Molecular assembly and biosynthesis of acetylcholinesterase in brain and muscle: the roles of t-peptide, FHB domain, and N-linked glycosylation

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INTRODUCTION

Cholinesterases (ChEs) are serine hydrolases that preferentially act on choline esters. Vertebrates possess two types of cholinesterases (ChEs), corresponding to two distinct genes: acetyl cholinesterase (AChE, EC 3.1.1.7) and butyryl cholinesterase (BChE, EC 3.1.1.8). These two enzymes are distinguished on the basis of the substrate specificities and their sensitivities to selective inhibitors (Mendel and Rudney, 1943; Austin and Berry, 1953). The primary function of AChE is to efficiently hydrolyze the neurotransmitter acetylcholine (ACh) at cholinergic synapses (Massoulié et al., 1993), whereas the physiological function of BChE in vertebrates remains a question of different speculations. Studies of AChE knockout mice suggested that BChE can partially compensate for the absence of AChE in the nervous system (Xie et al., 2000; Dysen et al., 2001); therefore BChE could hydrolyze acetylcholine functionally. Poisoning by ChE inhibitors, such as insecticides or nerve gas, results in accumulation of ACh, and uncontrolled activation of cholinergic receptors, which causes cholinergic crisis and potentially leads to death (Feyereisen, 1995; Bajgar, 2004). On the other hand, controlled treatment with ChE inhibitors are used in therapeutics for patients suffering from myasthenia gravis (Brenner et al., 2008; Mehndiratta et al., 2011), Alzheimer’s disease (Giacobini, 2000; Stone et al., 2011), and Parkinson’s disease (Hutchinson and Fazzini, 1996; Emre et al., 2004).

Acetylcholinesterase (AChE) is responsible for the hydrolysis of the neurotransmitter, acetylcholine, in the nervous system. The functional localization and oligomerization of AChE T variant are depending primarily on the association of their anchoring partners, either collagen tail (ColQ) or proline-rich membrane anchor (PRiMA). Complexes with ColQ represent the asymmetric forms (A12) in muscle, while complexes with PRiMA represent tetrameric globular forms (G4) mainly found in brain and muscle. Apart from these traditional molecular forms, a ColQ-linked asymmetric form and a PRiMA-linked globular form of hybrid cholinesterases (ChEs), having both AChE and BChE catalytic subunits, were revealed in chicken brain and muscle. The similarity of various molecular forms of AChE and BChE raises interesting question regarding to their possible relationship in enzyme assembly and localization. The focus of this review is to provide current findings about the biosynthesis of different forms of ChEs together with their anchoring proteins.

Keywords: acetylcholinesterase, butyrylcholinesterase, assembly, membrane trafficking, glycosylation

In vertebrates, AChE gene produces several types of coding sequences differing in an alternative choice of splice acceptor sites in the 3′ region. This process generates different AChE isoforms, named AChE_R, AChE_II, and AChE_T (Massoulié, 2002). They contain the same catalytic domain, but are associated with distinct C-terminal peptides. In contrast, BChE gene produces single type of transcript and generates single type of isoform BChE_T (Blong et al., 1997). AChE and BChE are well-known for their multiple molecular forms that have their specific localizations: AChE_R is a soluble monomer that is up-regulated in the brain under stress stimulation (Kaufe et al., 1998; Perrier et al., 2005); AChE_II is a glycosylphosphatidylinositol-anchored dimer that is mainly expressed in red blood cells (Li et al., 1991); AChE_T and BChE_T are present in collagen-tailed forms at the neuromuscular junction (nmj) and hydrophobic-tailed forms in the brain (Legay et al., 1995; Blong et al., 1997; Massoulié et al., 2005). The molecular forms of AChE_T and BChE_T in brain and muscle are of particular interest because they are associated with their anchoring proteins: collagen Q (ColQ) or proline-rich membrane anchor (PRiMA). Complexes with ColQ represent the collagen-tailed or asymmetric (A) forms in muscle (Krejci et al., 1997), while complexes with PRiMA represent membrane-bound tetrameric globular form (G4), mainly in brain (Perrier et al., 2002, 2003; Xie et al., 2009) and muscle (Xie et al., 2007). In addition, mixed cholinesterases, ColQ-linked AChE–BChE_A12 hybrid enzyme (Tsim et al., 1988a) and PRiMA-linked AChE–BChE_G4 hybrid enzyme (Chen et al., 2010) that contain both AChE and BChE homodimers in a single molecule are being found in avian system. Here, we summarized the recent studies on the assembly of oligomeric AChE and BChE, as well as the regulation of AChE and/or BChE biosynthesis in neurons and muscles.

