CHAPTER - II

INTERACTION OF TRITON X-100 WITH ACYL POCKET OF BUTYRYLCHOLINESTERASE: EFFECT ON ESTERASE ACTIVITY AND INHIBITOR SENSITIVITY OF THE ENZYME

1. SUMMARY

The effect of non-ionic detergents like Triton X-100, Lubrol PX, Brij 35 and Tween 80 on the esterase activity and inhibitor sensitivity of human serum butyrylcholinesterase (BChE) were studied. The results showed that though BChE is not a detergent dependent enzyme, the esterase activity and inhibitor sensitivity of it can be modulated by the presence of detergents. All the detergents caused a marginal activation of the esterase activity. The presence of Lubrol PX, Brij 35 or Tween 80 did not affect the 50% molar inhibition concentration ($IC_{50}$) of the inhibitors tested. But in the presence of Triton X-100 the $IC_{50}$ values were increased for neostigmine, eserine and tetraisopropylpyrophosphoramide (iso-OMPA; acylation site interacting inhibitors), whereas for inhibitors like ethopropazine, imipramine and procainamide (choline binding pocket specific inhibitors) the $IC_{50}$ values were unaltered. In addition, in the presence of Triton X-100 the bimolecular reaction constant for phosphorylation reaction ($k_t$) of BChE for the acyl pocket specific iso-OMPA was reduced. Triton X-100 partially
protected BChE against this iso-OMPA inactivation. These results indicate that Triton X-100 by interacting with the acyl pocket hydrophobic region is able to activate the esterase activity of BChE. Further it reduces the capacity of the enzyme to react with inhibitors that inactivate it by interacting with the serine residue of the acylation site.

2. INTRODUCTION

Human serum butyrylcholinesterase (BChE; 3.1.1.8), a soluble enzyme, though abundant has no well defined physiological function [36]. It is generally viewed as a backup for the catalytic activity of the homologous enzyme, acetylcholinesterase (AChE; 3.1.1.7) and as a scavenger of naturally occurring poisonous compounds targeted at acetylcholine binding sites [140,174]. Nevertheless, the reason for continued interest in BChE is its extraordinary sensitivity to insecticides like organophosphate esters and carbamates, certain therapeutic drugs such as echothiophate and nerve gases like sarin. Hence its activity is measured to aid in the diagnosis and prognosis of poisoning by these compounds [227-229].

Biochemical analysis and site-directed mutagenic studies on BChE and crystallographic studies of Torpedo AChE have revealed the active site of BChE to be within a 20 Å deep gorge lined by hydrophobic amino acids [113, 142, 143, 230]. The catalytic site is present at about 4 Å from the base of the gorge. It contains the reactive serine residue at the acylation site.
where acyl-enzyme intermediate is formed during hydrolysis of ester substrates. The differential substrate specificity and inhibitor sensitivity of BChE over AChE are defined primarily by two distinct regions, characterised by the presence of a cluster of hydrophobic amino acids [144]. One is the acyl pocket, present at the bottom of the gorge that interact with the acyl moieties of the substrates. Further the acyl pocket is characterised by the presence of two unique residues Leu and Val. The presence of these residues in the acyl pocket allows BChE to interact specifically with tetraisopropylpyrophosphoramide (iso-OMPA) and also is the reason for its broad substrate specificity [144, 168]. The second region is the choline binding pocket containing Ala and Trp along with a Glu residue. It is separated by about 10 Å from the acyl pocket. This choline binding pocket is responsible for the specificity of BChE towards tricyclic inhibitors (ethopropazine and imipramine) and for binding of carbamates (eserine and neostigmine) [145, 168, 175, 182]. Though BChE is hydrophilic [237], its substrate specificity and inhibitor sensitivity are defined by hydrophobic amino acids. Thus it was of interest to analyse the effect of non-ionic detergents on the esterase activity and inhibitor sensitivity of BChE. Deciphering detergent interacting site will throw more light on the active site region of the BChE. In this chapter the effect of various non-ionic detergents like Triton X-100, Lubrol PX, Brij 35 and Tween 80 on the esterase activity and inhibitor sensitivity of BChE have been probed. Using
kinetic data it has been demonstrate that the non-ionic detergent Triton X-100 interacts with the acyl pocket hydrophobic region of BChE in influencing both the esterase activity as well as inhibitor sensitivity of the enzyme.

