Natural Monomeric Form of Fetal Bovine Serum Acetylcholinesterase Lacks the C-Terminal Tetramerization Domain

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ABSTRACT: Acetylcholinesterase isolated from fetal bovine serum (FBS AChE) was previously characterized as a globular tetrameric form. Analysis of purified preparations of FBS AChE by gel permeation chromatography revealed the presence of a stable, catalytically active, monomeric form of this enzyme. The two forms could be distinguished from each other based on their molecular weight, hydrodynamic properties, kinetic properties, thermal stability, and the type of glycans they carry. No differences between the two forms were observed for the binding of classical inhibitors such as edrophonium and propidium or inhibitors that are current or potential drugs for the treatment of Alzheimer's disease such as (−) huperzine A and E2020; tacrine inhibited the monomeric form 2−3-fold more potently than the tetrameric form. Sequencing of peptides obtained from an in-gel tryptic digest of the monomer and tetramer by tandem mass spectrometry indicated that the tetramer consists of 583 amino acid residues corresponding to the mature form of the enzyme, whereas the monomer consists of 543−547 amino acid residues. The subunit molecular weight of the protein component of the monomer (major species) was determined to be 59 414 Da and that of the tetramer as 64 239 Da. The N-terminal of the monomer and the tetramer was Glu, suggesting that the monomer is not a result of truncation at the N-terminal. The only differences detected were at the C-terminus. The tetramer yielded the expected C-terminus, CSDL, whereas the C-terminus of the monomer yielded a mixture of peptides, of which LLSATDTLD was the most abundant. These results suggest that monomeric FBS AChE is trimmed at the C-terminus, and the results are consistent with the involvement of C-terminal amino acids in the assembly of monomers into tetramers.

Acetylcholinesterase (AChE; EC 3.1.1.7),[1] an essential component of cholinergic synapses, catalyzes the hydrolysis of acetylcholine at the postsynaptic junctions. This enzyme has been shown to exist in multiple molecular forms in a wide variety of mammalian tissues (*1*). The various molecular forms consist of monomers or oligomers of catalytic subunits that range in molecular weights of 70−80 kD. In addition to the catalytic subunits, the asymmetric (A) forms also contain noncatalytic structural subunits such as the collagenic Q subunits, which allows their association with the basal lamina (*2*). The AChE molecular forms that lack the collagen tail are termed globular (G) forms, which can be subdivided into amphiphilic and nonamphiphilic forms on the basis of their interaction with nonionic detergents (*3*). On the basis of their hydrodynamic properties, the globular forms of AChE can be identified as monomers G_1 (4.5 S), dimers G_2 (6−7 S), or tetramers G_4 (9−11 S).

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[1] Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; FBS, fetal bovine serum; AD, Alzheimer's disease; ATC, acetylthiocholine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); edrophonium, ethyl(*m*-hydroxyphenyl)dimethylammonium chloride; propidium, 3,8-diamino-5′-3′-(trimethylammonium)propyl-6-phenylphenanthridium iodide; 7-HQ, 7-hydroxy quinolinium; MEPQ, 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide; DEPQ, 7-(*O*,*O*-diethyl-phosphinyloxy)-1-methylquinolinium methyl sulfate; DTT, dithiothreitol; tacrine, 9-amino-1,2,3,4-tetrahydroacridine; E2020, (*R*,*S*)-1-benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methyl)piperidine hydrochloride; LC−MS, liquid chromatography−mass spectrometry; CAD, collision activated dissociation.

Globular forms represent the major fraction of AChE and butyrylcholinesterase (BChE) in most vertebrate tissues. For example, in bovine and human erythrocytes, AChE exists in a membrane-bound dimeric form (*4*−*7*), whereas rat erythrocytes contain monomeric AChE (*8*). Similarly, human and chicken serum contains soluble AChE and BChE in G_4, G_2, and G_1 forms (*9*, *10*), whereas rat blood contains BChE in G_4, G_2, and G_1 forms and AChE in the G_4 form only (*8*, *11*). The cerebrospinal fluid contains only soluble AChE in the G_4 form in man. The liver contains principally BChE (G_4 and G_1 forms in chicken; mostly G_1 in rat) and some AChE (G_4 and G_1 in chicken; G_4, G_2, and G_1 in rat) (*1*). A tetrameric membrane-bound G_4 form and a monomeric soluble G_1 form are the two predominant AChE species in mammalian brain (*12*). Several recent studies have examined the effect of aging and Alzheimer's disease (AD) on the distribution of AChE and BChE molecular forms in the brain. A significant age-related decline in the G_4 form of AChE was observed in the cerebral cortex, hippocampus, striatum, and hypothalamus and not in the medulla-pons or the cerebellum of rats (*13*). Inhibition studies with eight different AChE inhibitors indicated that there were differences in the sensitivity of these inhibitors toward the various molecular forms of AChE in rat brain (*14*). A comparison of the distribution of AChE molecular forms in the anatomical regions of post-mortem brains from patients with AD and nondemented age-matched controls demonstrated a selective loss of the G_4 form of AChE from several cortical areas of

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AD brains (*12*, *15*, *16*). Concomitant with a decrease in the G_4 form, a small increase in the G_1 form of AChE and BChE was observed in the most severely affected cases. The pattern for the distribution of AChE and BChE molecular forms and the kinetic properties of AChE resembled those of the developmental form of AChE present in human embryonic brain (*17*). In vitro studies with G_1 and G_4 forms of AChE isolated from post-mortem brain tissue from patients with AD demonstrated that although both forms were inhibited equally well by physostigmine and tacrine, differences in the inhibitory potency of some drugs such as heptylphysostigmine and SDZ ENA 713 were observed (*18*). Since different molecular forms of AChE may have specific physiological functions, an understanding of their biochemical and kinetic properties is imperative to clinical drug design.

To further understand the biochemical and kinetic properties of different molecular forms of AChE and elucidate the molecular basis for their existence, we isolated the monomeric form of fetal bovine serum (FBS) AChE and compared its catalytic properties with the tetrameric form that was characterized in our laboratory (*19*, *20*). The two forms could be distinguished from each other based on their molecular weight, hydrodynamic properties, kinetic properties, thermal stability, and the type of carbohydrate structures they carry. From the sequence analysis of peptides obtained from an in-gel tryptic digest of the monomer and tetramer by tandem mass spectrometry, the subunit molecular weights of the protein component for the major species were determined to be 59 414 and 64 239 Da, respectively. These results suggest that monomeric FBS AChE is truncated at the C-terminus and are consistent with the involvement of C-terminal amino acids in the assembly of monomers into tetramers.

