Choline acetyltransferase structure reveals distribution of mutations that cause motor disorders

Yiying Cai¹, Ciarán N Cronin²,
Andrew G Engel³, Kinji Ohno³,
Louis B Hersh^{1,*} and David W Rodgers^{1,*}

¹Department of Molecular and Cellular Biochemistry, and Center for Structural Biology, University of Kentucky, Lexington, KY, USA, ²SYRRX Inc., San Diego, CA, USA and ³Department of Neurology and Neuromuscular Research Laboratory, Mayo Clinic, Rochester, MN, USA

Choline acetyltransferase (ChAT) synthesizes acetylcholine in neurons and other cell types. Decreases in ChAT activity are associated with a number of disease states, and mutations in ChAT cause congenital neuromuscular disorders. The crystal structure of ChAT reported here shows the enzyme divided into two domains with the active site in a solvent accessible tunnel at the domain interface. A low-resolution view of the complex with one substrate, coenzyme A, defines its binding site and suggests an additional interaction not found in the related carnitine acetyltransferase. Also, the preference for choline over carnitine as an acetyl acceptor is seen to result from both electrostatic and steric blocks to carnitine binding at the active site. While half of the mutations that cause motor disorders are positioned to affect enzyme activity directly, the remaining changes are surprisingly distant from the active site and must exert indirect effects. The structure indicates how ChAT is regulated by phosphorylation and reveals an unusual pattern of basic surface patches that may mediate membrane association or macromolecular interactions.

The EMBO Journal (2004) 23, 2047–2058. doi:10.1038/sj.emboj.7600221; Published online 6 May 2004

Subject Categories: structural biology; neuroscience

Keywords: acetyltransferase; cholinergic; crystal structure; myasthenic syndrome; neurotransmission

Introduction

In 1943, Nachmansohn and Machado (1943) identified the enzymatic activity responsible for the synthesis of acetylcholine, the first neurotransmitter described in the literature (Loewi, 1921). This finding initiated decades of research into the biochemistry and molecular biology of choline acetyltransferase (ChAT; EC 2.3.1.6), which catalyzes the reversible transfer of an acetyl group between acetyl-coenzyme A (AcCoA) and choline. Cholinergic neurons are

distributed widely throughout the central and peripheral nervous systems where they are involved in motor function, the autonomic nervous system, and various integrative brain functions such as learning and memory (Karczmar, 1993). Acetylcholine produced by ChAT is also present in non-nervous tissues (Kawasima and Fujii, 2003; Wessler *et al*, 2003), and it is increasingly recognized as an important regulatory molecule in many basic cellular functions, underscoring the importance of the cholinergic system and acetylcholine synthesis.

ChAT belongs to the choline/carnitine acyltransferase family, which also includes carnitine acetyltransferase (CrAT), carnitine octanoyltransferase, and carnitine palmitoyltransferase, enzymes involved in fatty acid metabolism and maintenance of acyl-CoA pools (Ramsay *et al*, 2001). The reaction catalyzed by ChAT largely follows ordered sequential kinetics with AcCoA as the leading substrate (Hersh, 1982), and an active site histidine is proposed to function as a general base, enhancing the nucleophilicity of the choline hydroxyl for attack on the thioester bond in the forward reaction (Roskoski, 1974; Carhini and Hersh, 1993). Release of CoA is thought to be the rate-limiting kinetic step (Hersh *et al*, 1978a), and conditions that increase $K_m^{AcCoA/CoA}$ can therefore enhance activity *in vitro*. Substrate concentrations in axon terminals where acetylcholine synthesis takes place are probably limiting, however, and reductions in substrate affinity may decrease the rate of acetylcholine synthesis *in vivo* (Tucek, 1990).

The single ChAT gene produces multiple transcripts that primarily encode one form of the enzyme with an apparent molecular mass of ~68 kDa as judged by gel electrophoresis (Oda, 1999). In primates, an alternative splice variant may also be translated to produce an 82 kDa form with an 118-amino-acid N-terminal extension (Kong *et al*, 1989; Oda *et al*, 1995). ChAT is synthesized in the parakaryon and is transported by both the fast and slow axonal transport systems to the axon terminals (Oda, 1999). In cholinergic nerve terminals, ChAT is present primarily in a soluble form, but a significant pool is membrane associated (Martinez-Murillo *et al*, 1989; Carroll, 1994; Gabrielle *et al*, 2003). The nature of the membrane association and significance of this enzyme pool are unknown. The larger form of ChAT has been shown to localize to the nucleus when expressed in cultured cells, and both forms of the enzyme bear nuclear localization sequences (Resendes *et al*, 1999; Gill *et al*, 2003). Again, the significance of these observations has yet to be determined.

Diminished ChAT activity signals damage to cholinergic neurons in a number of neurodegenerative disorders, including Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Oda, 1999). Schizophrenia, Rett syndrome, and sudden infant death syndrome also correlate with decreased ChAT activity (Oda, 1999; Dunn and MacLeod, 2001). Recent work has shown that recessive

*Corresponding authors. Department of Molecular and Cellular Biochemistry, University of Kentucky, 800 Rose St, Lexington, KY 40536, USA. Tel.: +1 859 257 5205; Fax: +1 859 323 1037; E-mail: david.rodgers@uky.edu or lhersh@uky.edu

Received: 6 February 2004; accepted: 5 April 2004; published online: 6 May 2004

Table I Summary of crystallographic data and refinement

	rChAT	SeMet-rChAT	rChAT-CoA
<i>crystallographic data</i>			
Wavelength (Å)	0.97934	0.97923	0.97934
Resolution (Å)	30.0–2.5	15.0–3.3	20.0–3.7
Last shell (Å)	2.59–2.50	3.42–3.30	3.83–3.70
Average redundancy (last shell)	3.80 (3.76)	3.28 (3.09)	4.02 (3.59)
R _{sym} (last shell) (%)	7.5 (33.7)	14.2 (27.8)	14.4 (21.2)
I/σI (last shell)	8.1 (3.9)	7.9 (3.6)	5.9 (4.0)
Completeness (last shell) (%)	97.9 (95.2)	99.4 (99.1)	94.9 (95.9)
<i>Refinement</i>			
Resolution (Å)	30–2.5		
R _{work} /R _{free} (%)	0.223/0.252		
R.m.s.d. bond lengths (Å)	0.008		
R.m.s.d. bond angles (deg)	1.25		
R.m.s.d. improper angles (deg)	0.84		
R.m.s.d. dihedral angles (deg)	21.9		
B r.m.s.d. bonded atoms (main/side)	1.5/2.2		
Average B (Å ²)	31.7		
Number of solvent molecules	345		
Number of metal ions	2		

