Evidence for a Zn²⁺-binding site in human serum butyrylcholinesterase

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Purified human serum butyrylcholinesterase after treatment with either of the metal chelators EDTA or NaCN was able to bind to a Zn²⁺-chelate–Sephacryl column and was eluted from the column by EDTA or imidazole. Prior EDTA treatment of the enzyme was essential for binding to this affinity column. The enzyme could be labelled with ⁶⁵Zn²⁺ after EDTA treatment of the enzyme. Diethylpyrocarbonate modification of histidine residues in the EDTA-treated enzyme resulted in the abolition of both binding to the Zn²⁺-chelate–Sephacryl column and labelling by ⁶⁵Zn²⁺. Stoichiometry of ⁶⁵Zn²⁺ binding indicated approximately 0.85 mol of Zn²⁺/mol of subunit of the EDTA-treated

enzyme. EDTA or NaCN treatment resulted in the loss of thermal stability of the enzyme at 37 °C which could not be reversed by Zn²⁺. Whereas the cholinesterase activity of butyrylcholinesterase was not affected by EDTA, there was significant loss of its carboxypeptidase activity in the presence of EDTA, and the loss could be reversed by added ZnCl₂. These results suggest the presence of a Zn²⁺-binding site on human serum butyrylcholinesterase and the involvement of histidine residues in the metal binding. The presence in human serum butyrylcholinesterase of a sequence HXXE...H found in many known Zn²⁺-containing enzymes supports these findings.

INTRODUCTION

Human serum butyrylcholinesterase (BChE) is a tetrameric glycoprotein of four identical subunits (each of approximate molecular mass 90 kDa) with four active sites per molecule [1–4]. The complete amino acid sequence of BChE is known and it shows 53% amino acid sequence identity with acetylcholinesterase (AChE) [4]. BChE shows similar responses to a number of classical AChE inhibitors but differs from AChE in its substrate preference. The amino acid sequence at the active-site serine of AChE and BChE is well conserved. Along with an active-site serine residue, a histidine and a glutamate residue are considered important for the catalytic activity of both AChE and BChE [5,6].

Unlike AChE, the physiological significance of BChE is not fully understood. Roles for BChE in cell differentiation and mitosis [7] and in detoxification [8] have been proposed. Both AChE and BChE exhibit aryl acylamidase activity [9,10]. The aryl acylamidase activity found in BChE is sensitive to 5-hydroxytryptamine and tyramine [10,11]. Besides the aryl acylamidase activity, a metallopeptidase activity is also found associated with BChE [12,13]. The Zn²⁺-binding sequence HXXE...H (consisting of a sequence of histidine, any two amino acids XX and a glutamic acid followed by another histidine either adjacent or at a distance, with several amino acids of varying number intervening) found in many Zn²⁺-containing enzymes is present in both BChE (residues 438–548) and AChE (residues 440–550) [14,15].

In the present study we demonstrate a Zn²⁺-binding site in human serum BChE. Evidence for the involvement of histidine in the metal binding and the contribution of the metal to the maintenance of the thermal stability of the enzyme are also presented.

MATERIALS AND METHODS

⁶⁵ZnCl₂ (1800 mCi/g) was procured from Bhabha Atomic Research Centre, Bombay, India. EDTA (disodium salt) was from Qualigens Fine chemicals. Iminodiacetic acid, butane-1,4-diol diglycidyl ether, diethylpyrocarbonate, Sepharose 4B and imidazole were purchased from Sigma. The metal salts used were of

analytical grade of the highest purity available. Distilled deionized water was used in all experiments.

BChE was purified from outdated human plasma by the procedure described previously involving DEAE-cellulose chromatography followed by procainamide–Sephacryl affinity chromatography performed twice [16].

Preparation of metal ion-chelating gel

Iminodiacetic acid–Sephacryl 4B was prepared by coupling iminodiacetic acid to epoxy-activated Sepharose 4B obtained using butane-1,4-diol diglycidyl ether [17,18].