3. MATERIALS AND METHODS

Materials

Butyrylthiocholine iodide (BTCI), Lubrol PX, eserine hemisulfate, neostigmine bromide, tetraisopropylpyrophosphoramide (iso-OMPA), ethopropazine hydrochloride, imipramine hydrochloride and procainamide hydrochloride were purchased from Sigma Chemical Company, St.Louis, USA. Triton X-100 and Tween 80 were obtained from LOBA Chemie Pvt. Ltd., India. Brij 35 was from s.d. fine-chem Ltd., India. All other reagents used were of analytical grade.

BChE was purified essentially as described in appendix I (page 83), from outdated human plasma (obtained from G.K.Naidu Memorial Hospital, Coimbatore, India).

Effect of non-ionic detergents

The effect of non-ionic detergents such as Triton X-100, Lubrol PX, Brij 35 and Tween 80 on esterase activity of BChE were assayed by pre-incubating the enzyme in the assay mixture with different concentrations of
the detergents for 10 min. After pre-incubation, the activity was measured using the substrate BTCI.

Determination of 50% molar inhibition concentration ($IC_{50}$)

The residual activities observed after incubating BChE with different inhibitors (ethopropazine, imipramine, procainamide, eserine, neostigmine and iso-OMPA) were plotted against the respective concentrations of the inhibitors. The inhibitor concentration corresponding to 50% residual activity was taken as $IC_{50}$ value of that particular inhibitor. Similarly $IC_{50}$ values of the inhibitors were also determined in the presence of various non-ionic detergents.

Determination of rate constants

Different concentrations of iso-OMPA were incubated with BChE for varying time periods (5-30 min) and the residual activities measured. Similar assays were performed with enzyme pre-incubated with 60 μM Triton X-100; and also at a fixed iso-OMPA concentration with variable concentration of Triton X-100. The pseudo-first order rate constants, $k_i$, were then determined from the slopes of the plots of $\ln \left( \frac{V}{V_0} \times 100 \right)$ versus $t$, where $V_0$ and $V$ being the activity of BChE in the absence and presence of iso-OMPA for the specified time interval, $t$ min. The bimolecular reaction constants for phosphorylation reactions, $k_i$, were determined from the slopes of the plot of $1/\text{iso-OMPA}$ versus $1/k_i$ by regression analysis [231].
Enzyme activity

The activity of BChE was assayed according to the method of Ellman et al [232] as detailed in appendix IIa (page 84). One unit of esterase activity is the quantity of enzyme required to liberate 1 μmole of thiocholine per min from the substrate BTCI under the standard assay conditions.

4. RESULTS AND DISCUSSION

Non-ionic detergents like Triton X-100, Lubrol PX, Brij 35 and Tween 80 were used to evaluate their effect on the esterase activity and inhibitor sensitivity of BChE. These detergents vary in their structure with respect to the number of polyoxyethylene group and also the side chain that is attached to it. The side chain linked to polyoxyethylene group of Triton X-100, Lubrol PX, Brij 35 and Tween 80 are octylphenoxy, dodecyl, lauryl and sorbitane monooleate -group respectively (Table 5).

The purified BChE was pre-incubated with increasing concentrations of various detergents (the concentrations of detergents used were well below their critical micellar concentration [CMC]) and the activity assayed. The detergents in general caused a marginal increase in esterase activity of the enzyme (Figure 10). It has been reported that non-ionic detergents like hemolysin and Triton X-100 at low concentrations, inhibited BChE activity
<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>C.M.C (mM)</th>
<th>Average Mr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton X-100</td>
<td>C$<em>{12}$H$</em>{25}$H$_2$(O-CH$_2$-CH$<em>2$)$</em>{10}$-OH</td>
<td>0.3</td>
<td>628</td>
</tr>
<tr>
<td>Lubrol PX</td>
<td>C$<em>{12}$H$</em>{25}$H$_2$(O-CH$_2$-CH$<em>2$)$</em>{12}$-OH</td>
<td>0.1</td>
<td>582</td>
</tr>
<tr>
<td>Brij 35</td>
<td>C$<em>{12}$H$</em>{25}$H$_2$(O-CH$_2$-CH$<em>2$)$</em>{10}$-OH</td>
<td>0.012</td>
<td>1300</td>
</tr>
<tr>
<td>Tween 80</td>
<td>C$<em>{12}$H$</em>{25}$H$_2$(O-CH$_2$-CH$<em>2$)$</em>{10}$-OH</td>
<td>0.091</td>
<td>1204</td>
</tr>
</tbody>
</table>

