A peptidase activity exhibited by human serum pseudocholinesterase

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The identity of a peptidase activity with human serum pseudocholinesterase (PsChE) purified to apparent homogeneity was demonstrated by (a) co-elution of both peptidase and PsChE activities from procainamide-Sepharose and concanavalin-A-Sepharose affinity chromatographic columns; (b) comigration on polycrylamide gel electrophoresis; (c) co-elution on Sephadex G-200 gel filtration and (d) coprecipitation at different dilutions of an antibody raised against purified PsChE. The purified enzyme showed a single protein band on gel electrophoresis under non-denaturing conditions. SDS gel electrophoresis under reducing conditions, followed by silver staining, also gave a single protein band ($M_r \approx 90\,000$). Peptidase activity using different peptides showed the release of C-terminal amino acids. Blocking the carboxy terminal by an amide or ester group did not prevent the hydrolysis of peptides. There was no evidence for release of N-terminal amino acids.

Potent anionic or esteratic site inhibitors of PsChE, such as eserine sulphate, neostigmine, procainamide, ethopropazine, imipramine, diisopropylfluorophosphate, tetra-isopropylpyrophosphoramide and phenyl boronic acid, did not inhibit the peptidase activity. An anionic site inhibitor (neostigmine or eserine) in combination with an esteratic site inhibitor (diisopropylfluorophosphate) also did not inhibit the peptidase. However, the choline esters (acetylcholine, butyrylcholine, propionylcholine, benzoylcholine and succinylcholine) markedly inhibited the peptidase activity in parallel to PsChE. Choline alone or in combination with acetate, butyrate, propionate, benzoate or succinate did not significantly inhibit the peptidase activity. It appeared that inhibitor compounds which bind to both the anionic and esteratic sites simultaneously (like the substrate analogues choline esters) could inhibit the peptidase activity possibly through conformational changes affecting a peptidase domain.

Human serum pseudocholinesterase (PsChE) is a tetramer with a subunit $M_r$ of 90000 [1]. It has four identical subunits, two monomers covalently linked through a single disulfide bond into a dimer and two such dimers hydrophilically linked into a tetramer [2]. It is a less complex protein than acetylcholinesterase (AChE) from several sources [3–6]. However, there are several features common to PsChE and AChE. PsChE exhibits a serotonin-sensitive aryl acylamidase activity similar to AChE from diverse sources [7–10]. It bears a limited homology in its active-site amino acid sequence to AChE and some serine proteases [11]. Based on several lines of evidence it has been speculated that the cholinesterases may have more than one function [12, 13].

The hydrolysis of substance P by purified human serum PsChE was demonstrated by Lockridge [14] and Masson [15]. The cleavage products from substance P by the action of PsChE indicated the involvement of a dipeptidyl peptidase and a deamidase-like activity [14]. Nausch and Heymann [16] showed that the dipeptidyl peptidase activity was a contaminant present in the purified PsChE and suggested that the deamidase-like activity might be associated with the serum PsChE.

In the present work we have made a systematic study to establish the identity of a peptidase activity with human serum PsChE purified to apparent homogeneity. We also describe the nature of this peptidase and its inhibition characteristics in comparison to PsChE.

MATERIALS AND METHODS

Materials

Various amino acids, peptides, o-phthalaldehyde, butyrylthiocholine iodide, procainamide HCl, ethopropazine, imipramine, tetra-isopropylpyrophosphoramide, phenyl boronic acid, choline and choline esters were obtained from Sigma Chemical Co. (St Louis, USA). Sephadex G-200 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals and solvents were of high grade purity or procured as described earlier [17]. Ethyl esters of peptides were prepared by bubbling dry HCl gas through the peptides (2 mg) in 0.5 ml absolute ethanol for 45 min [18]. N-Acetyl-[Met]enkephalin was prepared by acetylation of [Met]enkephalin with acetic anhydride in NaHCO$_3$ solution under nitrogen according to George and Balasubramanian [19]. Diisopropylfluorophosphate (Dip-F) was a kind gift from Dr K. S. Krishnan, Tata Institute of Fundamental Research, Bombay.
Preparation of affinity media