Zn²⁺-chelate–Sephacryl chromatography

Iminodiacetic acid–Sephacryl was packed into a column (1.5 cm × 5 cm) and saturated with Zn²⁺ by passing through 100 ml of ZnCl₂ (3 mg/ml) solution. The column was then washed with 20 mM potassium phosphate buffer, pH 7.4. The purified enzyme (5 µg) was loaded on the column, washed with 500 ml of the buffer and eluted with either 10 mM EDTA or 10 mM imidazole in 20 mM phosphate buffer, pH 7.4. Fractions of 2 ml were collected and dialysed against 20 mM phosphate buffer, pH 7.4, before assay. A Co²⁺-chelate column was operated in a similar way.

Diethylpyrocarbonate treatment for histidine modification

Diethylpyrocarbonate (25 mM) was incubated with the enzyme in 20 mM potassium phosphate buffer, pH 7.0, at 22 °C for 15 min [16,19]. The enzyme was then dialysed against 20 mM phosphate buffer, pH 7.4, for 16 h. A 100% loss of cholinesterase activity was observed after diethylpyrocarbonate treatment under these conditions [13].

⁶⁵Zn²⁺-binding studies

The purified enzyme (1 µg) in 100 µl of 20 mM potassium phosphate buffer, pH 7.4, before or after pretreatment with

EDTA was incubated with 50 μM $^{65}\text{ZnCl}_2$ under various conditions for 4 h at 4 °C. The samples were concentrated under vacuum and dotted on to a nitrocellulose membrane, washed four times with 10 mM Tris/HCl buffer (pH 7.5)/0.1 M KCl [20,21]. The dried nitrocellulose membrane was exposed to X-ray film at -70 °C for autoradiography. Radioactivity was measured in a liquid-scintillation counter.

The stoichiometry of binding was studied by incubating different molar concentrations of $^{65}\text{ZnCl}_2$ with the same amount of EDTA-treated BChE (0.23 nmol). The required volume of $^{65}\text{ZnCl}_2$ solution was initially dried under vacuum in test tubes to which the purified enzyme was added in a final volume of 10 μl . After 12 h the samples were dotted on to nitrocellulose membrane and the radioactivity was determined as described above.

The purified enzyme (5 μg) in 20 mM potassium phosphate buffer, pH 7.4, pretreated with 10 mM EDTA followed by dialysis against the buffer, was incubated at 4 °C with 50 μCi of $^{65}\text{ZnCl}_2$ for 12 h. It was then loaded on the procainamide-Sepharose affinity column, previously equilibrated with 20 mM potassium phosphate buffer, pH 7.4. The column was washed exhaustively with the buffer to remove all traces of free $^{65}\text{ZnCl}_2$ and eluted with 50 mM procainamide in the buffer. Fractions (2 ml) were assayed for cholinesterase activity and radioactivity was measured in a liquid-scintillation counter.

Enzyme assays

BChE activity was assayed with butyrylthiocholine iodide as substrate as described by Ellman et al. [22]. Aryl acylamidase was assayed as described previously [10] using *o*-nitroacetanilide as substrate.

Carboxypeptidase activity using Leu-enkephalin as substrate was measured by HPLC. Leu-enkephalin (400 μM) was incubated with 2 μg of BChE in 50 μl of 20 mM potassium phosphate buffer, pH 7.4 [13]. The incubation mixture was boiled for 3 min to arrest the peptidase activity. Reverse-phase HPLC analysis was carried out in Shimadzu HPLC apparatus. A 0–80 % linear gradient of acetonitrile containing 0.05 % triethanolamine/0.1 % trifluoroacetic acid was employed at a flow rate of 1 ml/min and absorbance was monitored at 230 nm.

