Conformational Flexibility of the Acetylcholinesterase Tetramer Suggested by X-ray Crystallography*

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Acetylcholinesterase, a polymorphic enzyme, appears to form amphiphilic and nonamphiphilic tetramers from a single splice variant; this suggests discrete tetrameric arrangements where the amphipathic carboxyl-terminal sequences can be either buried or exposed. Two distinct, but related crystal structures of the soluble, trypsin-released tetramer of acetylcholinesterase from Electrophorus electricus were solved at 4.5 and 4.2 Å resolution by molecular replacement. Resolution at these levels is sufficient to provide substantial information on the relative orientations of the subunits within the tetramer. The two structures, which show canonical homodimers of subunits assembled through four-helix bundles, reveal discrete geometries in the assembly of the dimers to form: (a) a loose, pseudo-square planar tetramer with antiparallel alignment of the two four-helix bundles and a large space in the center where the carboxyl-terminal sequences may be buried or (b) a compact, square nonplanar tetramer that may expose all four sequences on a single side. Comparison of these two structures points to significant conformational flexibility of the tetramer about the four-helix bundle axis and along the dimer-dimer interface. Hence, in solution, several conformational states of a flexible tetrameric arrangement of acetylcholinesterase catalytic subunits may exist to accommodate discrete carboxyl-terminal sequences of variable dimensions and amphipathicity.

Differences in the molecular forms of the cholinesterases are the primary determinants of their tissue distribution and disposition within the cell; association of subunits also may govern the turnover of the enzyme (cf. Refs. 1 and 2). In mammals, the predominant form of acetylcholinesterase (AChE) in the central nervous system is an amphiphilic tetramer anchored to the membrane by a hydrophobic, noncatalytic subunit; at the neuromuscular junction, it is an asymmetric form containing 1–3 tetramers associated with the basal lamina by a collagen-like, structural subunit. Collagen-tailed forms are also predominant in the electric organ of the eel Electrophorus electricus. In the form containing three catalytic tetramers, three collagen-like subunits are disulfide-linked together, and each is attached to a tetramer in which two catalytic subunits forming a proximal dimer are disulfide-linked to the tail subunit and are associated with a peripheral dimer by quaternary interactions (3, 4) (Scheme 1).² Apart from the association as a dimer of disulfide-linked dimers, both amphiphilic and nonamphiphilic tetramers appear to form from a single splice variant. The eel AChE₉ subunits (EeAChE) present the capacity to form heteromeric quaternary associations (5). This suggests discrete tetrameric arrangements where the amphipathic carboxyl-terminal sequences (T peptides) are either buried or exposed.

Tetrameric arrangements of AChE subunits were observed in situ (6–8), and several models were proposed (4, 9–11); however, little structural information is available about the subunit orientation in the tetramer and the association of tetramers with anchor subunits. The crystal structure of recombinant mouse AChE (mAChE) revealed a compact, pseudo-square planar tetrameric arrangement of subunits (12); mAChE, however, lacks the carboxyl-terminal hydrophobic glycopospholipid or the amphipathic helix found on natural forms of AChE and is expressed as a monomer (13), a feature that poses the question of whether physiological forms of the enzyme would form a similar tetramer. In fact, crystals of EeAChE and diffraction experiments were reported earlier (14–16), but solution of a structure was precluded by the limited resolution achieved and failure in obtaining heavy atom derivatives, along with unavailability of a three-dimensional template for molecular replacement and of the primary structure of the eel species. Since then, crystal structures of Torpedo californica AChE (TcAChE) (17) and mAChE (18) were solved, and the cDNA-derived primary structure of EeAChE was determined (5).

Here we report two low resolution crystal structures solved from two distinct crystal forms grown in different conditions from the soluble, trypsin-released EeAChE tetramer. These structures reveal discrete but related tetrameric arrangements of catalytic subunits that are consistent overall with that found in the mAChE crystal and with arrangements observed in situ. Moreover, comparison of these arrangements suggests that, in solution, the AChE tetramer has significant conformational flexibility.