Concanavalin-A—Sepharose and lysine-Sepharose were prepared by coupling concanavalin A or lysine to cyanogen-bromide-activated Sepharose 4B [20] according to methods described earlier [21, 22]. Procainamide was coupled to Sepharose through a ten-carbon spacer arm [10, 17]. Zn$^{2+}$ and Co$^{2+}$-chelated iminodiacetic-acid—Sepharose was prepared as described by Porath et al. [23].

Purification of the PsChE

PsChE was purified by the procedure described earlier [17] involving the use of a procainamide-Sepharose affinity column twice. The enzyme eluted from the first procainamide-Sepharose chromatography with NaCl, after dialysis it was rechromatographed and eluted with 0.05 M procainamide in 20 mM potassium phosphate buffer, pH 7.0. The active fractions were pooled and used in further studies.

Chromatography on other affinity gels

The purified enzyme (1 ml) was applied on to a concanavalin-A—Sepharose column (4×0.5 cm) pre-equilibrated with 20 mM sodium acetate buffer, pH 5.0, washed with the same buffer and eluted with 0.5 M methyl x-gluco-side in the equilibrating buffer. The lysine-Sepharose column (4×0.5 cm), equilibrated with 20 mM potassium phosphate buffer pH 7.0, was washed with the same buffer and eluted with 0.6 M NaCl in the equilibrating buffer. In the case of Zn$^{2+}$ and Co$^{2+}$-chelated Sepharose columns (4×0.5 cm) the equilibration and washing were done with 20 mM potassium phosphate buffer, pH 7.0, and elution with 10 mM imidazole in the equilibrating buffer.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis of the purified enzyme (8 µg) was carried out according to Davis [24] in Tris/glycine buffer, pH 8.3, in tubes with 7% acrylamide. After electrophoresis the gels were either stained with Coomassie brilliant blue G or assayed for enzyme activities as follows. The gels were cut into slices of 2.0 mm thickness, extracted in 0.4 ml 20 mM potassium phosphate buffer pH 7.0 overnight, dialysed against the same buffer to remove Tris/glycine and assayed for PsChE and peptidase activity using suitable aliquots. SDS gel electrophoresis of the purified enzyme under reducing and non-reducing conditions [17] was carried out in 10% acrylamide slab gels according to Laemmli [25], which were stained with silver nitrate in an alkaline medium in the presence of formaldehyde [26]. Standard marker proteins used were immunoglobulin (Mr, 150,000), bovine serum albumin (Mr, 68,000), ovalbumin (Mr, 45,000) and myoglobin (Mr, 17,800).

Sephadex G-200 gel filtration

The concentrated purified enzyme (0.5 ml) was applied on a Sephadex G-200 gel filtration column (35×0.7 cm) pre-equilibrated with 20 mM potassium phosphate buffer, pH 7.0, containing 0.5 M NaCl. The enzyme was eluted by the same buffer at a flow rate of 2.7 ml/h and fractions of 1 ml were collected. Each fraction, after dialysis against the phosphate buffer, was assayed for both enzyme activities.

Immunological studies

Anti-PsChE serum was raised in a rabbit and purified by DEAE-cellulose chromatography as described earlier [10]. The purified antibody was free of any detectable PsChE or peptidase activity. Immunoprecipitation was done by cross-reacting different dilutions (0.03—0.5 mg) of antibody with the purified enzyme (0.004 mg) in 0.4 ml 20 mM potassium phosphate buffer, pH 7.2, followed by incubation at 4°C overnight. After centrifugation of the mixture at 10000×g for 30 min the PsChE and peptidase activities were determined in the supernatant.

