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Conversion of Acetylcholinesterase Hydrophilic Tetramers into Amphiphilic Dimers and Monomers

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Exposure of purified hydrophilic tetramers of acetylcholinesterase (AChE) from fetal bovine serum to various guanidinium chloride (Gdn) concentrations led to inactive tetramers (2 M Gdn) and dimers (6 M Gdn). The native tetramers were almost fully monomerized by reduction, a minor fraction of the released monomers remaining active. Sedimentation analysis and hydrophobic chromatography showed that the modified tetramers, dimers and monomers had amphiphilic properties. Intrinsic fluorescence spectra and binding of the amphiphilic probe, 1-anilino-8-naphthalene sulfonate (ANS), revealed that AChE subunits in the modified tetramers were in a 'molten globule' structure, the dimers in a denatured state, and the inactive monomers in a 'native-like' structure. These data show that AChE subunits possess a flexible conformation, which may be important for generating a full set of molecular forms. In addition, the behavior of the active monomers with amphiphiles may explain the interactions of type II AChE forms with membranes.

Acetylcholinesterase (AChE, E C 3.1.1.7) hydrolyzes the neurotransmitter acetylcholine. The enzyme exists in several molecular forms, which fall into two classes: collagen-like tailed asymmetric (A_{12}, A_8, and A_4) and globular (G_4, G_2, and G_1) species (1,2). Salt-soluble asymmetric forms are abundant at the neuromuscular junctions (1,3). Detergent-soluble (G_4^A) and salt-soluble (G_4^H) tetramers coexist in mammalian brain (4,5). In addition, fully hydrophilic (secretory) G_4^S forms are released from brain to the cerebrospinal fluid (6). There are two types of amphiphilic dimers and monomers: type I possess a glycosylphosphatidylinositol (GPI) anchor, whereas type II do not (1,7,8). Alternative splicing of a single AChE gene produces T and H subunits (9–12). Asymmetric, tetrameric and light type II AChE forms are made up of T subunits (2,13,14), and type I species of H subunits (13,15).

The membrane-bound G_4^A AChE is constituted by one pair of subunits disulfide-bound through Cys^{580}, the other two subunits being disulfide-linked to a hydrophobic structural 20-kDa subunit for membrane anchoring (16,17). G_4^H enzyme forms are made up of two pairs of subunits, which are disulfide-bound, the dimers being linked to each other by hydrophobic interactions (18). Although G_4^H forms are devoid of the hydrophobic subunit (18), they still show hydrophobic domains, as judged by their interaction with amphiphiles (5,17,19). The AChE subunits probably possess a notable conformational flexibility to allow a differential folding, which would explain the distinct behavior of G_4^H, G_4^S and type II G forms with amphiphiles.

To gain insight into the possible conformations adopted by the AChE subunits, fully hydrophilic tetramers were purified, chemically denatured or reduced. The modifications induced in the AChE subunits by these treatments were studied by centrifugation analysis, hydrophobic chromatography, changes in the intrinsic fluorescence spectra and binding of the amphiphilic probe 1-anilino-8-naphthalene sulfonate.

MATERIALS AND METHODS

Fetal bovine serum (FBS, Cat. No. F-2442), acetylthiocholine iodide, edrophonium chloride, 5,5′-dithio-bis-2-

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nitrobenzoic acid (DTNB), epoxy-activated Agarose, phenyl-Agarose, N-ethylmaleimide (NEM), dithiothreitol (DTT), guanidinium chloride (Gdn), bovine liver catalase, calf intestine alkaline phosphatase, polyoxyethylene-10-oleyl ether (Brij 96), 1-anilino-8-naphthalene-sulfonate (ANS) were all from Sigma (St. Louis, MO). Sucrose and Triton X-100 were from Merck (Rahway, NJ). [^{3}H]DFP (6 Ci/mmol, 1 mCi/ml) was from Du Pont (France).

