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Original Article

Evaluation and Characterization of Free and Immobilized Acethylcholinesterase with Fluorescent Probe, Differential Scanning Calorimetry and Docking

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Acetylcholinesterase (AChE) enzyme which catalyses the hydrolysis of choline esters, such as acetylcholine, is very important in nerve function. Previous structural studies showed the possible amyloid fibril formation on the AChE. Therefore it is important to understand interaction of ligands to prevent the formation of amyloid fibrils. The purpose of the present study was to characterize AChE structure using differential scanning calorimetry (DSC), fluorescent probe and docking methods. Immobilization of AChE was carried out on porous silica matrix. Fluorescence of free and immobilized form of AChE was measured by a spectrofluorometer at 435 nm excitation wavelength .Calorimetric measurements were carried out on a differential scanning calorimeter. AChE–ligand docking Version Lig plot was used for docking studies. Based on our data, thioflavin T (Th-T) binding to the peripheral site of AChE, increased its fluorescence in a dose-dependent manner. DSC showed that immobilized AChE form is probably more stable structurally than its free form. Protein docking study revealed that AChE interacts through different regions with matrix, and each of these interactions have its own binding energy. A comparison between DSC, fluorescence spectroscopy and docking results revealed excellent agreement between them.

Keywords: Acetylcholinesterase, thioflavin T, differential scanning calorimetry, protein docking

The enzyme acetylcholinesterase (AChE; EC 3.1.1.7) hydrolyzes acetylcholine at the postsynaptic terminal or motor end –plate. AChE is a key component of the cholinergic brain synapses and neuromuscular junctions. The major biological function of AChE is the termination of nerve impulse propagation by rapid hydrolysis of the one of the most useful fluorescent probes of AChE (1). Also, as repor-

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ted by many other investigators, Th-T is frequently used to detect amyloid structure in polypeptides, enzymes and proteins (2). It is already well known that differential scanning calorimetry (DSC) is a useful tool to measure the heat capacity of a system as a function of temperature in isobaric conditions (3). DSC is an experimental technique to simultaneously characterize the energetics of unfolding. According to the recent works, DSC is a useful tool to estimate binding parameters in multi-ligand binding proteins (4). Based on these data, it can be concluded that DSC is a technique which has allowed the study of thermal transformations in enzymes (5). It has been shown that DSC is the most useful technique for characterizing thermal stability of proteins in terms of their thermodynamic characteristics, and permits analysis of thermal transitions in proteins containing several domains (6). DSC is widely used for the study of thermal protein denaturation. Crystal structure data suggested that there is a potential locus of interaction for beta-amyloid on the AChE molecule (7). This locus is near AChE's peripheral site, on the external surface of the enzyme near the entrance to its catalytic gorge (8, 9). Furthermore, amyloid fibrils are involved in a number of diseases such as Alzheimer's disease and type 2 diabetes. Interestingly, in some of these diseases the amyloid fibril deposits cause the disease, but the function of amyloid fibrils is not clear (10). Accordingly, the ability of proteins to form amyloid fibrils has been observed for a large range of proteins (11). It is well established that the presence of amyloid fibrils is a therapeutic problem since amyloid fibrillated proteins are biologically inactive (12) and may cause immunological responses in patients (13). Therefore, understanding the fibrillation process and especially focusing on how to prevent and/or inhibit the fibril formation are important issues in medicine. Based on these data, it can be concluded that using experimental methods for detection of amyloid, provides increased knowledge about the fibrillation process.There are some reports in the literature showing that Th-T is a fluorophore that has been used to stain proteins. Th-T is a dye which is widely used to determine the presence of amyloid fibrils. Th-T (3,6-dimethyl-2-(4-dimethylaminophenyl)-benzothiazoliu-m), is a benzothiazolium dye (2). This fluorophore has been shown to bind to the peripheral site of acetylcholinesterase, and increase its fluorescence by more than 1000- fold over unbound Th-T (14), thereby constituting a valuable tool for studying ligand interactions with the peripheral anionic site (15). In the present study, the crystal structure of AChE subjected to various heat treatments, has been studied using differential scanning calorimetry. Furthermore, the same sample has been analyzed using fluorescent probe and docking method in order to correct the differential scanning calorimetry data.

