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COMPARISON OF KINETIC PARAMETERS FOR ACETYLTHIOCHOLINE, SOMAN, KETAMINE AND FASCICULIN TOWARDS ACETYLCHOLINESTERASE IN LIPOSOMES AND IN SOLUTION

GERTRUD PUU* and MONA KOCH
National Defence Research Establishment, Division of Experimental Medicine, S-901 82 Umeå, Sweden

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Abstract—Purified acetylcholinesterase from bovine brain was reconstituted by a detergent depletion technique into liposomes, prepared from soybean lecithin. The kinetics for the substrate acetylthiocholine and for three inhibitors with very different binding properties was studied. The results were compared with results from corresponding experiments with solubilized enzyme in detergent solution. The reconstituted enzyme showed a higher affinity for acetylthiocholine, ketamine and fasciculin. Parameters unaffected by the reconstitution were: turnover number for the substrate; the non-competitive component in ketamine inhibition and the kinetics for the active site-directed irreversible inhibitor soman.

Few enzymes have been so extensively studied as regards inhibition as acetylcholinesterase (EC 3.1.1.7). Usually, such studies have been done on enzyme, solubilized from the membrane, to which it is attached *in vivo*. Is the solubilized enzyme a good model for inhibition studies *in vitro*, from which one often wants to deduce the action of a substance to the *in vivo* situation?

It seems reasonable to answer the question with yes, as acetylcholinesterase is not an integral membrane protein, i.e. it does not penetrate through the lipid bilayer. Its catalytically active subunits are completely in the extracellular, aqueous environment and only a small, non-catalytic part of the enzyme is in the membrane. For the globular, tetrameric enzyme in mammalian brain, the insertion into the membrane is via a 20 kDa, non-catalytic subunit [1, 2]. The subunit has three sub-domains, one of which is small (7 kDa), pronase-resistant and fatty acid-containing and thus suggested to be the actual membrane-anchoring domain [3]. The globular, dimeric enzyme in e.g. erythrocytes is attached to the membrane via a glucosyl-phosphatidylinositol linkage [4, 6].

There are few reported comparative studies on substrate and inhibitor kinetics on membrane-bound, non-solubilized acetylcholinesterase (i.e. enzyme in membrane fragments or in intact tissue) and solubilized enzyme [7–11]. Unequivocal support for the assumption of identical kinetics is not given by those results. It should be kept in mind that the enzyme, left in its natural membrane, by definition is unpurified. Other proteins present in the preparation might bind the ligand and thus reduce the available concentration of ligand, which could result in a false impression of decreased affinity for the esterase [9]. However, the membrane-bound enzyme was found to be more sensitive than its solubilized counterpart to some inhibitors, and this was most pronounced for some ligands which bind at a peripheral site of the enzyme [7, 11].

We found it appropriate to investigate whether inhibitors behave differently against purified acetylcholinesterase in a well-defined lipid environment, i.e. reconstituted in liposomes, as compared to detergent (Triton X-100) solubilized enzyme. Such comparative studies have been done earlier for the substrate reaction, and the Michaelis constant could be manipulated in both directions, dependent on the lipid composition [12–15]. Some authors claim that the inhibition by substrate at higher concentrations is changed after reconstitution [15].

For this work we selected bovine brain as the enzyme source. The purified enzyme was reconstituted into liposomes, prepared from soybean lecithin, which is rich in phosphatidylcholine. For inhibition, we selected three inhibitors with very different properties: soman, ketamine and fasciculin. Soman (1,2,2-trimethylpropyl methylfluoro-phosphonate) is a very potent organophosphorus inhibitor, which acts by irreversible binding to the active site of the enzyme [16]. Previous studies on other organophosphorus inhibitors and membrane-bound and solubilized acetylcholinesterase, respectively, indicate that such inhibitors do not usually discriminate between the enzyme forms [7, 8].

Ketamine (2-(*o*-chlorophenyl)-2-methylaminocyclohexone), which is an anesthetic agent, has a direct effect on biological membranes, in which it dissolves and affects the fluidity [17]. The substance has several other biological effects, one of which is mixed type inhibition of acetylcholinesterase [18, 19]. It has been suggested that the fluidity changes cause or contribute to the inhibition [17], but the inhibition pattern seems to be the same for solubilized and synaptosomal enzyme [19].

