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Processing of Cholinesterase-like α/β -Hydrolase Fold Proteins: Alterations Associated with Congenital Disorders

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Abstract

The α/β hydrolase fold family is perhaps the largest group of proteins presenting significant structural homology with divergent functions, ranging from catalytic hydrolysis to heterophilic cell adhesive interactions to chaperones in hormone production. All the proteins of the family share a common three-dimensional core structure containing the α/β -hydrolase fold domain that is crucial for proper protein function. Several mutations associated with congenital diseases or disorders have been reported in conserved residues within the α/β -hydrolase fold domain of cholinesterase-like proteins, neuroligins, butyrylcholinesterase and thyroglobulin. These mutations are known to disrupt the architecture of the common structural domain either globally or locally. Characterization of the natural mutations affecting the α/β -hydrolase fold domain in these proteins has shown that they mainly impair processing and trafficking along the secretory pathway causing retention of the mutant protein in the endoplasmic reticulum. Studying the processing of α/β -hydrolase fold mutant proteins should uncover new functions for this domain, that in some cases require structural integrity for both export of the protein from the ER and for facilitating subunit dimerization. A comparative study of homologous mutations in proteins that are closely related family members, along with the definition of new three-dimensional crystal structures, will identify critical residues for the assembly of the α/β -hydrolase fold.

Keywords

α/β -hydrolase fold proteins; cholinesterases; neuroligins; thyroglobulin; ER-retention; chaperones; protein processing

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STRUCTURE AND FUNCTION OF THE α/β -HYDROLASE FOLD DOMAIN

The α/β -hydrolase fold family comprises a wide variety of structurally related proteins that share a common domain fold (Fig. 1). The canonical α/β -hydrolase fold is composed of 5–11 β -strands, with only strand β 2 arranged antiparallel [1]. The folded β -sheet comprises the core of the protein structure, which is surrounded by α -helices and loops interspersed among the β -strands in the primary sequence (Fig. 2) [2]. This core structural arrangement provides a stable scaffold for active sites of a wide variety of hydrolytic enzymes and for accommodation of different substrates. Because of this flexibility the α/β -hydrolase fold family encompasses a variety of enzymes including esterases, proteases, lipases, dehalogenases, peroxidases and epoxide hydrolases all of which contain a catalytic triad composed of a nucleophile, an anionic side chain and an intervening histidine [3].

In the cholinesterase subfamily of enzymes, the catalytic triad is composed of aligned serine, histidine, and glutamate residues that render Ser203 a nucleophile crucial for catalysis of the cholinergic neurotransmitter (Fig. 2) [4]. The nucleophile is not the same in all subfamilies of enzymes; in some instances, the serine can be replaced by cysteine or aspartate [5]. The α/β -hydrolase fold tolerates amino acid deletions and insertions of additional protein domains, allowing the canonical fold to diverge through evolution, adapting to serve distinct functions [6]. In the last 20 years, several catalytically inactive proteins containing the α/β -hydrolase fold domain have been identified by sequence homology to acetylcholinesterase (AChE) and have been grouped in a subfamily, often denoted as cholinesterase-like proteins. These inactive proteins lack the Ser residue properly positioned in the catalytic triad and therefore are devoid of catalytic function [7]. The α/β -hydrolase fold domain may also link to other functional domains forming an extended single peptide chain [8]. This is the case with thyroglobulin (Tg), the first homologous non-catalytic protein to be identified [9]. Tg possesses 28% amino acid identity with AChE only at its carboxyl end [4, 10]. Tg is a large homodimeric secretory glycoprotein produced in thyrocytes where it functions as a matrix for the synthesis of thyroid hormones (T3 and T4) [11]. Six cysteines residues in AChE that are involved in intrachain disulfide bonds are conserved in the AChE-homologous sequence of Tg indicating that both proteins share a common folding pattern in their respective tertiary structures.