EXPERIMENTAL PROCEDURES

Materials. Acetylthiocholine iodide (ATC), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), edrophonium, propidium, and tacrine were obtained from Sigma Chemical Co. (St. Louis, MO). DEPQ was provided by Drs. Yacov Ashani and Haim Leader (Israel Institute for Biological Research, Ness-Ziona, Israel). 7-Hydroxy quinolinium (7-HQ) was obtained by complete hydrolysis of 7-(methylethoxyphosphinyloxy)-1-methylquinolinium iodide (MEPQ), and its concentration was measured spectrophotometrically as described (*21*). (−) Huperzine A isolated from *Huperzia serrata* was purchased from Dr. Liu Jia-Sen (Alberta, Canada). (*R*,*S*)-1-Benzyl-4-[(5,6-dimethoxy-1-indanon)-2-yl]methyl)piperidine hydrochloride (E2020) obtained from Eisai Co., Tsukuba-shi, Ibaraki, Japan was a gift from Dr. Alan P. Kozikowski (Georgetown University, Washington, DC). Agarose bound lectins were obtained from Vector Laboratories (Burlingame, CA). Electrophoretically pure AChE from FBS was purified using the method of De La Hoz et al. (*22*).

Acetylcholinesterase Assay. AChE activity was measured by the Ellman method (*23*) in 50 mM sodium phosphate buffer, pH 8.0, at 25 °C, in the presence of 0.5 mM DTNB, using 0.5 mM ATC as the substrate.

Purification of Monomeric FBS AChE. The monomeric and tetrameric forms of FBS AChE were resolved by gel permeation chromatography of purified FBS AChE (*22*) on a Biogel A 1.5 m column (1.5 × 170 cm) equilibrated with 50 mM sodium phosphate, pH 8.0. The flow rate was 20 mL/h, and the fraction size was 1.2 mL. The elution positions of monomeric and tetrameric forms of FBS AChE were determined by an assay of AChE activity (*23*). Fractions containing AChE activity for the two forms were pooled separately and further purified by analytical procainamide affinity chromatography.

Sucrose Density Gradient Sedimentation Analysis of Monomeric and Tetrameric FBS AChE. Samples of monomeric or tetrameric forms of FBS AChE (10 U; 3 μg in 200 μL of 50 mM sodium phosphate, pH 8.0) were mixed with catalase (11.3S; used as a sedimentation marker) and applied to linear 5−20% sucrose gradients. The gradients were centrifuged at 75 000*g* for 18 h at 4 °C in an SW41Ti rotor (Beckman Instruments, Fullerton, CA). Gradients were fractionated from the top using an Auto Densiflow IIC (Buchler Instruments, Lenexa, KS), and fractions were assayed for AChE activity using the micro-Ellman assay (*24*).

Determination of Stokes Radii, Molecular Weights, and Frictional Ratios. The Stokes radii of native monomeric and tetrameric forms of FBS AChE were estimated from their elution positions on a calibrated Biogel A 1.5 m (1.5 × 170 cm) column. The proteins used for column calibration, thyroglobulin ($a = 85.0$ Å), catalase ($a = 52.2$ Å), aldolase ($a = 48.1$ Å), and bovine serum albumin ($a = 35.5$ Å) were purchased from Amersham Biosciences (Piscataway, NJ). The void and total volumes of the column were determined with blue dextran and potassium ferricyanide, respectively. From the elution position of standard proteins on this column, their K_{av} was calculated using the following equation:

$$K_{av} = (V_e - V_o)/(V_t - V_o)$$

where V_o and V_t are the void and total volumes, respectively, and V_e is the elution volume of the protein. The results of gel permeation experiments were plotted as described by Siegel and Monty (*25*) using the equation of Laurent and Killander (*26*). The molecular weights and frictional ratios for monomeric and tetrameric forms of FBS AChE were calculated using the following equations (*25*):

$$M = (6\rho\eta Nas)(1 - \upsilon\rho)^{-1}$$

$$f/f_o = a/(3M\upsilon/4\pi N)^{1/3}$$

where a is the Stokes radius, υ is the partial specific volume, N is Avogadro's number, s is the sedimentation coefficient, and M is the molecular weight of the protein obtained from velocity sedimentation experiments.

Nondenaturing Gel Electrophoresis. Samples (2.8 U) of purified FBS AChE prior to separation of monomeric and tetrameric forms or purified monomeric or tetrameric forms of FBS AChE were electrophoresed on a 10% polyacrylamide gel, 0.75 mm thick, with a 4% stacking gel, at 130 V for 3 h at 4 °C. The gel was stained for AChE activity by the method of Karnovsky and Roots (*27*), using 1.7 mM ATC as the substrate.

Titration of Active Sites with DEPQ. Active site titrations were conducted by adding increasing amounts of DEPQ (1−10 μL) to tubes containing the monomeric or tetrameric form of AChE (100 μL of 1 U/mL in 50 mM sodium phosphate,

pH 8.0, containing 0.1% BSA). Samples were incubated for 1 h at 22 °C, and residual activity was determined by assay for AChE activity as described (*23*). Titration curves were constructed by plotting residual enzyme activity versus concentration of DEPQ, and the concentration of the active sites was determined by extrapolation of the curve to zero activity.

Determination of Kinetic Parameters. Substrate inhibition was investigated by measuring enzyme activity of the monomeric and tetrameric forms of FBS AChE by the Ellman method using a substrate concentration range of 0.05−20 mM (*23*). The kinetic constants for the hydrolysis of ATC were determined by analyzing the data according to the following equation (*28*):

$$v = \left(\frac{1 + b[S]/K_{ss}}{1 + [S]/K_{ss}}\right)\left(\frac{V_{max}}{1 + K_m/[S]}\right)$$

where K_m is the Michaelis−Menten constant, K_{ss} is a substrate inhibition constant representing the dissociation of a second substrate molecule, and b reflects the efficiency of hydrolysis of the ternary complex, SES, as compared to the binary complex, ES (*28*).

