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(54) **LIGAND SENSING FLUORESCENT ACETYLCHOLINESTERASE FOR DETECTION OF ORGANOPHOSPHATE ACTIVITY**

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(57) **ABSTRACT**

Disclosed are methods for the preparation and use of labeled AChE and labeled AChE inhibitory conjugate compositions for detecting accumulation of toxic materials such as organophosphates, insecticides, and other nerve agents. Also disclosed are methods for the use of labeled AChE and labeled AChE inhibitory conjugate compositions in a variety of areas, including the detecting of toxic materials in biological samples, in the area of food and water analysis, in environmental monitoring, and in industrial settings.

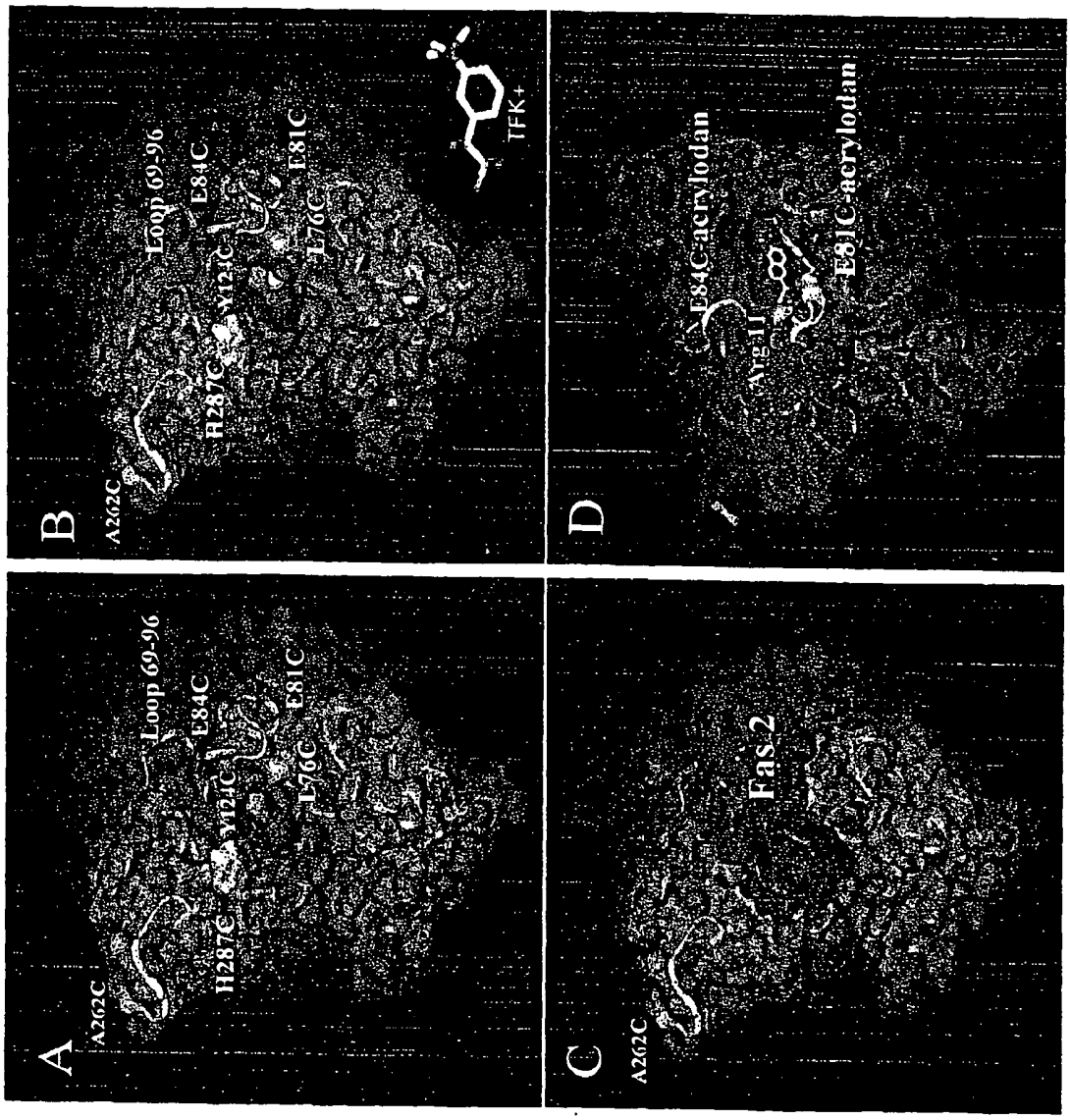
A
A262C
Loop 69-96
E84C
V124C
H287C
L76C
E81C
B
A262C
Loop 69-96
E84C
V124C
H287C
L76C
E81C
TFK+
C
A262C
Fas 2
D
E84C-acrylodan
Arg 11
E81C-acrylodan

FIGURE 1

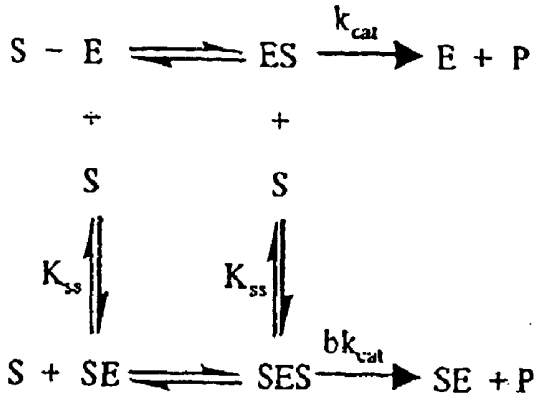

FIGURE 2

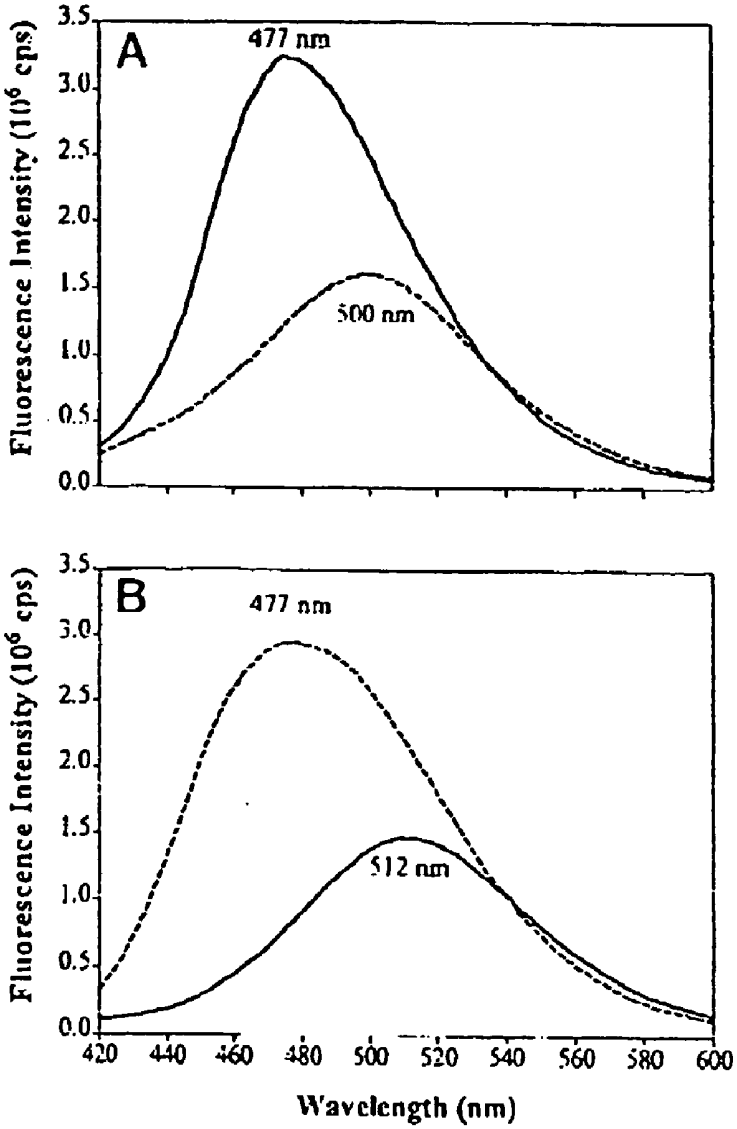
A
477 nm
500 nm
Fluorescence Intensity (10^6 cps)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B
477 nm
512 nm
Fluorescence Intensity (10^6 cps)
420
440
460
480
500
520
540
560
580
600
Wavelength (nm)

FIGURE 3

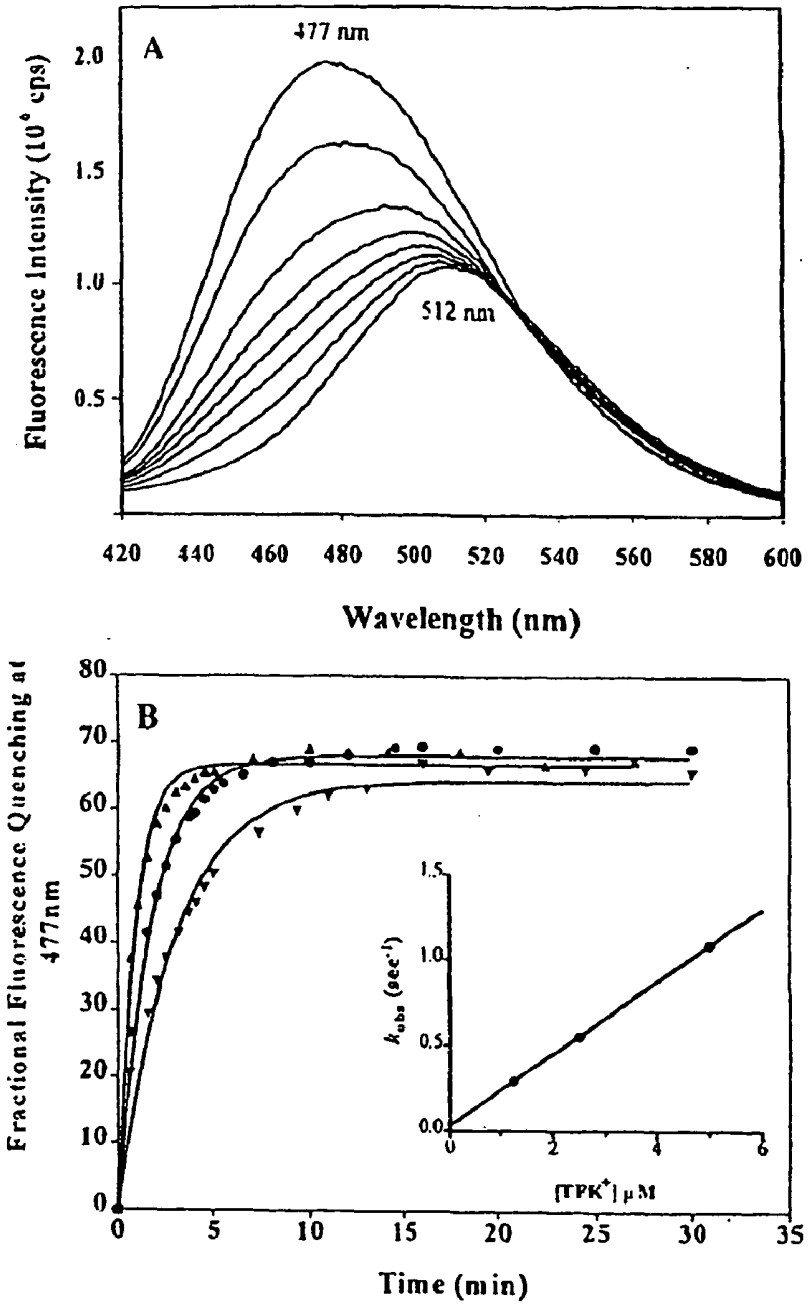
A
477 nm
512 nm
Fluorescence Intensity (10^6 cps)
2.0
1.5
1.0
0.5
420
440
460
480
500
520
540
560
580
600
Wavelength (nm)
B
Fractional Fluorescence Quenching at 477nm
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
30
35
Time (min)
k_{obs} (sec^{-1})
1.5
1.0
0.5
0.0
0
2
4
6
[TPK$^+$] μM

