Study of the peptidasic site of cholinesterase: preliminary results

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The peptidasic site of highly purified human plasma cholinesterase was investigated using active-site-directed inhibitors. Peptidase activity was assayed taking substance P as substrate. Inhibition by organophosphates indicated that the peptidasic site contained an active serine. The presence of essential histidine residues associated with serine was revealed by histidine modifications. Carboxyl group reagents showed that the active centre contained carboxyl groups in a non-polar environment. The removal of sialic acids did not alter peptidase activity. The peptidasic site of cholinesterase shared many properties with serine proteases sites and esteratic sites of cholinesterases. In addition, with the peptidasic site, as well as the esteratic site, there was always the possibility of 'aging' when inhibited by DFP or soman.

Cholinesterase *Peptidase activity* *Substance P* *Active-site labelling* *Protein-modifying reagents* *Serine peptidase*

1. INTRODUCTION

Butyrylcholinesterase (EC 3.1.1.8) is found in plasma as well as in various tissues of vertebrates, including nervous structures [1]. BuChE was assigned no definite function [2] and its distribution in organisms differs from that of acetylcholinesterase (EC 3.1.1.7) [1]. BuChE has been revealed in association with secretory products in the neurohypophysis and hypothalamus [3,4], and it has been suggested as a hypothesis that a neuropeptide could be its endogenous substrate [5,6]. It was demonstrated recently that cholinesterases catalyzed the hydrolysis of substance P [7,8] and other peptides [9]. Kinetic data do not support a role for ChE in substance P regulation [7,8,10]. However, studies are too fragmentary to allow attribution, or exclusion, of a functional significance to the peptidase activity of ChE in the processing or termination of the biological activity of peptides [11]. Arguments have been presented [12] for a physiological role of AChE as a peptidase in retinae.

In a previous work on the peptidase activity of human plasma BuChE, we found that sites responsible for the esterase activity and the SP-hydrolyzing property are different [10]. Here, we report further investigations on the nature of the peptidasic site of this enzyme. We have undertaken amino acid modification to identify this site. Experiments were carried out on native and desialylated BuChE using active-site directed irreversible inhibitors and other modifiers. Results showed that the peptidasic site has structural features very similar to those of the esteratic site.

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Abbreviations: BuChE, butyrylcholinesterase; AChE, acetylcholinesterase; DFP, diisopropyl fluorophosphonate; DATD, diallyltartardiamide; NANA, *N*-acetylneuraminic acid; SP, substance P; 2-PAM, pyridine aldoxime methiodide; DEPC, diethylpyrocarbonate; ω-BAP, ω-bromoacetophenone; TLCK, (tosylamido-1)-lysine chloromethyl ketone; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; EEDQ, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline; TMO, trimethyloxonium; NEM, *N*-ethylmaleimide

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2. MATERIALS AND METHODS

2.1. *Enzyme preparation*

BuChE was highly purified from pooled outdated human plasma by the procedure in [13] except that β-mercaptoethanol was omitted in buffers. The enzyme in solution in 20 mM phosphate buffer (pH 6.9) containing 1 mM EDTA (buffer A) had a specific activity of 200 μmol·min^{-1}·mg^{-1} protein. Cholinesterase activity was measured as described by Ellman et al. [14] with 1 mM butyrylthiocholine iodide in 0.1 M phosphate buffer, pH 7, 25°C. The preparation was stored at 4°C for several months without loss of activity.

The purified enzyme was considered to be homogeneous and free of peptidase contaminants. This assumption was supported by the following arguments:

(i) The purification procedure included an affinity chromatography stage on a gel to which procainamide, a BuChE ligand, had been coupled.

(ii) No peptidase activity was found with various peptidase substrates: *N*-benzoyl-L-arginine ethyl ester and *N*-benzoyl-L-tyrosine ethyl ester specific for trypsin and chymotrypsin-like proteases [15,16], Arg-Pro-*p*-nitranilide for X-prolyldipeptidylaminopeptidases (EC 3.4.14.1) [17], and benzoyloxycarbonyl-Gly-Pro-*p*-nitranilide for prolyl-endopeptidase (EC 3.4.21.26) [18].