The effect of pH on the activity of monomeric and tetrameric forms of FBS AChE was determined by measuring enzyme activity in buffers at various pH values as described (*23*). A substrate concentration range of 0.05−20 mM was used for the following buffer solutions: 50 mM sodium acetate at pH 5.0 and 5.5; 50 mM sodium phosphate in the pH range 6.0−8.0; and 50 mM glycine in the pH range 8.0−11. The kinetic constants for the hydrolysis of ATC were determined as described previously, and the pH dependence of V_{max} for the monomeric and tetrameric forms of FBS AChE was fit to a two-pK_a model described by the equation below:

$$v = \frac{L}{1 + 10^{(pK_1 - pH)} + 10^{(pH - pK_2)}}$$

Measurement of AChE Inhibition. Inhibition of enzyme activity was measured in 50 mM sodium phosphate, pH 8.0, over a substrate concentration range of 0.01−0.4 mM and at least three inhibitor concentrations to determine the components of competitive and noncompetitive inhibition as described (*29*). A plot of reciprocal velocities versus reciprocal substrate concentrations (Lineweaver−Burk plot) at a series of inhibitor concentrations yields a family of slopes and y-intercepts. Replots of the slope and y-intercept values versus inhibitor concentrations were used to determine the values of K_I and αK_I, the inhibition constants reflecting the interaction of inhibitor with the free enzyme and enzyme−substrate complex, respectively.

Thermal Stability. Aliquots of enzyme containing monomeric or tetrameric forms of FBS AChE at a concentration of 20 U/mL in 50 mM sodium phosphate, pH 8.0 were incubated at one of several temperatures (25−51 °C) in a water bath with constant shaking. Samples were monitored for AChE activity at 2 min intervals using the Ellman assay (*23*).

Lectin Affinity Chromatography. Agarose-bound lectin columns (0.5 mL bed volume) were equilibrated with 50 mM sodium phosphate, pH 8.0. In some cases, 1 mM $CaCl_2$ and 1 mM $MgCl_2$ were also added to the equilibration buffer. The columns were equilibrated with 1 mL of buffer containing 100 U (30 μg) of the monomeric or tetrameric form of FBS AChE overnight at 4 °C. The columns were washed with 10 mL of equilibration buffer and specifically eluted with 10 mL of equilibration buffer containing the appropriate haptenic sugar. Fractions (0.5 mL) were collected and assayed for AChE activity using the Ellman assay (*23*).

Mass Spectrometric Sequencing of FBS AChE from Polyacrylamide Gels. Sequence analysis of monomeric and tetrameric forms of FBS AChE were performed at the W. M. Keck Biomedical Mass Spectrometry Laboratory (Biomolecular Research Facility, University of Virginia School of Medicine, Charlottsville, VA) according to the following procedure (*30*). Samples of monomeric or tetrameric form of FBS AChE containing 10 μg of protein were heated for 5 min at 100 °C in sample buffer containing 2% SDS and 5% mercaptoethanol and electrophoresed in a 10% polyacrylamide slab gel using the buffer system of Laemmli (*31*). Bands were visualized by staining the gel with Coomassie brilliant blue R-250. Bands for both forms were excised from gels, transferred to siliconized tubes, and destained in 50% methanol overnight. The gel pieces were dehydrated in acetonitrile and reduced with 10 mM DTT in 0.1 M ammonium bicarbonate at room temperature for 30 min. The DTT solution was removed, and the samples were alkylated with 50 mM iodoacetamide in 0.1 M ammonium bicarbonate at room temperature for 30 min. The reagent was removed, the gel pieces were washed with 0.1 M ammonium bicarbonate, and the samples were digested with trypsin overnight at 37 °C. The peptides released from the digest were extracted with 50% acetonitrile/5% formic acid, concentrated, and subjected to LC−MS analysis.

The LC−MS system consisted of a Finnigan LCQ ion trap mass spectrometer equipped with a Protana nanospray ion source operated at 2.8 kV and interfaced to a C_{18} reverse phase capillary column (8 cm × 75 μm id). Peptides were separated by injecting 0.5−2 μL of extract and eluting the column with an acetonitrile/0.1 M acetic acid gradient at a flow rate of 0.25 μL/min. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine the amino acid sequence in sequential scans. This mode of analysis produces approximately 400 CAD spectra of ions ranging in abundance over several orders of magnitude. Not all CAD spectra are derived from peptides. The data were analyzed by database searching using the Sequest search algorithm. Peptides that were not matched by this algorithm were interpreted manually and searched versus the EST databases using the Sequest algorithm.

RESULTS

Purification of Monomeric FBS AChE. Figure 1 shows the separation of monomeric and tetrameric forms of FBS AChE by gel permeation chromatography of purified FBS AChE (*22*). Since the monomer comprises 1−2% activity of purified FBS AChE, it was enriched by repeated gel permeation chromatography of the monomer peak to eliminate most of the tetramer. The profile shown in Figure 1 was obtained after three to four repetitions of this chroma-

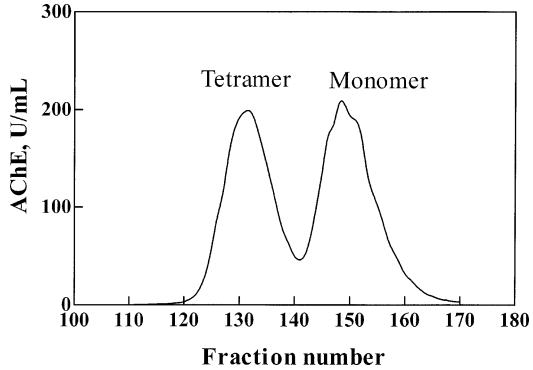

FIGURE 1: Gel permeation chromatography of monomeric and tetrameric FBS AChE. FBS AChE, purified by the method of De La Hoz et al. (*22*) was applied to a Biogel A 1.5 m column (1.5 × 170 cm) equilibrated with 50 mM sodium phosphate, pH 8.0. The flow rate was 20 mL/h, and fraction size was 1.2 mL. The elution position of monomeric and tetrameric forms of FBS AChE was determined by assay of AChE activity (*23*).