AChE activity was assayed spectrophotometrically (20). One unit (U) of AChE activity was the amount of enzyme which splits one μmol of acetylthiocholine per min, at 37°C. AChE activity in fractions recovered from sucrose gradients was determined by a microtiter assay (8), the enzyme activity being expressed in arbitrary units (A.U.).

Protein was determined by a modified biuret method (5) or by absorbance at 280 nm, using an $\epsilon^{mg/ml} = 1.1$, which had been calculated for FBS AChE (21).

AChE was purified by affinity chromatography in an edrophonium-Sepharose matrix (22). In short, 500 ml of FBS was applied to the matrix (15 ml), which was equilibrated with 50 mM phosphate buffer, pH 8.0 (buffer 1). About 95% of the enzyme was bound to the matrix and 8% eluted by washing with 500 mM NaCl in buffer 1. Nearly 50% of the bound AChE was detached with 12 mM edrophonium chloride dissolved in 500 mM NaCl, 50 mM phosphate buffer, pH 7.0. The AChE activity in the purest fraction was 2,640 U/mg which, when compared with that in FBS (0.037 U/mg), indicated that the protein was purified 71,000-fold.

The molecular weight of AChE was assessed by electrophoresis on 5–20% acrylamide gradient slab gels, in a Pharmacia-LKB electrophoresis system (Uppsala, Sweden). The running buffers, and those in the stacking and separation gels were those of the Laemmli method (23), but without SDS. Details on casting of gels, buffer solutions and operation conditions have already been described (24). Gels were histochemically stained (25). The AChE subunit size was determined by SDS-PAGE (10% acrylamide), under reducing conditions (23), using a Bio-Rad Mini Protean II system (Richmond, CA). Protein was stained with Coomassie Blue.

For AChE subunit labelling, [^{3}H]DFP (70 μl) was added to 1 ml-fractions of enzyme (180 U/ml) and incubated for 2.5 h, at 4°C. Free [^{3}H]DFP was removed by dialysis in 50 mM phosphate buffer, pH 8.0, and by desalting in Presto-desalting columns (Pierce, Rockford, IL). The labelled protein (25,000 cpm/25μl) was then subjected to denaturation or reduction.

For chemical denaturation, aliquots of native or [^{3}H]DFP-labelled AChE were incubated with increasing Gdn concentrations (0.6–6 M), 30 min at 25°C, this being followed by dialysis and desalting when necessary. Enzyme depolymerization induced by Gdn was determined by sedimentation analysis, and the possible amphiphilic properties of the denatured enzyme were assessed by its migration in sucrose gradients made with Triton X-100 or with Brij 96, and by hydrophobic chromatography.

Purified native and [^{3}H]DFP-AChE was reduced with 10 mM DTT, and alkylated with 40 mM NEM, each treatment being made for 2 h at 37°C. The excess of NEM was removed by desalting. The level of enzyme monomerization was assessed by sedimentation analysis.

Amphiphilic and hydrophilic AChE forms were separated by hydrophobic chromatography on phenyl-Agarose (8), and later identified by sedimentation analysis.

AChE forms were separated and characterized according to their sedimentation coefficients by centrifugation on 5–20% (w/v) sucrose gradients made up in 1 M NaCl, 50 mM $MgCl_2$, 10 mM Tris buffer, pH 7.0, without detergent, with 0.5% Triton X-100 or with 0.5% Brij 96 (5,8). Internal markers were catalase (11.4 S) and alkaline phosphatase (6.1 S). The tubes were centrifuged at 165,000 g_{max} (35,000 rpm), 18 h at 4°C, in a SW 41 Ti Beckman rotor (Fullerton, CA). Fractions of 275 μl were collected and assayed for AChE activity or [^{3}H]DFP-labelled protein, and enzyme markers.

Normalized intrinsic fluorescence spectra were obtained in a LS-50B Perkin Elmer luminescence spectrometer at 25°C using 100 μl quartz cells (Hellma, Müllheim, Germany). Excitation was at 295 nm, with a 2.5 nm slit width in both monochromators.