Fasciculin, isolated from the green mamba (*Dendroaspis angusticeps*), is a polypeptide, with a molecular weight of 6765 and a pronounced dipolar character [20–22]. It is a very tight-binding, non-competitive inhibitor against several but not all

* To whom correspondence should be addressed.

acetylcholinesterases. At neutral pH, there is an excess of positive charges. Most of those are located in one loop (position 22–39), while negatively charged amino acids are concentrated in another loop, close to the C-terminal end. As fasciculin can displace propidium in binding to the enzyme [21], the positively charged loop seems to be crucial for the anticholinesterase properties. We paid some extra attention to this inhibitor, as its action on purified acetylcholinesterase has not been studied before.

Finally we also studied the kinetics for the substrate, acetylthiocholine, including inhibition at higher concentrations.

MATERIALS AND METHODS

Fasciculin 1 was a gift from Dr Evert Karlsson, Institute of Biochemistry, University of Uppsala. Parke Davis Co. kindly gave us ketamine hydrochloride. Soman was synthesized at the Chemistry Division of this institute. Soybean lecithin type IV-S, *n*-octyl glucoside, proteinase K (EC 3.4.21.14) and acetylthiocholine iodide were from the Sigma Chemical Co. Affi-Prep™ 10 was from Bio-Rad and wheat germ lectin-Sepharose 6 MB from Pharmacia.

Purification of acetylcholinesterase. The membrane-bound form of the enzyme (mainly G4-form) was purified from bovine brain. The extraction of the enzyme from the tissue followed essentially the procedure of Sørensen *et al.* [23]. The purification scheme involved two affinity chromatography steps. The first one was on an Affi-Prep™ 10 gel, to which procainamide had been coupled via a spacer-arm. The enzyme was eluted by 10 mM decamethonium. The second step was on wheat germ lectin-Sepharose 6 MB, from which the enzyme was eluted by 1 M *N*-acetylglucosamine. The enzyme was purified 24,000 times and had a specific activity of 30 μkat/mg (1800 units/mg), with acetylthiocholine as substrate. The enzyme was stored in 20 mM sodium phosphate buffer pH 7.4, with 0.1% (w/v) Brij 35 and 0.05% (w/v) bovine serum albumin.

For studies on solubilized ("free") enzyme, the preparation was run through a small Sephadex G-25 column (Pharmacia PD 10), equilibrated with 20 mM Tris–HCl/100 mM NaCl/0.1% Brij 35/0.01% BSA, pH 7.4, in order to obtain the same experimental conditions as for the liposomal enzyme.

Preparation and characterization of acetylcholinesterase-containing liposomes. Essentially, the preparation of proteoliposomes followed the protocol of Mimms *et al.* [24]. Lecithin (5 mg) was dissolved in chloroform. The solvent was removed by rotary evaporation under a stream of nitrogen or argon. The thin film of lipids was left overnight in an argon atmosphere to permit evaporation of residual chloroform. Acetylcholinesterase (10–25 units) and 25 mg *n*-octyl glycoside in 1.4 mL 20 mM Tris–HCl/100 mM NaCl/0.01% bovine serum albumin (pH 7.4) was used for dissolution of the lipids. Ten small glass pearls were also added to improve the dissolution. The mixture was then applied to a column (42 × 1.6 cm), filled with Sephadex G-50, pre-equilibrated with 20 mM Tris–HCl/100 mM NaCl. The detergent depletion was run at 4°, with a flow rate of 7 mL/hr. Fractions of 1.8 mL were collected and analysed for liposomes (absorbance at 300 nm) and acetylcholinesterase activity.

The liposome-containing fractions were pooled, transferred to an Amicon concentration cell with a XM 300 membrane and concentrated and washed with buffer containing 0.01% BSA in three cycles to a final volume of 4 mL.

Proteinase K treatment. Liposomes were incubated, directly in a concentration cell with a XM 300 membrane, with proteinase K (8 μg/mL) for 40 min at room temperature. Degradation was stopped by dilution and free enzyme, including the protease, was removed by two washing cycles.

Enzyme assay. Enzyme activity was measured by the procedure of Ellman *et al.* [25] in an automatic enzyme analyzer Clinicon Corona, at 37° in 0.05 M sodium phosphate buffer, pH 8.0. For studies on free enzyme, 0.1% Triton X-100 was included in the buffer. Calculations of the kinetic constants for the enzyme were based on Eadie–Hofstee plots.