Abbreviations: A, asymmetric; ACh, acetylcholine; AChE, acetylcholinesterase; BChE, butyrylcholinesterase; ChEs, cholinesterases; ColQ, collagen tail; Endo H, endoglycosidase H; ER, endoplasmic reticulum; FHB, four-helix bundle; G4, tetrameric globular form; nmj, neuromuscular junction; PRAD, proline-rich attachment domain; PRiMA, proline-rich membrane anchor; WAT, tryptophan amphiphilic tetramerization.
MOLECULAR FORMS OF AChE AND BChE IN BRAIN AND MUSCLE

PRiMA-LINKED GLOBULAR FORMS OF ChEs

In *Xenopus* oocytes, COS-7 cells, neuroblastoma cells, and muscle cells, the expression of PRiMA has been identified as a limiting factor in organizing G4 AChE and targeting it to the cell membrane (Perrier et al., 2002; Xie et al., 2007), as well as in directing its membrane raft localization (Xie et al., 2010a). In the absence of PRiMA, the G4 AChE, or the G4 BChE, could not be formed. In the brain, the catalytic subunit contained in G4 AChE is AChET (Inestrosa et al., 1994). The expression of PRiMA mRNA and protein are increased with an increment of G4 AChE during the development of brain and spinal cord (Leung et al., 2009; Xie et al., 2010b).

The PRiMA-linked AChET is also present at the nmj, in which both motor neuron and muscle are the major suppliers. In rat muscles, the protein expression of PRiMA and AChET, as well as the G4 AChE, are dramatically increased during development (Leung et al., 2009). The parallel expression of PRiMA and G4 AChE during the development strengthens the importance of PRiMA in directing, and/or regulating the formation of G4 enzyme. The PRiMA-linked G4 BChE however has not been well studied, and therefore the expression profile of which in brain or muscle are not fully revealed.

ColQ-LINKED ASYMMETRIC FORMS OF ChEs

Asymmetric forms of AChE and BChE are characterized by the presence of collagen-like tail, which is formed by the triple helical association of three ColQ subunits (Feng et al., 1999). The cDNA encoding ColQ has been cloned in *Torpedo* (Krejci et al., 1991), rat (Krejci et al., 1997), human (Ohno et al., 1998), quail, and chicken (Ruiz and Rotundo, 2009a). The presence of the collagen-tailed forms of ChEs has been found in all classes of vertebrates, but not in invertebrates. They are specifically expressed in muscles and regulated by physiological activity (Sketelj and Brzin, 1985; Deprez et al., 2003; Lau et al., 2008). In human and rat, *COLQ* gene has two transcripts, ColQ-1 and ColQ-1a; they are differentially expressed in slow-twitch (ColQ-1) and fast-twitch (ColQ-1a) muscles (Ohno et al., 1998; Krejci et al., 1999). The differentiated expression patterns may account for the synaptic and non-synaptic expression profile of ColQ-linked AChE in fast slow-twitch and fast-twitch muscles, respectively (Lee et al., 2004; Crne-Finderle et al., 2005; Choi et al., 2007).