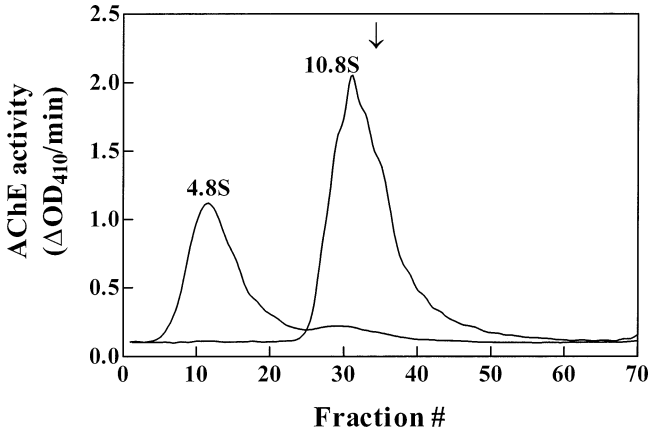

FIGURE 2: Sucrose density gradient centrifugation of purified monomeric and tetrameric FBS AChE. Samples of monomeric or tetrameric forms of FBS AChE (10 U/200 μL) were overlaid on linear 5−20% sucrose density gradients prepared in 50 mM sodium phosphate, pH 8.0, and centrifuged at 75 000*g* for 18 h at 4 °C in an SW41 rotor. The gradients were fractionated from top, and fractions were assayed for AChE activity as described (*24*). The arrow indicates the position of catalase (11S), which was used as an internal reference for all samples.

tography step, and the monomeric form was always found to contain ∼5−10% tetrameric form (Figures 2 and 3). The Stokes radii for the two forms of FBS AChE were determined based on their elution positions from a calibrated Biogel A 1.5 m column by plotting $(-\log K_{av})^{1/2}$ versus Stokes radius. From this plot, the molecular size of monomeric FBS AChE was estimated to be 45 Å and that of tetrameric FBS AChE as 75 Å. The Stokes radius of tetrameric FBS AChE was previously reported as 76 Å (*19*). Fractions containing AChE activity for the two forms were pooled separately and further analyzed by sucrose density gradient centrifugation (Figure 2) and nondenaturing polyacrylamide gel electrophoresis (Figure 3). As shown in Figure 2, the monomeric form sedimented with an $S_{20,w}$ of 4.8 S as compared to the $S_{20,w}$ of 10.8 S reported for the tetrameric form of FBS AChE (*19*). Assuming a partial molar volume of 0.714 ± 0.007 mL/g, a value determined for *Electrophorus* AChE (*32*), the molecular weights for monomeric and tetrameric FBS AChE were calculated to be 89 000 ± 2000 and 340 000 ± 8000 Da, respectively. Using these parameters, frictional ratios of

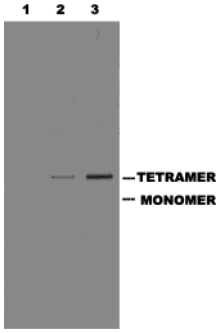

FIGURE 3: Nondenaturing polyacrylamide gel electrophoresis of purified monomeric and tetrameric FBS AChE. Samples (2.8 U) of each enzyme were loaded onto a 10% native polyacrylamide gel. After electrophoresis at 130 V for 3 h, the gel was stained for activity using ATC as described (*27*). Samples are purified monomer (lane 1); purified tetramer (lane 2); and purified FBS AChE prior to separation of the two forms (lane 3). Locations of monomer and tetramer are indicated.

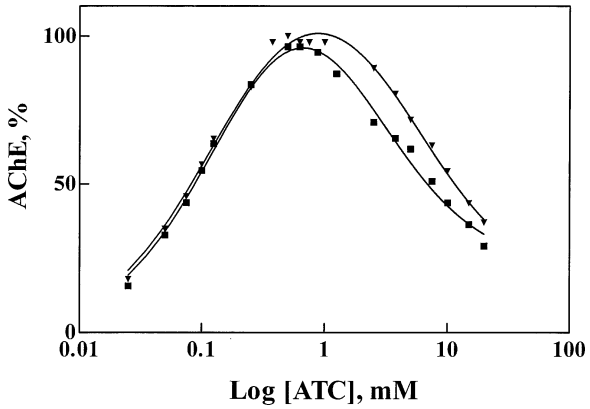

FIGURE 4: Dependence of catalytic activity on ATC concentration for monomeric and tetrameric FBS AChE. Enzyme activity for the two forms was measured by the Ellman assay in 50 mM sodium phosphate, pH 8.0, at 25 °C (*23*) and expressed as a percent of highest activity obtained for each form. The symbols represent monomeric (■) and tetrameric (▼) FBS AChE.

Table 1: Kinetic Constants[a] for Monomeric and Tetrameric FBS AChE

	K_m (μM)	K_{ss} (mM)	*b*	k_{cat} (min^{-1})
monomer	0.17 ± 0.02	2.0 ± 0.4	0.14 ± 0.02	270 000
tetramer	0.13 ± 0.01	5.0 ± 0.8	0.11 ± 0.03	240 000

[a] Values for K_m, K_{ss}, and *b* were calculated using nonlinear fitting of data (*28*). Substrate inhibition is observed when $b < 1$. k_{cat} was evaluated using DEPQ titrations. Enzyme assays were conducted in 50 mM sodium phosphate, pH 8.0 at 25 °C, as described (*23*).

3.3 and 1.6 were calculated for the monomeric and tetrameric forms of FBS AChE, respectively.

Substrate and Inhibitor Specificity. Figure 4 shows the substrate concentration dependence curves for the monomeric and tetrameric forms of FBS AChE in 50 mM sodium phosphate, pH 8.0. The data were analyzed to obtain the values for K_m and K_{ss}. A slightly higher K_m value was observed for the monomeric form as compared to the tetrameric form (Table 1), suggesting that the affinity for the substrate (ATC) was not significantly affected by the oligomeric state of the enzyme. The phenomenon of excess substrate inhibition, a characteristic unique to AChEs, was also exhibited by both forms of the enzyme. These results are consistent with those reported for the monomeric and

Table 2: Dissociation Constants for the Binding of Inhibitors to Monomeric and Tetrameric FBS AChE

	K_I[a] (μM)			
	5 mM phosphate, pH 8.0		50 mM phosphate, pH 8.0	
inhibitor	monomer	tetramer	monomer	tetramer
7-HQ	22.5 ± 2.0	100.8 ± 10.1	47.4 ± 10.5	33.2 ± 1.5
edrophonium	0.15 ± 0.02	0.65 ± 0.07	0.48 ± 0.04	0.46 ± 0.03
propidium[b]	1.45 ± 0.01	1.4 ± 0.1	2.9 ± 0.1	3.8 ± 0.1
tacrine			0.04 ± 0.007	0.11 ± 0.02
(−) huperzine A			0.008 ± 0.001	0.007 ± 0.3
E2020			0.007 ± 0.001	0.009 ± 0.001

[a,b] Values for K_I (competitive inhibition constant) and αK_I (noncompetitive inhibition constant) for propidium were determined from analyses of slopes of $1/v$ vs $1/s$ at various inhibitor concentrations. Values are from at least three experiments.