(iii) Electrophoretic data of DFP inhibited BuChE: 100 μl of the preparation were incubated with [^{3}H]DFP (4 μCi, 50 μM final concentration) for 24 h at 4°C. Under these conditions, esterase and peptidase activities were fully inhibited. Excess DFP was removed by continuous dialysis in a microcell as in [10]. Electrophoreses were carried out under denaturing and non-denaturing conditions using a solubilizable polyacrylamide gel (T = 5,10%) crosslinked with *N,N'*-diallyltartardiamide in place of methylenebisacrylamide (Bis). The molar amount of DATD was 3-times that of Bis. After electrophoresis, proteins were first stained with Coomassie brilliant blue R-250 [19] and then silver-stained [20]. At the same time, other gel rods were cut into 2-mm thick slices. Each slice was placed in a counting vial and incubated with 1 ml of 2% periodic acid for 10 min at room temperature. Scintillation fluid (10 ml of Lumagel, Lumac Systems) was then added to each vial and the radioactivity measured in an Intertechnique SL.4000 liquid scintillation spectrometer. Disc electrophoresis of 20 μl enzyme performed according to the standard alkaline discontinuous system [21] showed a single radioactive protein-staining band corresponding to the tetrameric form of BuChE (C4 component). SDS-electrophoresis with or without β-mercaptoethanol [19] revealed two radioactive protein-staining bands of unequal intensity: the dimer and a trace of monomer in the absence of reducing agent, the monomer and a minor residual covalent dimer [13,22] in the presence of β-mercaptoethanol.

2.2. *Neuraminidase treatment*

N-Acetylneuraminic residues were enzymatically removed from the carbohydrate moiety of BuChE. 150 μl of the preparation were incubated with 50 mU of a suspension of insolubilized neuraminidase from *Clostridium perfringens* (Sigma type VI-A) at 37°C under gentle stirring. The desialylation of BuChE was monitored by following the changes in electrophoretic mobility on polyacrylamide gel [21]. The reaction was stopped after 6 h incubation when no further change in the electrophoretic pattern could be detected. After treatment, desialylated BuChE was separated from neuraminidase by gentle centrifugation.

2.3. *Hydrolysis of substance P*

The peptidase activity of BuChE was measured with SP (Sigma) as substrate whose concentrations ranged from 0.052 to 0.5 mM. BuChE cleaves 3 peptide bonds of SP successively [8], but only the hydrolysis of the first site (Pro^2-Lys^3) was studied. The products of hydrolysis were separated by thin-layer chromatography on silica gel. Details of the experiment have been given [10].

2.4. *Inhibition by organophosphates*

The enzyme was inhibited by 0.015 mM DFP at 20°C. Inhibition was carried out for 6 h. Esterasic and peptidase activities were measured at various times. The enzyme was also inhibited by 0.1 mM DFP or soman (pinacolyl methyl fluorophosphonate) until both esterase and peptidase activities were zero. Organophosphate-inhibited cholinesterases can be reactivated by treatment with nucleophilic reagents. However, inhibited en-

zymes become progressively refractory to reactivation. This process, called 'aging', is due to the release of an alkoxy group from the enzyme phosphorus moiety [23]. Reactivation of completely inhibited activity was attempted, using pyridinealdoxime methiodide, a potent reactivator: the inhibited enzyme was dialyzed continuously in dialysis microcell as in [10] for 3 h against a solution of 0.1 M 2-PAM, 50 mM Tris, pH 7.8. Esterase and peptidase activities were finally measured after a 2 h continuous dialysis against buffer A to remove 2-PAM.

2.5. *Chemical modifications*

Chemical modifiers were used to designate amino acid residues involved in the peptidasic site or located in its immediate environment. The chemicals were prepared in buffer A and 100 μl enzyme added to 100 μl of their solutions. The reactions took place at 20°C. Excess reagent was removed as above prior to substrate hydrolysis.

2.5.1. Sulfhydryl alkylation

BuChE was reacted for 2 h with 10 mM *N*-ethylmaleimide.