Another functional group of proteins, the tactins, was also identified by protein sequence alignments to be part of the cholinesterase-like family of proteins. The tactins encompass a single extracellular domain that has 25–32% amino acid identity with AChE (Fig. 2) [12]. *Drosophila* neurotactin, gliotactin and glutactin as well as the mammalian neuroligins (NLGNs) are a family of cell adhesion molecules that are primarily expressed in the nervous system. With the exception of glutactin that is a secreted basement membrane glycoprotein of unknown function [13], NLGN [14], gliotactin [15, 16] and neurotactin [17] are all transmembrane cell surface proteins whose extracellular α/β -hydrolase fold domain appears to be involved in heterophilic interactions with specific protein partners [18]. The NLGN extracellular domain exhibits ~35% sequence identity with AChE, and as it has been recently shown to share the overall shape and structure of the globular domain of AChE [19–22]. Substantial sequence differences have been found between NLGNs and their cholinesterase-like α/β -hydrolase fold relatives and, to a lesser extent, between the five

NLGNs themselves [23]; in particular, the linkage of the third disulfide bond, divergent patterns of consensus sites for N-linked glycosylation, as well as the potential for insertion of two alternatively spliced sequences of 20 (insert A, NLGNs 1–4) and 9 (insert B, only NLGN 1) residues which generate several additional isoforms (Fig. 1) [14, 24]. The α/β -hydrolase fold domain of the NLGNs recognizes and interacts directly and specifically with presynaptic ligands belonging to the neurexin (NRXN) family; the interacting proteins form contact points connecting presynaptic and post-synaptic terminals [25]. The recent solution of the NLGN1 and 4 crystal structures, together with the structure of the NLGN1 and 4/NRXN1 β complexes, has added valuable information, allowing predictions of the influence of single mutations in altering protein-protein interactions [19, 21, 26].

α/β -HYDROLASE FOLD DOMAIN PROTEINS AND CONGENITAL DISORDERS

The correct folding of the α/β -hydrolase fold domain has been shown to be crucial for proper protein localization and function for members of the cholinesterase-like family as demonstrated by mutations affecting the integrity of its structure found in association with congenital disorders. In 2003, the first of several rare alterations have been reported in the genes encoding *NLGN3* and *NLGN4* in association with autism spectrum disorders and mental retardation [27, 28]. Non-synonymous single nucleotide polymorphisms, premature stop codons generated by frame shifts, deletions or missense mutations have been found to map in the α/β -hydrolase fold domain of NLGN3 and NLGN4 [29–33]. Alterations of the extracellular domain of the NLGNs are known to affect their binding affinity with the pre-synaptic partner protein NRXN, ultimately resulting in an imbalance in the excitatory/inhibitory networks, and leading to impaired neuronal signaling [34]. Polymorphisms involving cysteine substitutions have also been associated in the cytoplasmic domain of NLGN4 [32].

Whereas the α/β -hydrolase domain represents the main extracellular domain of the NLGNs that is tethered to a membrane spanning sequence by a single O-glycosylated chain [35], in Tg this domain represents only 20% of the primary sequence, corresponding to ~544 amino acids in the C-terminal region of the polypeptide. The rest of the molecule is made up of three repetitive disulfide-rich domains called I-II-III, which are held together by intra-domain disulfide bonds [10, 36]. The α/β -hydrolase fold domain of Tg is a region commonly affected by mutations linked to a spectrum of diseases ranging from euthyroidism to severe congenital hypothyroidism. At least six missense mutations map to the human α/β -hydrolase fold domain [37]. Tg missense mutations linked to hypothyroidism have also been found to occur naturally in the α/β -hydrolase fold domain of animal models: the goiter producing *cog/cog* mouse, and dwarf *rdw/rdw* rat. The three-dimensional structure of the Tg protein has not yet been solved, but it is possible to map mutations in the α/β -hydrolase fold domain using structural homology models with AChE and NLGN crystal structures (Fig. 2). There are only a few naturally occurring mutations detected in the AChE gene probably because of the important physiological role played by the AChE; the total absence of AChE activity is not compatible with prolonged life. For example, it has been observed in the mouse AChE total nullizygote strain that 50% of the mice die by postnatal day 14 and 100% die within 21

days after birth [38]. For the few single nucleotide polymorphisms (SNPs) that have been identified in the AChE gene, most are not likely to have a direct effect on the catalytic properties of AChE, but may affect protein stability and/or antigenicity, produce blood group antigenicity (YT antigen), or influence putative protein-protein interactions [39]. In a manner that is distinct from AChE, butyrylcholinesterase (BChE) not only hydrolyzes acetylcholine but also other choline esters, and choline ester-containing drugs such as the neuromuscular blocking agents, succinylcholine and mivacurium used during general anesthesia [40]. Seventy natural BChE mutations have been identified in patients with prolonged duration of action of succinylcholine that are responsible for approximately 65% of the cases of prolonged apnea during surgeries where succinylcholine is used for general anesthesia [41], an uncommon, but serious, adverse event that occurs in 1 out of 1800 patients [42]. Most of the BChE mutants that have been studied show absence of catalytic activity or lower than 10% of the average level of the wild-type enzyme [43].