Table 5: Structure of polyoxyethylene detergents
Figure 10. Effect of polyoxyethylene ether detergents on the esterase activity of BchE: Purified BchE was incubated for 10 min at 37°C with increasing concentrations of Triton X-100 (O), Lubrol PX (□), Brij 35 (△) and Tween 80 (▽) in the assay mixture at pH 7.0, thereafter the activity was assessed in terms of increase in absorbance at 412 nm. The concentration ranges were 20-100 μM for Triton X-100; 10-50 μM for Lubrol PX and Brij 35; and 2-10 μM for Tween 80. Each point represents the mean of three independent determinations.
(The value 0.00 depicts the absolute activity of the enzyme in absence of the detergent, which corresponds to 1U)
with acetylcholine as substrate [225]. Nevertheless, tryptamine, histamine and several alkyl amines, activate BChE hydrolysis of butyryl- and benzoylcholine [233, 234]. Also, long chain alkanols are known to activate the esterase activity of BChE [220]. Similar activation of RNA-instructed DNA polymerase by non-ionic detergents binding to the enzyme’s accessible hydrophobic region is also known [235]. The ability of the detergents to activate BChE in the present study would depend on their capacity to interact with the hydrophobic regions of the enzyme. Since the active site of BChE is rich in hydrophobic amino acids, the detergents could interact with the active site hydrophobic region inducing subtle conformational change leading to the activation of esterase activity. A similar scheme of substrate induced conformational change has been proposed for the substrate activation phenomenon in BChE catalysis [159]. To identify the detergent interacting site, further studies were carried out with active site specific inhibitors in the absence and presence of the detergents.

The $IC_{50}$ values of inhibitors having different binding specificity for BChE were determined in the absence and presence of the detergents. The $IC_{50}$ values of the inhibitors were unaffected by the presence of Lubrol PX, Brij 35 and Tween 80. In the presence of Triton X-100 the $IC_{50}$ values of ethopropazine, imipramine and procainamide (inhibitors specific for choline binding pocket) were not affected. However a 4.2, 5.5 and 6.9 fold increase
in the \( IC_{50} \) values was observed for neostigmine, eserine and iso-OMPA (inhibitors that interact with Ser of the acylation site) respectively (Table 6).

The possibility that the increase in \( IC_{50} \) of eserine, neostigmine and iso-OMPA in the presence of Triton X-100 could be due to the ‘apparent’ activation caused by the detergent rather than protection offered by the detergent is ruled out by the following observations. Only with inhibitors that specifically interact with the reactive serine of the acylation site there is an increase in \( IC_{50} \) value. Secondly, though Lubrol PX, Brij 35 and Tween 80 caused an increase in esterase activity, their presence did not affect the \( IC_{50} \) values of inhibitors tested. Lastly, the sequestering effect of Triton X-100 on inhibitors there by causing an increase in \( IC_{50} \) is ruled out by the fact that the concentration of Triton X-100 used in the present study was well below its CMC (CMC=300\(\mu\)M) [236]. Further up to 500\(\mu\)M concentration of Triton X-100 either activation or no change in activity was observed. However at higher concentrations (more than 1mM) there is inhibition.

The characteristic structural feature of Triton X-100 that distinguishes it from other detergents used is its aromatic (octylphenoxy) group. Hence the observed in \( IC_{50} \) values could mainly be due to the interaction of the octylphenoxy group of the monomeric form of the detergent with the active site hydrophobic regions of BChE. The possibility of Triton X-100 interacting with the hydrophobic region of choline binding pocket is ruled out by the fact that, in the presence of the detergent, the \( IC_{50} \) values of the choline binding pocket specific inhibitors ethopropazine and imipramine were unaltered. But for the acyl pocket specific iso-OMPA, there was a 6.9 fold increase in the IC50 value in
Table 6. *IC*$_{50}$ values of inhibitors for BChE in the absence and presence of Triton X-100.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Absence of Triton X-100</th>
<th>Presence of Triton X-100</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethopropazine</td>
<td>1.8 x10$^{-6}$</td>
<td>1.9 x10$^{-6}$</td>
<td>Nil</td>
</tr>
<tr>
<td>Imipramine</td>
<td>3.1x10$^{-4}$</td>
<td>3.3 x10$^{-4}$</td>
<td>Nil</td>
</tr>
<tr>
<td>Procainamide</td>
<td>1.1 x10$^{-3}$</td>
<td>1.2 x10$^{-3}$</td>
<td>Nil</td>
</tr>
<tr>
<td>Neostigmine</td>
<td>4.0 x10$^{-4}$</td>
<td>1.7 x10$^{-3}$</td>
<td>4.3</td>
</tr>
<tr>
<td>Eserine</td>
<td>4.5 x10$^{-8}$</td>
<td>2.5 x10$^{-7}$</td>
<td>5.5</td>
</tr>
<tr>
<td>Iso-OMPA</td>
<td>3.6 x10$^{-6}$</td>
<td>2.5 x10$^{-5}$</td>
<td>6.9</td>
</tr>
</tbody>
</table>