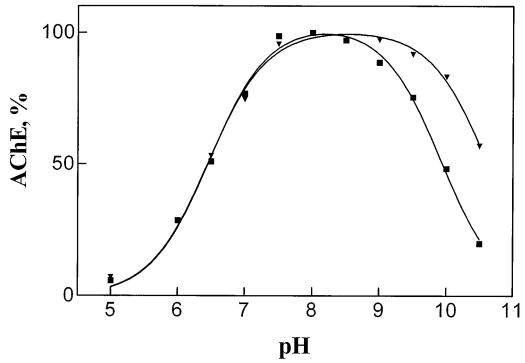

FIGURE 5: pH dependence of the catalytic activity of monomeric and tetrameric FBS AChE. Enzyme activity for the two forms was measured by the Ellman assay (*23*) in various buffers at 25 °C as described in Experimental Procedures and expressed as a percent of highest activity obtained for each form. The symbols represent monomeric (■) and tetrameric (▼) FBS AChE.

tetrameric forms of *Electrophorus electricus* AChE (*33*), bovine superior cervical ganglion AChE (*34*), and recombinant human AChE (*35*). The K_{ss} values of 2.0 ± 0.4 and 5.0 ± 0.8 mM for the monomeric and tetrameric forms, respectively, suggest that the accessibility of substrate to the peripheral anionic site was slightly affected by the association of monomers into tetramers. The catalytic activity of FBS AChE was not affected by subunit association as suggested by similar k_{cat} values (Table 1), which were determined by DEPQ titrations.

The effects of edrophonium and propidium, two positively charged ligands, that bind at the active site and at the peripheral anionic site of AChE, respectively (*36*), on the activity of monomeric and tetrameric forms of FBS AChE were also studied. There was no difference in the dissociation constant (αK_I) for propidium with respect to the monomeric and tetrameric forms of FBS AChE (Table 2). However, at low ionic strength, 4-fold lower K_I values for edrophonium and 7-HQ were observed with the monomeric form as compared to the tetrameric form. The K_I values for AChE inhibitors that are potential or current drugs for the treatment of AD (*37*) such as (−) huperzine A and E2020 were similar for both forms of FBS AChE, whereas the monomeric form was 2−3-fold more sensitive to inhibition by tacrine.

The catalytic activity of the monomeric and tetrameric forms of FBS AChE as a function of pH are shown in Figure 5. The shape of the pH profile for both forms of AChE is best described as a distorted bell with a maximum between pH 7.5 and 8.5, but the pH curve is much broader for tetrameric AChE. The curves were fit to a two pK_a model, and the calculated pK_as for the monomeric form were 6.47 ± 0.04 and 9.92 ± 0.04 as compared to values of 6.45 ± 0.04 and 10.62 ± 0.05 for the tetrameric form.

Table 3: Thermal Stability of Monomeric and Tetrameric FBS AChE

	% activity[a]			
	10 min		30 min	
T (°C)	monomer	tetramer	monomer	tetramer
25	100	100	100	100
40	82	94	82	94
45	82	94	58	90
48	50	90	28	88
51	12	60	1	58

[a] Aliquots of enzyme containing monomeric or tetrameric forms of FBS AChE at a concentration of 20 U/mL in 50 mM sodium phosphate, pH 8.0, were incubated at the indicated temperatures and assayed for AChE activity at 2 min intervals. AChE activity remaining at the end of 10 and 30 min is reported.

Thermal Stability. Aliquots of purified monomeric and tetrameric forms of FBS AChE were incubated at different temperatures, and enzyme activity remaining after 10 and 30 min is listed in Table 3. The results of this experiment show that the monomeric form of FBS AChE was thermally less stable than the tetrameric form in the temperature range of 40−51 °C. These differences became more pronounced as the incubation temperature was increased; for example, the tetrameric form lost only 42% of its activity after a 30 min incubation at 51 °C, whereas the monomeric form had lost 99% of its activity under similar conditions. The temperature sensitivity of the monomeric form as compared to the tetrameric form was previously demonstrated for recombinant and serum derived BChE (*38*−*40*) as well as recombinant and tissue-derived AChE (*35*, *40*).

Characterization of Carbohydrate Moieties. Studies on the glycosylation of monomeric and tetrameric forms of FBS AChE suggest that although both forms are similar in carbohydrate composition, they differ in the types of glycan moieties they carry (*41*). Lectin affinity chromatography was used for the qualitative structural characterization of the carbohydrate moieties on the monomeric and tetrameric forms of FBS AChE. Consistent with previous observations, the results of these studies summarized in Table 4 demonstrate that both forms lack O-linked glycans. The major difference between the two forms was in their binding to WGA, *R. communis* I, and *R. communis* II, suggesting that the monomeric form was deficient in GlcNAc/sialic acid and $(Gal\text{-}GlcNAc)_n$ structures.

Sequence Analysis by Tandem Mass Spectrometry. Three replicate samples for monomeric and tetrameric forms of FBS

Table 4: Specific Binding of Monomeric and Tetrameric FBS AChE to Lectin Columns

lectin	specificity	% AChE bound[a] monomer	% AChE bound[a] tetramer
WGA	GlcNAc, sialic acid	85	>99
ConA	mannose/glucose	96	>99
S. nigra L	α-2,6 sialic acid	0	0
R. communis I	(Gal-GlcNAc)$_n$	71	>99
R. communis II	(Gal-GlcNAc)$_n$	85	>99
jacalin	O-linked carbohydrate	0	0

[a] The lectin columns were equilibrated with 1 mL of buffer containing 100 U (30 μg) of monomeric or tetrameric form of FBS AChE overnight at 4 °C. The columns were washed with 10 mL of equilibration buffer and eluted with 10 mL of equilibration buffer containing the appropriate haptenic sugar. The data represent the percentage of AChE activity specifically eluted by the haptenic sugar, and each value is the mean of at least two experiments.