Inhibition by soman. Incubation took place at room temperature, in 20 mM Tris–HCl, 100 mM NaCl, pH 7.4 (for free enzyme also containing 0.1% Triton X-100). The concentrations of soman used were 0.5, 1.0, 2.0 and 4.0 nM. Aliquots were withdrawn in time series, 7–8 samples during a total time of 15–20 min, and residual activity measured as described above. The irreversible inhibition constant, k_i, was determined as the rate constant, obtained by linear regression, divided by the soman concentration used.

Inhibition by ketamine. The concentration of ketamine was varied between 0.2 and 2.0 mM, whilst the substrate concentration was varied between 0.05 and 0.8 mM. Calculation of K_{max}^{app} was based on Eadie–Hofstee plots. K_i and K_i' were obtained by plotting K_m^{app}/V_{max}^{app} and $1/V_{max}^{app}$, respectively, vs ketamine concentration, with linear regression.

Inhibition by fasciculin. Fasciculin was diluted in 50 mM sodium phosphate buffer, pH 8.0, containing 0.1% bovine serum albumin. The final concentration of inhibitor was in the range 8 to 200 pM. In some experiments the enzyme concentration was varied also. The interaction between inhibitor and enzyme was permitted to equilibrate for about 10 min before the substrate was added [20, 26]. The inhibition data were analyzed mainly algebraically, using the weighted Ackermann–Potter method [27, 28], to obtain both the inhibition constant, K_i', and the enzyme concentration.

RESULTS

Reconstitution and characterization of acetylcholinesterase in liposomes

We found that a detergent depletion technique, with *n*-octyl glycoside as detergent in a 12:1 molar ratio to the lipids, was the best way to obtain liposomes with acetylcholinesterase reconstituted in the membranes. This detergent was used although it has been reported to be inhibitory against bovine brain acetylcholinesterase [29]. We could confirm this with our enzyme preparation. The activity was completely abolished when measured in 2% *n*-octyl glycoside. Obviously this effect is transitory during the liposome

Table 1. Kinetic parameters for acetylthiocholine, soman, ketamine and fasciculin towards acetylcholinesterase in liposomes and in solution

	Solubilized	Liposomal
Acetylthiocholine		
(N = 10) K_m (mM)	0.082 (0.016)	0.057 (0.012)*
(N = 3) k_{cat} (sec^{-1})	10,700 (100)	10,200 (600)
(N = 3) K_{is} (mM)	30 (5)	23 (5)
Soman		
(N = 6) k_i (M^{-1} sec^{-1})	4.0×10^5 (0.6)	3.8×10^5 (0.5)
Ketamine		
(N = 4) K_i (mM)	0.53 (0.06)	0.31 (0.03)*
(N = 4) K'_i (mM)	3.2 (0.6)	3.9 (1.1)
Fasciculin		
K'_i (pM)	22	16†

The data are given, except for fasciculin, as mean values with standard deviations in parentheses. Differences were tested by Student's *t*-test and * denotes significant difference, $P < 0.001$.

For fasciculin the data are from an experiment with three concentrations of enzyme and 13 concentrations of inhibitor. The data were analysed by the method of Ackermann and Potter; † denotes that the difference is significant, $P < 0.05$.

preparation, and the detergent depletion technique used (gel filtration) effectively removes the detergent. We ended up with liposome preparations with a recovery of 40–60% as regards enzymatic activity. The liposomes obtained had diameters of 50–150 nm, as estimated from electron micrographs.

Gel filtration on Sepharose is the most widely used method in reconstitution studies to remove unbound protein, if there is any, from its liposomal counterpart. We obtained unacceptably high losses of liposomes when we tried this method, which might be explained by the rather small quantity of liposomes handled. As an attractive alternative we successfully tried ultrafiltration on a XM 300 membrane, through which free enzyme penetrates readily. The method was found to be gentle as well as fast. Calculations on enzyme activities in filtrates from the washings and in the remaining liposomal fraction indicated that 90% or more of the enzyme was membrane-bound. Most of the enzyme was exposed towards the outer side. Treatment with Triton X-100 to disrupt the liposomes and thus expose all enzyme resulted in less than 10% increase of activity. We also found that active enzyme could be released from the liposomes by proteinase K-treatment. Thus, the reconstituted enzyme seems to be oriented very much in the same way as in biological membranes [3, 30].