HOMOLOGOUS MUTATIONS IN PROTEINS OF THE α/β -HYDROLASE FOLD FAMILY

Among all the mutations affecting the NLGNs found associated with autism spectrum disorders, the R451C was the first to be identified in two autistic brothers [29]. The mutation maps in the α/β -hydrolase fold domain and affects the proper protein localization of NLGN3 that is mainly retained in the ER instead of being exposed to the cell surface [44–47]. By comparing the sequences of NLGNs and cholinesterases (ChEs) it was observed that the R451 in NLGN3 is conserved in the NLGNs and in the ChEs across mammalian species (Fig. 3). The homologous mutation in BChE, R386C, is reflected in the human population resulting in the absence of expressed activity in plasma and is associated with the pharmacogenomic phenotype of succinylcholine apnea [48]. A comparative study of the R451C NLGN3 mutation at the homologous position in BChE (R386C) and AChE (R395C) has shown that the substitution impairs protein trafficking of the three mutant proteins causing their intracellular retention. The mutation alters the conserved structure of the α/β -hydrolase fold domain of NLGN, BChE and AChE irrespective of protein function or oligomerization state. Studying processing deficiencies in the ChEs offers the advantage of being able to easily monitor sensitive catalytic parameters, quickly assessing the folding of the nascent protein, along with intracellular processing and transport to the cell membrane or secretion to an extracellular location. The R451C mutant NLGN3 produced a reduced binding affinity for NRXN- β 1 but partially maintained the ability to promote synapse formation [45, 49]. Similarly, the R395C AChE mutant protein shows compromised catalytic function [46]. Recently, the characterization of the R87W mutation in NLGN4 has also been shown to affect protein folding and stability causing ER retention and limiting NLGN4 transport to the cell surface [33]. As observed for the mutations found in the NLGNs and ChEs, most of the hypothyroidism-inducing Tg mutants characterized so far are defective for intracellular protein transport [50]. Studying the processing of human Tg R2223H [51, 52], mouse Tg L2263P [53], rat Tg G2300R mutations [54, 55] has shown that substitutions in the α/β -hydrolase fold domain impair Tg export from the ER of thyrocytes suggesting a critical function for this domain in the transport of the protein through the secretory pathway (Fig. 4). However, the α/β -hydrolase fold domain is not only required for

Tg secretion, but it appears to be necessary for Tg dimerization, serving as an intramolecular chaperone facilitating oxidative maturation of regions I-II-III that are processed to become the thyroid hormones [56–58]. The Tg residues that are associated with hypothyroidism, Tg L2263, G2300 and R2223, are highly conserved within the α/β -hydrolase fold domain of both NLGNs and ChEs across animal species (Fig. 3), suggesting that they are critical for the integrity of the domain. In *Torpedo californica* AChE, residue R44, homologous to Tg R2223, produces the formation of a salt bridge and is needed for correct folding and transport of the cell surface of *TcAChE*. In order to understand how Tg mutations can affect processing of the α/β -hydrolase fold domain, NLGN3 has been used as a template protein to predict the influence of mutations based on positions of homology in the crystal structure of NLGN4 [59] (Fig. 3). NLGN3 G221R, homologous to Tg G2300R, is deeply buried in the core of the extracellular domain in contrast to the rather superficial location of the NLGN3 R451C autism-associated mutation (Fig. 4). By studying the effects of the G221R and R451C mutations on the folding and glycosylation processing of the α/β -hydrolase fold domain of NLGN3, the prediction obtained from structural analysis was confirmed. G221R likely causes a global misfolding of the extracellular domain of NLGN3 entirely blocking glycosylation processing of the protein and trafficking through the secretory pathway. The nascent protein is routed from the ER to the proteasome for degradation [59]. On the other hand, the R451C mutation affects only the local folding of the protein near the mutation, allowing the protein to partially escape the ER quality control system, enabling it to reach the cell surface. This type of mutation could then reveal a gain of function as shown by the knock-in mice expressing the R451C mutation [47] (Fig. 4). Investigating the influence of structural mutations on the α/β -hydrolase fold domain processing for closely related proteins of the superfamily will allow the identification of a potential locus where human congenital mutations might naturally arise. To analyze phenotypes at a cellular level, it may be important to examine cellular response in the context of the specialized differentiated cell in the nervous or endocrine system. For example, neurons have specialized trafficking systems that export proteins from the soma to particular dendritic locations. Mutations that affect such trafficking might best reveal a disorder by studies in neuronal cell types rather than in undifferentiated embryonic cells that lack such specialized functions [59].