EXISTENCE OF AChE–BChE HYBRID ChEs

Most of our knowledge concerning ChEs derives from the studies on the classical AChE and BChE homogenous oligomers. However, the sequence similarity between AChET and BChET has been further emphasized regarding the existence of hybrid A12 forms in new-born chicken muscle (Tsim et al., 1988a), as well as a hybrid G4 from in the brain (Chen et al., 2010) and embryonic muscles of chicken (Chen et al., 2009). In these hybrid enzymes, both AChET and BChET are attached to the same anchoring protein, ColQ for A12 form and PRiMA for G4 form (Figure 1). The hybrid enzyme exists as a single type, with equivalent number of AChE and BChE catalytic subunits in a single molecule. Interestingly, the expression of these hybrid molecules in chicken brain and muscle was found to be developmentally regulated. In 1-day-old chicken muscle, the predominant form of AChE is A12 hybrid form; however, the proportion of BChE subunits in the hybrid molecules progressively disappear during the muscle development from embryonic, hatching to adult, and the homogeneous asymmetric AChE becomes the sole form upon the muscle maturation (Tsim et al., 1988b). On the other hand, a continuous increase of AChE–BChE G4 hybrid expression was observed in chicken brain, while an obvious decrease was found in the leg muscles. To date, we do not have conclusive idea about the underlying regulatory mechanism of these hybrid enzymes. We believe that AChE–BChE hybrid enzymes could take part in cholinergic functions, which includes (i) they carry the catalytic activities of AChE and BChE in hydrolyzing ACh; (ii) they are associated with the anchoring protein, PRiMA or ColQ, which can anchor the hybrid enzymes onto the plasma membrane in the brain or nmj in the muscles; and (iii) the level of BChET indirectly regulates the expression of homogenous G4 AChE.

In fact, the notion of having the existence of AChE–BChE hybrid enzyme is not new. An abnormal ChE species in the serum of patients suffering from carcinomas was reported (Zakut et al., 1988). This abnormal ChE species in human serum was inhibited by both AChE inhibitor BW284c51 and BChE inhibitor iso-OMPA. In addition, a collagen-tailed asymmetric hybrid AChE has been found relatively abundant in young chicken muscle (Ts tim et al., 1988b), but which tends to disappear at the adult stage (Tsim et al., 1988b). Moreover, a hybrid tetramer having AChE and BChE activity was also found in cyst fluids derived from a human astrocytoma (García-Ayllón et al., 2001). However, none of the physiological function of these abnormal ChEs has been elucidated.

ASSEMBLY MECHANISM OF AChE AND BChE

The presence of G4 and A12 hybrid ChEs raised interesting questions about the organization of the subunits in a hybrid ChE complex. The assembly of AChE and BChE in cells could provide a good model in revealing the protein assembly of oligomers. By using DNA transfected cell cultures, the organization of different subunits in the PRiMA-linked ChE tetramers has been studied (Chen et al., 2010). Interestingly, AChET and BChET could not form hybrid dimer in the absence of anchoring protein; on the
other hand, a single type of hybrid tetramer was clearly observed when the two catalytic subunits were co-expressed with PRiMA. Therefore, a "2 + 2 + 2" model is proposed for the organization of the four catalytic subunits in the PRiMA-linked ChE tetrarers (Figure 2). After protein synthesis, AChE-T and BChE-T spontaneously form AChE homodimer and BChE homodimer first. When two dimers (two AChE-T dimers, or two BChE-T dimers, or one AChE-T dimer plus one BChE-T dimer) encounter the anchoring protein, PRiMA, and thus ChE tetrarers, e.g., G4 AChE, G4 BChE, and G4 AChE/BChE hybrid, are formed.

T-PEPTIDE IS NECESSARY FOR THE OLIGOMERIZATION OF AChE-T AND BChE-T
AChE-T and BChE-T have a catalytic domain of approximately 500 amino acids, followed by a C-terminal t-peptide of 40 and 41 residues, respectively. The t-peptide on AChE-T and BChE-T presents a considerable sequence similarity, with 24 identical residues, including seven aromatic residues and one cysteine near the C-terminus, which are conserved in human, cat, rabbit, mouse, cow, rat, chicken, and Torpedo (Massoulie et al., 1993).

The t-peptide was reported to play an important role in the biosynthesis of ChEs, particularly in the protein folding and exportation. The presence of aromatic residues in the t-peptide induces the misfolding of newly synthesized AChET polypeptides, and this effect depends on the hydrophobic character of these residues, because the same effect occurs when they are replaced by leucines (Falasca et al., 2005). In the absence of a proline-rich attachment domain (PRAD)-containing anchoring protein, the t-peptide enhanced a pool of AChET molecules toward endoplasmic reticulum (ER)-associated degradation (Belbeoch et al., 2003).