Materials & methods

Immobilization of AChE

Immobilization of AChE (Sigma, St. Louis, MO, USA) was carried out based on previously described method (16, 17). An AChE solution with the concentration of 0.03 mg/ml in a final volume of 300 µl of phosphate buffer (50 mM, pH 7.4) was added to 1.8 mg of octadecyl macroporous silica (Merck, Darmstadt, FRG). The suspension was incubated for 1.5 h at room temperature with gentle stirring. The suspension was centrifuged at 500 rpm for 10 s, and then the supernatant was discarded. The pellet, which was immobilized AChE, was washed three times by vortexing with 1 ml of phosphate buffer solution (50 mM, pH 7.4) to remove loosely

adsorbed enzymes. After washing three times, the immobilized AChE was stored at 4 ºC until use. Immobilization of enzyme was carried out on porous silica matrix (Fractosil-500) and 25 nm matrix. Matrixes were hydrophobized by alkylation in dry system. Determination of chemical properties of the Fractosil-500 and approval of alkylation with C18 group was carried out by Thermogravimetric analysis (TGA) and infrared (IR) spectrophotometry, respectively. 20 mg octadecyl-substituted macroporous silica as carrier was washed twice with 300 µl of 50 mM phosphate buffer, pH 7.4, then was loaded with 300 µl of AChE at concentration range of 0 - 0.2 mg/ml in the same buffer. The mixtures were then incubated for 90 min at room temperature under gentle rotary stirring. Assessment of immobilization was carried out through estimation of protein and AChE activities in the supernatants after 5 min centrifugation at 3000×g. Pellets were washed three times with 1 ml buffer solution to ensure removal of the trapped or non-bound enzyme molecules from the adsorption product. The support bearing the immobilized AChE was used to directly assess the immobilization process and to perform discontinuous and continuous catalytic activity, respectively.

Acetylcholinesterase activity assay

AChE initial rate was measured according to Ellman's method (18). The substrate working solution (SWS) contained 25 mM phosphate buffer (pH 7.4), 0.33 mM ,DTNB (5,50-Dithio-bis(2-nitrobenzoic acid)) (Sigma, St. Louis, MO, USA) and the substrate acetylthiocholine iodide (Sigma, St. Louis, MO, USA) at the final desired concentrations. The reaction was started by adding 10 µl of free enzyme to 300 µl of SWS and the increase in optical density (OD) was followed spectrophotometrically (Camspect M550 double beam scanning UV/visible spectrophotometer) at 412 nm. Immobilized enzyme activity (in the duration of initial rate) was measured according to an end point method. The reaction was started by adding 1 ml of SWS to 1.8 mg of immobilized AChE. After 2 min, the reaction was stopped by precipitating immobilized AChE through centrifugation at 500 RPM for 10 s, then the supernatant was separated and the OD of supernatant was determined at 412 nm. When dividing OD by time, the quotient was the activity of immobilized AChE.

Binding measurements

Different concentrations of AChE solutions (0.0–0.3 mg/ml) were added to a constant amount of the octadecyl macroporous silica support (1.8 mg). The activity of the immobilized enzyme was determined by two methods: direct activity over the support (DAOS) and indirect activity over the support (IDAOS). In DAOS, the activity of immobilized enzyme was directly assayed by the end point method previously described (18). In IDAOS, the activity of the bound enzyme was determined by the difference between the total activity of enzyme applied and the activity remaining unbound in the reaction mixture (18). The stability of the immobilized AChE was studied under storage conditions (4 ºC and 25 ºC, in 50 mM phosphate buffer, pH 7.4), up to 70 days. The batch of the preparation was pre-washed with the same buffer followed by the end point assay (DAOS). After each measurement, the preparations were washed again by a phosphate buffer solution (50 mM, pH 7.4) followed by vortex and restored at the defined conditions until the next round of measurements. Each series of measurements were carried out twice for duplicate samples and the mean activities were reported.