Enhancement of ANS fluorescence was measured using a concentration of AChE active sites of 0.3 μM and 0.1 mM ANS. The excitation and emission wavelengths were 390 nm and 490 nm, the slit width of the excitation and emission monochromators being 5 nm and 10 nm.

RESULTS AND DISCUSSION

A protein band of 318 ± 15 kDa was identified in samples of purified AChE by nondenaturing electrophoresis, and bands of 154 ± 5 kDa and 81 ± 3 kDa were separated by SDS-PAGE under non-reducing and reducing conditions, respectively (protein profiles not shown). The native AChE had a sedimentation value of 10.6 ± 0.1S (Fig. 1), which was unmodified in detergent-containing gradients. Moreover, the enzyme was excluded (90%) from a phenyl-Agarose matrix (profile not shown). These results agreed with the structural parameters of hydrophilic AChE tetramers (21).

The native enzyme lost little activity (10%) after incubation with 0.6 M or 1.2 M Gdn, and was fully inactivated by treatment with 6 M Gdn. The [^{3}H]DFP-labelled AChE exposed to 6 M Gdn showed a peak of 5.8 ± 0.1 S or 4.8 ± 0.1 S in gradients with Triton X-100 or with Brij 96 (Fig. 1). These sedimentation values coincided with those reported for AChE dimers (14,26). Further-

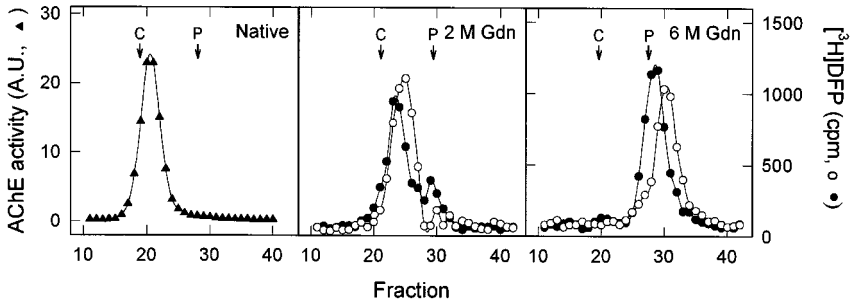

FIG. 1. Sedimentation profiles of FBS AChE before and after incubation with Gdn. The activity in native AChE is given in arbitrary units (A.U.); [^{3}H]DFP-AChE treated with 2 M or 6 M Gdn. Sucrose gradients made without or with detergent (▲), with 0.5% Triton X-100 (●), or with 0.5% Brij 96 (○). Sedimentation markers were catalase (C) and alkaline phosphatase (P).

more, the different migration patterns of the [^{3}H]DFP-labelled protein with the detergent added to the gradients suggested that the dimers displayed amphiphilic properties. Hydrophobic chromatography revealed that 95% of the [^{3}H]DFP-AChE exposed to 6 M Gdn bound to phenyl-Agarose, and about 50% of the absorbed protein eluted with a Triton X-100-containing buffer (elution profile not shown).

The *Torpedo* AChE dimers remain in a long-lived partially unfolded state (a molten globule state, MGS) either through oxidative stress (27), treatment with disulfides (28,29), or by mild denaturation with 1.0–1.5 M Gdn (27,28). The MGS is described as a compact structure, of slightly larger dimensions than the native protein, and possessing secondary conformational domains similar to those of the native protein. Proteins in the MGS display an increased overall flexibility of the polypeptide chain relative to the native state (30), while the hydrodynamic radius is increased by 10% (31). They are also prone to aggregation by exposure of hydrophobic domains to the external milieu (32), a shift of *ca* 6 nm in the fluorescence emission spectrum, an increased ANS binding, and a notable decrease in the ellipticity in the near-UV (28,33).