Table 5: C-Termini Peptide Sequences[a] for Monomeric and Tetrameric FBS AChE

AA residues	measured MW (Da)	sequence	calculated MW (Da)
		Monomer	
539−543	504.3	LLSAT	504.6
539−544	619.3	LLSATD	619.3
539−546	833.4	LLSATDTL	833.5
539−547	948.4	LLSATDTLD	948.5
		Tetramer	
580−583	494.3	CSDL	494.2
580−583	508.3	CSDL[b]	508.2
577−583	893.4	QDRCSDL	893.9
577−583	907.4	QDRCSDL[b]	907.9

[a] Peptides obtained from an in-gel, tryptic digest of monomeric and tetrameric forms of FBS AChE were sequenced by tandem mass spectrometry. [b] C designates *S*-carbamidomethyl cysteine.

AChE in gel were digested with trypsin, which produced the same peptides for both forms. The sequences of the peptides covered about 70% of the sequence for both forms. The subunit molecular weights of the protein components of monomeric (major species) and tetrameric FBS AChE deduced from these sequences were 59 414 and 64 239 Da, respectively. Both monomer and tetramer had the same N-terminal peptide, EGPEDPELLVMVR. The only differences detected were at the C-terminus, which are shown in Table 5. The monomer appeared to have a ragged C-terminus: LLSAT, LLSATD, LLSATDTL, and LLSATDTLD. Although it was difficult to quantitate the relative amount of these peptides by mass spectrometry, the peptide LLSATDTLD appeared to be the most abundant. The C-terminus for the tetramer continued past the C-terminus for the monomer and yielded five additional peptides, including the reported C-terminus, CSDL.

DISCUSSION

In vertebrates, ChEs exist in various molecular forms, which not only control their destiny within the cell but their distribution in various tissues. Although the existence of different molecular forms of AChE is well-documented, their physiological significance is not clearly understood. It has been reported that these molecular forms differ in their sensitivity to inhibitors, which is clinically important in the development of therapeutic drugs. Since stable, monomeric forms of ChEs are not abundant in mammalian tissues, few kinetic and biochemical studies on this form of enzyme are reported in the literature. Most of these studies were performed using monomeric forms of ChEs that were generated by proteolytic degradation of the tetrameric form of enzyme (*34*, *39*) or by the expression of a mutant cDNA in which the C-terminal Cys codon was substituted by an Ala codon (*35*, *38*).

During attempts to purify the tetrameric G_4 form of AChE from FBS, we observed the presence of a stable, naturally occurring, monomeric G_1 form of FBS AChE. The monomeric form of FBS AChE comprises 1−2% activity of purified FBS AChE; its presence in this preparation could not be detected by nondenaturing polyacrylamide gel electrophoresis. However, it could be isolated and separated from the tetrameric form by gel permeation chromatography. On the basis of the elution position, its molecular size was estimated to be 45 Å and that of tetrameric FBS AChE as 75 Å. Its sedimentation coefficient of 4.8 S as compared to that of 10.8 S for the tetrameric form and migration on a nondenaturing polyacrylamide gel established its identity as the monomeric form of FBS AChE. These results suggest that FBS is an excellent source for the isolation of reasonable quantities of the monomeric form of mammalian AChE and enabled us to characterize its biochemical properties and identify the molecular basis for its existence.

The catalytic properties of the monomeric form as indicated by the K_m and k_{cat} values were similar to those of the tetrameric form, suggesting that subunit association does not affect the binding of substrate to the active site. Inhibition by excess substrate was observed with both forms of FBS AChE, which is indicated by b values <1. However, a higher K_{ss} value for the tetrameric form and the displacement of the substrate curve to the right as compared to the monomeric form suggest that the binding of substrate to the peripheral anionic site was somewhat hindered upon subunit association. This result is not surprising in light of the reported X-ray crystal structure of the mouse AChE tetramer, which shows that an association between loop Cys257−Cys272 of subunit A and the peripheral anionic site of the facing subunit C sterically occludes the entrance to the active site gorge (*42*).

The oligomeric state of the enzyme does not affect the binding of various classical inhibitors of AChE such as edrophonium, which binds to the active site, or propidium, which binds to the peripheral anionic site. Similarly, no differences between the two forms were observed for the binding of (−) huperzine A or E2020; tacrine inhibited the monomeric form slightly more potently than the tetrameric form. This result is similar to that reported for rat cortical AChE and post-mortem AD brain AChE, where some inhibitors were found to inhibit the G_1 form 2−6-fold more potently than the G_4 form (*14*, *18*). A 4-fold lower K_I value for edrophonium and 7-HQ was observed for the monomeric form in 5 mM sodium phosphate buffer. Since the peripheral anionic site is sensitive to ionic strength, these results suggest that this effect on the binding of inhibitors to the active site is a result of perturbations in the peripheral anionic site. Therefore, it appears that the peripheral anionic site of the monomer is more sensitive to changes in ionic strength.

Although kinetic evidence suggests that the association of monomeric subunits into a tetramer does not affect the catalytic efficiency of the enzyme, it appears to affect the pH dependence of the catalytic reaction as indicated by the

broader pH profile curve for the tetramer. The bell-shaped pH-rate profiles can be analyzed by dissecting the curve into two titration curves. The ascending leg represents an increase in rate of the reaction due to the rising concentration of the conjugate base form of an acid. The descending leg of the curve represents the effect on rate of the reaction due to the declining concentration of a weak conjugate acid form of a base. Each titration curve yields an inflection point corresponding to the pK_a of an ionizing residue catalyzing the reaction. The pK_a of the residue acting as a base catalyst is ~6.4 for both forms of AChE. This pK_a value is similar to intrinsic pK_a values of the catalytic His440 for acylation and deacylation in the reaction of substrates of AChE (*43*−*46*). The pK_a of the residue acting as an acid catalyst increases from 9.9 in the monomeric form to 10.6 in the tetrameric form. The curve is broader in the tetramer because of the increased difference in the pK_a values of the basic and acidic groups catalyzing ATC hydrolysis.