The major function of t-peptide is directing the assembly of tetrarers of AChE-T (Bon and Massoulie, 1997) and BChE-T (Blong et al., 1997), as well as the association with the structure proteins, ColQ and PRiMA (Krejci et al., 1997; Perrier et al., 2002; Bon et al., 2004). The t-peptide is also named as tryptophan (W) amphiphilic tetramerization (WAT) domains, which contains a sector with seven aromatic residues that are strictly conserved between AChET and BChET. The association between AChE-T or BChE-T catalytic subunits and anchoring proteins, ColQ and PRiMA, is mostly based on the interaction between four WAT domains on the t-peptides and a PRAD on ColQ or PRiMA (Bon et al., 1997; Dvir et al., 2004; Noureddine et al., 2007). A crystallographic analysis of the complex of synthetic t-peptide and PRAD peptide indicated that four α-helical t-peptide form coiled-coil structure around the PRAD, which is arranged in a poly-proline II helix (Dvir et al., 2004). In addition, the formation of disulfide bonds through the cysteine residues near the end of the t-peptides stabilizes the quaternary association, and in fact this association appears to be critical in the case of AChE dimer formation. Dimers could be hardly observed after mutagenesis of the C-terminal cysteine residues in H or T- peptides of Torpedo and rat AChE (Morel et al., 2001; Chen et al., 2010).

The importance of t-peptide in the assembly of AChE-T and BChE-T was also reported by DNA mutagenesis studies. The truncated mutants, AChEAT and BChEAT, in which the t-peptides were deleted from the catalytic subunits, produced only monomers (Duval et al., 1992). AChE-T and AChEβChET, BChE-T and BChEαChET-T, in which the catalytic domain of each enzyme was swapped with the t-peptide of each other, presented similar assembly ability to form oligomers (Liang et al., 2009; Chen et al., 2010).

THE FHB DOMAIN IS INVOLVED IN THE SELECTION OF CATALYTIC SUBUNITS DURING DIMERIZATION
Although the nature of AChE-T and BChE-T oligomers depends on the presence of the t-peptides, the catalytic domains also influence the oligomerization patterns. The X-ray crystallography studies of Torpedo AChE dimers showed that the contact zone between two AChET subunits could be a “four-helix bundle” (FHB), formed by two α-helices from each catalytic domain (Sussman et al., 1991). In addition, rat AChE-T was also demonstrated to dimerize through FHB inter-subunit contact zone (Morel et al., 2001). Based on these findings, we compared the predicted FHB sequences that are responsible for the dimeric contact zone of AChET and BChET from different species. Indeed, FHB domains are highly conserved across different species for either AChE or BChE, including human, mouse, rat, chicken, and Torpedo (Figure 3). On the other hand, the similarity between FHB domains of AChE-T and BChET is very low. An inter-species hybrid dimer could be formed between human AChET and chicken AChET, but not between mammalian AChET and BChET, in transfected HEK293T cells (Chen et al., 2010). The selectivity of dimerization seems to be based on the feature that the FHB domains of AChET are highly conserved among different species of vertebrates, but are distinguished from vertebrate BChETs. Moreover, another hybrid dimer, between human AChEβChET and chicken AChET, which contained the similar FHB but different t-peptides, was formed when they were co-expressed together in HEK293T cultured cells (Chen et al., 2010). This further confirmed that the catalytic domains, possibly the FHB domains, should play a critical role in the selection of subunits during the dimerization of ChEs.