mutations in ChAT cause a motor disorder known as congenital myasthenic syndrome associated with episodic apnea (CMS-EA) (Ohno *et al*, 2001; Kraner *et al*, 2003; Maselli *et al*, 2003; Schmidt *et al*, 2003), which results in severe muscular weakness and respiratory insufficiency. Characterization of heterologously expressed mutant forms of ChAT showed that the mutations decrease the catalytic efficiency of the enzyme (Ohno *et al*, 2001). In particular, the most common effect is an increase in K_m^{AcCoA} of up to 30-fold over wild type, which would likely cause a decrease in acetylcholine synthesis in nerve terminals.

Here we report the crystal structure of rat ChAT along with a low-resolution view of the complex with coenzyme A. The three-dimensional model reveals the architecture of the enzyme and provides insights into substrate binding, catalysis, and regulation. In addition, the known disease-causing mutations are seen to be widely distributed over the enzyme, with many of the residue positions far from the active site despite their effect on substrate binding and catalysis. Recently, the crystal structures of CrAT alone (Jogl and Tong, 2003; Wu *et al*, 2003) and in complex with CoA or carnitine (Jogl and Tong, 2003) were reported, and a comparison with ChAT shows similar CoA binding with the possibility of an additional protein contact and a mechanism for the ability of ChAT to discriminate between choline and carnitine.

Results and discussion

Overview of the structure

The crystal structure of recombinant rat ChAT (rChAT) was determined at 2.5 Å resolution (Table I) by molecular replace-

ment using the structure of CrAT (PDB accession number 1NDB) (Jogl and Tong, 2003). The model consists of residues 18–617 of the full-length, 645-residue enzyme used for crystallization (Figure 1). ChAT contains a total of 22 α helices and 18 β strands, which can be divided into two structural domains: residues 102–401 make up the N domain following the nomenclature established for CrAT (Jogl and Tong, 2003), and residues 18–101 and 402–617 form the C domain. Both domains have $\alpha + \beta$ folds and share a common core structure of six β strands (strands 1,10,9,8,6,7 in the N domain corresponding to strands 12,18,17,15,13,14 in the C domain) and three helices (helices 6,8,13 in the N domain corresponding to helices 16,18,22 in the C domain) that lie on one side of the sheet. In the N domain, additional helices cover the other face of the central sheet, but the corresponding face of the C domain sheet forms a large part of the interface between the two domains. The central sheets have the same topology, but β 11 adds to one side of the N domain central sheet and β 16 from the C domain adds to the other side for a total of eight strands. Both domains are structurally similar to the monomers of the trimeric acetyl transferases chloramphenicol acetyltransferase (Leslie *et al*, 1988) and dihydrolipoyl transacetylase (Mattevi *et al*, 1992) as noted for CrAT (Jogl and Tong, 2003).

The short β 11 strand that forms one end of the N domain sheet follows a long open coil region (residues 353–372) and precedes a short open coil segment (residues 376–379). These three elements form an extended chain that wraps around the outside of the N domain, connecting α 13, which extends out of the domain interface, and α 14, the helix that leads into the C domain. The first extended coil segment of this element is

Figure 1 Overview of the rat ChAT structure. (A) Stereo ribbons view of ChAT. The N domain (residues 102–401) is shown in green and the C domain (residues 18–101 and 402–617) is in gold. Secondary elements are labeled. The catalytic histidine (His334) is highlighted in orange, and the bound metal is shown as a blue sphere. This figure and other ribbon figures were made with the program RIBBONS (Carson, 1987). (B) Topology diagram of ChAT with α helices and β strands numbered sequentially from the N terminus. Residue numbers for the beginning and end of each secondary element are given whenever possible. Domain colors are the same as in panel A. (C) Sequence alignment of ChAT in different mammalian species and CrAT from mouse. Nonconserved residues are highlighted in blue and conservative changes are in yellow. Red stars mark the positions of mutations found in patients suffering from congenital myasthenic syndrome with episodic apnea. Green disks indicate phosphorylated residues. The catalytic histidine is in magenta.

known to be a site of proteolysis that affects ChAT activity (Wu *et al*, 1995).

The domain interface comprises some 2500 Å² of surface area. The contact area is reduced by the presence of a solvent-accessible tunnel that runs through the center of the molecule. The previously identified catalytic histidine residue (Carbini and Hersh, 1993) is located in a turn between β10 and α13 where it points into the central tunnel at about the midpoint of the domain interface. In CrAT and the other acetyltransferases, this tunnel is known to be the binding site for the substrates AcCoA and the acetyl group acceptor, which is choline for ChAT.

As expected from their 42% sequence identity (Figure 1C), the same acetyltransferase family members ChAT and CrAT share similar backbone structures, with an r.m.s. deviation of 1.6 Å on 588 overlapping Cα positions. The central sheets and helices of the two domain cores align well as do nearly all other regular secondary elements. Significant deviations in backbone conformation occur in the short coil, turn, or loop regions between β5 and β6, α8 and α9, β8 and α12, and α17 and α18, and in the extended coil region between α13 and β11. In general, these conformational differences are associated with small insertions or deletions. The largest insertion in ChAT extends by four residues (153–156) the β hairpin formed from strands β2 and β3 relative to CrAT, and the possible significance of this extension for AcCoA binding is discussed below.