RESULTS

Zn^{2+} -chelate-Sepharose chromatography of human serum BChE

Purified BChE without prior treatment with EDTA when applied to a Zn^{2+} -chelate-Sepharose column did not bind to the column, 100 % being recovered in the unbound fractions (Figure 1a). However, pretreatment of the enzyme with 10 mM EDTA for 30 min at 4 °C followed by dialysis against buffer to remove the excess EDTA resulted in total binding of the enzyme to the Zn^{2+} -chelate column. The procedure involving pretreatment with EDTA and dialysis did not result in any significant loss of BChE activity. The bound enzyme could be eluted with 10 mM EDTA (Figure 1b). Aryl acylamidase activity exhibited by BChE showed an elution profile similar to that of BChE activity (Figures 1a and 1b). A mild metal chelator such as Tris (used as 20 mM Tris/HCl buffer, pH 7.4) or NaCl up to 50 mM did not elute the enzyme from the column.

The EDTA-treated enzyme did not bind to a Co^{2+} -chelate column as observed in repeated experiments.

The enzyme pretreated with 10 mM NaCN, another metal chelator, followed by dialysis against 20 mM phosphate buffer also bound to the Zn^{2+} -chelate-Sepharose column and was eluted with 10 mM EDTA.

Figure 1 Zn^{2+} -chelate-Sepharose chromatography: elution profile of cholinesterase (∇) and aryl acylamidase ($\square$) activities of the human serum BChE enzyme (a) not treated with EDTA or (b) pretreated with EDTA

Pretreatment with EDTA involved keeping the enzyme in the presence of 10 mM EDTA at 4 °C for 30 min followed by dialysis against 200 vol. of 20 mM potassium phosphate buffer, pH 7.4, for 4 h with four changes. Details of Zn^{2+} -chelate chromatography are given in the Materials and methods section. The arrow indicates the fraction where elution was started with 10 mM EDTA.

Evidence for the involvement of histidine residues of the enzyme in the binding to the Zn^{2+} -chelate-Sepharose column

The EDTA-treated enzyme that bound to the Zn^{2+} -chelate-Sepharose column was eluted with 10 mM imidazole in phosphate buffer, pH 7.4, as efficiently as with EDTA in buffer. The histidine residues in the EDTA-treated enzyme were modified with diethylpyrocarbonate as described in the Materials and methods section, and the modified enzyme was subjected to Zn^{2+} -chelate-Sepharose chromatography. As there was a 100 % loss of cholinesterase activity, protein was measured at 280 nm in the unbound fractions, washings and eluate. The modified enzyme was recovered in the unbound fractions, suggesting its failure to bind to the column. In additional experiments described below, the diethylpyrocarbonate-modified BChE also failed to bind to $^{65}\text{Zn}^{2+}$.

Binding of $^{65}\text{Zn}^{2+}$ to the EDTA-treated enzyme

The ability of $^{65}\text{ZnCl}_2$ to bind to the enzyme was tested under various conditions as described in the Materials and methods section. $^{65}\text{Zn}^{2+}$ labelling was maximum with EDTA-pretreated enzyme (Figure 2a). When the enzyme was not pretreated with EDTA, labelling was only 20 % of the maximum. In the presence of unlabelled ZnCl_2 or CoCl_2 only about 3 % of maximum labelling was observed. At pH 4.0, which is unfavourable for metal binding, $^{65}\text{Zn}^{2+}$ binding was reduced to 2 % of the maximum (Figure 2a). The enzyme modified by diethylpyrocarbonate treatment showed no detectable labelling by $^{65}\text{Zn}^{2+}$ (Figure 2b).