2.5.2. Histidine alkylation

Histidyl residues were modified using diethylpyrocarbonate and two halomethyl ketones, ω-bromoacetophenone and (tosylamido-1)lysine chloromethylketone. Carbethoxylation of the imidazole ring was continued for 1 h using 10 mM DEPC. The reaction was stopped at the desired degree of inactivation by the addition of 30 mM imidazole before dialysis. ω-BAP or TLCK (10 mM) reacted for 2 h with BuChE. After dialysis, the modified enzyme was tested for any activities remaining. At the same time, reactivation of the modified enzyme was attempted by treatment with nucleophilic reagents: hydroxylamine (100 mM) or 2-PAM (40 mM).

2.5.3. Reactions on carboxyl groups

BuChE was reacted with 3 carboxyl group reagents: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline and trimethyloxonium tetrafluoroborate. The enzyme was incubated with 10 mM EDAC or EEDQ for 6 h. For the TMO reaction, 20–100 μg reagent were weighed out and immediately diluted in an appropriate volume of the enzymatic preparation at 0°C, to obtain initial concentrations of TMO ranging from 0 to 20 mM. Since the half-life of hydrolysis of TMO was found to be 1.8 min at pH 7 [24], the effective concentrations of reagent were unknown. The reaction was considered to be complete after 5 min of incubation.

3. RESULTS AND DISCUSSION

The present results concerning the effect of organophosphate inhibitors and modifiers on the SP-hydrolyzing property of BuChE throw some light on the nature of the peptidasic site and its surrounding areas. The main data are summarized in table 1.

Desialylation of BuChE did not induce any change in the kinetics of hydrolysis of SP. The apparent kinetic parameters of the desialylated enzyme for the peptidase activity were the same as those of the untreated enzyme. It should be remembered that esterase activity of BuChE remains unchanged after neuraminidase treatment [25]. Our result indicates that NANA does not take part either in the binding of SP or in the enzymic reaction and that modification of the surface of BuChE consecutive to desialylation does not alter the peptidasic site conformation.

Esterase activity as well as peptidase activity may be inhibited by DFP. However, the rates of inhibition of the two activities are widely different (fig.1). This result, in agreement with our previous results obtained with soman and methanesulfonyl fluoride [10], argues in favour of an active serine as the basic residue target at the peptidasic site; moreover, it proves that this site is different from the esteratic site. The reactivity of the peptidasic site with organophosphates is very low compared with that of the esteratic site of BuChE, and even with that of the active site of various serine proteases. This could indicate a modification in the amino acid arrangement and in the topography of the active site compared to other serine proteases. But the possibility of a defect in the 'charge-relay system' must be regarded as has been shown recently for tonin, a serine protease which operates in the processing of various peptides [26]. Finally, treatment of DFP- or soman-inhibited enzyme with 2-PAM did not restore the peptidase activity.

Table 1

Effect of irreversible inhibitors and amino acid modifiers on peptidase activities of human plasma butyrylcholinesterase

Reagent (group attacked)	Effect on peptidase activity
Neuraminidase (enzymic removal of NANA units)	no effect
Methanesulfonyl fluoride (OH from active serine)	progressive inhibition [10]
Soman (OH from active serine)	progressive inhibition [10] with aging
Diisopropyl fluorophosphonate (OH from active serine)	progressive inhibition with aging
N-Ethylmaleimide (SH > NH_2)	no effect at 10 mM
Diethylpyrocarbonate (His > NH_2)	progressive inhibition (75% of inhibition in 3 min), reactivation by hydroxylamine
ω-Bromoacetophenone (His)	25% of inhibition in 2 h at 10 mM
TLCK (His)	100% of inhibition in 2 h at 10 mM
EDAC (COOH > SH, NH_2, OH)	no effect in 6 h at 10 mM
EEDQ (COOH)	75% of inhibition in 4 h at 10 mM
Trimethyloxonium (Glu, Asp)	50% of inhibition in 1 min when initial reagent concentration was 12.5 mM[a]
	100% of inhibition in 1 min when initial reagent concentration was 20 mM[a]