FIGURE 4

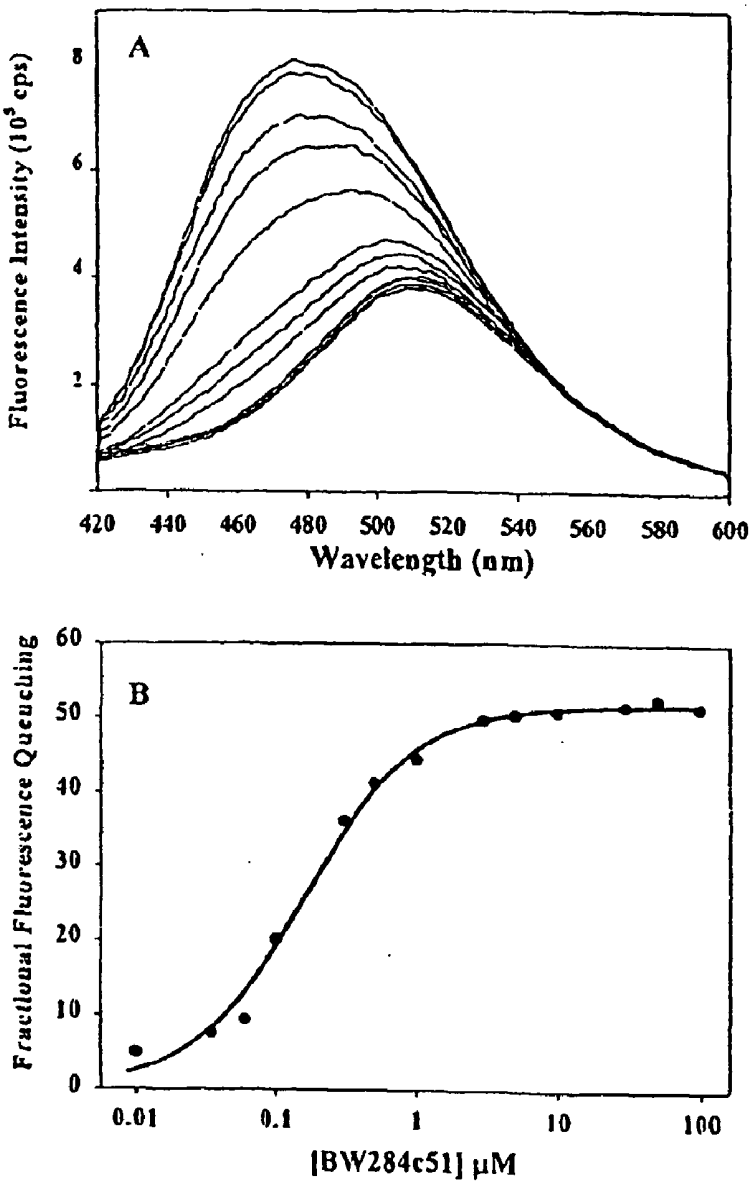
A
Fluorescence Intensity (10^5 cps)
8
6
4
2
420
440
460
480
500
520
540
560
580
600
Wavelength (nm)
B
Fractional Fluorescence Quenching
60
50
40
30
20
10
0
0.01
0.1
1
10
100
[BW284c51] μM

FIGURE 5

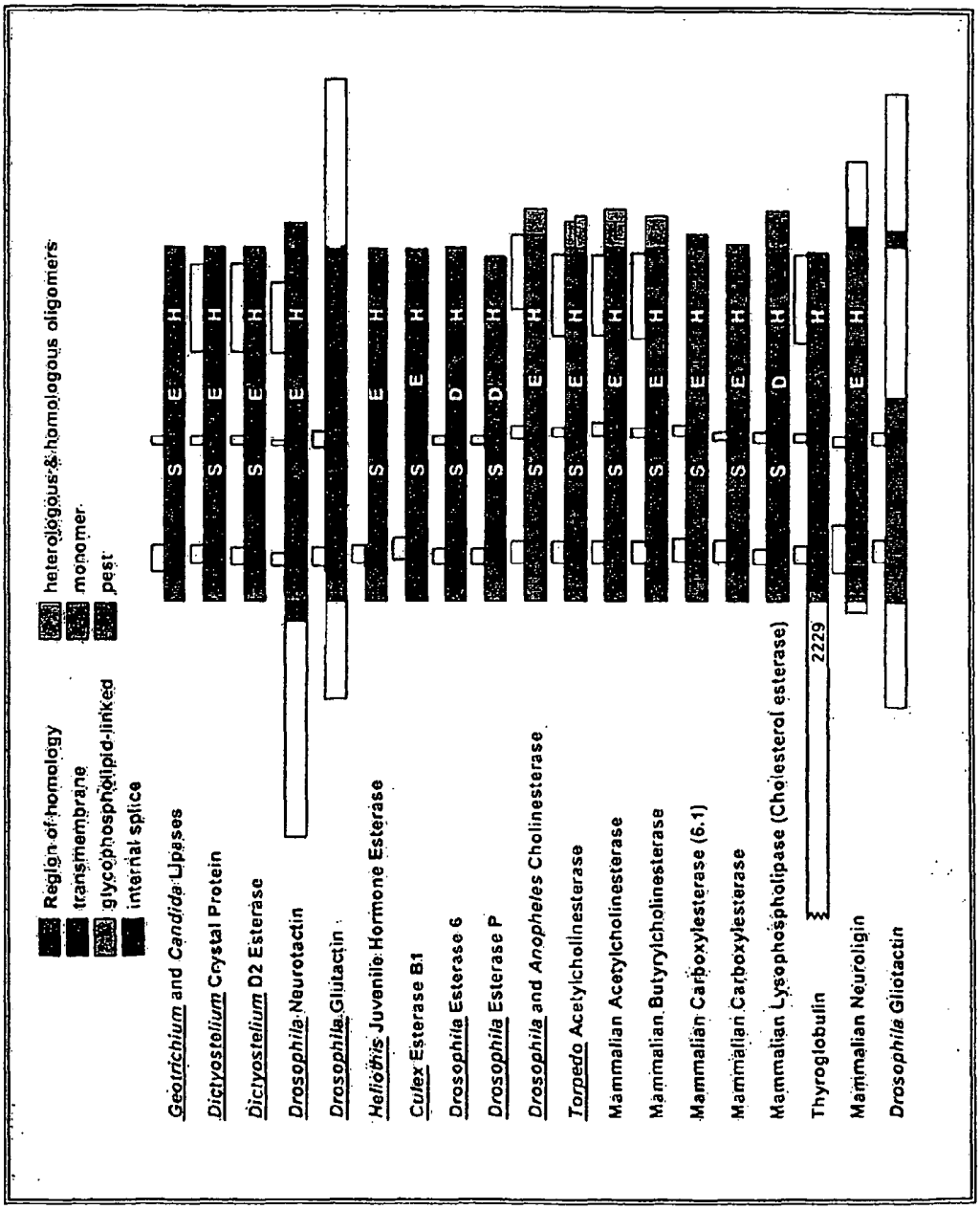
Region of homology
transmembrane
glycophospholipid-linked
internal splice
heterologous & homologous oligomers
monomer
pest
Geotrichium and Candida Lipases
Dictyostelium Crystal Protein
Dictyostelium D2 Esterase
Drosophila Neurotactin
Drosophila Glutactin
Heliothis Juvenile Hormone Esterase
Culex Esterase B1
Drosophila Esterase 6
Drosophila Esterase P
Drosophila and Anopheles Cholinesterase
Torpedo Acetylcholinesterase
Mammalian Acetylcholinesterase
Mammalian Butyrylcholinesterase
Mammalian Carboxylesterase (6.1)
Mammalian Carboxylesterase
Mammalian Lysophospholipase (Cholesterol esterase)
Thyroglobulin
2229
Mammalian Neuroligin
Drosophila Gliotactin

FIGURE 6

LIGAND SENSING FLUORESCENT ACETYLCHOLINESTERASE FOR DETECTION OF ORGANOPHOSPHATE ACTIVITY

[0001] This invention was made in part with government support under Grant Nos. R37-GM18360 and 17-1-8014 awarded by the United States Public Health Service (USPHS) and the Department of the Army Medical Defense Command, respectively. The government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] This invention relates generally to the field of detecting hazardous chemicals and chemical analysis, to include portable automatic sensing devices. More particularly, the present invention relates to methods, compositions, devices and kits thereof useful in the detection of chemical agents, insecticides and other acetylcholinesterase (AChE) inhibitors, modifiers and ligands.

[0004] 2. Background Information

[0005] Acetylcholinesterase (AChE), a serine hydrolase in the α/β-fold hydrolase protein superfamily, terminates nerve signals by catalyzing hydrolysis of the neurotransmitter acetylcholinesterase at a diffusion-limited rate. A number of nerve toxins, including insecticides and organophosphates, act through binding to and inhibiting AChE.

[0006] Organophosphorus and organosulfur compounds, are used extensively in insecticides and are highly toxic to many organisms including humans. Insecticide residues are found in soil and groundwater, and the detection of these residues is important for their elimination from the environment and to protect the health of both humans and animals. Organophosphorus compounds are also used in nerve agents, such as sarin, phosphine, soman, and tabun, for chemical warfare purposes.

[0007] These agents are some of the most potent toxic agents and are specific inhibitors of acetylcholinesterase (AChE).

[0008] Acetylcholine is an essential neurotransmitter that affects parasympathetic synapses (autonomic and CNS), sympathetic preganglionic synapses, and the neuromuscular junction (see, e.g., Taylor et al., in *Basic Neurochemistry,* 5th ed., 1993, (Siegal et al., eds.), Chapter 11, pp. 231-260, Raven Press, New York, N.Y.). Hydrolysis of acetylcholine by acetylcholinesterase, present in nervous tissue, normally limits the duration of action function. Organophosphate (e.g., Malathion, Parathion, Diasinon, Dursban) and carbamate (e.g., Sevin, Furadan) insecticides exert their toxicity by inhibiting the action of acetylcholinesterase and thereby causing a pronounced cholinergic response (Arron et al., Insecticides: Organophosphate and Carbamates in *Goldfrank's Toxicologic Emergencies,* 1994, (Goldfrank et al., eds.), Appleton & Lange, Norwalk, Conn.). Enzyme inhibition is the consequence of phosphorylation (organophosphates) or carbamylation (carbamates) of the cholinesterase-active site serine residue. The resulting phosphoroyl-serine bond is stable; therefore, enzyme inhibition is physiologically irreversible, whereas the carbamyl-serine bond undergoes spontaneous hydrolysis with regeneration of enzyme activity (24-48 h). For this reason and because of poor CNS penetration, carbamate insecticide neurotoxicity is less severe and of shorter duration than that for the organophosphates (*Tietz Textbook of Clinical Chemistry,* 1999, (Burtis et al., eds.), W. B. Saunders Company, Philadelphia, Pa.).

[0009] Excess synaptic acetylcholine stimulates muscarinic receptors (peripheral and CNS) and stimulates but then depresses and paralyzes nicotinic receptors. The CNS neurotoxic effects include restlessness, agitation, lethargy, confusion, slurred speech, seizures, coma, cardiorespiratory depression, or death.