Figure 2 Dot-blot analysis of ⁶⁵ZnCl₂ binding to BChE

The binding of ⁶⁵ZnCl₂ to purified BChE (1 μg) under various conditions was analysed by dot-blot as described in the Materials and methods section. (a) (I) ⁶⁵ZnCl₂ incubated in the absence of protein at pH 7.4; (II) ⁶⁵ZnCl₂ incubated with enzyme not treated with EDTA at pH 7.4; (III) ⁶⁵ZnCl₂ incubated with EDTA-treated enzyme at pH 7.4; (IV) ⁶⁵ZnCl₂ incubated with EDTA-treated enzyme at pH 7.4 in the presence of 1 mM ZnCl₂; (V) ⁶⁵ZnCl₂ incubated with EDTA-treated enzyme at pH 7.4 in the presence of 1 mM CoCl₂; (VI) ⁶⁵ZnCl₂ incubated with EDTA-treated enzyme at pH 4.0 (100 mM sodium acetate buffer). (b) (I) ⁶⁵ZnCl₂ incubated with EDTA-pretreated enzyme unmodified by diethylpyrocarbonate at pH 7.4; (II) ⁶⁵ZnCl₂ incubated with diethylpyrocarbonate-modified EDTA-pretreated enzyme at pH 7.4.

Figure 3 Procainamide-Sepharose affinity chromatography of BChE incubated with ⁶⁵ZnCl₂

BChE (5 μg) pretreated with EDTA was incubated at 4 °C with 50 μCi of ⁶⁵ZnCl₂ for 12 h and loaded on a procainamide-Sepharose affinity column. The column was washed extensively to remove unbound ⁶⁵ZnCl₂ and the enzyme was eluted with 50 mM procainamide as described in the Materials and methods section. Fractions (2 ml) were assayed for cholinesterase activity (▼) and radioactivity (●).

The binding of ⁶⁵Zn²⁺ to BChE was further confirmed when the EDTA-treated enzyme incubated with ⁶⁵ZnCl₂ was passed through the procainamide-Sepharose affinity column. In the eluted fractions the peak of enzyme activity corresponded to the peak of radioactivity (Figure 3).

Stoichiometry of binding

The stoichiometry of binding was determined by incubating increasing concentrations of ⁶⁵ZnCl₂ with a known amount of protein (0.23 nmol of protein). The binding reached a plateau at 0.2 nmol of ⁶⁵ZnCl₂ bound after which there was no significant increase (Figure 4). The stoichiometry of binding was found to be approx. 0.85 mol of Zn²⁺ to 1 mol of protein monomer.

Figure 4 Saturation curve for ⁶⁵Zn²⁺ binding to the purified BChE enzyme

BChE (0.23 nmol of monomer) in each tube was incubated with various concentrations of ⁶⁵ZnCl₂ in a final volume of 10 μl as described in the Materials and methods section and blotted on to nitrocellulose membrane, and the radioactivity incorporated was measured.

Effect of EDTA or Zn²⁺ on BChE activity and on the thermal stability of the enzyme

EDTA in the reaction mixture during the assay of the purified enzyme did not affect BChE activity significantly. The normal reaction period of assay was 10 min at 37 °C. This was verified by adding EDTA at four different concentrations from 0.05 to 10 mM to the reaction mixture. The loss of activity was less than 5% at the highest concentration of EDTA used. Addition of ZnCl₂ at 15 different concentrations from 0.2 μM to 1 mM to the reaction mixture also did not affect the BChE activity.

The stability of the purified BChE before and after EDTA treatment was compared by keeping the enzymes at 37 °C and assaying for cholinesterase activity at specific intervals. The untreated enzyme showed less than 5% loss of activity after 3.5 h at 37 °C (Figure 5). On the other hand the EDTA-treated enzyme

Figure 5 Thermal stability at 37 °C of EDTA- and NaCN-treated enzyme

EDTA-treated BChE (▽), NaCN-treated BChE (▼) and BChE without any treatment (●) were kept at 37 °C, and enzyme activity was measured at specific intervals. Pretreatment with EDTA or NaCN was carried out as described in Figure 1.