Interestingly, the crystal structure of ChAT revealed a metal-binding site in the C domain (see Figure 1A). Main-chain carbonyl oxygen atoms from residues 588, 591, and 594 in the turn from β18 to α22 serve as three of the metal ion ligands, and Oγ of Thr594 is the fourth coordinating group contributed by the protein. Two water molecules also ligate the metal, completing a nearly ideal octahedral coordination shell. Valence calculations (Brown and Wu, 1976; Brown, 1992; Nayal and Di Cera, 1994, 1996) indicate that the metal is most likely a sodium ion, which is consistent with the prevalence of main-chain carbonyl coordinating groups (Harding, 2002) and the presence of sodium ions in the

crystallization buffer. The metal is therefore modeled as a sodium ion in the crystal structure. Although ChAT activity is modulated by the concentration of monovalent and divalent ions, this effect has largely been attributed to nonspecific effects on product release (Hersh and Peet, 1978; Hersh, 1979), and the role of the structural metal site awaits further investigation. The structure and sequence of the metal-binding turn are conserved in CrAT, but a six coordinated solvent molecule has been modeled at this position in the reported crystal structures (Jogl and Tong, 2003; Wu *et al*, 2003).

ChAT active site

The catalytic histidine, residue 334 in rChAT, is conserved in related acyltransferases, and structural and biochemical evidence suggests that it acts as a general base, extracting a proton from the attacking hydroxyl group of choline (in ChAT) or the sulfhydryl group of acyl-CoA depending on the direction of the reaction (Currier and Mautner, 1974; Carbini and Hersh, 1993; Ramsay *et al*, 2001). His334 is located at the interface between the two domains in the turn between β10 and α13 with its side chain extending into the solvent-filled tunnel where it could interact directly with substrate (Figure 1A). The position and conformation of the catalytic histidine are nearly identical to those found for the corresponding residue of CrAT (Jogl and Tong, 2003; Wu *et al*, 2003), and the overall structure of the active site region is similar in the two related enzymes. In particular, the side chains of the catalytic histidines in both enzymes are in a strained rotamer conformation, with both torsion angles around 40° from ideal values (Figure 2A). Tight packing with the side chain of Tyr95 on one side and Pro108 (from β strand 1) on the other forces this strained conformation, which permits the side-chain N^{δ1} (or N^τ) atom to donate a hydrogen bond to its own carbonyl oxygen. This interaction likely serves to stabilize the nonprotonated, nucleophilic state of N^{ε2} (or N^τ), supporting the role of this histidine in catalysis. A nearby acidic residue in CrAT, Glu347, has been proposed to play a role in polarizing the catalytic histidine (Wu *et al*, 2003). In ChAT, the equivalent residue, Asp338, points

Figure 2 The ChAT active site. (A) Stereo view of experimental electron density (F_o map computed with density-modified phases) for the catalytic histidine (His334) and nearby residues contoured at the r.m.s. deviation of the map. The figure was made in PDBVIEWER (Guex and Peitsch, 1997). (B) Ribbon diagram showing the active site histidine and a number of active site residues. Elements arising from the N domain are in green and elements from the C domain in gold. Distances from the active site histidine to nearby cysteine residues are indicated.

away from the active site making a bidentate contact with Arg458 and is therefore unlikely to function in catalysis.

The observation that sulfhydryl-modifying reagents inactivate ChAT raised the possibility that acetyl transfer in ChAT may proceed through a cysteine-linked covalent intermediate, with the active site histidine serving to extract a proton from the cysteine S γ rather than from substrate (Mautner, 1977). Indeed there are three cysteine residues (Cys332, Cys560, Cys573) located in the active site region near His334 (Figure 2B), the closest of which, Cys332, is not present in other family members. The distance from His334 to any of the cysteine residues, however, is too large for it to extract a proton without a substantial conformational change in the active site. The proximity of the cysteine residues to the substrate-binding sites does explain why bulky sulfhydryl-modifying reagents inhibit ChAT, and the structure is consistent with the observation that small modifications, particu-

larly the addition of methyl groups, do not have a profound effect on ChAT activity (Hersh *et al*, 1979).

CoA binding

In CrAT and the structurally related trimeric acetyltransferases, coenzyme A binds in one side of the central tunnel (or its multimeric equivalent), with the adenosine and phosphate groups interacting with the molecular surface at the tunnel opening and the pantothenic arm of the molecule extending through the narrow opening leading to the active site region (Figure 3A) (Leslie *et al*, 1988; Mattevi *et al*, 1992; Jogl and Tong, 2003). The thiol group of CoA is then in position to hydrogen bond with the catalytic histidine side chain, which is consistent with the proposed role of histidine as a general base for catalysis of the reverse reaction.

While structural alignment of the CrAT–CoA complex (Jogl and Tong, 2003) places CoA in a similar position in ChAT, comparison of the electrostatic potential at the tunnel

Figure 3 Coenzyme A binding. (A) Molecular surface of CrAT around the CoA-binding site computed from the CrAT–CoA crystal structure (Jogl and Tong, 2003) using the program GRASP (Nicholls *et al*, 1991). Positive potential is shown in blue and negative potential in red. The bound CoA is shown with phosphorus atoms in green. (B) The corresponding surface in ChAT. (C) Averaged difference electron density from the ChAT–CoA complex crystals. $F_o - F_c$ density was calculated using phases from the rigidly refined unliganded ChAT structure and displayed in the vicinity of the putative CoA-binding site with a cutoff level of 2.5 times the r.m.s. deviation of the map. Density for bound CoA was strong and well defined in both ChAT molecules present in the crystallographic asymmetric unit, and the density averaged over both molecules is presented. The superimposed CrAT–CoA complex is shown with the α trace in green and a wireframe representation of the bound CoA, which shows a good fit to the CoA difference density from the ChAT complex. The active site histidine of ChAT and basic residues referred to in the text are indicated. (D) Stereo view of electron density for the ChAT–CoA complex. Density was calculated with density-modified phases using the rigidly placed unliganded ChAT as a phase start and displayed with a cutoff level of 0.5 times the r.m.s. deviation of the map. CoA from the CrAT–CoA complex (Jogl and Tong, 2003) superimposed on the ChAT structure is shown in a wireframe representation.

entrance shows substantial differences between the enzymes in this region (Figure 3A and B). In CrAT, the side of the tunnel entrance that binds the adenosine and phosphate groups of CoA has a relatively neutral or slightly positive electrostatic potential at the surface, and the opposite side of the entrance is strongly electronegative, preventing interaction of the CoA phosphates with this surface. In contrast, this side of the tunnel is electropositive in ChAT, with arginines 452 and 453 as well as Lys152 contributing to the potential. The side in ChAT that corresponds to the CoA interaction site in CrAT is slightly electronegative, largely due to the substitution of Thr500 for Arg504 present in CrAT. These differences in surface potentials raise the possibility that the negatively charged nucleoside-phosphate portion of CoA may bind on the opposite side of the tunnel entrance in ChAT. This possibility is further supported by the observation that mutations at R452 increase the K_m for CoA up to ~50-fold and the double mutant R452Q/R453Q increases the K_m for CoA more than 170-fold (Wu and Hersch, 1995). These mutations do not greatly affect the K_m for 3'-dephospho-CoA, suggesting that the interaction is primarily with the 3' phosphate group.