[0010] The need for the reliable determination of these cholinesterase inhibitors has led to the development of a number of sophisticated instrumental methods, mostly involving the use of gas and liquid chromatography and mass spectrometry. Also a number of liquid phase chemiluminescence procedures have been developed for the determination of inorganic and organic species mostly utilizing the luminol and peroxyoxalate reactions. See Robards K. and Worsfold P. J., Anal Chem Acta (1992) 266:147.

[0011] These traditional methods are not practical for individual use as the methods are time consuming and complicated and the instruments utilized are expensive, non-portable and require high maintenance. Additionally, the measurement of nerve agents in mixtures with these traditional methods requires cumbersome extraction and manipulation procedures.

[0012] Thus, biosensors were developed as an alternative to the traditional gas and liquid chromatography and mass spectrometry technology. Generally, biosensors include those which are enzyme-based and bioaffinity-based. An enzymatic biosensor uses an enzymatic or metabolic process to detect a reaction product which occurs between an incoming substrate and an immobilized enzyme. A bioaffinity sensor relies on a biological binding event of a target substance.

[0013] Many existing methods for the detection of organophosphates and cumulative inhibition of cholinesterases lack sensitivity since they are based on inhibition of basal activities rather than accumulation of the inhibitory conjugate. Basal activities vary substantially between subjects resulting in inconsistency in present assays.

[0014] Existing monitoring methods routinely require expensive laboratory procedures involving sample transport or preparations of samples for assay.

[0015] Rapid analysis of toxic materials in the areas of food and water analysis, environmental monitoring, and in industrial settings is a problem that continues to exist and is currently addressed by time-consuming, expensive methods or by techniques that may be described as inadequate.

[0016] Many problems associated with exposure to toxic materials could be avoided or minimized by a detection procedure which gives near "real-time" indication of the presence of toxic gases. Equally important are the characteristics of economy, small size, and ease of use for the successful application of such devices.

[0017] Accordingly, there is a need for a method of detecting, quantifying, and evaluating hazards which provides for early detection and which can detect low levels of toxic materials. The present invention satisfies this need, as well as others.

SUMMARY OF THE INVENTION

[0018] The present invention overcomes one or more of the drawbacks in the prior art by providing compositions and methods for their use in the detection of specific inhibitors, modifiers, or ligands of acetylcholinesterase (AChE). Disclosed are methods for the preparation and use of labeled AChE and labeled AChE inhibitory conjugate compositions which are useful in detecting accumulation of toxic materials, which include but are not limited to, organophosphates, insecticides, nerve agents, such as sarin, phosphine, soman, and tabun, and other materials used for chemical warfare purposes. Also disclosed are methods for the use of labeled AChE and labeled AChE inhibitory conjugate compositions in a variety of areas, including the detecting of toxic materials in biological samples, the areas of food and water analysis, environmental monitoring, and in industrial settings. Embodiments are disclosed which describe methods for making and using labeled AChE and labeled AChE inhibitory conjugate compositions comprising fluorescing compounds, including but not limited to, dimethoxyphosphoryl and diethoxyphosphoryl labels.

[0019] In one embodiment of the invention, methods are disclosed for measuring accumulation of inhibitory conjugates comprising labeled AChE and an inhibitor, modulator or ligand (i.e., cognate partner). In a related aspect, such methods may comprise contacting a sample suspected of containing such cognate partners with labeled AChE in order for labeled AChE binding to occur between cognate partners in the sample and the enzyme. In a further related aspect, conjugated and unconjugated AChE may be separated by chromatographic methods. For example, such chromatographic methods may include, but are not limited to, capillary electrophoresis.

[0020] In another embodiment, the conjugated and unconjugated, labeled AChE are detected and differentiated by fluorochromic emission shift, where conjugated AChE shows a detectable shift in emission signal. In a related aspect, the shift in emission is a Stokes' shift upon conjugate formation.

[0021] In a related aspect, the cognate partner is an inhibitor, where the inhibitor may be designated as a carbamylating inhibitor or a phosphorylating inhibitor. In a further related aspect, the inhibitor may be an insecticide or an organophosphate.

[0022] In another related aspect, the presence of the cognate partner is estimated by determining the ratio of conjugated to unconjugated AChE.

[0023] In another embodiment, the sample may be obtained from the atmosphere, soil, water, industrial sites or environmental sites. Further, the sample may be biological or non-biological. In a related aspect, a biological sample may include, but is not limited to, the integumentary system, sputum, feces, blood, urine, plasma, lacrimal secretions, cerumen, and semen.

[0024] In one embodiment, the AChE is labeled on at least one site. In a related aspect, the AChE is labeled at multiple sites. In a further related aspect, the at least one label is peripheral to the active center of the AChE.

[0025] In one embodiment, a device comprising AChE is envisaged, where the AChE is compartmentalized in a mobile or stationary phase. In a related aspect, the stationary phase is a chip. In another related aspect, the mobile phase is a suspension.

[0026] In one embodiment, the device is a biosensor for analyzing a sample for at least one organophosphorous, nerve agent and/or insecticide, where at least one enzyme is immobilized on or within the device. In a related aspect, the immobilized enzyme is either covalently or non-covalently bound to the device. In a further related aspect, the biosensor is divided into multiple zones, where each zone differentiates between one or more organophosphorous, nerve and/or insecticide agents.

[0027] In another embodiment, the present invention also provides kits which contain the present labeled AChE for use in the present identification method.

[0028] In another embodiment, a labeled AChE composition comprising at least one fluorophore located peripherally to the active center of the AChE, where the at least one fluorophore possesses an emission signal that shows a Stokes' shift upon conjugate formation. In a related aspect, the fluorophore comprises a dimethoxyphosphoryl label or a diethoxyphosphoryl label.

[0029] In another embodiment, a labeled AChE molecule comprises at least one fluorophore on at least one site present at the periphery of the active site of the enzyme, wherein the at least one site is selected from the group consisting of residues 76, 81, 84, 124, 262 and 287 of AChE or equivalents thereof.

[0030] Exemplary methods and compositions according to this invention, are described in greater detail below.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

[0032] FIGS. 1A-D show locations of introduced cysteines for fluorophore modification. Residues 76, 81, and 84 are at the tip (76) and outer portion (81, 84) of the Q loop. Residues 124 and 287 are on an opposing face of the gorge and make up part of the peripheral anionic site. Residue 262 is on a peripheral disulfide loop and in the crystal has a large thermal factor. A-D, Connolly surface representations of structure. A, unligated AChE (6); B, TFK^+ conjugated with AChE; note partial exposure of the white molecule TFK^+, at the base of the gorge (33); C, fasciculin 2 bound AChE at the mouth of the gorge (5); D, fasciculin 2 complex with AChE, rotated 90°. Acrylodan conjugated to E84C is shown in yellow; and acrylodan conjugated to E81C is shown in green. (Note in D the proximity between arginine 11 on Fas 2 and the acrylodan side chain at position 84).

[0033] **FIG. 2** shows a scheme showing combining of substrate at two discrete sites to form two binary complexes, ES and SE (where S is substrate; E is enzyme; and P is product). Only ES results in substrate hydrolysis. For simplicity, S is assumed to combine equally well with E and ES. The efficiency of substrate hydrolysis of the ternary complex

SES, as compared with ES, is reflected in the value of the parameter, b, the relative catalytic turnover of the ternary complex (26).

[0034] FIGS. 3A-B show fluorescence emission spectra of acrylodan-labeled Y124C(A) and E84C (B) AChE free in solution (dashed line) and complexed with fasciculin (solid line). A, for acrylodan-labeled Y124C, fasciculin produces a hypsochromic shift and enhancement of fluorescence quantum yield. The large shift for Y124C reveals a clear isoemissive point indicative of the two (free and fasciculin bound) species. Equivalent concentrations of enzyme (215 nM) were present for all conditions. The concentration of fasciculin was 215 nM. B, for acrylodan-labeled E84C, fasciculin produces a bathochromic shift and reduction of fluorescence quantum yield. Equivalent concentrations of enzyme (270 nM) were present for all conditions. The concentration of fasciculin was 800 nM.

[0035] FIGS. 4A-B show association of TFK^+ with acrylodan-modified E84C AChE. A, fluorescence emission spectra of acrylodan-labeled E84C AChE following addition of excess TFK^+. TFK^+ produces a bathochromic shift and reduction of fluorescence quantum yield. The large chromic shift reveals a clear isoemissive point indicative of the two (free and TFK^+ bound) species. Initial enzyme concentration was 130 nM. Excess TFK^+ (1.25 μM) was added, and fluorescence spectra were recorded at the following times: 0, 1, 2.5, 4.3, 5.8, 7.4, 10.6 and 22 min. B, time course of the fluorescence changes. Initial acrylodan-modified E84C AChE concentration was 150 nM. Excess TFK^+ was added, and the decrease in fluorescence signal at 477 nm was monitored using an ISA Jobin Yvon-Spec Fluoromax fluorometer. The three TFK^+ concentrations were 1.25 (▼), 2.5 (○), and 5.0 (Δ) μM. Control enzyme samples, to which buffer rather than TFK^+ was added, did not show decreases in fluorescence signals over the time intervals measured. The inset shows rates plotted as a function of TFK^+ concentration. k_{on} for $TFK^+$is calculated based on ratios of the hydrated and unhydrated ketone (21).

[0036] FIGS. 5A-B show association of BW284c51 with acrylodan-modified E84C AChE. A, fluorescence emission spectra of acrylodan-labeled E84C AChE following titration with BW284c51. BW284c51 produces a bathochromic shift and reduction of fluorescence quantum yield. Initial enzyme concentration was 70 nM. BW284c51 concentrations were 0, 0.01, 0.035, 0.06, 0.1, 0.3, 0.5, 1, 3, 5, 10, 30, 50, and 100 μM. B, the decrease in fluorescence emission curves is plotted as a function of BW284c51 concentration. K_d is determined by fitting the data with Equation 1 as outlined below (EXAMPLES, Materials and Methods).

[0037] **FIG. 6** shows an illustration showing regions of homology between AChE and various enzyme species.

DETAILED DESCRIPTION OF THE INVENTION

[0038] Before the present compositions and methods are described, it is understood that this invention is not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be described by the appended claims.

[0039] It must be noted that as used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a subject" includes a plurality of such subjects, reference to "an enzyme" includes one or more enzymes and equivalents thereof known to those skilled in the art, and so forth.

[0040] Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the methods, devices, and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the proteins, compounds, and methodologies which are reported in the publications which might be used in connection with the invention. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

[0041] As used herein acetylcholinesterase (AChE) means a serine hydroxylase in the α/β-fold hydrolase protein superfamily which terminates nerve signals by catalyzing the hydrolysis of the neurotransmitter acetylcholine. In one embodiment, AChE is from mouse (e.g., but not limited to, Accession Nos. IMAAA, IAAB, IMAAC, IMAAD and IMAHA). In a related aspect, the mutagenized residues of AChE as envisaged in the instant invention are denoted by the wild type residue, followed by the residue number (based on, for example the Accession Numbers, supra), with the mutated residue last (i.e., cysteine, "C"). For example, based on Accession No. IMAAA, conversion of residue 81 (i.e., Glu or E) to cysteine (or C) would be denoted as follows: E81C. Corresponding structures on equivalent AChE molecules may be determined by alignment of homologous regions (see, e.g., **FIG. 6** and Soreq et al., Trends Biochem Sci (1992) 17(9):353-8).