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‡ The abbreviations used are: AChE, acetylcholinesterase; EeAChE, E. electricus AChE; mAChE, recombinant mouse AChE; PAGE, polyacrylamide gel electrophoresis; TcAChE, T. californica AChE.

1 The atomic coordinates (code IC2B for structure A and code IC20 for structure B) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org ).

2 Scheme 1 is not intended to describe the crystal structures reported in this study.
**EXPERIMENTAL PROCEDURES**

*Materials—* The prepacked Superdex-200 HiLoad 26/60 column and the gel-filtration calibration markers were from Amersham Pharmacia Biotech. N-Tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (EC 3.4.21.4) and the protein molecular weight standards for SDS-PAGE were from Sigma. Other reagents and the salts used for crystallization were of the highest grade available.

*Purification and Analysis of the Tetramer—* EeAChE, as a mixture of asymmetric forms, was isolated from homogenized electric organs by affinity chromatography and subjected to tryptic cleavage as described previously (19). The released tetramer was purified by size-exclusion fast performance liquid chromatography (Amersham Pharmacia Biotech) on a Superdex-200 column equilibrated and eluted with 100 mM NaKPO₄, pH 7.5, 400 mM NaCl, 0.01% NaN₃ (w/v) at a flow rate of 0.5 ml min⁻¹ and at 20 °C. The purified tetramer was dialyzed against 100 mM NaKPO₄, pH 7.5, 100 mM NaCl, 0.01% NaN₃ (w/v) and concentrated to 10–15 mg ml⁻¹; it was stored on ice.

AChE activity measurements were conducted spectrophotometrically (20) with 0.5 mM acetylthiocholine and 0.33 mM dithiobis(2-nitrobenzoic acid) in 100 mM NaPO₄, pH 7.0, 0.1 mg ml⁻¹ bovine serum albumin (λ = 412 nm) (13).

SDS-PAGE was performed on homogeneous 7.5% gels using a PhastSystem apparatus (Amersham Pharmacia Biotech). The samples were boiled for 5 min in the presence of 2.5% (w/v) SDS with (reducing conditions) or without (nonreducing conditions) 5% (v/v) β-mercaptoethanol. Staining was by silver nitrate according to the manufacturer’s method number 210.

*Crystallization and Data Collection—* Crystallization was achieved by vapor diffusion using hanging drops of 4 μl and a protein-to-well solution ratio of 1:1. Form A crystals were grown at 4 °C using 1.1–1.5 mM NaKPO₄, pH 8.0–8.5, as the well solution; form B crystals were grown at 20 °C using 1.4 mM ammonium sulfate, pH 5.5–6.0, as the well solution. Data were collected at 20 °C on mounted crystals and using a 300 mm MarResearch imaging plate detector equipped with a Siemens rotating anode (50 kV × 80 mA). Oscillation images were integrated with DENZO (21) and scaled and merged with SCALA (22) (Table I). Amplitude factors were generated with TRUNCATE (23). Form A crystals belonged to the orthorhombic space group P2₂2₁ with unit cell dimensions a = 118 Å, b = 215.9 Å, c = 229.4 Å, giving a Vₐₐₐ value of 4.5 Å³/Da (73% solvent) for one EeAChE subunit (~80 kDa) in the asymmetric unit (24). Form B crystals belonged to the monoclinic space group C2 with unit cell dimensions a = 211 Å, b = 129.7 Å, c = 195.4 Å (β = 103.2°), giving a Vₐₐₐ value of 4.1 Å³/Da (69.5% solvent) for one EeAChE tetramer (~320 kDa) in the asymmetric unit.

*Structure Solution and Refinement—* Initial phases for structure A were obtained by molecular replacement using the EeAChE subunit from the mAChE structure (Protein Data Bank code 1MAA) (12) as a search model with the AMoRe program package (25) (Table I). The phases calculated from a positioned catalytic subunit were improved by solvent flattening using program DM (23) and a mask built around the subunit. The same procedure with additional averaging was used for structure B. For the two structures, rigid body refinement was applied to the whole subunit using the program CNS (26). Accuracy of the structures was further checked by omitting from the starting model and before rigid body calculation the entire 17-residue helix α₁₀ (mAChE residues 526–543), a key structure element for the formation of the dimer four-helix bundle (12, 18); difference electron density maps calculated after rigid body refinement unambiguously revealed the presence of helix α₁₀ (cf. Fig. 4).