Despite these indications, however, difference density calculated with data collected from ChAT-CoA complex crystals to 3.7 Å (Table I) demonstrates that CoA binds to ChAT and CrAT in similar positions and conformations (Figure 3C). The density is well defined and unambiguous for the entire CoA molecule, and CoA placed by superimposing ChAT and CrAT-CoA complex fits the density with only minor conformational adjustments indicated. In addition, the data show that no substantial conformational changes occur in ChAT upon CoA binding (Figure 3D). Unliganded ChAT placed by rigid-body refinement was used as a phase start for density modification, and electron density was calculated with the resulting phases and experimental amplitudes from the ChAT-CoA complex. The bound CoA molecule is well defined, and the rigidly placed unliganded ChAT fits the density well, showing that at most only small rearrangements accompany CoA binding.

With CoA binding on the same side of the tunnel as seen in the CrAT complex structure, it seems unlikely that arginines 452 and 453 would be able to make direct contacts to bound CoA, leaving open the basis for mutational effects at these sites. In unliganded ChAT, Arg452 plays an important structural role, stabilizing the base of the β 4-5 hairpin, which forms one side of the substrate tunnel opening, by hydrogen bonding to Asp149. Interestingly, this β hairpin is extended by four residues in ChAT relative to the corresponding element in CrAT (see Figure 3C), and based on its position in the refined unliganded structure, Lys152 could potentially interact with the CoA 3' phosphate. The dependence of the effect of mutations at positions 452 and 453 on the presence of the 3' phosphate of CoA (Wu and Hersch, 1995) argues for this mechanism. Also the approximately 10-fold lower K_m^{CoA} in ChAT versus CrAT (Farrell *et al*, 1984; Bloisi *et al*, 1990; Wu *et al*, 1995) may reflect additional interactions between CoA and the extended hairpin present in ChAT.

Model for choline binding and substrate discrimination

In the CrAT complex structure, carnitine binds in the active site tunnel in a position that does not overlap the CoA-binding site, forming a hydrogen bond between its hydroxyl group and the side chain of the catalytic histidine residue (Jogl and Tong, 2003). By analogy, choline should occupy a similar site in ChAT, and superimposing the protein backbone of the two enzymes places carnitine at an equivalent position in the ChAT active site (Figure 4A).

There are no significant sequence or conformational changes in ChAT near the choline portion of the superimposed carnitine molecule. As in CrAT, the active site histidine, His334, could hydrogen bond to the hydroxyl group of choline, and the positively charged trimethylammonium group of choline would be in position to form a cation- π interaction with the side chain of an aromatic residue, in this case Tyr562 rather than the phenylalanine in CrAT. The conformation of the conserved Ser-Thr-Ser motif, known to be important for carnitine-binding (Cronin, 1997), is the same in ChAT and the CrAT-carnitine complex. Overall, then, the structure suggests

Figure 4 Choline-carnitine discrimination. (A) Superposition of the ChAT and CrAT choline- or carnitine-binding sites. Carnitine from the CrAT-carnitine complex structure is in gray, and side chains in the substrate-binding site are shown in standard colors for ChAT and in green for CrAT. (B) Diagram of choline and side chains in ChAT binding site. Likely interactions are indicated. The additional carboxylate group in carnitine is indicated by dashed lines.

that ChAT binding interactions with choline mimic the interactions between CrAT and the choline-like portion of carnitine.

Despite the similarity of the ChAT and CrAT choline-binding sites, ChAT has a strong preference for choline as a substrate, with a greater than 6000-fold difference in K_m values (Cronin, 1998). Thus the presence of the acetyl group in carnitine greatly diminishes its affinity for ChAT. The structure of rChAT suggests that three sequence differences between it and CrAT near the acetyl group of superimposed carnitine are key in establishing this level of substrate discrimination (Figure 4A and B). In CrAT, the negatively charged carboxylate of carnitine is stabilized in part by an electrostatic interaction with Arg518. ChAT has a neutral asparagine at this position, and therefore would lose the electrostatic interaction. Mutation to arginine at this position in ChAT is known to improve the binding affinity for carnitine (Cronin, 1998). In addition, Met94 in ChAT substitutes for the much smaller alanine in CrAT, and this substitution forces the side chain of Trp90 to adopt a different rotamer conformation. These changes alter the shape of the binding pocket near the acetyl group, placing a hydrophobic and aromatic surface up against, and even sterically overlapping, the position that would be occupied by the charged carboxylate group of carnitine. Finally, Val459 substitutes for the threonine present in CrAT, eliminating a hydrogen bond to the carnitine carboxyl oxygen. Mutating the valine to threonine, along with the nearby change N461T, greatly increases the affinity of ChAT for carnitine (Cronin, 1998).

Mutations in ChAT that cause congenital motor disorders

While decreases in ChAT activity have been associated with a number of diseases (Oda, 1999), the most direct link between

abnormal ChAT activity and disease etiology is in a particular class of the motor disorders known as congenital myasthenic syndromes (Ohno *et al*, 2001; Engel *et al*, 2003). Heritable mutations in ChAT that affect its function or expression level are one of the most common causes of the presynaptic forms of this disorder, giving rise to a disease subclass known as congenital myasthenic syndrome with episodic apnea (CMS-EA) (Ohno *et al*, 2001).