[0042] Equivalents of AChE may include, but are not limited to, *Candida* lipase (Acc. No. CAD86495), *Dictyostelium* crystal protein (Acc. No. CAA36702), *Dictyostelium* D2 esterase (Acc. No. A60531), *Drosophila* neurotactin (Acc. No. S12005), *Drosophila* glutactin (Acc. No. S12519), *Heliothis* juvenile hormone esterase (Acc. No. P12992), *Culex* esterase BI (Acc. No. A35986), *Drosophila* esterase 6 (Acc. No. AAD39965), *Drosophila* esterase P (Acc. No. B34089), *Drosophila* cholinesterase (Acc. No. A25363), *Torpedo* acetylcholinesterase (ACC. No. ACRYE), mammalian acetylcholinesterase (e.g., *homo sapiens*, ACC. No. AAA53473), mammalian butyrylcholinesterase (e.g., *homo sapiens*, Acc. No. AAH18141), mammalian carboxylesterase (6.1) (e.g., *rattus norvegicus*, Acc. No. P16303), mammalian lysophospholipase (e.g., *mus musculus*, Acc. No. AAH13536), thyroglobulin (e.g., *rattus norvegicus*, Acc.No. NP_112250), mammalian neuroligin (e.g., *homo sapiens*, Acc. No. AAH34018), and *Drosophila* gliotactin (NP_723931). In a related aspect, derivatives, fragments and variants of these AChE equivalents are also envisaged for the methods, compositions and devices of the present invention. (See, also e.g., **FIG. 6** and Soreq et al., 1992).

[0043] In one embodiment, mutants can be prepared by site-directed mutagenesis (or evolution methods, see e.g.,

Devlin et al., Science (1990) 249:404-406; and Scott & Smith, Science (1990) 249:386-390) using a conventional oligonucleotide directed in vitro mutagenesis system such as that described by Eckstein et al., Nucleic Acids Research (1985) 13:8749-8785. (See also, U.S. Pat. No. 6,001,625). Other conventional PCR techniques known in the art may be used.

[0044] In one embodiment, the selection of residues for replacement is based on a molecular model, using the crystal structure of AChE published by Sussman, et al. on an Evans and Sutherland PS390 platform with Biosym software.

[0045] As used herein, "cognate partner," including grammatical variations thereof, means a molecule that stereoselectively binds within the active site of AChE. For example, a ligand (e.g., acetylcholine), modulator (e.g., galantamine) or inhibitor (e.g., organophosphates) of AChE would be considered a cognate partner.

[0046] The instant invention is a sensitive method for the detection of AChE ligands, modulators and inhibitors using fluorescent labeled forms of AChE. The AChE of the present invention is labeled at sites peripheral to the active center with a fluorophore whose emission signal shows a large Stokes' shift upon binding of ligands. The attached fluorophore does not interfere with ligand binding, so the high affinity for inhibitors, modifiers or ligands seen in the native enzyme is maintained. The labeled AChE of the present invention can be used to detect interaction with inhibitors, modifiers and ligands. This is particularly useful for the detection of organophosphates such that insecticides or nerve agents and provides a method to determine if the concentrations of a substance present in the atmosphere or ground are sufficient to inhibit AChE.

[0047] After a dye molecule has been electronically excited due to absorption of light, it may almost instantaneously emit light. This very fast process occurring on the nanosecond time scale (10^{-9} sec) is called fluorescence. A fluorescence dye is characterized by its spectral characteristics (excitation and emission spectra, lex and lem respectively), its quantum yield (QY) and its fluorescence lifetime (t).

[0048] The spectral characteristics of fluorescence dyes depend strongly on their molecular backbone. For absorption and emission in the UV, near the visible spectral range, at minimum, a system of conjugated double bonds is needed. The wavelengths of absorption and emission are simply stated as shifted towards red or longer wavelengths. Additional electron donating substituents are necessary for the spectral characteristics of the dye to finally occur in the visible spectrum of light (400-700 nm), which is called the bathochromic shift.

[0049] The emitted light occurs normally at a longer wavelength with respect to the absorbed light due to a loss in energy while the dye molecules remain in their electronically excited state. This so-called Stokes' shift makes the maximum emission occur at a few or up to several tens of nanometers (10^{-9} m) red-shifted with respect to the absorption maximum. These spectral properties of a dye are widely used for the identification of differently labeled probes. In a related aspect, the fluorophores of the present invention exhibit large Stokes' shift (i.e., the difference in wavelength between the excitation wavelength and the emission wavelength).

[0050] One of the most important properties of a fluorescence dye is the quantum yield (QY). The quantum yield is the ratio of emitted light to the light absorbed prior to the observed fluorescence. Hence the quantum yield is a measure of the efficiency of fluorescence, which is concurring with other so-called dark processes. The higher the quantum yield of a fluorescence dye the better it can be observed.

[0051] Due to the quantum nature of fluorescence, the emission of light from each molecule occurs arbitrarily after its excitation. In other words, the dye molecules remain in the electronically excited state for a very short but random time span. Nevertheless the statistics of emission follows a known model, which in the simplest case is an exponential decay. This well known kinetic law contains a time constant called fluorescence lifetime, mostly abbreviated as t, which is characteristic for the given dye and may be used for its identification.

[0052] Fluorescence lifetime and the closely related quantum yield may strongly depend on the molecular environment of the individual dye molecule, such as the surrounding solvent or the local pH. Therefore a change of these properties indicates a change of the local environment of the dye molecules. Close investigations of this phenomenon have revealed several well-defined processes, often referred to as quenching, which can be used for switching the dye properties, allowing for the development of intelligent probes (GenePin).

[0053] As used herein, "hypsochromic," including grammatical variations thereof, means a shift of a spectral band to higher frequency or shorter wavelength upon substitution or change in medium (e.g., solvent). It is informally referred to as a blue shift, and is opposite to bathochromic shift.

[0054] Sensitivity of assays and devices envisaged by the present invention is high, because the method of evaluating the presence of a conjugate (e.g., a labeled AChE-inhibitory conjugate) is based on detection with high quantum yield fluorophores. In one embodiment, the label is acrylodan.

[0055] Fluorescence emission of acrylodan is exquisitely sensitive to the dielectric constant of the solvent. In general, the fluorescence emission spectrum of acrylodan shifts toward the red (bathochromic), and the quantum yield decreases as the polarity of solvent increases (20, 40-42). This sensitivity to solvent polarity arises from the interaction of the excited state of acrylodan with its surrounding solvent. The excited state is more polar than the ground state and, as such, will interact with a polar solvent so as to align solvent dipoles. This alignment lowers the energy of the excited state and causes the red shift of the emission spectrum. Hence, an acrylodan-labeled enzyme with an emission maximum of 510-525 nm likely reflects exposure of the side chain to solvent (20, 42). On the other hand, acrylodan emission maxima in the range of 475-500 nm likely reflect solvent exclusion and a more hydrophobic environment surrounding the fluorophore. For example, the time course of TFK^+ reaction with acrylodan-E84C (**FIG. 3**) reveals a large spectral shift from 477 to 512 nm, indicating acrylodan conjugated at this position has moved to a more hydrophilic environment with TFK^+ bound. The large spectral shift yields a clear isoemissive point (i.e., the wavelength at which the intensity of emission of a sample does not change during a chemical reaction or physical change. The term derives from the Greek word for 'same lumines-

cence'), which arises when only two distinct emitting species are present, in this case the free enzyme and the TFK^+ conjugate.

[0056] As used herein, "accumulation of conjugates," including grammatical variations thereof, means continuous increase in the number of AChE molecules bound (i.e., combine by chemical action) by a ligand, modulator or inhibitor.

[0057] As used herein, "conditions sufficient for binding of an inhibitor to the enzyme" includes, but is not limited to, contacting a labeled AChE (or equivalents thereof) comprising a carrier (e.g., bovine serum albumin) in an appropriate buffer (e.g., sodium phosphate buffer, pH 7.0) with various concentrations of cognate partners, for example between about 0.05 mins to about 0.1 mins, about 0.1 mins to about 0.5 mins, about 0.5 mins to about 1 min, about 1 min to about 2.5 mins, about 2.5 mins to about 3 mins, about 3 mins to about 4.5 mins, about 4.5 mins to about 5.5 mins, about 5.5 mins to about 7 mins, about 7 mins to about 10 mins, about 10 mins to about 15 mins, about 15 mins to about 20 mins, or about 20 mins to 25 mins, at about 15° C. to about 20° C., about 20° C. to about 25° C., about 25° C. to about 30° C. to about 32° C., about 32° C. to about 37° C., about 37° C. to about 40° C. or about 40° C. to about 45° C.

[0058] As used herein, "biological sample," including grammatical variations thereof, means materials obtained from living organisms. For example, such samples include, but are not limited to, an integumentary system sample, sputum, feces, blood, urine, plasma, lacrimal secretions, cerumen, and semen.

[0059] As used herein, "non-biological sample," including grammatical variations thereof means materials obtained from inanimate objects or non-living materials. For example, such samples include, but are not limited to, soil, water, air and environmental (e.g., walls, floors, furniture etc.) surfaces.

[0060] Methods are described which distinguish between various classes of organophosphates by analyzing the fluorescence shift of labeled-AChE of the present invention. In a related aspect, the AChE may be labeled with various fluorophores, including but not limited to, dimethoxyphosphoryl and diethoxyphosphoryl labels. In a further related aspect, fluorophores yield different emission maxima.

[0061] In one embodiment, the labeled AChE can be immobilized on a chip to detect cumulative AChE inhibition. In a related aspect, such a chip can serve as a remote sensor for inadvertent or planned contamination.

[0062] In general, the simplest biosensor for compounds as envisaged for the present invention comprises a material having AChE immobilized upon or within a stationary phase, secured upon a carrier such as plastic, glass, cloth, nylon, rubber, etc. Detection of cognate partners may be qualitatively determined by separation of conjugated enzyme from unconjugated enzyme. To test for cognate partners, the biosensor is exposed to the sample to be tested. Since the accumulation of conjugated-immobilized enzyme is substantially similar to that observed for the soluble form, only a short duration of exposure to the sample is required. In a related aspect, a shift in fluorescence indicates the presence of a conjugate.

[0063] In one embodiment, the biosensor may be washed to remove compounds and/or compositions which may cause interference since the immobilized enzyme does not leach and the cognate partner is irreversibly bound to the immobilized enzyme. An appropriate buffer may be applied to the material. In a related aspect, in the field of drugs and testing of samples, it will be preferred to apply detectable cognate partners providing optimal signal in an aqueous system.

[0064] In one embodiment, biosensors may be disposed after a single use or may be reused. The biosensor may be regenerated using a reagent to displace the cognate partner, e.g. fluoride salts. Accuracy of the biosensor is assured if it is recalibrated prior to use.

[0065] The present invention also provides kits. Such kits will contain a container means which contains the instant enzyme. The container means may be any suitable container, but will typically be a glass vial or jar, a plastic pack, etc. In one embodiment, the container means may be a foil or plastic pouch which contains the enzyme immobilized on a microtitre plate or a chip. For example, a method of immobilization may include, but is not limited to, the method as described in Taylor et al. (U.S. Pat. No. 5,192,507). In other embodiments, the container means may be a plastic, glass, or metal tube which contains the enzyme, and the tube may possess an inlet means at one end and an outlet means at the other end; this type of container means may be used as a column in a flow biosensor and may itself be contained in a second container means.

[0066] The kit may further comprise a negative control sample. Such a negative control sample will contain either no cognate partner or a very low amount of cognate partner. The kit may also comprise a positive control sample, which will comprise, typically, an amount of cognate partner which is equal to or greater than the amount of cognate partner which is considered a positive result. The kit may also contain chemicals, such as buffers or diluents, and sample handling means, such as pipettes, reaction vials, vessels, tubes, or filters.

[0067] In addition, the kit may comprise written instructions on a separate paper, or any of the container means, or any other packaging. These instructions will usually set forth the conditions for carrying out the detection method, such as mixing ratios, amounts, incubation times, etc., and criteria for evaluating the results of the method, including spectra charts.

[0068] The following examples are intended to illustrate but not limit the invention.

