1997. Expression of a single dimeric membrane-bound acetylcholinesterase in *Parascaris equorum*. *Parasitology 115*:653–660.

Tarrab-Hazdai R, Levi-Schaffer F, Gonzales G, Arnon R. 1984b. Acetylcholinesterase of *Schistosoma mansoni*: Molecular forms of the solubilized enyme. *Biochim Biophys Acta 790*:61–69.

Tarrab-Hazdai R, Levi-Schaffer F, Smolarsky M, Arnon R. 1984a. Acetylcholinesterase of *Schistosoma mansoni*: Antigenic cross-reactivity with *Electrophorus electricus* and its functional implications. *Eur J Immunol 14*:205–209.

Tarrab-Hazdai R, Toker L, Silman I, Arnon R. 1999. Acetylcholinesterase from *Schistosoma mansoni*: Interaction of globular species with heparin. *Biochem J*. Forthcoming.

Taylor P. 1990. Anticholinesterase agents. In: Gilman AG, Rall TW, Nies AS, Taylor P, eds. *The pharmacological basis of therapeutics*, 8th ed. New York: MacMillan. pp 131–149.

Viratelle OM, Bernhard SA. 1980. Major component of acetylcholinesterase in *Torpedo* electroplax is not basal lamina associated. *Biochemistry 19*:4999–5007.

Weise C, Kreienkamp HJ, Raba R, Pedak A, Aaviksaar A, Hucho F. 1990. Anionic subsites of the acetylcholinesterase from *Torpedo californica*. *EMBO J 9*:3885–3888.