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Figs. 2, 3, and 4 were generated with the ALSCRIPT (27), RIBBON (28), and TURBO-FRODO (29) programs, respectively.

**RESULTS AND DISCUSSION**

*Characterization of the Purified EeAChE Tetramer—* The trypsin-released tetramer eluted from the gel filtration column as a single, symmetric absorbance peak of apparent mass of ~360 kDa; analysis of the specific AChE activity throughout the peak suggested functional homogeneity of the tetramer (data not shown). However, electrophoretic analysis suggested structural heterogeneity. Indeed, although SDS-PAGE performed in reducing conditions yielded the three broad bands of ~80, 50, and 30 kDa, characteristic for reduced EeAChE (30, 31), SDS-PAGE performed in nonreducing conditions yielded a weak, thin band of ~320 kDa representing residual tetramer and two pairs of closely migrating, intense bands in a ratio of about 1:1 and average apparent masses of ~160 and 80 kDa, values consistent with dimers and monomers, respectively (Fig. 1). The purified tetramer thus appears not only to be composed of two equal populations of dimers differing slightly in their masses (as expected from a dimer of dimers where one set of disulfides links with the residual tail and the other set forms between monomers; cf. Scheme 1) but also to contain, in the same proportion, two equal populations of slightly different monomers. Trypsin cleaves peptides and proteins at the ester linkages of arginines and lysines, two residues that are found upstream of the linking cysteines not only in the amino-terminal end of the tail (32, 33) but also in the carboxyl-terminal end of the EeAChE catalytic subunit (T peptide, Fig. 2) (5). In the presence of SDS, dimers that lack the disulfide dissociate into monomers. Hence, the EeAChE tetramer subjected to crystallization is composed of two equal populations of covalent and noncovalent dimers, either proximal or distal to the tail.

The apparent mass of the EeAChE tetramer estimated from chromatography is ~12% higher than that estimated from
electrophoresis, a difference that suggests either dimensional asymmetry or a high level of hydration of the tetramer (or both). Substantial dimensional asymmetry was reported earlier for the EeAChE tetramer (3), the homologous 11S TcAChE species (34), and the fetal bovine serum AChE tetramer (35). Alternatively, two highly solvated crystal forms were grown from the purified EeAChE tetramer (cf. “Experimental Procedures”).

Tetrameric Arrangements of EeAChE Subunits

For each of the two EeAChE structures herein reported, the low resolution achieved does not reveal details in the positions of the side chains in the catalytic subunit nor in those located at the subunit interfaces. As well, structure elements that are unique to the eel enzyme, peptide Ile418–Gln446 and the carboxyl-terminal T peptide Glu571–Leu610 (5) (Fig. 2), are not Cys256(257)–Cys267(272), which is located between helices roughly perpendicular to the four-helix bundle axis, loop in EeAChE (13, 37). In EeAChE structure A, the two dimers are aligned antiparallel, and the main axes of the two dimers may interact with the peripheral site regions of the facing two subunits, a situation reminiscent of the recently reported intersubunit interaction of mAChE (38–40). The EeAChE-specific peptide Ile418–Gln446, which is located between the very long helix α12, and the short strand β8 of a subunit, protrudes at the surface of the subunit and to the solvent, an exposition that, along with the high flexibility imparted by the high content in Gly residues, likely accounts for the absence of density in these regions.

Structure B—In EeAChE structure B, the two dimers arrange as a compact, square nonplanar tetramer, also of a marked dimensional asymmetry (Fig. 3). Compared with structure A, the tetramer folds as to position the two four-helix bundle axes 60° from each other, and one dimer rotates relative to the second one by ~40° as to reorient the main axes of the two dimers antiparallel. At the dimer-dimer interface, which now extends in a direction perpendicular to the plane made by the two four-helix bundle axes, the facing loops Cys256/257, Cys267/272 are still separated by 13 Å, consistent with bridges possibly imparted by the facing oligosaccharide moieties linked to Asn161(162) and Asn260(265), and the four Thr570(543) residues, which are terminal to helices α10, by 32 Å.