Enzyme assay and protein estimation

PsChE activity was assayed with butyrylthiocholine iodide as substrate according to Ellman et al. [27]. 1 unit of PsChE activity was defined as the absorbance change of 1/min at 412 nm under standard assay conditions. The peptidase activity was assayed in 50 mM potassium phosphate buffer, pH 7.2, using 400 µM of either Phe-Leu or [Leu']enkephalin as substrate and 0.1 ml enzyme (4 µg) in a total volume of 0.2 ml reaction mixture. After incubation at 37°C for different time periods of up to 6 h the reaction mixture was heated in a boiling water bath for 1 min and the peptide activity was measured by the released amino acids as determined by the o-pthalaldehyde fluorimetric method [28]. Briefly, to 0.05—0.1 ml reaction mixture, 0.3 ml o-pthalaldehyde reagent (2 mg o-pthalaldehyde/0.2 ml ethanol mixed with 2 µ1 2-mercaptoethanol/0.2 ml ethanol and made up to 12 ml in 50 mM borate buffer, pH 9.5) was added in a final volume of 0.4 ml. Fluorescence was measured in a Hitachi spectrofluorimeter (model 204-A) using an excitation wavelength of 345 nm and an emission wavelength of 455 nm. The amount of the substrate (for Phe-Leu) hydrolysed was determined from a standard curve obtained with known concentrations of an equimolar mixture of phenylalanine and leucine. 1 unit of peptidase activity is defined as 1 nmol each of phenylalanine and leucine liberated/h.

Protein was determined according to Lowry et al. [29] with crystalline bovine serum albumin as standard or by absorbance at 280 nm (for column effluents).

Paper chromatography

One dimensional paper chromatography (descending) on Whatman no. 1 paper was done using the solvent system
Enzyme activities were measured using the substrate butyrylthiocholine (○—○) for PsChE and Phe-Leu (●—●) and [Leu³]enkephalin (□—□) for the peptidase. Details of the electrophoresis and assay procedure are given in Materials and Methods.

Fig. 3. Sephadex G-200 gel filtration of the purified enzyme and elution profile of PsChE and peptidase activities. Each fraction was assayed for PsChE (○—○) and peptidase using Phe-Leu (●—●) or [Leu³]enkephalin (□—□) as substrate. Details of chromatography and assay procedures are given in Materials and Methods. Arrows indicate the elution peaks of (a) blue dextran, (b) immunoglobulin (150000), (c) bovine serum albumin (68000) and (d) ovalbumin (45000).

The elution profiles of PsChE and peptidase activities of the purified enzyme from the procainamide-Sepharose affinity column chromatographic procedure is shown in Fig. 1. Both enzyme activities were bound 100% to the column and co-eluted and the ratios of the two activities were similar in all the active fractions.

In order to verify the identical behaviour of the PsChE and peptidase activities the purified enzyme was passed through different types of affinity columns. Both the enzyme activities were completely bound to a concanavalin-A-Sepharose column, indicating the glycoprotein nature of the enzyme, and were co-eluted more than 90% with methyl α-glucoside in buffer. Both enzymes bound to a lysine-Sepharose column and were completely elutable with NaCl. Zn²⁺ and Co²⁺ metal chelate columns did not bind both enzyme activities.

RESULTS

Binding and co-elution of PsChE and peptidase activity in various affinity column chromatographic procedures

The elution profiles of PsChE and peptidase activities of the purified enzyme from the procainamide-Sepharose affinity column chromatographic procedure is shown in Fig. 1. Both enzyme activities were bound 100% to the column and co-eluted and the ratios of the two activities were similar in all the active fractions.

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Comigration on polyacrylamide gel electrophoresis

Fig. 2 shows the gel electrophoresis pattern of the purified enzyme under non-denaturing conditions and the profile of the enzyme activities in the gel slices. A single protein band, which corresponded to both PsChE and peptidase activities, was observed indicating comigration of both activities.

Sephadex G-200 gel filtration

The Sephadex G-200 gel filtration pattern of the purified enzyme is shown in Fig. 3. The elution profile showed that...
Fig. 5. Immunoprecipitation of PsChE (○) and peptidase activities at different dilutions of the antibody raised against purified PsChE. Peptidase was assayed using Phe-Leu (●) or [Leu']-enkephalin (□) as substrate. Details of the experiment are given under Materials and Methods.