Km and Vm values evaluation

The substrate acetylthiocholine iodide (AChE) was used at seven concentrations ranging from 50 to 700 µM in order to evaluate Km and Vm values

under different tested conditions. The data were given as the average of three separate experiments at room temperature.

Soluble AChE was assayed according to the method of Ellman. The assay mixture contained acetylthiocholine iodide at 500 µM in Ellman reagent. The Ellman reagent contained 9.1 mg DTNB [5, 5-Dithiobis (2-Nitrobenzoic acid)] (Sigma, St. Louis, MO, USA), 3.75 mg sodium bicarbonate in 100 ml phosphate buffer (25 mM, pH 7.4). The enzymatic reaction was monitored at 412 nm using a Camspect M550 double beam spectrophotometer. Activity of the immobilized AChE was determined directly on the immobilized preparations. 1 mL Ellman reagent was added to 1.8 mg immobilized AChE. The reaction mixture was incubated at room temperature for desired time periods under gentle stirring. Then reaction was terminated by removing the immobilization product using centrifugation at 500 RPM for 10 s. To assess the immobilized enzyme activity, the absorbance of supernatant was read out at 412 nm.

Stability of the immobilized preparation

Immobilization products were considered for their functional stability at storage conditions (4 ºC and 25 ºC, in 50 mM phosphate buffer solution, pH 7.4), up to 70 days. The esterase activity of the immobilized AChE on octadecyl-substituted porous silica was monitored at one-day intervals. The batch of the preparation was pre-washed with the same buffer followed by direct assay as mentioned above. After each measurement, the preparations were washed again by phosphate buffer solution (50 mM, pH 7.4) followed by vortex and restored at defined conditions until the next round of measurement. Each series of measurements were carried out twice for duplicate samples and the mean activities were considered.

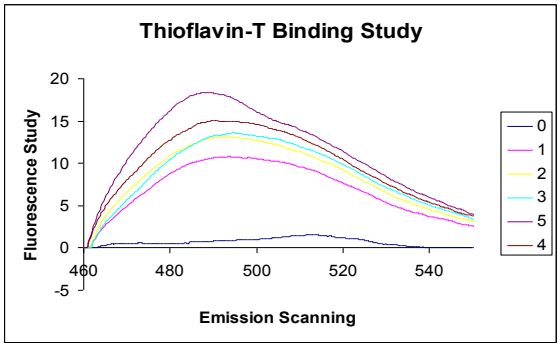


Figure 1. Fluorescence determination of Th-T binding to AChE. Fluorescence was measured in the presence of increased concent-rations of Th-T (0, 1.824, 3.648, 5.472, and 9.120 µM) and 0.0456 µM AChE for 30 min at 23 ºC in 0.02% TritonX-100. The fluorescence of Th-T is enhanced when this ligand binds to the AChE peripheral site.

Results

Fluorescence measurements

Aliquots of enzyme at 0.0456 µM concentration were incubated for 0, 15 and 30 min at 23 ºC in 50 mM Phosphate buffer, (pH 7.0). To quantitate amyloid formation, a Th-T fluorescence method was used. Th-T binds specifically to amyloid and this binding produces a shift in its emission spectrum and a fluorescent signal proportional to the amount of amyloid formed. After incubation, 0.0456 µM AChE enzyme was added to 1.824 µM Th-T (pH 7.0), in a final volume of 400 µl. Fluorescence was monitored at 435 nm excitation and 460-550 nm emission using a Hitachi spectrofluorometer. A time scan of fluoresc-ence was performed, and three values after the decay reached the plateau (10 min) were averaged after µM Th-T. For co-incubation experiments, fluorescence of pre-incubated 0.0456 µM AChE

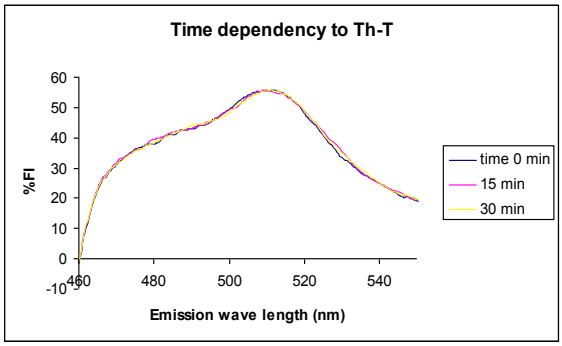


Figure 2. A time scan of fluorescence of AChE with Th-T.