In tetramer B, the axes of the EeAChE active center gorges are oriented antiparallel within a dimer but are tilted by 120° from a dimer to the second one. Of the four peripheral sites, two, from diagonally opposed subunits, are exposed at the surface of the assembly and are freely accessible to the outside solvent; the other two peripheral sites, from the second pair of diagonally opposed subunits, face the tetramer internal space. A symmetric situation applies to the four peptides Ile118–Gln446 of which two are exposed to the outside solvent and are disordered and two face the internal space. As a result, residue Val147, at the same position as mAChE-Ala420 in subunit A in the first dimer, is separated by 17 Å from peripheral site residues Tyr336(341) and Trp281(286) at the gorge entrance of subunit C in the second dimer. The presence of a weak, loop-shaped electron density in this region between subunits A and C suggests that, in the internal space, the peptide undergoes stabilizing interactions. The same weak density is observed between the peptide of subunit D and the peripheral site region of subunit B. Hence, in tetramer B, peptides Ile418–Gln446 of two subunits may interact with the peripheral site regions of the facing two subunits, a situation reminiscent of the recently reported intersubunit interaction of mAChE loop Cys257–Cys272, also rich in Gly residues, with the facing peripheral site...
As a result, the surface area buried at the dimer-dimer interface is larger, and the space between the two dimers is 2-fold narrower than in tetramer A.

The Carboxyl-terminal T Peptides

The EeAChE carboxyl-terminal T peptide, Glu571(544)–Leu610, located in the extension of helix $\alpha_{10}$, has no counterpart in the crystallized mAChE and TcAChE. Indeed, monomeric mAChE was generated as a soluble enzyme truncated after residue 548 (13), whereas dimeric TcAChE was enzymatically solubilized from a dimer of AChE subunits (41). The T peptide, containing 40 residues, of which nine are hydrophobic residues conserved among cholinesterases, was predicted to be organized as one or several helical regions forming one or two four-helix bundles (9, 10).

The amphipathic character of the T peptide precludes its full exposition to the solvent and total disorder. In addition, in contrast to the large surface area buried at the mAChE dimer-dimer interface (12), the limited surface areas buried at the dimer-dimer interfaces of the EeAChE tetramers appear insufficient for cohesion of the two dimers (42). These constraints, along with the large internal space observed in each of the two tetramers, raise the questions of the positions of the four T peptides in the EeAChE structures and of their contribution to the dimer-dimer interface. In the loose tetramer A, the four carboxyl-terminal T peptides could either be buried at the center of the arrangement, as proposed based on a square-planar model for association of nonamphiphilic AChE subunits (10), or pair off and exit on either side of the tetramer plane, as recently suggested from analysis of the compact, pseudo-square planar tetramer of crystalline mAChE (12). In the folded, compact tetramer B, the four T peptides could be exposed on the same side of the arrangement, roughly similar to a recent model for tetrameric human butyrylcholinesterase (9). Either option would be expected to stabilize the four T peptides and provide the tetramer with the locking points, internal or external to the arrangement, that are required for dimer-dimer cohesion.