Fig. 6. The degradation of Phe-Leu (●) and [Leu']-enkephalin (□) by the peptidase activity of PsChE at different time intervals of incubation. The amino acids released from the peptides were monitored by the 3-phthalaldehyde fluorometric method. Details of the assay procedures are given in Materials and Methods.

both PsChE and peptidase activities co-eluted as a single peak from the column. From the elution pattern of marker proteins the relative molecular mass of the native enzyme was calculated to be approximately 170,000 corresponding to the dimeric form of PsChE. The ratio of PsChE to peptidase activity remained approximately constant in all the eluted fractions.

SDS gel electrophoresis

The SDS gel electrophoretic pattern of the purified enzyme under reducing and non-reducing conditions is shown in Fig. 4. Under non-reducing conditions protein bands corresponding to both the monomeric and dimeric forms of enzyme appeared, whereas under reducing condition only the monomeric form ($M_r \approx 90,000$) was seen.

Immunoprecipitation studies

Immunoprecipitation of the purified enzyme showed that both the peptidase and PsChE activities could be co-precipitated by the purified antibody. The ratio of peptidase to PsChE activity precipitated at different dilutions of the antibody was constant (Fig. 5).

Table 1. Release of amino acids from various peptides by the peptidase associated with PsChE

<table>
<thead>
<tr>
<th>Peptide substrate used</th>
<th>Amino acids released</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phe-Leu</td>
<td>Phe, Leu</td>
</tr>
<tr>
<td>[Leu']-enkephalin (Tyr-Gly-Gly-Phe-Leu)</td>
<td>Leu, Phe</td>
</tr>
<tr>
<td>[Met']-enkephalin (Tyr-Gly-Gly-Phe-Met)</td>
<td>Met, Phe</td>
</tr>
<tr>
<td>Tyr-Gly-Gly</td>
<td>not detectable</td>
</tr>
<tr>
<td>N-Acetyl-[Met']-enkephalin</td>
<td>Met, Phe</td>
</tr>
<tr>
<td>Phe-Leu NH$_2$</td>
<td>Phe, Leu</td>
</tr>
<tr>
<td>Phe-Leu ethyl ester</td>
<td>Phe, Leu</td>
</tr>
<tr>
<td>[Leu']-enkephalin ethyl ester</td>
<td>Leu, Phe</td>
</tr>
<tr>
<td>N-Acetyl-[Met']-enkephalin ethyl ester</td>
<td>Met, Phe</td>
</tr>
</tbody>
</table>

Substance P (Arg-Pro-Lys-Pro-Gln-Phe-Gly-Leu-MetNH$_2$) Met, Leu

Bradykinin (Arg-Pro-Arg-Phe-Arg-Phe-Arg-Phe-Arg) Arg, Phe

Angiotensin I (Asp-Arg-Tyr-Ile-His-Pro-Phe-His-Leu) Leu

Tyr-bradykinin (Tyr-Arg-Pro-Gly-Phe-Ser-Pro-Phe-Arg) Arg, Phe

Characterization of the peptidase associated with PsChE

The peptidase activity in the purified enzyme was labile to freezing and thawing, as was PsChE (100% loss of peptidase and 82% loss of PsChE after two freezing and thawing). The activity was linear up to 4 h for hydrolysis of either Phe-Leu or [Leu']-enkephalin (Fig. 6). A Lineweaver-Burk plot for hydrolysis of Phe-Leu and [Leu']-enkephalin showed $K_m$ values of 303 $\mu$M and 211 $\mu$M respectively. The peptidase activity was inhibited 55-65% by 1 mM Cd$^{2+}$, Co$^{2+}$ or Zn$^{2+}$. EDTA inhibited the peptidase 50-15% in the concentration range of 0.1-1 mM (data not shown). The inhibitory effect of EDTA was verified by paper chromatography of the reaction mixtures using different peptides as substrate.