To date, 14 ChAT mutations have been identified in CMS-EA patients (Ohno *et al*, 2001; Kraner *et al*, 2003; Maselli *et al*, 2003; Schmidt *et al*, 2003). Two of the mutations abolish ChAT expression, either through a frameshift insertion (after nucleotide 523 of the human 82 kDa ChAT coding sequence) or through conversion of the codon for human residue R548 to a stop codon. The other 12 mutations produce single residue changes, and most of these mutant enzymes have been characterized for changes in their kinetic parameters and for their expression level in COS cells (Table II) (Ohno *et al*, 2001). By far, the most frequent effect (eight of the nine characterized mutants) is to increase the K_m of ChAT for AcCoA in the range of 2.5- to 30-fold (or more). Four of these mutants also showed substantial changes in their K_m for choline and/or their catalytic constants (k_{cat}). The one mutant (E441K, human) not classified as having an increased K_m^{AcCoA} showed no catalytic activity and therefore could not be further characterized. This mutant and two others were present at much lower levels than the wild-type enzyme when expressed in COS cells, implying a lower level of production, decreased stability, or both. All the mutations are located at positions conserved in mammals (see Figure 1C).

The locations of the 12 point mutations in the ChAT three-dimensional structure are shown in Figure 5A. Most of the mutations (nine out of 12) are at positions distributed

Table II Summary of congenital ChAT mutations

Amino-acid change (human)	Corresponding residue (rat)	Effect on ChAT activity	Reference	Likely structural effect of mutation
V194L	V86	ND	Maselli <i>et al</i> (2003)	Alter AcCoA- and choline-binding sites
L210P	L102	$\uparrow \uparrow K_m^{AcCoA}$, $\uparrow \uparrow K_m^{chol}$, $\downarrow k_{cat}$	Ohno <i>et al</i> (2001) and Maselli <i>et al</i> (2003)	Alter active site and choline-binding site
P211A	P103	$\uparrow \uparrow K_m^{AcCoA}$, $\uparrow k_{cat}$	Ohno <i>et al</i> (2001) and Maselli <i>et al</i> (2003)	Alter active site and choline-binding site
I305T	I197	$\uparrow K_m^{AcCoA}$, $\downarrow E$	Ohno <i>et al</i> (2001)	Destabilize fold, distant from active and binding sites
I336T	I228	ND	Schmidt <i>et al</i> (2003)	Destabilize fold, distant from active and binding sites
R420C	R312	$\uparrow \uparrow K_m^{AcCoA}$, $\uparrow \uparrow K_m^{chol}$, $\uparrow k_{cat}$, $\downarrow E$	Ohno <i>et al</i> (2001)	Destabilize fold, distant from active and binding sites
E441K	E333	No activity, $\downarrow E$	Ohno <i>et al</i> (2001)	Alter active site and choline-binding site
R482G	R374	$\uparrow K_m^{AcCoA}$	Ohno <i>et al</i> (2001)	Alter extended open coil, distant from binding sites
S498L	S390	$\uparrow K_m^{AcCoA}$	Ohno <i>et al</i> (2001)	Effect not apparent, distant from active and binding sites
V506L	V398	$\uparrow K_m^{AcCoA}$	Ohno <i>et al</i> (2001) and Maselli <i>et al</i> (2003)	Effect not apparent, distant from active and binding sites
R560H	R452	$\uparrow \uparrow K_m^{AcCoA}$, $\uparrow \uparrow K_m^{chol}$, $\downarrow k_{cat}$	Ohno <i>et al</i> (2001)	Alter AcCoA-binding site
S694C	S586	ND	Maselli <i>et al</i> (2003)	Alter choline-binding site

Residue numbers in humans are based on the 82 kDa version of ChAT.

k_{cat} : catalytic constant; K_m^{AcCoA} and K_m^{chol} : Michaelis–Menten constants for the substrates acetyl coenzyme A and choline; E: expression level in COS cells (Ohno *et al*, 2001); $\uparrow$: increase in the value of kinetic constant; $\uparrow \uparrow$: large increase in the value of kinetic constant; $\downarrow$: decrease in the value of kinetic constant or expression level; ND: kinetic constants and expression level not determined.

Figure 5 ChAT mutations found in patients suffering from congenital myasthenic syndrome with episodic apnea (CMS-EA). (A) Locations of point mutants on the ChAT C α trace are indicated by red spheres. The view is down the substrate-binding tunnel. (B–G) Environments of individual mutations as described in the text.

throughout the N domain, all but one in helical regions or the strands of the central sheet. The three mutations that map to the C domain are located relatively close to the domain interface and thus close to the active site.

Based on the rChAT crystal structure, the CMS-EA mutations fall into two broad classes: those likely to affect substrate binding and in some cases active site conformation, and those distant from the active and substrate-binding sites (Table II). Six of the 12 mutations (hChAT residues V194L, L210P, P211A, E441K, R560H, S694C) fall into the first class, and the remaining six mutants (hChAT residues I305T, I336T, R420C, R482G, S498L, V506L) are in the latter. For example, the R560H mutation in hChAT, which corresponds to position 452 in rChAT (Figure 5B), alters a residue near the projected binding site for AcCoA as described above, consistent with the increase in K_m^{AcCoA} found for this mutant (Table II) (Ohno *et al*, 2001). On the other hand, the increase in K_m^{AcCoA} associated with hChAT I305T, which corresponds to rChAT position 197 (Figure 5C), is less easily rationalized based on the structure. This mutation substitutes a polar side chain in the hydrophobic core and is therefore likely to destabilize the fold. (I336T, which has not been characterized, would have a similar structural effect.) It has been shown to lower enzyme levels in COS cells (Ohno *et al*, 2001), possibly reflecting this decrease in stability. But given its location approximately 2 Å from the nearest atom of AcCoA, any effect on AcCoA interaction with the protein must be transmitted in some way by conformational changes across the center of the N domain.

In fact, despite the prevalence of changes in K_m^{AcCoA} , only two of the mutations, R560H and V194L, would likely alter the CoA-binding site. For the other mutations located near the active site, changes in K_m^{AcCoA} could result from altered choline binding given the ordered sequential kinetic mechanism. Like I305T, however, the other four remote mutations that increase K_m^{AcCoA} (Figure 5D–G) would not obviously affect

AcCoA binding. The R420C substitution, position 312 in rChAT, might decrease stability (Figure 5D), but local effects would have to be transmitted a considerable distance to affect AcCoA binding. Interestingly, R374, the rChAT residue corresponding to the human R482G mutation site, forms part of the small β 11 strand (Figure 5E), which is at one edge of the central sheet and to some extent anchors the extended coil region known to be proteolytically sensitive (see below). Side-chain interactions from this residue help to stabilize what is otherwise a weak strand–strand interaction of just three hydrogen bonds. Loss of the arginine side-chain interactions in the hChAT R482G mutant could destabilize association of β 11 with the central sheet and alter the conformation of the extended coil. Since alteration of the extended coil by proteolysis is known to increase K_m for both substrates (Wu *et al*, 1995), it is possible that the effect of this mutation could be transmitted in some way through the coil to the AcCoA-binding site.