[a] Identical effect on the desialylated enzyme

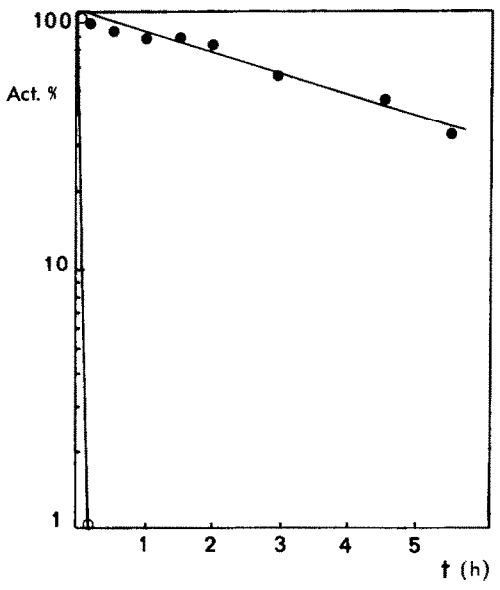

Fig.1. Progressive inhibition of peptidase and esterase activities of highly purified human plasma butyrylcholinesterase (BuChE) by 15×10^{-5} M diisopropyl fluorophosphonate at pH 6.9, 20°C. Peptidase activity (●) with substance P (0.25 mM) as substrate (rate constant of inhibition $k_i = 29 \times 10^{-3}$ min^{-1}, bimolecular rate constant $k_i/[I] = 196.5$ $mol^{-1} \cdot min^{-1}$). Esterase activity (○) with butyrylthiocholine (1 mM) as substrate.

This implies that this site is susceptible to aging as is the esteratic site. Unfortunately, we have not yet any direct evidence for the dealkylation of the phosphonylated peptidasic site: after [^{3}H]DFP inhibition, a search for the [^{3}H]isopropyl group, removed during the aging process, failed.

Treatment of BuChE with the sulfydryl reagent NEM did not affect peptidase activity. It is unlikely therefore, that SH groups are essential. On the other hand, the existence of histidine associated with serine in the charge-relay system was shown. Whereas 20 mM DEPC was necessary to inhibit 50% of the esterase activity in 30 min, more than 70% of the peptidase activity was inhibited by 10 mM DEPC in the same time. The progressive

loss of enzyme activity followed pseudo-first-order kinetics; however, the curves showed deviations presumably due to hydrolysis of DEPC. For this reason, the rate constants were not determined. Peptidase activity could be restored by treatment with nucleophilic agents. Since hydroxylamine removes the carbethoxy group from *N*-carbethoxyhistidyl residues [27], the inactivation of peptidase activity by DEPC may be attributed to the modification of an essential histidine. This assumption was reinforced by the results of halomethyl ketone treatment. At 10 mM, ω-BAP slightly inactivated the peptidase activity; but at the same concentration, TLCK appeared to be a more potent inactivator. Once again, reactivation of the halomethyl ketone-inhibited enzyme by hydroxylamine argues in favour of a modification of an active site histidine.

EDAC (10 mM), a rather hydrophilic water-soluble carbodiimide, had no effect on the basal peptidase activity. On the other hand, EEDQ and TMO, which are less polar, inhibited this activity. This fact suggests that the peptidasic site contains carboxyl groups in a hydrophobic environment. Reactivity to the sterically bulky reagent EEDQ points to the relative accessibility of these groups. Finally, the fast inactivation of native or desialylated enzyme by TMO confirms that NANA units are not involved in the reactivity and conformation of the peptidasic site.

In conclusion, the peptidasic site appears to have structural features and some properties very similar to those of the esteratic site. This finding suggests that these sites may perhaps be related. To establish their relationship, several hypotheses can be proposed as a subject for investigation. The first is that these sites are supported by the same subunit. In this case, sites may perhaps result from gene duplication during evolution. This hypothesis is very improbable, because the results of active-site titration have shown that the number of peptidasic sites is very low compared with the number of esteratic sites (unpublished). This evidence provides support for alternative hypotheses which state that these sites are to be found on different subunits. It is conceivable that an unknown cholinesterase locus, distinct from known ChE loci, encodes for an isozyme devoid of cholinesterase activity but having peptidase properties. This constitutes the isozyme subhypothesis. However, studies on the peptidase activity of BuChE were carried out on enzymes purified from pooled plasma ([8], Lockridge, personal communication, [10]) and preparations may contain a small number of rare cholinesterase variants, some of these having peptidase activity. Within the framework of this allozyme subhypothesis, the peptidasic site should be considered as a mutated site rather than another active site. These hypotheses are being put to the test in our laboratory.

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