The products released by the peptidase from various peptide substrates, as identified by paper chromatography, are given in Table 1. From both [Leu']-enkephalin and [Met']-enkephalin only two amino acids from the carboxy terminal, namely Leu and Phe or Met and Phe, were released. This was also verified in separate experiments by subjecting the reaction mixtures, incubated for periods from 30 min to 4 h to paper chromatography. Tyr-Gly-Gly was a poor substrate and did not show any release of amino acids. Blocking the N-terminal by an acetyl group, as in N-acetyl-[Met']-enkephalin, also gave the two C-terminal amino acids released. Surprisingly blocking the C-terminal amino acid by an amide or ester group did not prevent the release of the amino acids as in Phe-Leu NH$_2$, Phe-Leu ethyl ester, [Leu']-enkephalin ethyl ester or N-acetyl-[Met']-enkephalin ethyl ester.

Use of other peptide substrates like substance P, bradykinin, angiotensin I and Tyr-bradykinin also resulted in the release of C-terminal amino acids (Table 1). Gly, His and Pro appeared to be unfavourable amino acids to be released.

Inhibition characteristics

Potent inhibitors of PsChE, like ethopropazine, imipramine, eserine sulphate, neostigmine, Dip-F, tetra-isopro-
Table 2. Effect of PsChE inhibitors on peptidase activity

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Activity PsChE</th>
<th>Activity peptidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eserine sulphate</td>
<td>0.02 mM</td>
<td>36%</td>
<td>124%</td>
</tr>
<tr>
<td></td>
<td>0.10 mM</td>
<td>12%</td>
<td>138%</td>
</tr>
<tr>
<td></td>
<td>2.00 mM</td>
<td>0%</td>
<td>141%</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>0.10</td>
<td>8%</td>
<td>108%</td>
</tr>
<tr>
<td>Procainamide</td>
<td>20.00</td>
<td>22%</td>
<td>102%</td>
</tr>
<tr>
<td></td>
<td>100.00</td>
<td>7%</td>
<td>91%</td>
</tr>
<tr>
<td>Ethopropazine</td>
<td>0.10</td>
<td>0%</td>
<td>92%</td>
</tr>
<tr>
<td>Imipramine</td>
<td>0.10</td>
<td>0%</td>
<td>86%</td>
</tr>
<tr>
<td>Diisopropylfluorophosphate</td>
<td>0.02</td>
<td>0%</td>
<td>132%</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>0%</td>
<td>142%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0%</td>
<td>152%</td>
</tr>
<tr>
<td>Tetra-isopropylpyrophosphoramid</td>
<td>0.10</td>
<td>0%</td>
<td>88%</td>
</tr>
<tr>
<td>Phenyl boronic acid</td>
<td>0.02</td>
<td>29%</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>4%</td>
<td>128%</td>
</tr>
<tr>
<td></td>
<td>2.00</td>
<td>0%</td>
<td>128%</td>
</tr>
<tr>
<td>Eserine sulphate + Dip-F</td>
<td>0.10</td>
<td>0%</td>
<td>129%</td>
</tr>
<tr>
<td>Neostigmine + Dip-F</td>
<td>0.10</td>
<td>0%</td>
<td>163%</td>
</tr>
</tbody>
</table>

DISCUSSION

The identity of the peptidase with the purified human serum PsChE is based on the following observations. (a) Both PsChE and peptidase co-eluted with constant ratios of activity in the procaainamide-Sepharose affinity column chromatographic procedure and showed identical characteristics in other types of affinity chromatography. (b) Both the activities migrated on gel electrophoresis under non-denaturing conditions and both the activities corresponded to the only protein band detectable on the gel. (c) Both activities showed similar elution profiles on Sephadex G-200 gel filtration. (d) Both enzyme activities were coprecipitated at different dilutions of the antibody raised against purified PsChE enzyme. (e) Procainamide, which is a specific reversible inhibitor of PsChE, inhibits PsChE more than 90% at 100 mM concentration but does not significantly inhibit the peptidase activity (Table 2). The fact that both peptidase and PsChE activities bind 100% to the procaainamide-Sepharose column and that both are co-precipitable with 0.05 M procainamide suggested that the peptidase must be associated with the PsChE enzyme.