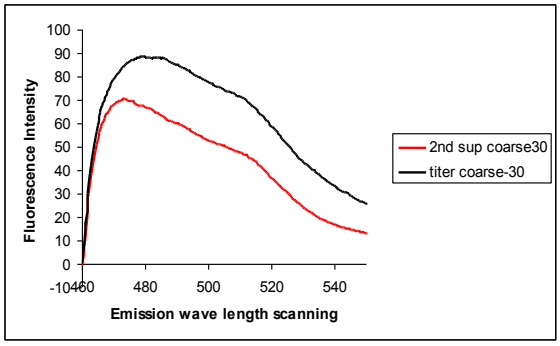


Figure 3. Liberation of attached Th-T into the solutions due to binding C18.

subtracting the background fluorescence of 1.824 alone was determ-ined. The AChE with or without 0.02% Triton X-100 (Sigma, St. Louis, MO, USA) gave fluorescence values. To study the effect of the AChE concentration on the ligand aggregation, Th-T at 1.824 µM in 50 mM phosphate buffer, (pH 7.0) was incubated for 2 h in the presence of increasing concentrations of AChE, and fluorometry was performed as described above. All experiments were performed in triplicate.

Discussion

Fluorescence was monitored on a spectrometer (MPF-4, Hitachi, Japan) in 50 mM sodium phosphate buffer (pH 7.0) and 1, 2, 3, 4, and 5 mM of Th-T in 0.02% Triton X-100 thermostated at 23 °C. Th-T fluorescence was measured with excitation at 435 nm and emission from 460 to 500 nm with excitation and emission slits of 10 nm. Total areas under the fluorescence emission curves were calculated and fluorescence contributions from scatter in the buffer and AChE enzyme were subtracted. In experiments with Th-T and AChE alone, the fluorescence (*F*) was recorded over 3–4 min**,** with Th-T or enzyme alone.

Calorimetric experiments

Calorimetric experiments were carried out on a differential scanning calorimeter (Q100, TA instrument's) and thermograms were obtained. A nitrogen pressure of 0.1 MPa was applied to the cell compartments to prevent degassing of the sample during the heating. Scan rates of 1 ºC/min were used with a temperature range from 10 to 85 ºC. To prevent the formation of air bubbles, samples were degassed exhaustively before filing the calorimeter cell. The reference cell was filled with buffer. The differential scanning calorimetry (DSC) curves were obtained by scanning AChE solution of 8.3 mg/ml in 50 mM sodium phosphate buffer at pH 7.0, in the presence and absence of Th-T. Irreversibility of the thermal transition was checked by reheating the samples after cooling from the first run. Instrumental baselines measured under identical conditions than the samples were subsequently subtracted.

Steady state inhibition of enzyme-catalyzed substrate hydrolysis

Substrate hydrolysis rates were measured in buffer (50 mM sodium phosphate and 0.02% Triton

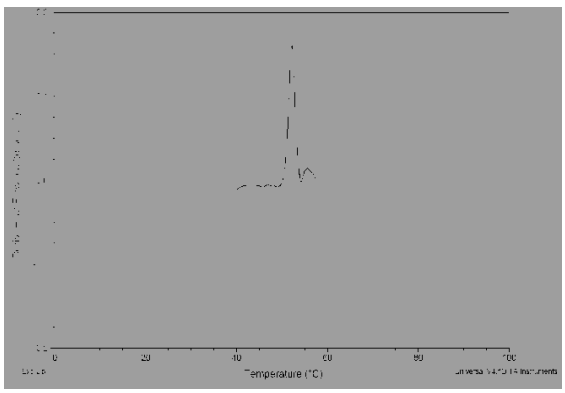


Figure 4. Differential scanning calorimeter curve of immoblized enzyme.