The monomeric and tetrameric forms of AChE were also found to differ in their thermal stability and the types of glycans they carry. The monomer was thermally more labile than the tetrameric form. The increased thermal stability of the tetrameric form of the enzyme as compared to the monomeric form was previously demonstrated in ChEs from various species (*35*, *39*, *40*). Consistent with a previous observation that the glycans on tetrameric FBS AChE were of the complex biantennary type, the efficiency of binding of this enzyme to lectin columns specific for mannose, Gal-GlcNAc, and sialic acid was >99% (*47*). On the other hand, the monomeric form bound efficiently only to the Con A column and demonstrated a decreased binding to columns specific for Gal-GlcNAc and sialic acid. These results suggest that the glycans on monomeric FBS AChE lack complex biantennary type of structures and correlate with the results of our recent study in which differences in the oligosaccharide profiles for monomeric and tetrameric forms were observed (*48*). These results also suggest that the catalytic properties of the active site gorge are not affected by the type of carbohydrate structures present on the surface of the enzyme molecule.

The presence of monomeric AChE is not unique to FBS. Monomers were shown to constitute a major fraction of the intracellular AChE pool in various cells and tissues, and the presence of free extracellular monomers of AChE and BChE was also noted in the culture media of cell lines as well as CSF and sera of animals. Monomers could be products of (a) unassembled tetrameric subunits, (b) degradation of extracellular multimers, or (c) alternatively spliced AChE mRNA species lacking the C-terminal Cys residue. It was demonstrated that the monomeric form of recombinant wild-type BChE secreted from Chinese hamster ovary cells resulted from proteolysis at the C-terminus (*38*). To elucidate the molecular nature of the monomeric form of FBS AChE, we conducted sequence analysis of tryptic peptides from both forms using mass spectrometry. Using this method, the subunit molecular weight of the protein component of the monomer (major species) was determined to be 59 414 Da and that of the tetramer as 64 239 Da. This method yielded about 70% of the sequence for both forms with the remaining 30% being comprised of large heavily glycosylated peptides that could not be sequenced. The sequences of the common region of the monomer and tetramer were 100% identical. The tetramer consists of 583 amino acid residues corresponding to the mature form of the enzyme, whereas the monomer consists of 543−547 amino acid residues. The N-terminal of the monomer was Glu, which is also the N-terminal amino acid residue for the tetramer (*20*), suggesting that the monomer is not a result of truncation at the N-terminal. The only differences detected were at the C-terminus. The tetramer yielded the expected C-terminus, CSDL, whereas the C-terminus of the monomer yielded a mixture of peptides, of which LLSATDTLD was the most abundant. These results suggest that like monomeric BChE secreted from Chinese hamster ovary cells, monomeric FBS AChE may have resulted from the proteolysis of tetrameric FBS AChE at the C-terminus. Since the splicing site between exon 4 and 6 is at position 543, it is also possible that the monomer is a product in which exon 6 was not spliced into exon 4. This possibility is supported by the presence of a peptide LLSAT only in monomeric FBS AChE. Since FBS AChE contains four glycosylation sites at positions 61, 265, 350, and 464 (*49*), C-terminal truncation did not remove any glycosylation sites, suggesting that carbohydrate residues did not affect oligomerization. The only other natural monomeric AChE characterized to date is that from *Bungarus fasciatus* venom (*50*). The sequence of this enzyme deduced from cDNA clones demonstrated that its catalytic subunit is highly homologous to other AChEs (*51*). However, the C-terminal region lacks a Cys residue, and the sequence is different from the C-terminal regions of hydrophobic (H) or tailed (T) subunits. The C-terminal consists of a short hydrophilic peptide of 15 residues with the sequence VDPPRADRRRR-SARA. Taken together, these results demonstrate that the monomer is a result of truncation at the C-terminus, and the results are consistent with the involvement of C-terminal amino acids in the assembly of monomers into tetramers (*38*, *42*).

On the basis of multiple sequence alignment, analysis of three-dimensional structural data, and mutagenesis and biochemical data, two models for the organization of monomeric subunits into tetramers were proposed (*52*). In the square planar model, the active site gorges would be freely accessible to the substrate, whereas in the pseudo-tetrahedral model, the gorges would be orthogonal to each other. The association of subunits is stabilized through the formation of a four-helix bundle by the C-terminal regions. The results of biochemical studies presented here with the monomeric and tetrameric forms of FBS AChE support the square planar arrangement of subunits in the tetramer, which are held together at the C-terminus. The assembly of monomeric subunits into a tetramer does not have a significant effect on the accessibility of substrate and inhibitors to the active site gorge. Our conclusions are supported by the X-ray crystal structures of mouse AChE and *E. electricus* AChE, which show a pseudo-square planar arrangement of monomeric subunits in the tetramer that is stabilized through interactions at the C-terminus (*42*, *53*).