To test whether the AChE subunits in the FBS dimers detached from tetramers by Gdn treatment were in a partially or fully unfolded state, the intrinsic fluorescence spectra and the ANS binding of native and Gdn-incubated enzyme were analyzed. In normalized spectra, the maximum emission peak in native AChE and in samples incubated with 0.6 M or 1.2 M Gdn occurred at 338 nm (Fig. 2). In contrast, the maximum emission peak shifted to 343 nm or 355 nm in samples exposed to 2 M or 6 M Gdn, respectively (Fig. 2). Little ANS binding was measured in the native enzyme and in samples incubated with 1.2 M Gdn. However, ANS fluorescence was notably enhanced in samples incubated with 2 M Gdn and decreased thereafter (Fig. 2). This suggested that the enzyme subunits remained in the native conformation by exposure to 1.2 M Gdn, and were converted into a partly unfolded state by 2 M Gdn and into a denatured state at higher denaturant concentrations. Since 1.0–1.5 M Gdn suffices to transform the *Torpedo* AChE dimers into a MGS (27,28), the above results show that the non-covalent forces which keep the native structure in subunits are stronger in tetramers than in dimers.

A crucial point of this study was to establish whether the formation of the 'molten globule' state preceded enzyme dimerization. To answer this question, the [^{3}H]DFP-labelled AChE was equilibrated with 2 M Gdn and then subjected to centrifugation analysis. The profiles revealed a protein peak of 10.0 ± 0.1 S or 9.5 ± 0.1 S in gradients with Triton X-100 or with Brij 96 (Fig. 1). This showed that the quaternary structure was maintained in AChE tetramers, although the subunits were in a MGS. Furthermore, the higher buoyancy of the modified enzyme than of the native protein agreed with the reported 10% increase in the hydrodynamic radius of proteins in a MGS (31). Moreover, the different migration patterns of the modified tetramers with the detergent added

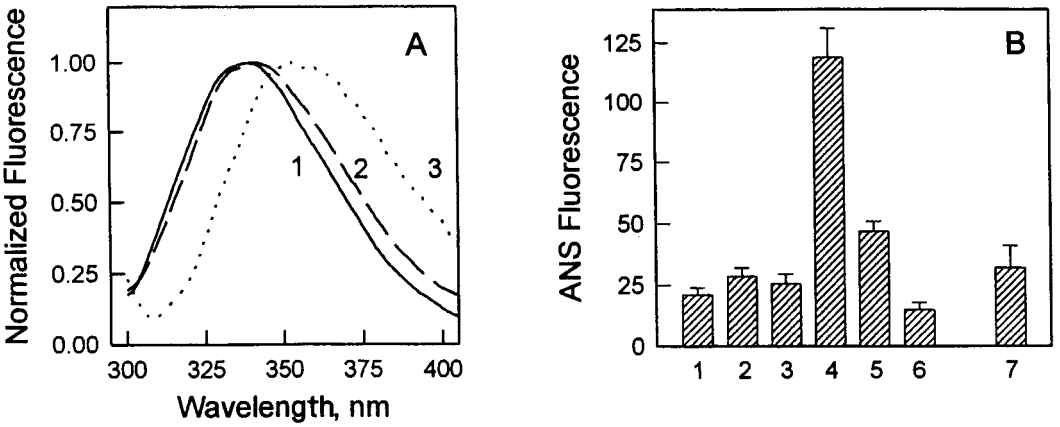

FIG. 2. Spectroscopic comparison of native and modified AChE. Traces: A, normalized intrinsic fluorescence spectra of AChE native or reduced (1), and incubated with 2 M (2) or 6 M Gdn (3). B, ANS binding in native AChE (1), equilibrated in 0.6 M (2), 1.2 M (3), 2 M (4), 3 M (5) or 6 M Gdn (6), and in reduced and alkylated enzyme (7).

to the gradients suggested that the hydrophilic AChE was converted into an amphiphilic form, which is a property of proteins in a MGS (32).