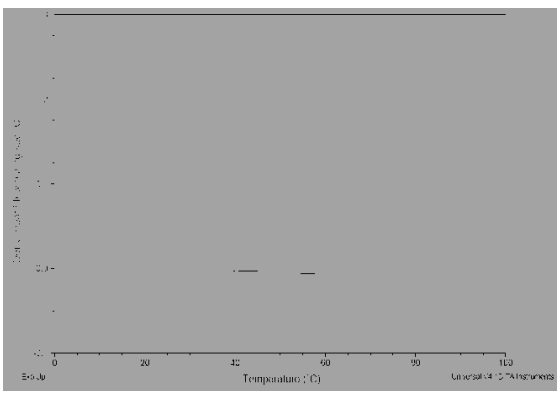


Figure 5. Differential scanning calorimeter curve of support medium or carrier and sodium phosphate buffer.

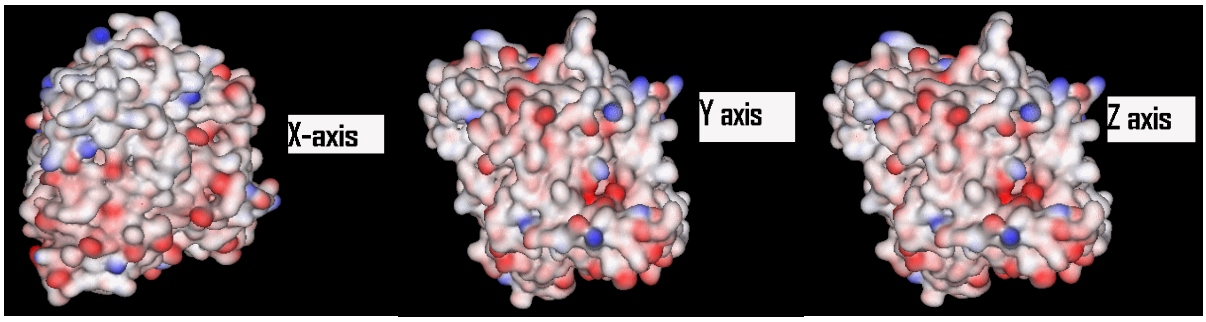
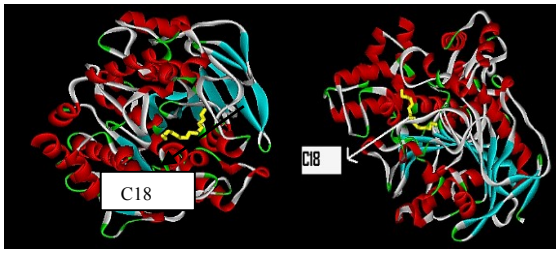


Figure 6. 3-dimension representation of the C18 alkyl group used to dock.

X-100 at pH 7.0) at 23 °C after addition of small aliquots of Th-T, acetylthiocholine, and 5,59-dithiobis-(2-nitrobenzoic acid) (Sigma, St. Louis, MO, USA) to a final concentration of 0.33 mM in a total volume of 1.0 ml. An Ellman assay was used to measure formation of the thiolate dianion of 5,59-dithiobis-(2-nitrobenzoic acid) at 412 nm for 1–5 min on a (M550 double beam scanning UV/visible) spectrophotometer (18).

Docking studies

0AChE–ligand docking version Lig plot was used for docking studies. This program generates an ensemble of different rigid body orientations for the C18 alkyl group–AChE within the binding pocket and then passes each molecule against a negative image of the binding site. We determined the binding pocket using the ligand-free AChE structure and a box enclosing the binding site of enzyme. Each of the best-scored compounds was saved for subsequent steps. The C18 alkyl group used to dock were converted into 3-dimension. To this end, C18 alkyl group correspo-nding to the modeled protein was added.