Substrate kinetics

The liposomal enzyme was found to have the same optimal substrate concentration as the free one, i.e. about 1 mM for acetylthiocholine. The membrane-bound enzyme did show a higher affinity for the substrate, with a K_m of 57 μM as compared to 82 μM for the solubilized species (Table 1).

Acetylcholinesterase is inhibited by its substrate at high concentrations and displays a very typical bell-shaped activity vs pS curve. We obtained very similar profiles for the two enzyme forms. Calculation of the substrate inhibition constant, K_{is}, indicated that substrate inhibition was essentially the same for the liposomal and the solubilized enzyme (Table 1).

From the experiments on fasciculin binding and inhibition (see below) reproducible estimates of the concentration of enzyme in our preparations could be obtained. We could thus calculate the turnover number for acetylthiocholine and found that the two enzyme forms were equally effective (Table 1).

Inhibition by soman

The two enzyme forms behaved very much the same towards this irreversible inhibitor. The bimolecular reaction constants obtained (Table 1) were approximately equal. The identical progressive inhibition of the two enzyme forms at 2×10^{-9} and 4×10^{-9} M soman are shown in Fig. 1.

Inhibition by ketamine

Ketamine acts as an inhibitor of mixed type. For the free enzyme we obtained inhibition constants (Table 1) very much the same as we have reported earlier [18]. The liposomal enzyme was found to be more susceptible towards ketamine, with a lower K_i. The non-competitive component of the inhibition, expressed as K'_i, was the same for both enzyme forms.

Inhibition by fasciculin

Fasciculin 1 was found to be a purely non-competitive inhibitor towards acetylcholinesterase, both in free and liposomal form. Michaelis constant was as given in Table 1, even at high concentrations of the inhibitor. Nor could we find any activation of the enzyme at very low concentrations [20].

We could confirm the very tight binding properties of this inhibitor against acetylcholinesterase from mammalian brain. The binding constant obtained for the free enzyme (Table 1) was even lower than that reported for acetylcholinesterase in rat brain homogenate [20]. The potency of the inhibitor was

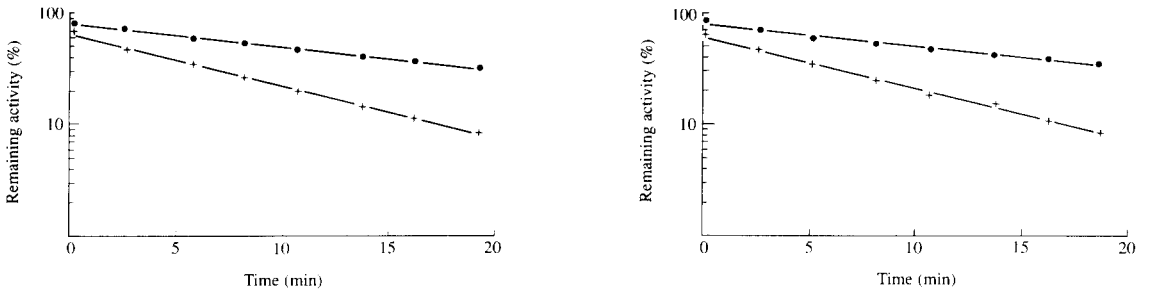

Fig. 1. The progressive inhibition of solubilized (left panel) and liposomal (right panel) acetylcholinesterase by 2×10^{-9} M (upper curve) and 4×10^{-9} M soman (lower curve) in 20 mM Tris–HCl, 0.1 M NaCl, pH 7.4, 23°.

still more pronounced when the enzyme was in the liposomal environment (Table 1).

There are many methods to calculate the inhibition constant for a tightly binding inhibitor, i.e. an inhibitor so potent that the ratio between enzyme and inhibitor concentrations lies between 0.1 and 100. Several of those methods are lacking in precision [27]. In this context, where we wanted to test the hypothesis that there is a difference in inhibition constant for the free and liposomal enzyme, respectively, the choice of method is of greatest importance. The values given in Table 1 are based on a series of experiments, in which both the enzyme and the inhibitor concentration were varied, and which was analysed by the Ackermann–Potter method.

Essentially the same relation in inhibition constants were obtained in other experimental series, in which only the concentration of fasciculin was varied, and also when data were analysed by Dixon plots or by the method of Henderson [27].