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REFERENCES

1. Massoulié, J., and Bon, S. (1982) *Annu. Rev. Neurosci. 5*, 57−106.
2. Massoulié, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F.-M. (1993) *Prog. Neurobiol. 41*, 31−91.
3. Lazar, M., and Vigny, M. (1980) *J. Neurochem. 35*, 1067−1079.
4. Grossman, H., and Liefländer, M. (1979) *Z. Naturforsch., C: J. Biosci. 34*, 721−725.
5. Niday, E., Wang, C. S., and Alaupovic, P. (1977) *Biochim. Biophys. Acta 459*, 180−193.
6. Römer-Luthi, C. R., Hajdu, J., and Brodbeck, U. (1979) *Hoppe-Seylers Z. Physiol. Chem. 360*, 929−934.
7. Römer-Luthi, C. R., Ott, P., and Brodbeck, U. (1980) *Biochim. Biophys. Acta 601*, 123−133.
8. Rieger, F., Faivre-Bauman, A., Benda, P., and Vigny, M. (1976) *J. Neurochem. 27*, 1059−1063.
9. Atack, J. R., Perry, E. K., Bonham, J. R., and Perry, R. H. (1987) *J. Neurochem. 48*, 1845−1850.
10. Lyles, J. M., Barnard, E. A., and Silman, I. (1980) *J. Neurochem. 34*, 978−987.
11. Fernandez, H. L., Duell, M. J., and Festoff, B. W. (1979) *J. Neurobiol. 10*, 441−454.
12. Fishman, E. B., Siek, G. C., MacCallum, R. D., Bird, E. D., Volicer, L., and Marquis, J. (1986) *Ann. Neurol. 19*, 246−252.
13. Meneguz, A., Bisso, G. M., and Michalek, H. (1992) *Neurochem. Res. 17*, 785−790.
14. Ogane, N., Giacobini, E., and Messamore, E. (1992) *Neurochem. Res. 17*, 489−495.
15. Schegg, K. M., Harrington, L. S., Neilsen, S., Zweig, R. M., and Peacock, J. H. (1992) *Neurobiol. Aging 13*, 697−704.
16. Siek, G. C., Katz, L. S., Fishman, E. B., Korosi, T. S., and Marquis, J. K. (1990) *Biol. Psychiatry 27*, 573−580.
17. Arendt, T., Bruckner, M. K., Lange, M., and Bigl, V. (1992) *Neurochem. Int. 21*, 381−396.
18. Enz, A., Chappuis, A., and Probst, A. (1992) in *Multidisciplinary Approaches to Cholinesterase Functions* (Shafferman, A., and Velan, B., Eds.) pp 243−249, Plenum Press, New York.
19. Ralston, J. S., Rush, R. S., Doctor, B. P., and Wolfe, A. D. (1985) *J. Biol. Chem. 260*, 4312−4318.
20. Doctor, B. P., Chapman, T. C., Christner, C. E., Deal, C. D., De La Hoz, D. M., Gentry, M. K., Ogert, R. A., Rush, R. S., Smyth, K. K., and Wolfe, A. D. (1990) *FEBS Lett. 266*, 123−127.
21. Levy, D., and Ashani, Y. (1986) *Biochem. Pharmacol. 35*, 1079−1085.
22. De La Hoz, D., Doctor, B. P., Ralston, J. M., Rush, R. S., and Wolfe, A. D. (1986) *Life Sci. 39*, 195−199.
23. Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. (1961) *Biochem. Pharmacol. 7*, 88−95.
24. Doctor, B. P., Toker, L., Roth, E., and Silman, I. (1987) *Anal. Biochem. 166*, 399−403.
25. Siegel, L. M., and Monty, K. T. (1966) *Biochim. Biophys. Acta 112*, 346−362.
26. Laurent, T. C., and Killander, J. (1964) *J. Chromatogr. 14*, 317−330.
27. Karnovsky, M. J., and Roots, L. (1964) *J. Histochem. Cytochem. 12*, 219−221.
28. Radić, Z., Pickering, N., Vellom, D. C., Camp, S., and Taylor, P. (1993) *Biochemistry 32*, 12074−12084.
29. Saxena, A., Redman, A. M. G., Jiang, X., Lockridge, O., and Doctor, B. P. (1997) *Biochemistry 36*, 14642−14651.
30. Shevchenko, A., Wilm, M., Vorm, O., and Mann, M. (1996) *Anal. Biochem. 68*, 850−858.
31. Laemmli, U. K. (1970) *Nature (London) 227*, 680−685.
32. Bon, S., Huet, M., Lemonnier, M., Rieger, F., and Massoulié, J. (1976) *Eur. J. Biochem. 68*, 523−530.
33. Bon, S., and Massoulié, J. (1976) *FEBS Lett. 67*, 99−103.
34. Vigny, M., Bon, S., Massoulié, J., and Gisiger, V. (1979) *J. Neurochem. 33*, 559−565.
35. Velan, B., Grosfeld, H., Kronman, C., Leitner, M., Gozes, Y., Lazar, A., Flashner, Y., Marcus, D., Cohen, S., and Shafferman, A. (1991) *J. Biol. Chem. 266*, 23977−23984.
36. Taylor, P., and Lappi, S. (1975) *Biochemistry 14*, 1989−1997.
37. Giacobini, E. (1996) in *Alzheimer Disease: From Molecular Biology to Therapy* (Becker, R., and Giacobini, E., Eds.) pp 187−204, Birkhäuser Press, Boston.
38. Blong, R. M., Bedows, E., and Lockridge, O. (1997) *Biochem. J. 327*, 747−757.
39. Cauet, G., Friboulet, A., and Thomas, D. (1987) *Biochim. Biophys. Acta 912*, 338−342.
40. Edwards, J. A., and Brimijoin, S. (1983) *Biochim. Biophys. Acta 742*, 509−516.
41. Saxena, A., Ashani, Y., Raveh, L., Stevenson, D., Patel, T., and Doctor, B. P. (1998) *Mol. Phamacol. 53*, 112−122.
42. Bourne, Y., Taylor, P., Bougis, P. E., and Marchot, P. (1999) *J. Biol. Chem. 274*, 2963−2970.
43. Rosenberry, T. L. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3834−3838.
44. Rosenberry, T. L. (1975) *Croat. Chem. Acta 47*, 235−250.
45. Quinn, D. M. (1987) *Chem. Rev. 87*, 955−975.
46. Taylor, P., and Radić, Z. (1994) *Annu. Rev. Pharmacol. Toxicol. 34*, 281−320.
47. Saxena, A., Raveh, L., Ashani, Y., and Doctor, B. P. (1997) *Biochem. 36*, 7481−7489.
48. Saxena, A., Ashani, Y., Raveh, L., Stevenson, D., Patel, T., and Doctor, B. P. (1998) *Mol. Pharmacol. 53*, 112−122.
49. Mendelson, I., Kronman, C., Ariel, N., Shafferman, A., and Velan, B. (1998) *Biochem. J. 334*, 251−259.
50. Cousin, X., Créminon, C., Grassi, J., Méflah, K., Cornu, G., Saliou, B., Bon, S., Massoulié, J., and Bon, C. (1996) *FEBS Lett. 387*, 196−200.
51. Cousin, X., Bon, S., Duval, N., Massoulié, J., and Bon, C. (1996) *J. Biol. Chem. 271*, 15099−15108.
52. Giles, K. (1997) *Protein Eng. 10*, 677−685.
53. Raves, M. L., Giles, K., Schrag, J. D., Schmid, M. F., Phillips, G. N., Jr., Chiu, W., Howard, A. J., Silman, I., and Sussman, J. L. (1999) in *Structure and Function of Cholinesterases and Related Proteins* (Doctor, B. P., Taylor, P., Quinn, D. M., Rotundo, R. L., and Gentry, M. K., Eds.) pp 387−392, Plenum Press, New York.

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