According to our current knowledge, the oligomerization of AChE-T or BChE-T with their associated anchoring proteins could rely on three types of interactions (Figure 4): (i) the FHB interaction for the formation of dimer through hydrophobic interaction; (ii) intercatenary disulfide bonds between the t-peptide of AChET or BChET subunits; and (iii) tight hydrophobic interaction between the WAT domains of AChET or BChET subunits and the PRAD on PRiMA or ColQ.
**FIGURE 3** | Comparison of FHB sequences of AChET and BChET among different species. The sequences of the two alpha helices (FHB-1 and FHB-2) forming the dimeric contact zone of AChE and BChE are shown. The residues conserved across species are highlighted in bold. The amino acid sequences of human, mouse, rat, chicken, and *Torpedo* AChE catalytic subunits were deduced from nucleotide sequences accessed from GenBank™ AAA68151, CAA39867, EDM13278, P36196, and CAA27169, respectively. The amino acid sequences of human, mouse, rat, and chicken BChE catalytic subunits were deduced from nucleotide sequences accessed from GenBank™ AAA99296.1, AAH99977, NP_075231, and NP_989977, respectively.

**FIGURE 4** | Three types of interaction involved in the oligomerization of AChE and BChE. (A) The FHB domains of AChE or BChE direct the dimer formation. (B) The t-peptides of AChE or BChE form intercatenary disulfide bonds. (C) WAT domains of AChET and BChET interact with PRAD on PRiMA or ColQ.

**N-GLYOSYLATION IS NOT REQUIRED FOR THE ASSEMBLY OF AChET BUT IS REQUIRED FOR THE MEMBRANE TRAFFICKING**

AChET and BChET are well-known highly glycosylated enzymes, which carry various amounts of N-linked carbohydrate side chains attached to their core polypeptides (Liao et al., 1992; Kolarich et al., 2008). Mature human AChET monomer possesses three potential N-linked glycosylation sites (Soreq et al., 1990; Velan et al., 1993). Mature human BChET carries nine potential N-linked glycosylation sites (Lockridge et al., 1987). These N-linked glycosylation sites, both in AChE and BChE, are highly conserved in mammals, which implies the physiological importance of these glycans for ChEs.

Glycosylation is proposed to be used as a marker for the progression of ChE forms through different subcellular compartments, since it is known that the glycans added in ER are remodeled and matured in Golgi apparatus. In chicken muscles, the assembly of catalytically active dimer and tetramer occurred in the rough ER, with a subset of tetramers being further assembled with ColQ in Golgi apparatus into asymmetric forms (Rotundo, 1984). Once assembled, these catalytically active AChE oligomers were stable, acquired complex oligosaccharides in Golgi apparatus, and were transported to plasma membrane or secreted into medium (Rotundo, 1984). All of these exported AChE molecules contain complex oligosaccharides, because they could bind to lectins such as wheat germ agglutinin and ricin, and were endoglycosidase H (Endo H) resistant (Rotundo et al., 1989). In contrast, 70–80% of the newly synthesized AChE polypeptide chains in chicken muscle appeared to be catalytically inactive and Endo H sensitive, and they were degraded intracellularly with a half-life of about 1.5 h (Rotundo, 1988). Recently, Ruiz and Rotundo (2009a,b) reported that the expression of AChE in quail muscles is regulated by muscle activity through post-translational controls: the over-expression of ER molecular chaperons, such as calnexin, ER protein 72, and protein disulfide isomerase results in an increase of catalytic active ColQ-linked AChE in quail muscles.

The biological function of the glycans on ChEs was elucidated by site mutagenesis studies. Elimination of N-glycosylation sites did not interfere with the ability of AChET to form a soluble dimer (Velan et al., 1993), or to assembly with PRiMA to form a PRiMA-linked AChE tetramer (Chen et al., 2011). It appears therefore that the oligosaccharide side chains do not affect the structural elements that are responsible for the interaction of different subunits. Indeed, none of the N-glycosylation sites on AChE is close to or within the FHB domain implied in dimerization of AChE subunits (Sussman et al., 1991; Morel et al., 2001), or the t-peptide that allows the oligomerization of AChET with the anchoring
proteins, e.g., ColQ and PRiMA (Bon et al., 2004). Moreover, the glycosylation of AChET can greatly affect the protein folding and membrane trafficking. When the glycosylation is eliminated, the folding of AChET fails, leading to a severe loss of the enzymatic activity (Chen et al., 2011). In the absence of glycosylation, the secreted G₁ and G₂ AChE are dramatically reduced in transfected cells, and the PRiMA-linked G₄ AChE is retained in ER and fails to be exported to plasma membrane (Chen et al., 2011).