EXAMPLES

[0069] The following examples are to exemplify certain embodiments of the invention. Those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

[0070] Materials and Methods

[0071] Inhibitors and Substrates-Acetylthiocholine iodide, 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), dithiothreitol, tacrine (9-amino-1,2,3,4-tetrahydroacridine hydrochloride hydrate), BW284c51, decamethonium, and edrophonium were purchased from Sigma. m-(N,N,N-trimethylammonio)trifluoromethylacetophenone (TFK^+) and (−)huperzine A were purchased from Calbiochem. Acrylodan was obtained from Molecular Probes (Eugene, Oreg.). Fasciculin 2 (purified from the venom of *Dendroaspis*

angusticeps) was a gift of Dr. Pascale Marchot (University of Marseille, France). Drs. Yacov Ashani and Bhupendra P. Doctor (Walter Reed Army Research Center, Washington, D.C.) kindly provided 7-((methylethoxy) phosphinyl)-oxyl)-1-methylquinolinium iodide (MEPQ) and procainamide-linked Sepharose CL-4B resin. m-tert-Butyl trifluoromethylacetophenone (TFK^+) was synthesized as described (21) and kindly provided by Dr. Daniel Quinn, University of Iowa, Iowa City, Iowa. All other chemicals were of the highest grade commercially available.

[0072] Expression, Mutagenesis, and Purification of mAChE-Mouse AChE was produced by transfection of an expression plasmid (pCDNA3, Invitrogen, San Diego, Calif.) containing an encoding cDNA where the AChE sequence was terminated at position 548. The plasmid was transfected into HEK293 cells. Cells were selected with G418 to obtain stable producing cell lines, and AChE was expressed as a secreted soluble enzyme in serum-free media (20). Mutant enzymes were generated by standard mutagenesis procedures, and cassettes containing the mutation were subcloned into pCDNA3 (20). Nucleotide sequences of the cassettes were confirmed by double-stranded sequencing to ensure that spurious mutations were not introduced into the coding sequence. Affinity chromatography using (m-aminophenyl)trimethylammonium linked through a long chain to Sepharose CL-4B resin (Sigma) permitted one-step purification of AChE. From 4 to 6 liters of media, mutant and wild type enzyme were purified in quantities ranging between 5 and 25 mg, as described previously (22-24). Purity was ascertained by SDS-PAGE and by measurements of specific activity.

[0073] Assay of Catalytic Activity—The spectrophotometric method of Ellman was used (25), and kinetic constants for acetylthiocholine hydrolysis were determined by fitting the observed rates as described (26). Titration of active sites with known concentrations of the irreversible phosphorylating agent, MEPQ, was accomplished by the method of Levy and Ashani (27).

[0074] Acrylodan Labeling—Mutant enzymes were pretreated with 0.25 mM dithiothreitol for 30 min at room temperature to ensure reduction of the introduced cysteine. Excess dithiothreitol was removed by use of a G-50 Sephadex spin column (Roche Molecular Biochemicals) equilibrated in 10 mM Tris, 100 mM NaCl, 40 mM $MgCl_2$, pH 8.0. A volume of 1 μl of acrylodan at 100 times the enzyme concentration was slowly mixed with the enzyme to achieve a ~5-fold molar excess of acrylodan to mutant enzyme. Labeling was allowed to proceed for at least 12 h at 4° C., and unreacted acrylodan was removed by size exclusion chromatography using Sephadex G-25 (Amersham Pharmacia Biotech) in 0.1 M sodium phosphate buffer, pH 7. Concentrations of acrylodan-labeled enzyme were determined from the maximal absorbance found between 360 and 380 nm (ϵ~16,400 M^{-1} cm^{-1}). Stoichiometry of labeling of the various preparations, estimated from a comparison of enzyme concentration by protein (280 nm) to acrylodan (360-380 nm) absorbance, ranged as follows: L76C, 0.7-0.8; E81C, 0.79-1.0; E84C, 0.77-1.0; Y124C, 0.79-1.0; A262C, 0.69-0.85; and H287C, 0.82-0.88. Specificity of labeling was assessed by comparison of areas under the fluorescence emission curves for acrylodan-treated mutant and wild type enzymes. Specific labeling for each mutant was as follows; L76C, 70-85%; E81C, 81-91%; E84C, 85-93%; Y124C, 83-90%, A262C, 80-90%; H287C, 70-76%.

[0075] Trifluoroacetophenone Inhibition—Picomolar amounts of enzyme in 0.01% bovine serum albumin in 0.1 M sodium phosphate buffer, pH 7.0, were reacted with TFK^+ in the absence of substrate. Inhibition was monitored by measuring residual enzyme activity by removal of aliquots during the course of the reaction. Bimolecular rate constants of inhibition were determined by nonlinear fit of the data (28).

$$\Delta F=\Delta F_{max}(E_t+I_t+K_d-\sqrt{(E_t+I_t+K_d)^2-4\,E_tI_t})(2E_t)^{-1} \quad \text{(Eq. 1)}$$

[0076] ΔF and ΔF_{max} are the change and maximum change in fluorescence, respectively; E_1 is the total enzyme concentration, and I_1 is the total inhibitor concentration. Association of TFK^+ with acrylodan-labeled E81C and E84C was assessed from the kinetics of decrease in fluorescence at 470 and 477 nm respectively, following addition of a stoichiometric excess TFK^+ at several concentrations. Data were fitted to a single exponential approach to equilibrium.

[0077] Association and dissociation rate constants of edrophonium and BW286c51 with E81C and E84C AChEs were determined from changes in the tryptophan fluorescence using a stopped-flow spectrophotometer as described previously (29). Time-dependent decreases in tryptophan fluorescence were observed upon excitation at 276 nm by means of a 305 nm emission cut-off filter.

EXAMPLE 1

Characteristics of Substrate Hydrolysis and Fasciculin 2 Inhibition

[0078] Crystallographic Structures and Solution Dynamics of the Acetylcholinesterase Complex—In the several crystal structures of AChE with conjugated or reversible bound ligands that have been studied, little evidence for change in enzyme conformation has been detected with a difference of less than a root mean square of 2 Å for the α-carbon backbone between the apoenzyme and the various complexes (4-6, 13, 14, 33-35, 44). Changes in side chain orientation occur most notably in the phenyl ring at position 337 for certain reversible complexes (34) and phenylalanine 297 when bulky organophosphates are conjugated to the active site serine (44). However, based on the multiple positions of the outer trimethylammonio moiety in decamethonium for mouse (6) and Torpedo crystal structures (34), some flexibility may exist particularly within the gorge itself. Brownian dynamics often require lining the radii of the attacking ligands or the residues lining the gorge in order to simulate the kinetics of diffusion-limited substrate access observed experimentally (45). Thus, all of crystal structures reported to date reveal a closed gorge with constrained dimension. The solution-based fluorescence studies reported herein provide the first physical evidence for localizing the ligand-induced conformational change to the Cys^{69}-Cys^{96} Ω loop. This finding raises an interesting possibility that the unliganded enzyme exists in a rapidly converting conformational equilibrium between open and closed states, and both ligand binding and conditions of crystallization favor formation of a closed gorge state. In fact, analysis of the molecule dynamics of a solvated mouse AChE shows fluctuations yielding an average widening of the motions of the gorge, which may also be integral to the catalytic cycle of transacylation and deacylation during ester hydrolysis.

[0079] The cysteine-substituted enzymes show kinetics of acetylthiocholine hydrolysis similar to wild type enzyme (Table I and **FIG. 2**) suggesting that all mutant enzymes fold correctly despite the presence of the newly introduced cysteine. The K_m value of E84C shows slightly less than a 4-fold increase, whereas the change in turnover rate, k_{cat}, is

minimal. Similar changes in kinetic constants were observed previously for E84Q mAChE (28). Since K_m, in the diffusion-limited catalysis, depicts the initial encounter between substrate and enzyme, an increase in K_m likely arises from the reduction of negative charge that electrostatically steers the cationic substrate into the active center gorge. Interestingly, a similar E81C mutation has little or no effect on substrate hydrolysis. Not all negatively charged residues around the active center appear to be involved equivalently in electrostatic steering.

[**0080**] Association and dissociation rates of fasciculin with A262C, H287C, and Y124C mutant enzymes were also found to be close to the rates of wild type enzyme (20). Fasciculin, at low concentrations, is also capable of associating with the mutant enzymes after acrylodan conjugation (**FIG. 2**). In addition, enzyme activity measurements of fasciculin-bound acrylodan conjugates show greater that 99% inhibition.

EXAMPLE 2

Influence of Residue Modification of Inhibition by m-Trimethylammoniotrifluoromethylacetophenone

[**0081**] TFK^+ binding to cysteine-substituted enzymes, both free and modified with acrylodan, was also examined (Table II). For E81C and E84C, the association rate constants (k_{on}) for TFK^+ were obtained from measurements of enzyme activity. Although positions 81 and 84 are both spatially removed from the TFK^+ binding site, k_{on} for E84C is slightly slower than that for wild type enzyme. By contrast, E81C shows no difference in the kinetic constants. Conjugation of acrylodan, a neutral naphthalene derivative, with E84C reduces k_{on} of TFK^+ 7-fold compared with unconjugated E84C, whereas conjugation of E81C with acrylodan only reduces k_{on} of TFK^+ slightly. For acrylodan-labeled mutants, k_{on} was measured from the time-dependent decrease of fluorescence signal (**FIG. 3**).

[**0082**] Influence of Residue Modification of Ligand Binding—The changes in emission spectra of acrylodan-labeled Ω loop residues 81 and 84 have been exploited to monitor ligand binding (Table II). Cysteine substitution and acrylodan conjugation at position 84, but not at position 81, affect ligand binding kinetics. Cysteine substitution at position 84 has little influence on catalytic parameters derived from steady-state catalysis (Table I).

TABLE I

Constants for acetylthiocholine hydrolysis by wild type and mutant mouse AChEs.*

Enzyme	K_m (μM)	K_{SS} (mM)	b	k_{cat} (10^5/min)	k_{cat}/K_m (10^9/M-min)
WT#	54 ± 16	14 ± 5	0.2 ± 0.07	1.6 ± 0.4	3.0
Y124C#	65 ± 17	20 ± 14	0.2 ± 0.09	1.4 ± 0.3	2.2
H287C#	58 ± 7	12 ± 6	0.2 ± 0.06	1.8 ± 0.2	3.1
A262C#	59 ± 4	11 ± 3	0.2 ± 0.04	1.6 ± 0.1	2.7
L76C	97 ± 19	17 ± 1	0.2 ± 0.03	1.8 ± 0.1	1.9
E81C	57 ± 6	11 ± 1	0.2 ± 0.03	1.6 ± 0.1	2.9
E84C	190 ± 9	26 ± 2	0.2 ± 0.05	1.9 ± 0.4	1.0

*Data shown as means ± S.D. typically from three measurements. Data were fit to the Equation $v = (1 + b[S]/K_{SS})/V_{max}/(1 + [S]/K_{SS})(1 + K_m/[S])$, where [S] is substrate concentration, K_{SS} is the substrate inhibition or activation constant, and b is the relative catalytic turnover of the ternary complex (12).

Data are from Boyd et al., J Biol Chem (2000) 275: 22401–22408.

[**0083**] The K_m of E84C increases less than 4-fold compared with the wild type enzyme. By contrast, a similar substitution at position 81 has no effect of ATCh steady-state catalysis. Precise quantitation of these catalytic parameters for the acrylodan-conjugated enzyme is complicated by incomplete modification by acrylodan. However inhibitor association can be measured using the change in fluorescence signal (Table II).