Figure 6 Loss of carboxypeptidase activity in BChE caused by treatment with EDTA and recovery of activity in the presence of Zn^{2+} as measured by HPLC

Leu-enkephalin ($400 \mu\text{M}$) was incubated with BChE, and HPLC was performed by injected $25 \mu\text{l}$ of the reaction mixture on to a reverse-phase HPLC column as described in the Materials and methods section. Peak 1 corresponds to Tyr-Gly-Gly and peak 2 to Leu-enkephalin. (a) BChE incubated with Leu-enkephalin, (b) BChE incubated with Leu-enkephalin in the presence of 1 mM EDTA; (c) BChE incubated with Leu-enkephalin in the presence of 1 mM EDTA and 2 mM ZnCl_2 .

showed 50% loss of activity after 1 h and 90% after 3.5 h at 37°C (Figure 5). ZnCl_2 (1 mM) was added to the EDTA-treated enzyme, kept for 12 h at 4°C and the enzyme analysed for thermal stability as described above. There was no change in thermal stability, indicating that addition of Zn^{2+} did not reverse the loss of activity caused by pretreatment with EDTA.

Similar observations on thermal stability were made with NaCN-treated enzyme (Figure 5).

Effect of EDTA on the metallocarboxypeptidase activity of BChE

Carboxypeptidase activity of purified human serum BChE stimulated by Zn^{2+} was observed in our earlier studies [12–14]. The catalytic activity of the peptidase was located at a site different from those of cholinesterase and aryl acylamidase activities by limited protease digestion of the BChE enzyme [12]. We studied the effect of EDTA on the carboxypeptidase activity of BChE. In contrast with its ineffectiveness on the cholinesterase activity, EDTA significantly inhibited the carboxypeptidase activity at different concentrations from 0.01 to 1 mM, and the inhibition could be reversed by the addition of Zn^{2+} . Figure 6 shows the inhibition by EDTA (1 mM) of Leu-enkephalin hydrolysis to Tyr-Gly-Gly by the carboxypeptidase activity in BChE and its reversal by Zn^{2+} .

DISCUSSION

Prior treatment of purified BChE with EDTA was necessary for its binding to the Zn^{2+} -chelate-Sepharose column. This indicated that the binding of the enzyme to the column was not due to non-specific forces but truly involved metal-affinity binding. The binding of NaCN-treated enzyme to the Zn^{2+} -chelate column further supported the metal-affinity binding. The inability of the enzyme to bind to Co^{2+} -chelate-Sepharose suggests a lower affinity of the EDTA-treated enzyme for this bivalent metal.

Additional evidence to suggest that there is a Zn^{2+} -binding site in BChE comes from co-elution of $^{65}\text{Zn}^{2+}$ radioactivity with the BChE activity from the procainamide-Sepharose affinity column and the binding of $^{65}\text{Zn}^{2+}$ to the EDTA-treated enzyme as found in the dot-blot experiments.

The low but detectable level of $^{65}\text{Zn}^{2+}$ labelling of the enzyme not treated with EDTA compared with the EDTA-treated enzyme (Figure 2a) could result from an exchange of $^{65}\text{Zn}^{2+}$ with the Zn^{2+} present in the enzyme [23]. Unlabelled Zn^{2+} and Co^{2+} were efficient in competing with $^{65}\text{Zn}^{2+}$ and preventing labelling of the EDTA-treated enzyme. Stoichiometry studies show a ratio of approx. 0.85 mol of $^{65}\text{Zn}^{2+}$ to 1 mol of subunit of the EDTA-treated enzyme, suggesting one site for Zn^{2+} binding per subunit.

Although EDTA in the reaction mixture did not inhibit the catalytic activity of BChE, prior EDTA treatment considerably reduced the thermal stability of the enzyme. This indicated that the metal was essential for maintaining the secondary and/or tertiary structures of the protein. Similar instances of the requirement of a metal ion for the conformational stability of an enzyme are found in the Zn^{2+} -containing *Escherichia coli* glutamyl-t-RNA synthetase, aminoacylase and bovine 5-aminolaevulinate dehydratase [24–26], two of which contain more than one Zn^{2+} -binding site [24,26]. On the other hand, the carboxypeptidase activity in BChE was inhibited by EDTA and the inhibition could be reversed by Zn^{2+} .