The inhibition characteristics of the peptidases are of interest. Several potent inhibitors of PsChE did not inhibit the peptidase activity (Table 2). These inhibitors are known to act at either the anionic or esteratic site of PsChE [30, 31]. In fact a slight activation of the peptidase activity was observed with diisopropylfluorophosphate (Dip-F), eserine and phenyl boronic acid. It has been reported [30] that an aziridinium derivative, which alkylates the anionic site of AChe while inhibiting the hydrolysis of acetylcholine, can activate the hydrolysis of the very poor substrate indophenyl acetate. Apparently any inhibitor of PsChE which acted only at either the anionic or esteratic site of PsChE did not inhibit the peptidase. Use of an anionic-site inhibitor (eserine or neostigmine) together with an esteratic-site inhibitor (Dip-F) also did not inhibit the peptidase activity. On the other hand inhibitors of PsChE which occupied both the anionic and esteratic sites, like the substrate analogues acetylcholine, butyrylcholine, propionylcholine, benzoylcholine or succinylcholine, effectively inhibited the peptidase activity. Use of the acyl moiety of the choline esters (acetate, propionate, butyrate, benzoate or succinate) together with choline did not affect the peptidase. It was therefore evident that chemical entities which simultaneously occupied both esteratic and anionic sites and which were covalently linked to each other alone could bring about an inhibition of the peptidase activity. Under such conditions it is possible that a conformational alteration occurs resulting in the inhibition of a peptidase domain in the PsChE molecule. Evidence for alteration in conformational stability arising from structural modification of the active center of PsChE has been reported [32]. The tetrameric form of PsChE appears to be an asymmetric prolate ellipsoid [33] but the folded conformation of the enzyme is still unknown. Multistate denaturation of PsChE, caused by the possible unfolding and refolding of each domain independently at different concentrations of urea, is also known [32].

The peptidase activity in PsChE was able to release amino acids from the carboxy terminal of several peptides. There was no evidence for removal of N-terminal amino acids. Blocking of the C-terminal amino acid by an amide group or ester group did not prevent the release of the C-terminal amino acids. This would suggest that the enzyme has an esterase, amidase and peptidase activity. The reported aryl
peptidase activity was a contaminant in the PsChE preparation by Nausch and Heymann [17] showed that the dipeptidyl peptidase activity was a contaminant in the PsChE preparation. Dip-F inhibited this dipeptidyl peptidase activity [17]. Later work by Nauch and Heymann [17] showed that the dipeptidyl peptidase activity was a contaminant in the PsChE preparation. Dip-F inhibited this dipeptidyl peptidase activity [17]. The present peptidase activity of PsChE reported by us is not inhibited by Dip-F. Lockridge observed the release of the amide -NH2 group of methionine from substance P by the purified PsChE but did not report the release of methionine and leucine from the C-terminal of substance P. This might be because of the use of EDTA by Lockridge in the reaction mixture [14]. In the present studies we have found that EDTA significantly inhibits the peptidase activity of PsChE with Phe-Leu, [Leu'lenkephalin or substance P as substrate.

It is of interest that AChE from diverse sources has also been shown to have a peptidase activity [34]. The multiple types of reaction catalysed by AChE [9, 35, 36] may be attributed to the highly complex and possible multidomain nature of the AChE protein [37, 38]. Recent evidence for several mRNAs, each generating distinct monomer of AChE [39 - 41], suggests that such multiple monomers present in AChE can also provide the multiple catalytic sites of AChE. PsChE, being a less complex protein containing four identical monomers, may be considered to have a specific domain responsible for its peptidase activity.

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REFERENCES