TABLE II

Kinetic and equilibrium constants for reaction of enzymes with TFK+, edrophonium, and BW284c51 in the presence of fluorescent (acrylodan) cysteine labeling compound.*

	TFK+		Edrophonium		BW284c51	
Enzyme	k_{on} $10^9 M^{-1}min^{-1}$	$\frac{k_{on}\ WT}{k_{on}\ mutant}$	K_d nM	$\frac{K_d\ mutant}{K_d\ WT}$	K_d nM	$\frac{K_d\ mutant}{K_d\ WT}$
Wild Type	150		250[a]		2.0[a]	
E81C	150	1	260[b]	1	2.6[b]	1.3
E81C-acrylodan	94	1.6	640	2.6	6.9	3.5
E84C	93	1.6	550[b]	2.2	35[b]	18
E84C-acrylodan	13	11	6300	25	130	65

*Data are shown as means from two to three measurements. Individual determinations are within 35% of the mean. Rates for TFK^+ are calculated based on ratios of the hydrated and unhydrated ketone (21).

[a]Data are from Radic et al. J Biol Chem (2001) 276: 4622–4633.

[b]Equilibrium dissociation constants are derived from the ratio of k_{off}/k_{on} using stopped-flow measurement of tryptophan quenching.

[0084] Reductions in binding kinetics were observed for several ligands (Table II) ranging between 1 or 2 orders of magnitude at position 84 but very little change at position 81. Although a portion of the reduction at position 84 is due to the cysteine substitution, acrylodan conjugation has a small, but significant (3-10-fold), influence on ligand binding. Even though both the 81 and 84 residues reside on the enzyme surface removed from the active center gorge, modification only at position 84 appreciably affects the energetics of ligand binding. The acrylodan moiety, whose dimension is slightly larger than the indole moiety of tryptophan, may impart steric restrictions to the region around the 84 site contributing to the energy cost in ligand binding. A small alteration in ligand binding energy (1.5-3.0 kcal/mol) is not unexpected if the conformation of the Ω loop plays a role in ligand binding.

[0085] Velan et al. (18) examined steady-state kinetics for a large number of Ω loop substitutions and truncations. Modification of Glu^{84} and its neighboring residues was found to have limited effect on steady-state kinetics. Faerman et al. (19) inserted a cysteine at position 82 to pair with a second cysteine residing proximally in the body of the enzyme. Although it could not be firmly established that a disulfide bond formed, little change in kinetic parameters (K_m and k_{cat}) was observed. Because of compensating contributions of the component primary constants, it is often difficult to correlate changes in steady-state kinetic parameters with structural perturbations. Our site-directed fluorophore labeling provides a physical assessment of the localized conformational change in the Ω loop. In cases where the fluorophore makes direct contact with the ligand, as the acrylodan-labeled Y124C and H287C with fasciculin, the energetic perturbations from substitution are larger, since complementarity of the binding site may be altered through the insertion of acrylodan side chain at the interface between the ligand and its binding site (20).

[0086] Acrylodan Modification at a Site Distal to the Active Center Core—The A262C modification was selected as a positional reference for a site distal to the active center. This residue is also located at the tip of the disulfide loop but is located ~30 Å away from the rim of the active center gorge. Crystallographic studies show this region to have a high temperature coefficient (B factor), indicative of substantial molecular motion of this surface residue. In fact, the position of this residue and its immediate neighbors is the only secured in crystal forms where proximity of the symmetry-related AChE molecule limits its movement in the crystal structure (6).

[0087] Acrylodan substitutions at this position show a long wavelength emission (λ_{max}=517 nm) indicative of exposure to a hydrophilic environment (Table III).

TABLE III

Fluorescence emission parameters of mouse AChE mutants labeled with acrylodan in the presence of fasciculin.*

Enzyme	Acrylodan Emission Maxima (nm) No Fasciculin	Saturating Fasciculin	Chromic Shift (nm)	Relative Quantum Yield
L76C	505	505	0	1.40
E81C	489	510	21	1.16
E84C	477	512	35	0.47

TABLE III-continued

Fluorescence emission parameters of mouse AChE mutants labeled with acrylodan in the presence of fasciculin.*

Enzyme	Acrylodan Emission Maxima (nm) No Fasciculin	Saturating Fasciculin	Chromic Shift (nm)	Relative Quantum Yield
[a]Y124C	500	477	−23	1.78
[a]A262C	517	517	0	0.97
[a]H287C	524	507	−17	5.0

*Data are shown as mean values of at least three determinations. Relative quantum yields were determined by comparison of areas of the fluorescence emission curves.

[a]Data are from Boyd et al., J Biol Chem (2000) 275: 22401–22408.

[0088] Moreover, none of the ligands studied, whether they are covalently attached to the active center (TFK or alkylphosphates), reversibly bound to the active center (edrophonium), span between the active center and peripheral site (decamethonium and BW286c51), or bind only to peripheral site (fasciculin), affect the spectroscopic properties of acrylodan conjugated at site 262 (Tables III-VI).

TABLE IV

Fluorescence emission parameters of mouse AChE mutants labeled with acrylodan in the presence of covalent active site inhibitors.*

Enzyme	Acrylodan Emission Maxima (nm) Conjugated TFK[o]	Chromic Shift (nm)	Relative Quantum Yield
L76C	509	4	0.87
E81C	510	21	0.89
E84C	507	30	0.59
Y124C	478	−22	1.15
A262C	517	0	0.97
H287C	524	0	0.90

Enzyme	Acrylodan Emission Maxima (nm) Conjugated TFK+	Chromic Shift (nm)	Relative Quantum Yield
L76C	511	6	0.92
E81C	510	21	0.89
E84C	512	35	0.52
Y124C	503	3	0.70
A262C	517	0	0.97
H287C	524	0	1.07

Enzyme	Acrylodan Emission Maxima (nm) Conjugated DDVP	Chromic Shift (nm)	Relative Quantum Yield
L76C	503	−2	1.27
E81C	510	21	0.19
E84C	496	19	0.39
Y124C	496	−4	1.27
A262C	517	0	0.97
H287C	524	0	1.03

*Data are shown as mean values of at least three determinations. Relative quantum yields were determined by comparison of the areas of the fluorescence emission curves. Data for the unconjugated enzymes are found in Table III.

[0089]

TABLE V

Fluorescence emission parameters of acrylodan-labeled mouse AChE mutants in the presence of reversible active site inhibitors.*

Enzyme	Acrylodan Emission Maxima (nm) Saturating Edrophonium	Chromic Shift (nm)	Relative Quantum Yield
L76C	509	4	0.92
E81C	510	21	0.91
E84C	510	33	0.60
Y124C	500	0	0.79
A262C	517	0	0.97
H287C	524	0	1.13

Enzyme	Acrylodan Emission Maxima (nm) Saturating Huperzine A	Chromic Shift (nm)	Relative Quantum Yield
L76C	511	6	0.86
E81C	510	21	0.88
E84C	510	33	0.55
Y124C	500	0	0.63
A262C	517	0	0.97
H287C	524	0	1.13

Enzyme	Acrylodan Emission Maxima (nm) Saturating Tacrine	Chromic Shift (nm)	Relative Quantum Yield
L76C	509	4	0.87
E81C	510	21	0.91
E84C	510	33	0.45
Y124C	497	−3	0.51
A262C	517	0	0.97
H287C	524	0	1.13

*Data are shown as mean values of at least three determinations. Relative quantum yields were determined by comparison of the areas of the fluorescence emission curves. Data for the unliganded enzymes are found in Table III.

[0090]

TABLE VI

Fluorescence emission parameters of acrylodan-labeled mouse AChE mutants in the presence of bisquaternary ligands.*

Enzyme	Acrylodan Emission Maxima (nm) Saturating BW284c51	Chromic Shift (nm)	Relative Quantum Yield
L76C	508	3	1.13
E81C	510	21	0.98
E84C	512	35	0.47
Y124C	487	−13	1.05
A262C	517	0	0.97
H287C	510	−14	2.73

Enzyme	Acrylodan Emission Maxima (nm) Saturating Decamethonium	Chromic Shift (nm)	Relative Quantum Yield
L76C	508	3	1.05
E81C	510	21	0.94
E84C	505	28	0.59
Y124C	465	−35	1.85
A262C	517	0	0.97
H287C	517	−7	1.76

TABLE VI-continued

Fluorescence emission parameters of acrylodan-labeled mouse AChE mutants in the presence of bisquaternary ligands.*

*Data are shown as mean values of at least three determinations. Relative quantum yields were determined by comparison of the areas of the fluorescence emission curves. Data for the unliganded enzymes are found in Table III.

[0091] This pattern indicates a lack of global conformational change affecting residue environments in a disulfide loop well removed from the active center (**FIG. 1**).

[0092] Residues Residing on the Active Center Gorge in Apposition with the Ω Loop—Residues 124 and 287 lie in close proximity to the Ω loop with H287C at the rim of the gorge and Y124C, residing just below the rim in the gorge interior (**FIG. 1**). The crystal structure of the complex shows fasciculin to "cap" these residues, hypsochromic shifts of acrylodan upon fasciculin binding (20). None of the reversibly bound active center ligands (edrophonium, huperzine, and tacrine) induce a spectral shift at position 124 or 287. However, modest quenching is observed at position 124 upon binding of these active center ligands. The bisquaternary ligands, which should approach or come in close apposition with these residues, cause significant hypsochromic shifts. The large shift for decamethonium at position 124 may reflect the ability of the cluster of aromatic residues to collapse around the methylene chain of decamethonium enlodged within the active center gorge. Crystallographic studies show one quaternary ammonium of decamethonium to be consistently positioned in the vicinity of Trp^{84}; however, both the flexible side chain and the outermost quaternary group are found to assume multiple positions in the decamethonium-AChE complexes studied to date (6, 29).

[0093] Covalent inhibition of cationic trifluoroacetophenone (TFK^+) produces very little spectral shift of acrylodan at either position 124 or 287. This is consistent with the crystal structures where the trimethyl ammonio moiety of TFK^+ forms a cation −π interconnection with Trp^{86}, and the trifluoroacetophenone moiety forms a hemiketal bond with the active center serine 203 (33). However, the isosteric t-butyl congener (TFK^0) shifts the environment of residue 124 to that resembling a hydrophobic state. This difference suggests that the orientation of this hemiketal conjugate differs where the t-butyl group extends toward the gorge exit. TFK^0 inhibits the wild type enzyme 70-fold slower that TFK^+, presumably due to lack of cation-π interaction and slightly different ligand orientation (21). Alkyl phosphorylation with small alkyl groups also has little influence on the environment at position 124 (Table IV).

[0094] Ω Loop Substitutions—The residues modified, 76, 81 and 84, are all on the outer surface and do not form the inner gorge wall. Since residues 81 and 84 carry acidic side chains, they might be expected to show solvent exposure in the native enzyme and not be involved in the internal stabilization of the loop, as is evident in the crystal structure of the mouse enzyme (5, 6). In the absence of ligand, the spectra of the conjugated acrylodan moiety reveal different degrees of solvent exposure with the acrylodan at position

84 being the most protected in an hydrophobic environment, acrylodan at 81 being intermediate, and acrylodan at 76 being most exposed. Examination of the crystal structures of mouse enzyme revealed a surface cavity near the side chain of the 84 site (5, 6). The observed λ_{max} likely reflects acrylodan buried in this surface cavity when conjugated to the 84 site (**FIG. 1**.).

[**0095**] The presence of fasciculin causes a large bathochromic shift of acrylodan fluorescence at both the 81 and 84 positions, as well as an increase in quantum yield of acrylodan at 76. The lack of shift in emission seen in quantum yield of acrylodan at the 76 position may simply reflect a balance between small environmental changes at 76 upon ligand binding by fasciculin. In the case of Glu^{84}, the bathochromic shift likely reflects Arg^{11} of fasciculin loop I coming in van der Waals contact with the 84 side chain and displacing acrylodan into a more polar environment. However, an explanation of the bathochromic shift at position 81 requires a more involved analysis. Although 81 is removed from the fasciculin-binding site, fasciculin has a sufficient molecular dimension to restrict the Ω loop so that the entire loop freezes or closes upon fasciculin binding. Thus, fasciculin binding may confer strain on the α-carbon backbone structure of the Ω loop such that the acrylodan side chain at positions 81 and 84 becomes exposed to the hydrophilic environment. The fact that substitutions at both positions yielded acrylodan spectra with equivalent emission maxima after ligand binding suggests a conformational involvement of the entire loop.