The remaining two distant mutants, hChAT S498L and V506L, seem unlikely to have a large effect on the stability of the enzyme and, again, would have no direct effect on the AcCoA-binding site. The side chain of S390 in rChAT, equivalent to position 498 in hChAT, forms a hydrogen bond with Asp248 (Figure 5F). Since the aspartate is solvent exposed, however, loss of this interaction in the leucine mutant should not have a great effect, and there are nearby hydrophobic residues that would interact favorably with the leucine side chain. The conservative hChAT V506L mutant, residue 398 in rChAT (Figure 5G), also would not be predicted to have a large effect on conformation, and the mechanism by which it affects AcCoA binding on the other side of the molecule therefore remains mysterious.

Regulation and subcellular localization

ChAT activity is regulated at the level of transcription (Eiden *et al*, 1998; Shimojo *et al*, 1998; Oda, 1999; Prado *et al*, 2002),

Figure 6 ChAT regulation and localization. The surface of ChAT around the CoA-binding site is shown with positive and negative electrostatic potentials in blue and red, respectively. The locations of the serine (S) and threonine (T) residues known to be phosphorylated are indicated. Arrows point out the periodically spaced basic patches along a surface ridge.

but post-translational modification of ChAT may also play a role in regulating acetylcholine synthesis (Prado *et al*, 2002; Dobransky and Rylett, 2003). Two phosphorylation sites (Bruce and Hersh, 1989) have been identified and characterized recently (Dobransky *et al*, 2001, 2003). Protein kinase C (PKC) phosphorylates hChAT at Ser558, which corresponds to Ser450 in rChAT, and Thr574 of hChAT, Thr466 in rChAT, is modified by calcium/calmodulin-dependent protein kinase. Phosphorylation at the serine residue increases *in vitro* activity by around two-fold, and modification of the threonine, while having no effect on activity by itself, further enhances the activity of the doubly phosphorylated enzyme. In the rChAT crystal structure, Ser450 is located adjacent to the CoA-binding site, just under the hairpin that is extended in ChAT relative to CrAT (Figure 6). Phosphorylation of Ser450 might be expected to decrease CoA affinity because of charge repulsion with the nearby CoA phosphate groups. Correspondingly, the rate of product release would increase, which might account for the elevated ChAT activity under the reported assay conditions (Dobransky *et al*, 2001). Note that this kinase recognition sequence would be disrupted by the R560H CMS-EA mutation in hChAT (rChAT position 452), suggesting that this mutation might affect regulation as well as CoA binding. Interestingly, the second identified phosphorylation site, Thr466 in rChAT, is located at the N terminus of $\alpha 16$, well away from the active site region (Figure 6), and this residue is not surface accessible. A substantial conformational change in the region would be required for kinase access to this site, and any effect of phosphorylation would have to be transmitted through the C domain to the active site.

The surface of ChAT near the CoA-binding site and the site of phosphorylation at Ser450 bears a striking pattern of basic

patches spaced periodically along a ridge that runs the length of the enzyme (Figure 6). Each patch contains between two and four basic residues, which are all conserved in ChAT orthologs but not in CrAT. It is possible that this basic surface plays a role in electrostatically steering the negatively charged CoA substrate, but it may also be involved in the interaction with PKC. The PKC family members are acidic molecules and at least some substrates are basic proteins such as histones and myelin basic protein (Newton, 1995).

The basic surface ridge may also be involved in the reported peripheral interaction of ChAT with membranes (Oda, 1999). Approximately 20% of the ChAT in axonal terminals is thought to be associated with membrane structures, either synaptic vesicles (Carroll, 1994) or plasma membrane (Gabrielle *et al*, 2003) or both (Martinez-Murillo *et al*, 1989), and membrane association could play a role in the axonal transport of ChAT (Dziegielewska *et al*, 1976). The fact that phosphorylation at Ser450 appears to be required for membrane association (Dobransky *et al*, 2001) implicates the nearby ChAT basic surface in the interaction, although the structure does not immediately suggest a mechanism for the dependence on phosphorylation. The basic ridge might directly interact with negatively charged phospholipid head groups, or it may help to mediate an interaction between ChAT and a membrane-associated protein (Gabrielle *et al*, 2003).

Transport of ChAT into the nucleus of cells has been reported. The larger 82 kDa form of hChAT carries an N-terminal nuclear localization signal (NLS) and accumulates in the nucleus (Resendes *et al*, 1999), but the smaller form corresponding to our construct also contains an NLS sequence, RRLRXK, and has recently been shown to be imported into the nucleus if that sequence is intact (Gill *et al*, 2003). The first three residues of this sequence (residues 373–375 in rChAT) form the short β strand, $\beta 11$, that interacts with the central sheet of the N domain and divides the extended coil wrapping around the molecule (see Figure 1A). The solvent-exposed β conformation of this sequence is consistent with the extended β conformation adopted by peptides bound to karyopherin carrier molecules that mediate this form of nuclear transport (Conti and Kuriyan, 2000). The function of nuclear ChAT, if any, is not known. As such, it is interesting to note that the periodic basic patches along the ChAT surface ridge (Figure 6) have the correct spacing to interact with the backbone of two successive turns of B-form DNA, suggesting a possible nucleic acid-binding role.