The residual activities obtained after preincubating BChE for 10 min with different concentrations of inhibitors were plotted against the concentrations of the respective inhibitors. The inhibitor concentration corresponding to 50% residual activity was taken as *IC*$_{50}$ value of that particular inhibitor. Similarly *IC*$_{50}$ values were determined in the presence of 60 μM of Triton X-100. Each value represents the average of three independent determinations.
the presence of Triton X-100. Since the substrate specificity and irreversible inhibitor sensitivity are primarily determined by the hydrophobic amino acids of acyl pocket, the above results indicate that the Triton X-100 could modulate the inhibitor sensitivity of BChE by interacting with the acyl pocket hydrophobic region. To further substantiate these results, time course of BChE inactivation by iso-OMPA in the absence and presence of Triton X-100 were performed.

The time course of inactivation of BChE by different concentrations of iso-OMPA in the absence and presence of Triton X-100 is shown in Figure 11-A & B. The pseudo-first order rate constants, \( k_i \), were reduced in the presence of the detergent. Even with the narrow range of the inhibitor concentrations used, the bimolecular reaction constant for phosphorylation reaction, \( k_t \) of BChE for iso-OMPA in the presence of Triton X-100 was reduced to \( 1.63 \times 10^4 \text{ M}^{-1} \text{ min}^{-1} \) from \( 4.16 \times 10^4 \text{ M}^{-1} \text{ min}^{-1} \) (Figure 11-C). This decrease in phosphorylation constant of BChE for iso-OMPA and the increase in \( IC_{50} \) value in the presence of the detergent reflect the decreased affinity of the enzyme towards iso-OMPA. From the binding specificity of iso-OMPA, which is specified by the amino acids of the acyl pocket [168], it is apparent that Triton X-100 indeed interacts with the acyl pocket hydrophobic region, which is large enough to accommodate aromatic choline esters and aromatic hydrocarbons [221].
To assess the degree of protection conferred by Triton X-100 against iso-OMPA inhibition, time course of inactivation of BChE was followed at a fixed iso-OMPA concentration, but with varying detergent concentrations (Figure 12-A). This indicated that the detergent binds to the enzyme and in doing so protects the enzyme against the inactivation by iso-OMPA. However the detergent confers only partial protection against iso-OMPA inactivation of BChE. This is evident from the fact that the pseudo-first order rate constant, $k_1$, decreased upto 40 μM of Triton X-100, with further increase in the detergent concentration causing no apparent decrease in the $k_1$ value (Figure 12-B). Such results are expected from a compound that interacts non-competitively with the enzyme [226]. Thus, Triton X-100 by virtue of its aromatic group binds to the acyl pocket hydrophobic region of the active site of BChE so as to confer protection against iso-OMPA inactivation.

In conclusion, though BChE does not require the presence of detergents or any amphiphiles to exhibit its activity, its esterase activity and inhibitor sensitivity can be modulated by the presence of detergents. The non-ionic detergents used in the present study were able to activate the esterase activity of BChE. Further, Triton X-100, having an aromatic group in its structure, by interacting with the acyl pocket hydrophobic region of BChE reduces the capacity of the enzyme to react with compounds that irreversibly inactivates it. Thus, in general compounds with aromatic group...
Figure 12 A. Time course of inactivation of BChE by $3 \times 10^{-6} M$ iso-OMPA in the presence of, 0 (O), 20 (□), 40 (Δ), 60 (▽), 80 (◇) and 100 (○)μM of Triton X-100. B. The reciprocal of $k_i$ values calculated from the data of A are plotted against Triton X-100 concentration.
can be made to bind to BChE in such a way as to protect the enzyme against organophosphates, carbamates and nerve gases. Studies of this type, in addition to developing antidotes for anticholinesterase agents, should help in gaining information about the topology of the active site until direct evidence arises from crystallographic studies.