Statistical analysis

To calculate the significance of the difference, t-test for paired values was applied with $P<0.05$ as the minimal level of significance. Values given are Mean ±SD from at least three experiments.

Fluorescence titrations of AChE with thioflavin T

AChE was titrated in the presence of increasing amounts of Th-T by measuring Fluorescence values. Increasing concentrations of Th-T caused the increase of fluorescence values Figure1.

A time scan of fluorescence of AChE with Th-T was performed and showed that the interaction of AChE with Th-T was not time dependent Figure 2. Figure 3 shows the liberation of attached Th-T, into the solutions due to C18 binding and .figure 4 shows

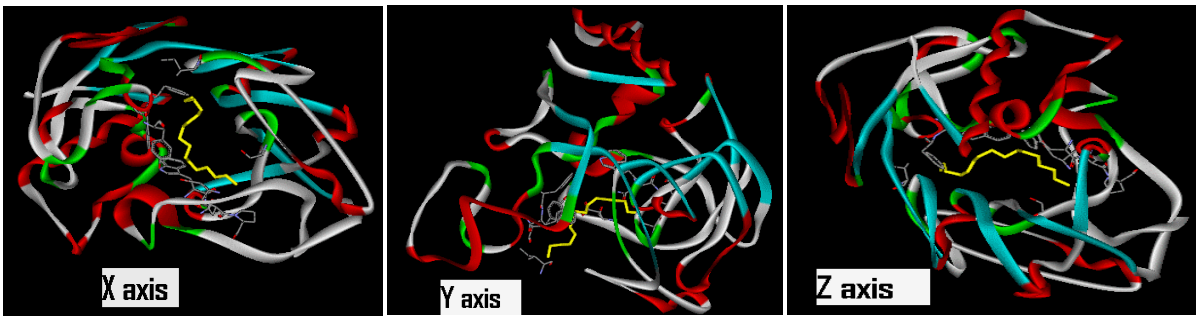


Figure 7. Different rigid body orientations for the C18 alkyl group–AChE within the binding pocket.

the thermograms of immobilized AChE. Thermograms of support medium or carrier, without enzyme are shown in figure 5.

AChE–ligand docking studies

Docking study of C18 group of ligand on active site of AChE is shown in figure 6 while different rigid body orientations for the C18 alkyl group–AChE within the binding pocket are represented in figure 7. Table 1 represents the Gibbs free energy (ΔG) of AChE with Th-T. The total energies of shape score of the best-docked conformations of each interaction region of the enzyme are indicated. Lig plot was used to study the interaction of AChE amino acids with C18 group Figure 8.

Preliminary studies revealed that several human neurodegenerative diseases, such as Alzheimer are associated with protein misfolding that leads to protein aggregation. The aggregation process may be attributed to the formation of amyloid fibril. Based on these data, it can be concluded that fibrillated amyloid may be the molecular basis of and the common link between a variety of pathological conditions and human neurodegenerative syndromes. Accordingly, as amyloid fibrillated proteins are biologically inactive, emphasizing amyloid fibrils formation is important. In this regard, amyloid fibril formation and/or prevention on AChE is important, because AChE is an enzyme found in the synaptic cleft. The role of this enzyme is to terminate the nerve impulse by hydrolyzing acetylcholine to choline and acetic acid. In the synaptic cleft, AChE is bound to a network of collagen and glycosaminoglycans derived from the posts-ynaptic cell. The degradation of acetylcholine is necessary to the depolarization of the nerve so that it can be repolarized in the next conduction event. Crystal structure data have suggested possible amyloid fibril formation on the AChE. Therefore, it is important to understand the mechanism of AChE amyloid formation or prevent the amyloid fibril process. It seems that using experimental models for understanding amyloid formation, gain increased knowledge about the fibrillation mechanism. The stability of enzymes and their interactions with ligands is a topic of special interest in biochemistry, because many cellular processes depend on that. In the present study, the crystal structure of AChE subjected to various heat treatments was studied using DSC. Furthermore, the same sample of AChE was analyzed using fluorescent probe and docking. The fluorescence values of mixtures of AChE and Th-T were determined. When AChE was titrated with varying concentration of Th-T, the fluorescence enhancement for Th-t bound to AChE was increased. Increasing the fluorescence value may be attributed to concentration of Th-T. This observation is in agreement with the results obtained by other investigators (7, 14) . The two rings of Th-T are planar, and this loss of rotational mobility is thought to provide the fluorescence enhancement in Th-T-AChE complexes. The results of time scan of fluorescence Figure 2 demonstrate that there are no differences in fluorescence values in different interaction times. The fluorescence of Th-T is enhanced when this ligand binds to the AChE peripheral site. These data indicate that Th-T binds to the AChE peripheral site. Fluorescence studies