We also investigated the reversibility of the binding, by classical dilution and dialysis experiments. Regardless of method and enzyme form (free or liposomal) we found that enzymatic activity could be only partly restored (20–50%), even after dialysis overnight. Fasciculin does not behave as a true irreversible inhibitor such as soman, which binds covalently and thus displays progressive inhibition. Rather fasciculin could be regarded as a pseudo-irreversible inhibitor [28]. Typical for such tight-binding inhibitors is, as shown by Cha [26], that dissociation and association reactions are very slow, and enzyme and inhibitor should be preincubated before assay.

DISCUSSION

We have, in this work, studied whether the kinetic properties of acetylcholinesterase are changed after reconstitution of the enzyme into liposomes. The enzyme in solubilized form would, if those properties were drastically changed, be regarded as a less relevant model for inhibition studies, from which one wants to deduce the action *in vivo*. It is essential for this work that the reconstituted enzyme is oriented in the liposomes in the same way as in the natural membrane. We were successful in preparing proteoliposomes which fulfil this criteria. The very small activity increase after disrupting the liposomes with detergent indicates that the enzyme, in the intact liposomes, exposes its catalytical sites towards the external medium. Treatment with proteinase K showed the same and also that only a non-catalytic part of the enzyme is responsible for the membrane attachment [2, 3]. The liposome preparation can be regarded as a relevant model for testing if the kinetics of the esterase, in a lipid bilayer, vs substrate and inhibitors is different from the kinetics of the enzyme in the detergent-solubilized state.

We found that in only one case, inhibition by soman, did the two enzyme forms display identical kinetics. For the other three compounds tested—acetylthiocholine, ketamine and fasciculin—the incorporation of the enzyme in a bilayer resulted in moderately increased affinity.

The result with the organophosphorus compound soman is in accordance with results obtained with other organophosphorus inhibitors in their actions towards solubilized acetylcholinesterase as compared to enzyme in membrane fractions [7, 8]. Preliminary experiments with the closely related inhibitor sarin (isopropyl methylphosphonofluoridate) also showed equipotency.

The affinity for the substrate, expressed as the Michaelis constant, has in several reports been shown to be modified by the hydrophobic environment [12–15]. In comparison to the values obtained for free enzymes, the K_m can be modulated in both directions after reconstitution in liposomes. The charge of the lipids seems to be crucial, with a tendency towards lower K_m for negatively charged phospholipids and vice versa. The maximal velocity is, as we also found, unchanged after reconstitution [12].

We found it pertinent to study the action of ketamine in our model system, as ketamine has direct effects on membranes. We found that the reconstituted enzyme was more susceptible towards ketamine. The difference was small and of the same magnitude as the difference between the Michaelis constants and furthermore restricted to the competitive component of the inhibition. To us it seems less likely that ketamine, through its action on the membrane, induces a conformational change in the enzyme with implications on the activity, as has been suggested [17].

For the third inhibitor, fasciculin 1, we also found

a small but significant increase in affinity after reconstitution. The inhibition constants we obtained were only slightly lower than those obtained for acetylcholinesterase in rat brain homogenate [20]. This indicates that fasciculin binds very selectively to acetylcholinesterase and has negligible affinity to other proteins in the brain. In mamba venom there are two fasciculins [20], denoted 1 and 2, and they have very similar amino acid sequences, probably differing in only one position. Published data on inhibition of acetylcholinesterase concern type 2, but the two have very similar properties (Evert Karlsson, personal communication) and bind to a peripheral site of the enzyme. We also noted that the binding is pseudo-irreversible, which might at least in part explain the long-lasting effect of the toxin *in vivo* [21, 31].

A general feature of the substances shown to behave differently against solubilized as compared to liposomal acetylcholinesterase is that they are positively charged at physiological pH. In all cases, the liposomal enzyme had a greater affinity for these compounds. On the other hand soman, an apolar active site-directed inhibitor, with a reaction mechanism very much analogous to that of the substrate, was equally potent against both enzyme forms. Velocity of the substrate reaction, substrate inhibition and K_i' values for ketamine were all indifferent to the enzyme form. We draw the conclusion that the reconstitution of acetylcholinesterase into a lipid bilayer does not induce a conformational change, with implications on activity or susceptibility towards inhibitors, in the active subunits of the enzyme. A second possibility, that the detergent is bulky and shields the active site, resulting in less accessibility for interacting substances, is less likely in view of the results obtained by the organophosphates. We find it more plausible that the surface potential, particularly for the liposomes [12], is decisive for the affinity. In conclusion, our results and interpretations suggest that the solubilized enzyme is good enough as model for studying inhibitors *in vitro*.

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