In addition to modulation by phosphorylation, ChAT is also activated by proteolytic cleavage, a possible regulatory mechanism. The cleavage site mediating activation is thought to be residues 360–364 (Wu *et al*, 1995) in the N-terminal segment of the extended coil region that wraps around the molecule. The density for this surface coil region is poor, and only α positions could be assigned for residues 356–367 in the structure, consistent with its proteolytic sensitivity. This site follows helix 13, which forms the ceiling of the CoA-binding site and is adjacent to the turn that contains the catalytic histidine (His334) and some choline-binding residues. It is therefore possible that proteolysis at this site alters the position of $\alpha 13$, accounting for the known effect on Michaelis constants for both substrates (Wu *et al*, 1995). Further structural work with substrate complexes and mutants of ChAT should resolve the mechanism of proteolytic

activation as well as many of the remaining questions concerning ChAT functional mechanisms.

Materials and methods

Expression and purification of recombinant rat ChAT

Recombinant rat ChAT in the protein expression vector pLENTY (Cronin, 1998) was overexpressed in DH5 α F'IQ cells. The cultured cells were grown overnight (12–18 h) at 30°C after induction with 0.1 mM isopropyl-1-thio- β -D-galacto-pyranoside (IPTG). Harvested cells were suspended in PDE buffer (10 mM phosphate, pH 7.0, 0.2 mM DTT, 1 mM EDTA) containing protease inhibitor cocktail (Boehringer Mannheim). Cells were lysed in a French press and the debris removed by centrifugation at 12 000 g for 20 min. The crude extract was applied to a 30 ml Accell⁺ CM column (Waters), washed overnight with 600 ml of PDE buffer, and then ChAT was eluted by applying a 300 ml linear gradient of 0–0.5 M KCl in PDE buffer. The eluted enzyme was dialyzed and then purified further using a 1 ml Mono S column (Pharmacia) equilibrated in 25 mM Tris-HCl, 1 mM EDTA, and 0.2 mM DTT, pH 7.2. ChAT was eluted by a 40 ml linear gradient of 0–0.5 M NaCl in the same buffer.

Selenomethionyl-ChAT was obtained by the methionine pathway inhibition technique (Doublé, 1997) with the same vector and *Escherichia coli* strain used for native ChAT. A 120 mg/l portion of DL-selenomethionine was added to culture medium. The purification procedure for selenomethionyl-ChAT was the same as that used for the native enzyme except that 2 mM DTT was added to all of the buffers.

ChAT activity determination

During the purification procedure, recombinant ChAT activity was monitored by a fluorometric assay (Hersh *et al*, 1978b; Wu *et al*, 1995; Wu and Hersh, 1995). The reverse ChAT reaction, transfer of an acetyl group from acetylcholine to CoA, is coupled by citrate synthase and malate dehydrogenase to the production of NADH. Assay mixtures contained 10 mM potassium phosphate buffer (pH 7.4), 250 mM sodium chloride, 0.125 mM NAD⁺, 0.5 mM L-malate, 0.1 mM DL-dithiothreitol, 25 mM acetylcholine chloride, 0.1 mM CoA, 1.5 U of pig heart citrate synthase (Sigma), and 4 U of pig heart malate dehydrogenase (Sigma). Reactions were initiated by the addition of acetylcholine chloride, and NADH formation was monitored continuously using an Optical Technologies fluorometer.

Crystallization and data collection

Crystals of both native and selenomethionyl-ChAT were grown by the sitting-drop vapor diffusion method at 4°C. The reservoir solution contained 9–10% (w/v) PEG 20000 and 75 mM MES (pH 6.5), and the protein at 10–15 mg/ml was diluted 1:1 with reservoir solution. Crystals were cryoprotected by rapidly passing them through a solution of mother liquor containing 20% glycerol, mounting in a nylon loop, and plunging the mounted crystal into liquid nitrogen. X-ray diffraction data were collected on a CCD detector at the SER-CAT and SBC beamlines of the Advanced Photon Source (APS), Argonne National Laboratory. Data were processed with the HKL (Otwinowski, 1997) and CCP4 packages (Science and

Engineering Research Council Collaborative Computing Project, Daresbury Laboratory). The crystals of native ChAT form in space group P₂₁, with unit cell dimensions of $a = 84.70$ Å, $b = 78.88$ Å, $c = 139.36$ Å, $\alpha = 90^\circ$, $\beta = 98.4^\circ$, and $\gamma = 90^\circ$. ChAT-coenzyme A complex crystals were prepared by hanging-drop vapor diffusion at 4°C using a 1:2 molar ratio of rChAT to CoA over a well of 7–8% PEG 12000 and 50 mM KH₂PO₄ (pH 4.5). The crystals form in space group P₂₁2₁2₁ with unit cell dimensions $a = 77.59$ Å, $b = 153.32$ Å, and $c = 152.83$ Å. Under these conditions, CoA is present at a concentration over 100-fold greater than the K_m for CoA in the reverse enzymatic reaction (Wu and Hersh, 1995). Attempts to soak CoA into existing crystals destroyed their order, and we were unable to grow crystals in the presence of CoA under conditions used for the unliganded ChAT crystals. Data for the complex were collected to 3.7 Å resolution as described above.

Structure determination and refinement

The ChAT structure was determined by molecular replacement using the CNS software package (Brünger *et al*, 1998) and the coordinates of unliganded CrAT (Jogl and Tong, 2003) (PDB identification code 1NDB) as a search object. Two molecules of ChAT in the asymmetric unit were identified. The backbone trace and registration were checked against an anomalous difference map calculated with data from the selenomethionine-substituted ChAT crystals. Initial manual rebuilding with the program O (Jones *et al*, 1991) was followed by simulated annealing refinement in CNS and subsequent cycles of manual rebuilding, addition of ordered solvent, and energy minimization. Noncrystallographic symmetry restraints were applied during refinement. The observed metal-binding site was analyzed for valence with various metal ions using the program WASP (Nayal and Di Cera, 1996). The ChAT-CoA complex electron density was calculated by molecular replacement with the unliganded ChAT structure followed by rigid-body refinement in CNS. Difference density was calculated directly in CNS, and F_o density for the entire complex was calculated with density modification improved phases using the unliganded model as a phase start. No further refinement of the complex was attempted because of the limited resolution of the data.

Coordinates

The coordinates of unliganded ChAT have been deposited in the Protein Data Bank (identification code 1Q6X).