Evidence for the involvement of histidine residues in Zn^{2+} binding came from two observations: (a) imidazole was as effective as EDTA in eluting the enzyme from the Zn^{2+} -chelate-Sepharose column and (b) diethylpyrocarbonate treatment which modifies histidine residues resulted in the non-binding of the enzyme to the Zn^{2+} -chelate-Sepharose column and also abolished $^{65}\text{Zn}^{2+}$ binding to the enzyme. An examination of the possible metal-binding sequences in human serum BChE indicates a potential sequence HXXE...H (residues 438–548). Such a sequence is also found in AChE, the zinc exopeptidases

carboxypeptidases A and B as well as several other recently reported enzymes [27–31]. In some of the enzymes (insulin-degrading enzymes from human and *Drosophila* and carboxypeptidase T), the last histidine residue follows immediately after the glutamic acid residue whereas in others (AChE, BChE, carboxypeptidases A and B and γ -D-glutamyl mesodiaminopimelic acid-hydrolysing peptidase) the last histidine is at a distance from the glutamic acid with several amino acids intervening [27–31]. This sequence is also conserved in serum BChE from several species [32]. Apart from the HXXE...H sequence, there is a cysteine residue (residue 66) in BChE which is inaccessible to alkylation [33]. Lockridge et al. [33] have suggested that this inaccessibility could be due to either steric hindrance caused by adjacent disulphide bonds or the presence of an unknown protective agent such as a metal.

It is not certain that the metal removed by EDTA from BChE is Zn²⁺ and it is possible that the Zn²⁺ binding demonstrated reflects only the ability of Zn²⁺ to substitute for another unknown metal naturally present in the enzyme. In many Zn²⁺-containing enzymes the metal has been located at more than one site and their roles are expressed in catalytic, structural and co-catalytic motifs [34]. Preliminary studies by us using the Zn²⁺ chelators, 1,10-phenanthroline and *N,N,N',N'*-tetrakis-(2-pyridylmethyl)-ethylenediamine have indicated at least one additional Zn²⁺-binding site in BChE apart from the one demonstrated in the present studies using EDTA as the chelator (C. D. Bhanumathy and A. S. Balasubramanian, unpublished work). Accessibility to the metal-containing sites in the protein is governed among other factors by the polarity of the chelators. Both 1,10-phenanthroline and *N,N,N',N'*-tetrakis-(2-pyridylmethyl)ethylenediamine are less polar than EDTA and therefore they are more permeant to the hydrophobic sites [35,36]. In addition to the Zn²⁺-binding sequence HXXE...H and the cysteine residue already discussed, two co-catalytic sequences GDV and GDY [34] are also found at residues 390–392 and 394–396 in human serum BChE. In AChE, regions ideal for cationic binding that help stabilize the active-site conformation have been reported [37].

The identification of the actual histidine residue(s) involved in Zn²⁺ binding in BChE will need further investigations. AChE shows a high degree of sequence homology to BChE and has also the HXXE...H sequence (residues 440–550), the histidine at 440 being involved in the catalytic triad of the enzyme. It is therefore pertinent to consider the crystal structure of AChE in which no bound metal is reported [6]. A dimeric form of AChE from *Torpedo californica* after solubilization and purification has been exhaustively dialysed against buffer containing 0.01% (w/v) NaN₃ before crystallization [38]. NaN₃ is a metal-complexing agent [39] and it has been shown to interact with several metalloenzymes and metalloproteins [40–43]. Further, the histidine residue at 550 in the carboxyl end beyond the HXXE sequence is missing from the dimeric AChE used for crystal studies [6]. For these reasons, the possibility exists that no bound metal was detected in the crystal structure of AChE.

In conclusion, we have shown that Zn²⁺ binds to human serum BChE after EDTA treatment, suggesting that a metal-binding site is present in the enzyme. Histidine residues are involved in the metal binding and the metal is required for the stability of the enzyme. To our knowledge, a metal-binding site on this enzyme has so far not been demonstrated. The implications of the metal-binding site on the non-cholinergic functions attributed to BChE remain to be elucidated [14].

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