EXAMPLE 3

Influence of Residue Modification on Inhibition by Noncovalent Active Site Inhibitors

[**0096**] A similar trend in inhibition kinetics was seen with noncovalent active site inhibitors such as edrophonium and BW286c51 (Table II). An increase over wild type K_d of 2-fold occurs for edrophonium binding to E84C, and an 18-fold increase in K_d is observed for BW286c51 binding. Similar increases in K_d of edrophonium and BW286c51 were seen E84Q human AChE (18). By comparison, E81C showed no alterations in ligand binding constants. For acrylodan-labeled mutants, K_d was measured from the fluorescence signals of an equilibrium titration (**FIG. 4**). Acrylodan-labeled E84C shows K_d increases of 10-fold for edrophonium and 3-fold for BW286c51 as compared to unreacted E84C. For acrylodan-labeled E81C, only a slight increase in K_d is seen for both ligands. The high concentration of acrylodan-labeled E81C required for equilibrium titrations precludes an accurate estimate of K_d for high affinity ligands such as BW286c51.

[**0097**] Similar to fasciculin, small ligands that bind to the active center produce a similar strain. All of the small ligands, whether reversibly bound or covalently attached, elicit marked changes in acrylodan emission with the largest spectral shift seen for E84C, an intermediate value seen for E81C, and only small change observed for L76C. In each case the conformational change induced by the ligand causes the acrylodan to move into a region of higher dielectric constant, presumably being more solvent-exposed. The pattern is remarkably consistent among the ligands, and only the small organophosphate when conjugated induces a shift of smaller magnitude. A likely explanation for the observed conformational changes is that ligand binding to the active center induces gorge closure, which is meditated throughout the Ω loop. The strain placed on the α-carbon backbone upon gorge closure causes the side chains to shift positions and become exposed to a hydrophilic environment.

[**0098**] DeFari et al. (43) has noted that the peripheral site inhibitor, thioflavin T, when bound to AChE, shows a large enhancement of fluorescence. Simultaneous binding of an active center ligand and thioflavin T partially quenches the enhanced fluorescence of bound thioflavin T. Radic and Taylor (29) have observed that bound active center ligands cause a partial quenching of the native tryptophan fluorescence in AChE. Since these ligands lack the spectral overlap for fluorescence resonance energy transfer, the bound ligand is likely to influence the connectivity between aromatic residues present in the gorge, thereby influencing fluorescence quantum yields. Taken together, these studies suggest that ligands induce conformational changes in AChE giving rise to a gorge conformation collapsed around the bound ligand. The site-directed cysteine mutagenesis and fluorescence labeling studies herein suggest the involvement of particular resides on the Ω loop in this conformational change.

EXAMPLE 4

Effect of Fasciculin on Acrylodan Fluorescence Emission

[**0099**] The peptide toxin, fasciculin, inhibits AChE by tightly capping the mouth of the active center gorge (**FIG. 1**) (11, 30-32). Table III shows changes in emission maxima of acrylodan-labeled AChE mutants in the presence of fasciculin. There is no discernible change in fluorescence emission of acrylodan-conjugated A262C (20), consistent with the position 262 being distal to the fasciculin-binding site. The large hypsochromic shifts seen at both the 124 and 287 positions reflect solvent exclusions and an increase in hydrophobicity experienced by the fluorophores in the gorge upon fasciculin binding (20). For the Ω loop mutant, L76C, fasciculin binding produces a 40% increase in quantum yield but no change in emission maximum. Bathochromic shifts are found at both the 81 and 84 positions, with position 84 producing a shift of larger magnitude (**FIG. 2** and Table III).

EXAMPLE 5

Effect of Covalently Conjugated Active Site Inhibitors on Acrylodan Fluorescence Emission

[**0100**] Changes in emission maxima of acrylodan-labeled AChE mutants in the presence of conjugating trifluoroacetophenones are shown in Table IV. The trifluoroacetophenones inhibit the enzyme by conjugating to form a hemiketal at the active site serine without dissociation of leaving group (33). Both the isosteric neutral and cationic trifluoroketones (TFK° and TFK^{+}) produced no discernible changes in emission spectra of acrylodan conjugated at H287C and A262C, consistent with a fluorophore position distant from gorge base and hence not in direct contact with ligand. Remarkably, both TFK° and TFK^{+} produce a substantial bathochromic shift (at least 30 nm) with acrylodan-E84C. The trifluoroketones also produce a spectral shift of intermediate

value (20 nm) for E81C and a much smaller change (4-6 nm) for L76C. Interestingly, neutral TFK° produces a large 22 nm of hypsochromic shift with the Y124C acrylodan conjugate.

[0101] O,O-Dimethyl-O-(2,2-dichlorovinyl)phosphate, a smaller achiral organophosphonate, phosphorylates the active site serine of mAChE, with subsequent departure of the dichlorovinyloxy group (34, 35). The small and symmetrical dimethyl phosphoryl conjugate remaining at the active site serine might lead one to suspect very little perturbation, if any at all, in fluorescence spectra. Indeed, acrylodan conjugated at positions 124, 262, and 287 showed very little or no change in spectrum. However, bathochromic shifts at positions 81 and 84 were observed, although of smaller magnitude for E84C when compared with other ligands (Table IV).

EXAMPLE 6

Effect of Noncovalent Active Site Inhibitors on Acrylodan Fluorescence Emission

[0102] Noncovalent active site inhibitors, such as edrophonium, tacrine, and huperzine, associate primarily with the choline subsite at the base of the active site gorge. Crystal structures of inhibitors bound to *Torpedo californica* AChE revealed that these ligands should have no direct contact with the conjugated fluorophore at all six cysteine-substituted sites (36, 37). Upon edrophonium, tacrine, or huperzine association, alteration of acrylodan emission maxima is undetectable for positions 124, 287, and 262 (Table V). However, as seen for other ligands, acrylodan conjugated at E84C surprisingly shows a bathochromic shift of 33 nm (from 477 to 510 nm) upon inhibitor binding. A change of smaller magnitude is seen in the case of acrylodan-L76C (from 505 to 509 nm) and acrylodan-E81C (from 480 to 510 nm) with noncovalent active site inhibitors. Ligand binding results in a common emission maximum (λ_{max}~510 nm) for acrylodan at the three Ω loop positions.

EXAMPLE 7

Effect of Bisquaternary Inhibitors on Acrylodan Emission Spectrum

[0103] Extended bisquaternary inhibitors, such as BW286c51 and decamethonium, belong to a class of inhibitors that interact with two binding sites of AChE simultaneously (32, 38-39). The quaternary ammonium moiety on one end of the molecule associates with the Trp^{86} residue that characterizes the choline-binding site, whereas the other end resides near Trp^{286} at the active site gorge rim. Table VI shows changes in emission maxima of acrylodan-labeled AChE mutants in the presence of bisquaternary inhibitors. No changes are observed at position 262. By contrast, both decamethonium and BW284c51 caused a pronounced hypsochromic shift and increase in quantum yields with acrylodan conjugated at Y124C and H287C. Addition of decamethonium produced a hypsochromic shift of 35 nm at position 124, and a modest 7 nm shift at position 287. BW284c51 has a similar effect; for the Ω loop mutants, L76C, E81C, and E84C, bathochromic shifts of similar magnitude to the monoquaternary ligands were observed (Tables V and VI).

EXAMPLE 8

Determination of the Presence of Potential Gaseous Cognate Partners in the Field Using a Portable Detection Device Containing Acrylodan-Labeled Mouse Acetylcholinesterase (Accession No. IMAAA)

[0104] A chip is configured to comprise multiple compartments containing various forms of labeled, immobilized acetylcholinesterase (AChE). Acrylodan-labeled mouse AChE is generated by the method as outlined in Shi et al. (J Biol Chem (2001) 276(45):42196-42204 and see above in Materials and Methods)). Alternatively, the mouse AChE is mutagenized by the method as outlined in U.S. Pat. No. 6,001,625 and labeled as outlined in Shi et al. (2001).

[0105] Picomolar amounts of labeled AChE in 0.01% bovine serum albumin and 0.01 M phosphate buffer (pH 7.0) are immobilized on a solid phase by the method as described in U.S. Pat. No. 6,406,876. The immobilized enzyme is then lyophilized as described in U.S. Pat. No. 5,354,654 and sealed under a gas permeable membrane. Prior to use, the enzyme is reconstituted by rehydration with an appropriate aqueous buffer. Alternatively, the enzyme is immobilized directly on a gas permeable resin and affixed to the chip surface (see, e.g., U.S. Pat. No. 4,619,897). In the latter system, the appropriate buffer system is added just prior to exposure to the deployment area.

[0106] The chip is configured for both standard, right-angled fluorescence detection or for epifluoresence (i.e., comprising windows for incident and emission of excitation wavelengths at 90° relative to the incident [excitation] light source and/or detection of emitted wavelengths at 180°).

[0107] The forms of immobilized AChE are as follows: acrylodan-labeled unconjugated AChE (comprising both single-site and multiple-site labeled enzymes, labeled at residues Leu^{76}, Glu^{81}, Glu^{84}, Tyr^{124}, Ala^{262} and/or His^{287}), acrylodan-labeled conjugated AChE (comprising the labels as above and further comprising various organophosphates such as sarin, phosphine, soman, or tabun as positive controls), and unlabeled forms of both conjugated and unconjugated AChE.

[0108] The chip is deployed in an area suspected of containing one or more airborne organophosphate agents. The chip remains in the area for a sufficient time (under ambient temperature conditions) to allow for diffusion of the surrounding gases across the gas permeable membrane and to contact/interact with the immobilized AChE.

[0109] The chip is then removed from the area and placed in a device that can detect fluorescence (e.g., spectrofluorimeter). The fluorescence detected in compartments containing newly formed conjugates is compared with the fluorescence detected in the corresponding controls. Estimation of ratios of unconjugated to conjugated forms of the AChE is measured. Subsequent determination of an accumulation of conjugated AChE demonstrates the presence of one or more organophosphate agents in the deployment area.

[0110] All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of illustrative embodiments, it will be

apparent to those of skill in the art that variations may be applied to the composition, methods and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. Although the invention has been described with reference to the above examples, it will be understood that modifications and variations are encompassed within the spirit and scope of the invention.

REFERENCES

[0111] 1. Cygler et al., Protein Sci (1993) 2:366-382.

[0112] 2. Rosenberry T. L., Adv Enzymol Relat Areas Mol Biol (1975) 43-:103-218.

[0113] 3. Quinn, D. M., Chem Rev (1997) 87:955-979.

[0114] 4. Sussman et al., Science (1991) 253:872-879.

[0115] 5. Bourne et al., Cell (1995) 83:503-512.

[0116] 6. Bourne et al., J Biol Chem (1999) 274:2963-2970.

[0117] 7. Ripoll et al., Proc Natl Acad Sci USA (1993) 90:5128-5132.

[0118] 8. Tan et al., Biochemistry (1993) 32:401-403.

[0119] 9. Wlodek et al., Biopolymers (2000) 53:265-27.

[0120] 10. Shou et al., Proc Natl Acad Sci USA (1998) 95:9280-9283.

[0121] 11. Eastman et al., J Biol Chem (1995) 270:19694-20399.

[0122] 12. Radic et al., J Biol Chem (1995) 270:20391-20399.

[0123] 13. Harel et al., Structure (1995) 3:1355-1366.

[0124] 14. Kryger et al., Acta Crystallogr Sec D Biol Crystallogr (2000) 56:1385-1394.