The importance of N-glycosylation in the biosynthesis of AChE could explain the abnormality of glycosylation status in some pathological conditions. Proper glycosylation of AChE is important for normal brain function. Accumulation of molecular forms of AChE with altered patterns of glycosylation has been observed in the brain and cerebrospinal fluid of Alzheimer’s patients (Sáez-Valero et al., 1999, 2000). Moreover, characteristics of AChE found in the senile plaques are different from those in normal brain with a higher degree of glycosylation, which is proposed to be one of the factors facilitating formation of amyloid fibrils in the senile plaques (Mimori et al., 1997). These abnormalities in the glycosylation of AChE are very specific for Alzheimer’s disease and are not detected in other dementia illness, which suggests that glycosylation of AChE may have a diagnostic value.

![Proposed model for the assembly and membrane processing of G₄ ChEs.](https://www.frontiersin.org)

**FIGURE 5** | Proposed model for the assembly and membrane processing of G₄ ChEs. G₄ AChE, G₄ AChE-BChE hybrid, and AChE-BChE G₄ hybrid molecules are assembled in ER where both AChE and BChE subunits have initial glycosylation. These G₄ complexes are subsequently transported to Golgi apparatus where the catalytic subunits can have further glycosylation, and finally anchored onto the plasma membrane. The AChE glycosylation mutant, in which the glycosylation is completely abolished, is still able to assemble with PRiMA and BChE to form G₄ AChE and G₄ hybrid. However, both of them are retained in ER, which possibly will be subjected to the degradation pathway.
To summarize the assembly and membrane processing of G4 ChEs, a model is being proposed as Figure 5. After the mRNAs are translated into peptides, AChET and BChET polypeptides undergo initial glycosylation, presumably in a co-translation manner. Shortly after the synthesis, the glycosylated AChET and BChET are assembled into homodimers spontaneously. When these homodimers encounter PRiMA, they form PRiMA-linked G4 AChE, or G4 BChE, or AChE–BChE G4 hybrid in ER. Afterward, PRiMA targets these G4 complexes to Golgi apparatus where AChET and BChET subunits have further glycosylation. This trafficking allows the G4 enzymes to be fully functional and finally become anchored onto the plasma membrane. During the whole process, the proper glycosylation of AChET is the key point for the membrane targeting. Without glycosylation, AChET polypeptides cannot fold properly, resulting in inactive AChE molecules. These un-glycosylated and inactive AChET molecules can still form PRiMA-linked G4 AChE and BChE–BChE G4 hybrid, but both of them are retained in ER, failing to be exported to Golgi apparatus, and finally they are subjected to degradation most possibly.

SUMMARY

The assembly of ChEs constitutes a fascinating model to study numerous biological processes, such as post-translational modification, protein–protein interactions, membrane trafficking, and protein degradation. The physiological function of ChEs depends on the catalytic property of the enzymes and the restricted subcellular localization. In brain and muscles, ChEs display extremely rich molecular polymorphisms, possessing soluble, membrane-bound and basal lamina-anchored forms, and additionally, hybrid ChEs containing both AChE and BChE catalytic subunits also curiously exist. During the assembly of these ChE complexes, dimer is believed to be the precursor for the PRiMA-linked tetramers and ColQ-linked asymmetric forms. The dimer formation of AChE or BChE depends on recognition between the FHB domains in their catalytic domains, and the assembly of tetramers with PRiMA or ColQ requires the interaction of WAT domain on the C-terminal t-peptides with the PRAD domain on PRiMA or ColQ. N-linked glycans of AChE are employed as both maturation and quality control tags that dictate the destination of the enzyme being exported or not, which inspires that the control of glycosylation may be as a means in regulating the level of functional AChE in pathological conditions. However, there are still several questions out there, which have not been resolved. These questions are: (i) the possible control mechanism in directing the formation of different forms of ChEs; (ii) the regulatory mechanism for the protein trafficking of different states of ChEs; (iii) the fate of the active and inactive ChEs; and (iv) the possible non-cholinergic function of different forms of ChEs.

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