Table 1. Gibbs free energy (ΔG) of AChE with Th-T

	ΔG (kcal/mol)
1	-5.68
2	-5.52
3	-5.18
4	-5.34
5	-4.77
6	-4.96
7	-4.30
8	-3.71
9	-3.48
10	-3.52

indicate that C18 group binds to the peripheral site of enzyme. Binding C18 causes liberation of attached Th-T, into the solution as shown in figure 3. The ligand binds to active site of AChE and the substituent containing C18 alkyl group binds to peripheral site of the enzyme. Protein docking studies revealed that the enzyme interacts by different regions with matrix, and each interaction has its own binding energy (Table 1). Our findings are consistent with those reported elsewhere (11, 14).

According to calorimetric results, enthalpy changes observed in figure 4 may be associated with molecular changes as a result of AChE unfolding. Carrier enthalpy changes are different from enzyme (Fig. 5),. The changes may be due to a combination of endothermic reactions, such as disruption of hydrogen bonds and exothermic reactions, such as disruption of hydrophobic interactions in AChE enzyme. The thermograms showed that temperature change may be attributed to denaturation of AChE. DSC shows that structurally, immobilized AChE is probably more stable than free enzyme. Results of AChE docking in 3-dimension show different rigid body orientations for the C18 alkyl group–AChE

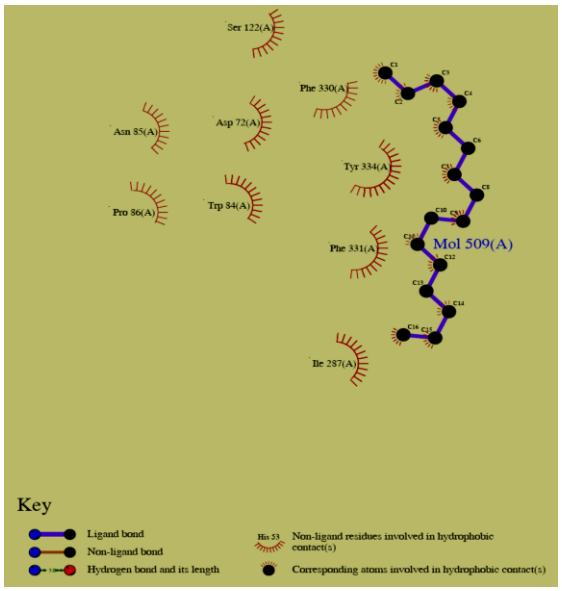


Figure 8. Use of Lig plot for studying the interaction of AChE amino acides with C18 group.

within the binding pocket. Lig plot was used for studying the interaction of AChE amino acides with C18 group and revealed that AChE's peripheral site may be a locus of protein interaction with amyloid. Therefore, a compound able to block this interaction, may prevent amyloid fibril formation process. With results of the present study based on characterization of AChE structure using DSC, fluorescent probe and docking methods, it should be possible to discover new drugs disrupting more effectively interactions between AChE and amyloid, ideally with minimal enzyme inhibition. Our findings showed that results obtained from the DSC, fluorescence spectroscopy and docking are in agreement with each other. However, the small size and other enzymatic parameters which may produce different results are among limitations of the present study.

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Conflict of interest

The authors declared no conflict of interests.

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