[0125] 15. Schrag et al., J Mol Biol (1993) 230:575-591.

[0126] 16. Grochulski et al., J Biol Chem (1993) 268:72843-72847.

[0127] 17. Grochulski et al., Protein Sci (1993) 3:82-91.

[0128] 18. Velan et al., FEBS Lett (1996) 395:22-28.

[0129] 19. Faerman et al., J Biol Chem (2000) 275:22401-22408.

[0130] 20. Boyd et al., J Biol Chem (2000) 275:22401-22408.

[0131] 21. Nair et al., Biochemistry (1994) 33:8566-8576.

[0132] 22. Marchot et al., Protein Sci (1996) 5:672-679.

[0133] 23. Berman et al., Proc Natl Acad Sci USA (1971) 68:395-398.

[0134] 24. De la Hoz et al., Life Sci (1986) 39:195-199.

[0135] 25. Ellman et al., Biochem Pharmacol (1961) 7:88-95.

[0136] 26. Radic et al., Biochemisrty (1993) 32:12074-12084.

[0137] 27. Levy et al., Biochem Pharmacol (1986) 35:1079-1085.

[0138] 28. Radic et al., J Biol Chem (1997) 272:23265-23277.

[0139] 29. Radic et al., J Biol Chem (2001) 276:4622-4633.

[0140] 30. Redic et al., J Biol Chem (1994) 296:11233-11239.

[0141] 31. Taylor et al., Annu Rev Pharmacol Toxicol (1994) 34:281-320.

[0142] 32. Marchot et al., J Biol Chem (1993) 268:12458-12467.

[0143] 33. Harel et al., J Am Chem Soc (1996) 118:2340-2346.

[0144] 34. Wilson, I. B. (1960) in The Enzymes (Boyer, P. D., Lardy. H., and Myrback, K. eds.), (1960) Vol. 4, 2nd Ed., pp. 501-520, Academic Press, New York.

[0145] 35. Wong et al., Biochemistry (2000) 39:5750-5757.

[0146] 36. Harel et al., Proc Natl Acad Sci USA (1993) 90:9031-9035.

[0147] 37. Raves et al., Nat Struct Biol (1997) 4:57-63.

[0148] 38. Taylor et al., Biochemistry (1975) 14:1989-1997.

[0149] 39. Taylor et al., Mol Pharmacol (1974) 10:93-107.

[0150] 40. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 185-210, Kluwer Academic Publishers and Plenum Publishing Corp., New York.

[0151] 41. Lew et al., J Biol Chem (1997) 272:1507-1513.

[0152] 42. Pendergast et al., J Biol Chem (1983) 258:7541-7544.

[0153] 43. De Ferrai et al., J Biol Chem (2001) 246:23282-23287.

[0154] 44. Millard et al., Biochemistry (1999) 38:7032-7039.

[0155] 45. Tara et al., Biopolymers (1998) 46:465-474.

[0156] 46. Tai et al., Biophys J (2001) 81:715-724.

What is claimed is:

1. An in vitro method for identifying an acetylcholinesterase (AChE) cognate partner comprising;

a) contacting a sample suspected of containing a cognate partner with a labeled AChE and

b) measuring accumulation of a conjugate between the AChE and the partner,

wherein detection of the conjugate is indicative of the presence of the cognate partner.

2. The method of claim 1, wherein the cognate partner is selected from the group consisting of a ligand, a modulator and an inhibitor of AChE.

3. The method of claim 2, wherein the inhibitor is a carbamylating inhibitor or a phosphorylating inhibitor.

4. The method of claim 3, wherein the inhibitor is a nerve toxin.

5. The method of claim 4, wherein the inhibitor is selected from the group consisting of insecticides and organophosphates.

6. The method of claim 1, wherein the AChE is labeled with a fluorophore.

7. The method of claim 6, wherein AChE comprises at least one fluorophore.

8. The method of claim 7, wherein at least one label is peripheral to the active center of the AChE.

9. The method of claim 6, wherein the fluorophore shows a Stokes' shift upon binding of ligand, modulator or inhibitor to the labeled enzyme.

10. The method of claim 1, further comprising measuring the ratio of conjugated to unconjugated AChE.

11. The method of claim 10, wherein the measuring is by chromatography or spectroscopy.

12. The method of claim 11, wherein chromatography comprises separation of conjugated and unconjugated AChE by capillary electrophoresis.

13. The method of claim 11, wherein spectroscopy comprises detecting conjugated and unconjugated AChE by fluorescence.

14. The method of claim 6, wherein the fluorophore is selected a dimethoxyphosphoryl compound or a diethoxyphosphoryl compound.

15. A device for measuring accumulation of a conjugate between acetylcholinesterase (AChE) and a cognate partner comprising labeled AChE, wherein at least one site of the AChE is labeled peripheral to the active site of the enzyme.

16. The device of claim 15, wherein the cognate partner is selected from the group consisting of a ligand, a modulator and an inhibitor of AChE.

17. The device of claim 15, wherein the label is a fluorophore.

18. The device of claim 17, wherein the fluorophore shows a Stokes' shift upon binding of a ligand, modulator or inhibitor to AChE.

19. The device of claim 15, wherein the AChE is compartmentalized in a mobile or stationary phase.

20. The device of claim 19, wherein the mobile phase is a suspension.

21. The device of claim 19, wherein the stationary phase is a chip.

22. The device of claim 21, wherein the AChE is covalently or non-covalently immobilized on the stationary phase.

23. The device of claim 15, wherein the AChE can detect multiple AChE cognate partners.

24. The device of claim 15, wherein the cognate partner is a nerve toxin.

25. The device of claim 24, wherein the nerve toxin is selected from the group consisting of insecticides and organophosphates.

26. A labeled acetylcholinesterase (AChE) molecule comprising at least one fluorophore present at the periphery of the active site of AChE, wherein the at least one site is selected from the group consisting of residues 76, 81, 84, 124, 262 and 287 of AChE or equivalents thereof.

27. The labeled AChE of claim 26, wherein the fluorophore shows a Stokes' shift upon binding of a ligand to the labeled enzyme.

28. The labeled AChE of claim 26, wherein the enzyme is recombinantly produced.

29. The labeled AChE of claim 28, modified by mutagenesis or evolution methods, wherein the modification comprises substitution of one or more selected amino acid residues with cysteine residues.

30. The labeled AChE of claim 26, wherein the fluorophore is a dimethoxyphosphoryl compound or a diethoxyphosphoryl compound.

31. The labeled AChE of claim 30, wherein the enzyme is conjugated to an inhibitor.

32. The labeled AChE of claim 31, wherein the conjugate is detectable by antibody.

33. A conjugate comprising a labeled acetylcholinesterase (AChE) molecule, an inhibitor of AChE and an antibody.

34. A method for evaluating the presence of an inhibitor of acetylcholinesterase (AChE), wherein the inhibitor is selected from the group consisting of carbamates and organophosphates, comprising the steps of:

a) providing a biological sample;

b) contacting the sample with a labeled AChE molecule comprising at least one fluorophore present at the periphery of the active site of the enzyme under conditions sufficient for binding of an inhibitor to the enzyme; and

c) measuring accumulation of a conjugate between the AChE and the inhibitor.

35. The method of claim 34, wherein the biological sample is selected from the group consisting of an integumentary system sample, sputum, feces, blood, urine, plasma, lacrimal secretions, cerumen, and semen.

36. The method of claim 35, wherein the inhibitor is a carbamylating inhibitor.

37. The method of claim 35, wherein the inhibitor is a phosphorylating inhibitor.

38. The method of claim 34, wherein the inhibitor is a nerve toxin.

39. The method of claim 34, wherein in the fluorophore shows a Stokes' shift upon binding of a ligand, modulator or inhibitor to the labeled enzyme.

40. The method of claim 34, further comprising measuring the ratio of conjugated to unconjugated AChE.

41. The method of claim 40, wherein the measuring is by chromatography or spectroscopy.

42. The method of claim 41, wherein chromatography comprises separation of conjugated and unconjugated AChE by capillary electrophoresis.

43. The method of claim 41, wherein spectroscopy comprises detecting conjugated and unconjugated AChE by fluorescence.

44. The method of claim 34, comprising a labeled AChE, wherein the fluorophore is a dimethoxyphosphoryl compound or a diethoxyphosphoryl compound.

45. A method for evaluating the presence of an inhibitor of acetylcholinesterase (AChE), wherein the inhibitor is selected from the group consisting of carbamates and organophosphates, comprising the steps of:

a) providing a non-biological sample;

b) contacting the sample with a labeled AChE molecule comprising at least one fluorophore present at the periphery of the active site of the enzyme under conditions sufficient for binding of an inhibitor to the enzyme; and

c) measuring accumulation of a conjugate between the AChE and the inhibitor.

46. The method of claim 45, wherein the non-biological sample is selected from the group consisting of soil, water, air and non-biological surfaces.

47. The method of claim 46, wherein the inhibitor is a carbamylating inhibitor.

48. The method of claim 46, wherein the inhibitor is a phosphorylating inhibitor.

49. The method of claim 45, wherein the inhibitor is a nerve toxin.

50. The method of claim 45, wherein in the fluorophore shows a Stokes' shift upon binding of an inhibitor to the labeled enzyme.

51. The method of claim 45, comprising measuring the ratio of conjugated to unconjugated AChE.

52. The method of claim 51, wherein the measuring is by chromatography or spectroscopy.

53. The method of claim 52, wherein chromatography comprises separation of conjugated and unconjugated AChE by capillary electrophoresis.

54. The method of claim 52, wherein the spectroscopy comprises detecting conjugated and unconjugated AChE by fluorescence.

55. The method of claim 45, wherein the fluorophore is a dimethoxyphosphoryl compound or a diethoxyphosphoryl compound.

56. A kit comprising:

a) a labeled acetylcholinesterase (AChE) molecule comprising at least one fluorophore present at the periphery of the active site of AChE and

b) a container comprising the labeled enzyme.

57. The kit of claim 56, wherein the container is selected from the group consisting of a glass vial, a glass jar, a plastic pack, a plastic tube, glass tube, a metal tube, a foil pouch and a plastic pouch.

58. The kit of claim 56, wherein the labeled AChE is immobilized on a microtitre plate or a chip.

59. The kit of claim 56, further comprising:

c) a control sample.

60. The kit of claim 59, wherein the control sample is a negative control sample.

61. The kit of claim 59, wherein the control sample is a positive control sample.

* * * * *

专利名称(译)	配体感应荧光乙酰胆碱酯酶用于检测有机磷酸酯活性		
公开(公告)号	US20050089926A1	公开(公告)日	2005-04-28
申请号	US10/469731	申请日	2003-04-16
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摘要(译)

公开了制备和使用标记的AChE和标记的AChE抑制性缀合物组合物的方法，用于检测有毒物质如有机磷酸酯，杀虫剂和其他神经毒剂的积累。还公开了在多个领域中使用标记的AChE和标记的AChE抑制性缀合物组合物的方法，包括在食品和水分析领域，环境监测领域和工业环境中检测生物样品中的有毒物质。

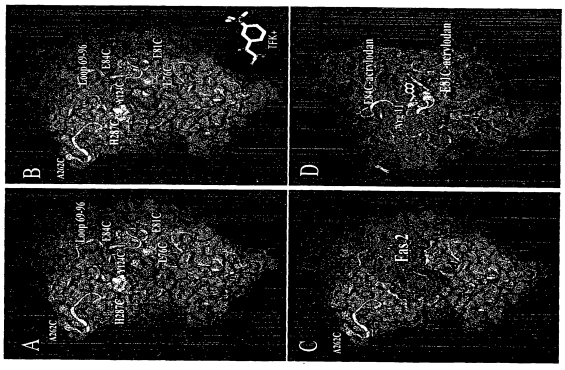

FIGURE 1