To test whether monomerization of enzyme tetramers yielded subunits in a MGS, the native enzyme was subjected to reduction and alkylation. Nearly 80% of the enzyme activity was lost by this treatment. AChE tetramers of 10.6 S (70%) and monomers of 3.6 ± 0.1 S or 2.6 ± 0.2 S, in gradients with Triton X-100 or with Brij 96, were identified in the active fraction (Fig. 3). These data seemed to show that the tetramers had not been fully monomerized by reduction. Moreover, the shift in the sedimentation values of the monomers with the detergent added to the gradients (Fig. 3) and their complete adsorption to phenyl-Agarose revealed that they had amphiphilic properties. This demonstrates that the association of hydrophobic residues, i.e., a fatty acid or a phospholipid, to the polypeptide is not required for amphiphilicity to be induced in the AChE subunits. This is especially relevant when considering the nature of the hydrophobic domains in type II dimers and monomers.

In order to investigate the conformation of the inactive monomers released by reduction, AChE tetramers were first labelled, then reduced, alkylated and later subjected to sedimentation analysis. A single [^{3}H]DFP-labelled protein of 3.7 ± 0.1S or 2.7 ± 0.1S was identified in gradients with Triton X-100 or with Brij 96 (Fig. 3). This demonstrated that most enzyme tetramers were converted into amphiphilic monomers by reduction, in contrast with the maintenance of the quaternary

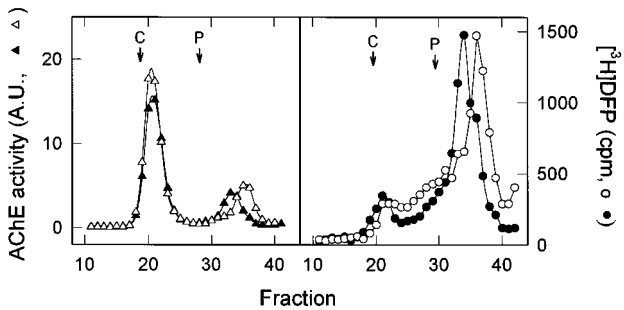

FIG. 3. Sucrose gradient profiles of AChE after reduction and alkylation. (Left) Enzyme forms which remained active (the activity is expressed in arbitrary units, A. U.); (right) enzyme components separated by reduction of [^{3}H]DFP-AChE. Sucrose gradients with added Triton X-100 (filled symbols) or Brij 96 (empty symbols). Internal enzyme markers as in Fig. 1.

structure in membrane-bound G_4 AChE from bovine brain (17) and in G_4 BuChE from human plasma (34,35).

The possible appearance of a MGS in the monomers isolated by reduction was analyzed by measuring their fluorescence spectra and ANS binding. No significant differences in the maximum emission peak of intrinsic fluorescence between native and monomerized enzyme was measured, nor was enhancement of ANS fluorescence observed in inactive monomers (Fig. 2). These data suggested that the AChE subunits in inactive monomers were not in a MGS or in a denatured state, but in a 'native-like' conformation resembling the metastable state of *Torpedo* AChE exposed to mercurials (29). However, the AChE subunits in FBS monomers were in a long-lived inactive state, although their coexistence with active forms suggested that the native polypeptide structure was maintained in a fraction of the enzyme subunits.

Summarizing, the AChE subunits in hydrophilic tetramers were modified by Gdn exposure to yield tetrameric assemblies with subunits in a MGS, or dimeric forms with subunits in a denatured state, the extent of the structural changes depending on the denaturant concentration. Most enzyme tetramers were monomerized by reduction, the inactive subunits remaining in a quasi-native conformation. AChE tetramers with subunits in the MGS, denatured dimers and inactive monomers displayed amphiphilic properties probably by exposing to the aqueous environment the hydrophobic domains which were buried and/or external, but covered by subunit overlapping, in native enzyme.

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