CONCLUSIONS

Naturally occurring mutations continue to provide unique insights into understanding pathophysiologic mechanisms underlying diseases. Studying disease-associated mutations in the cholinesterase-like family of proteins, together with the higher resolution and always more numerous three-dimensional protein structures, will provide important insights in structure-function relationships of the α/β -hydrolase fold domain in different proteins. Here we summarize how disease-related mutations in the α/β -hydrolase fold domain of NLGN, AChE, BChE and Tg share common processing deficiencies yet lead to discrete congenital disorders of differing severity in the endocrine and nervous systems. The challenge will be investigating the mechanism of impaired trafficking caused by mutations affecting the α/β -hydrolase fold domain. This should offer avenues towards disorder management or possible cures of different, yet related, pathologies sharing common molecular processing mechanisms.

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ABBREVIATIONS

NLGN	Neurologin
NRXN	Neurexin
ChE	Cholinesterase
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
Tg	Thyroglobulin
ER	Endoplasmic reticulum

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Figure 1. Primary Structure and Function of Proteins of Cholinesterase-like α/β -hydrolase fold family of proteins

This schematic drawing shows the domain organization and regions of homology shared between the neuroligins, *Drosophila* tactins (gliotactin, neurotactin, glutactin), acetylcholinesterase, butyrylcholinesterase, and thyroglobulin, all members of the Cholinesterase-like α/β -hydrolase fold family of proteins. The grey bar represents the homologous region common to all proteins. Neuroligins splice inserts A (neuroligins 1–4) and B (neuroligin 1) are shown in the white rectangle within the bar. Acetylcholinesterase exists as a soluble form that can be tethered on the cell surface by an anchoring subunit or a glycophospholipid anchor whereas the neuroligins are tethered on the cell surface by a transmembrane domain in the protein. Varying protein functions are also shown. Whereas the α/β -hydrolase fold domain comprises a major portion of the protein sequence in the neuroligins, tactins, acetylcholinesterase and butyrylcholinesterase, in thyroglobulin it represents only ~20% of the sequence.

Figure 2. Structural Definition of the α/β -Hydrolase Fold Proteins

A. Stereo view of acetylcholinesterase structure [60]. When the two helices of the dimerization domains of two subunits are in contact, they form a four-helix bundle which has been observed also for neuroligins and other members of the Cholinesterase-like α/β -hydrolase fold family. The catalytic triad in the core of acetylcholinesterase is formed by Ser203, Glu334 and His447, indicated by the oval. **B.** A single subunit of neuroligin 4 is shown in the same orientation. **C.** A homology model for thyroglobulin folding is based on acetylcholinesterase and was created using ModWeb (ModWeb version SVN.r1278). **D.** butyrylcholinesterase is shown in the same orientation as acetylcholinesterase. β -strands, α -helices, loops and coils are shown in the structure.

Figure 3. Conserved Residues within the α/β -Hydrolase Fold Domain of the Neuroligins, Cholinesterases and Thyroglobulin

Top panel: Regional sequence alignment shows conservation of residues in proteins of the α/β -hydrolase fold family across several species. Residue numbers refer to Neuroigin3. Underscored residues are where the disease-related mutation was originally found to occur: human thyroglobulin R2223H (here R83), mouse thyroglobulin L2263P (here L142), rat thyroglobulin G2300 (here G221), human Neuroigin3 R451C. **Lower panel:** Stereo view of the homology model of the α/β -hydrolase fold domain of rat neuroigin3. Three-dimensional model of neuroigin 3 shows the positioning of the conserved residues described in the alignment above. Amino acid numbers in both panels refer to neuroigin3. Homology model was built with ModWeb version SVN.r1278 using neuroigin 4 as template.

Figure 4. Schematic Model showing the Trafficking of Wild Type and Mutant Cholinesterase-like α/β -Hydrolase Fold Proteins

Wild type, membrane bound or secreted proteins are represented by white empty dimers, globally misfolded mutant proteins (G221R NLGN3) are dimers marked with a cross and locally misfolded mutant proteins (R451C NLGN3) have a black dot. Wild type proteins are correctly folded in the ER, translocated to the Golgi apparatus, and then inserted into the cell membrane or secreted. Globally misfolded proteins are never correctly folded in the ER, and are rapidly transported to the proteasome for degradation. The locally misfolded proteins follow an intermediate fate, only a small amount of this protein is fully processed and inserted into the cell membrane or secreted.