Actually, an additional density is apparent in the EeAChE structures, which is made of a loop-shaped portion located in the extension of helix $\alpha_{10}$ in the internal space and followed by a rod-shaped portion aligning antiparallel to helix $\alpha_{10}$; the same density is found at the end of each of the four helices $\alpha_{10}$ and on either sides of each of the two four-helix bundles in tetramer A (Fig. 4). It is also found in the corresponding regions of tetramer B (not shown). The good fit of the mAChE carboxyl-terminal segment Ala544–Ala547 into the loop-shaped portion and of a theoretical 10-residue $\alpha$-helix into the rod-shaped portion suggests that this density represents the T peptide amino terminus up to (about) residue Phe584. Hence, in a dimer, the amino termini of two T peptides pack against the dimer four-helix bundle, made of helices $\alpha_{7,8}$ and $\alpha_{10}$ from two subunits, perhaps to form a six-helix bundle. The orientation of the T peptide amino terminus appears to dictate the exit of the rest of the T peptide out of the tetramer in the extension of the bundle, where tiny density is detected. This suggests that the disulfide set that links one dimer with the residual tail and the disulfide that forms between monomers are distal to each other, a geometry consistent with the earliest models for AChE tetramers (cf. Fig. 11 in Ref. 4). In the crystallized EeAChE tetramers, however, some of the T peptides may be truncated carboxyl-terminal to residue Arg586 (cf. above); hence in the crystals, the residual T peptide amino-terminal parts may undergo stabilizing interactions that differ from those occurring in the physiological tetramer. Because of the current resolutions, second solutions of the two structures, alternative to tetramers A and B, might also be considered; however, the higher number of putative contacts at the dimer-dimer inter-
Fig. 3. Overall views of the loose, pseudo-square planar EeAChE tetramer A and the compact, square nonplanar EeAChE tetramer B. Top left, ribbon diagram of tetramer A viewed perpendicular to the four-helix bundle axis; top right, tetramer A viewed parallel to the four-helix bundle axis, 90° from the left view. Bottom left, ribbon diagram of tetramer B viewed perpendicular to the four-helix bundle axis of dimer AB (which is oriented as in the top left panel and is masked by dimer CD); bottom right, tetramer B viewed perpendicular to the dimer-dimer interface, 90° from the left view. The italicized labels A and B refer to the subunits in the left dimer and labels C and D to subunits in the right dimer for the top left and bottom right orientations. In tetramer A (overall dimensions: 132 Å × 132 Å × 55 Å), the main axes of the two dimers are tilted by ~40° from each other and the axes of the two four-helix bundles, made of helices α_1,α_8 and α_10, from two subunits and displayed in black, are aligned antiparallel with convergent helices α_10. The four peripheral sites, of which two from diagonally opposed subunits are oriented above the plane of the figure (circled), are accessible to the outside solvent; the apparent free space in the center of tetramer A is 75 Å long × 35 Å wide. In tetramer B (130 Å × 100 Å × 55 Å), the main axes of the two dimers are aligned antiparallel and the two four-helix bundle axes are positioned 60° from each other with convergent helices α_10 of the four peripheral sites, two are accessible to the outside solvent (circled) and two face the central, internal space (75 Å × 15 Å). For each of the two structures, the backbone shown is that of mAChE but residue numbering is that of EeAChE (cf. Fig. 2); the position of peptide Ile^{418}–Gln^{446}, which is unique to EeAChE and has not been modeled, is indicated by an arrow; the putative N-glycosylation sites are indicated by black spheres; and the labels N and C indicate the amino and carboxyl termini of subunit A, respectively.
faces and striking resemblance with the subunit arrangement found in the mAChE structure (12) support tetramers A and B as the more convincing solutions.

A Flexible AChE Tetramer?

Overall, each of EeAChE tetramers A and B is consistent not only with the tetrameric arrangement of subunits of crystalline mAChE (12) but also with tetrameric arrangements observed in situ (cf. Fig. 2c in Ref. 6). Also, a structure similar to structure A but showing a larger tilt (by −12°) of the two dimers in the tetramer was recently solved by Raves et al. (43) (Protein Data Bank code 1EEA) from a data set earlier collected on a crystal of the large peripheral site ligand, fasciculin (16). The existence of two extreme conformational states of an EeAChE tetramer undergoing, in solution, significant flexibility about the four-helix bundle axis and along the dimer-dimer interface axis. Most importantly, the possibility for several conformations of an overall tetrameric but malleable arrangement of AChE subunits would make the tetramer able to fit either of the carboxyl-terminal sequences, differing in length and amphipathic character, which characterize the diverse AChE molecular forms. Whether this flexibility is also related to regulation of catalysis is unknown. Because the high solvent content of the crystal forms used in this study precludes achievement of a higher resolution structure, even if synchrotron radiation were used, efforts to grow crystal forms of a higher diffraction potency are underway.

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A Flexible Acetylcholinesterase Tetramer?

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