Acknowledgements

We thank the staffs of Advanced Photon Source beamlines 22ID (SER-CAT), 19ID (SBC), and 14-BMC (BioCARS) for their help with data collection. Use of the Advanced Photon Source is supported by the US Department of Energy. We acknowledge support from the United States Public Health Service to DWR (NS38041), LBH (AG05893), and AGE (NS6277), the National Science Foundation to DWR (MCB-9904886), the American Chemical Society Petroleum Research Fund to DWR (37135-AC4), and the Muscular Dystrophy Association to AGE.

References

- Bloisi W, Colombo I, Garavaglia B, Giardini R, Finocchiaro G, Didonato S (1990) Purification and properties of carnitine acetyltransferase from human liver. *Eur J Biochem* **189**: 539–546
- Brown ID (1992) Chemical and steric constraints in inorganic solids. *Acta Crystallogr B* **48**: 553–572
- Brown ID, Wu KK (1976) Empirical parameters for calculating cation-oxygen bond valence. *Acta Crystallogr B* **32**: 1957–1959
- Bruce G, Hersh LB (1989) The phosphorylation of choline acetyltransferase. *Neurochem Res* **14**: 613–620
- Brünger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T, Warren GL (1998) Crystallography & NMR system: a new software suite for macromolecular structure determination. *Acta Crystallogr D* **54**: 905–921
- Carbini LA, Hersh LB (1993) Functional analysis of conserved histidines in choline acetyltransferase by site-directed mutagenesis. *J Neurochem* **61**: 247–253
- Carroll PT (1994) Membrane-bound choline-O-acetyltransferase in rat hippocampal tissue is associated with synaptic vesicles. *Brain Res* **633**: 112–118
- Carson M (1987) Ribbon models of macromolecules. *J Mol Graphics* **5**: 103–106
- Conti E, Kuriyan J (2000) Crystallographic analysis of the specific yet versatile recognition of distinct nuclear localization signals by karyopherin alpha. *Struct Fold Des* **8**: 329–338
- Cronin CN (1997) The conserved serine-threonine-serine motif of the carnitine acyltransferases is involved in carnitine binding and transition-state stabilization: a site-directed mutagenesis study. *Biochem Biophys Res Commun* **238**: 784–789

- Cronin CN (1998) Redesign of choline acetyltransferase specificity by protein engineering. *J Biol Chem* **273**: 24465–24469
- Currier SF, Mautner HG (1974) On the mechanism of action of choline acetyltransferase. *Proc Natl Acad Sci USA* **71**: 3355–3358
- Dobransky T, Brewer D, Lajoie G, Rylett RJ (2003) Phosphorylation of 69-kDa choline acetyltransferase at threonine 456 in response to amyloid-beta peptide 1–42. *J Biol Chem* **278**: 5883–5893
- Dobransky T, Davis WL, Rylett RJ (2001) Functional characterization of phosphorylation of 69-kDa human choline acetyltransferase at serine 440 by protein kinase C. *J Biol Chem* **276**: 22244–22250
- Dobransky T, Rylett RJ (2003) Functional regulation of choline acetyltransferase by phosphorylation. *Neurochem Res* **28**: 537–542
- Doublé S (1997) Preparation of selenomethionyl proteins for phase determination. *Methods Enzymol* **276**: 523–530
- Dunn HG, MacLeod PM (2001) Rett syndrome: review of biological abnormalities. *Can J Neurol Sci* **28**: 16–29
- Dziegielewska KM, Saunders NR, Evans CA, Skacel PO, Haggendal CJ, Heiwall PO, Dahalstrom AB (1976) Effects of colchicine and vinblastine on axonal transport of choline acetyltransferase in rat sciatic nerve. *Acta Physiol Scand* **96**: 486–494
- Eiden LE, Anouar Y, Hsu CM, MacArthur L, Hahm SH (1998) Transcription regulation coupled to calcium and protein kinase signaling systems through TRE- and CRE-like sequences in neuropeptide genes. *Adv Pharmacol* **42**: 264–268
- Engel AG, Ohno K, Sine SM (2003) Sleuthing molecular targets for neurological diseases at the neuromuscular junction. *Nat Rev Neurosci* **4**: 339–352
- Farrell SO, Fiol CJ, Reddy JK, Bieber LL (1984) Properties of purified carnitine acyltransferases of mouse liver peroxisomes. *J Biol Chem* **259**: 13089–13095
- Gabrielle P, Jeana M, Lorenza EC (2003) Cytosolic choline acetyltransferase binds specifically to cholinergic plasma membrane of rat brain synaptosomes to generate membrane-bound enzyme. *Neurochem Res* **28**: 543–549
- Gill SK, Bhattacharya M, Ferguson SS, Rylett RJ (2003) Identification of a novel nuclear localization signal common to 69- and 82-kDa human choline acetyltransferase. *J Biol Chem* **278**: 20217–20224
- Guex N, Peitsch MC (1997) SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* **18**: 2714–2723
- Harding MM (2002) Metal-ligand geometry relevant to proteins and in proteins: sodium and potassium. *Acta Crystallogr D* **58**: 872–874
- Hersh LB (1979) The lack of specificity towards salts in the activation of choline acetyltransferase from human placenta. *J Neurochem* **32**: 991–996
- Hersh LB (1982) Kinetic studies of the choline acetyltransferase reaction using isotope exchange at equilibrium. *J Biol Chem* **257**: 12820–12825
- Hersh LB, Barker LA, Rush B (1978a) Effect of sodium chloride on changing the rate-limiting step in the human placental choline acetyltransferase reaction. *J Biol Chem* **253**: 4966–4970
- Hersh LB, Coe B, Casey L (1978b) A fluorometric assay for choline acetyltransferase and its use in the purification of the enzyme from human placenta. *J Neurochem* **30**: 1077–1085
- Hersh LB, Nair RV, Smith DJ (1979) The reaction of choline acetyltransferase with sulfhydryl reagents. Methoxycarbonyl-CoA disulfide as an active site-directed reagent. *J Biol Chem* **254**: 11988–11992
- Hersh LB, Peet M (1978) Effect of salts on the physical and kinetic properties of human placental choline acetyltransferase. *J Neurochem* **30**: 1087–1093
- Jogl G, Tong L (2003) Crystal structure of carnitine acetyltransferase and implications for the catalytic mechanism and fatty acid transport. *Cell* **112**: 113–122
- Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* **47** (Part 2): 110–119
- Karczmar AG (1993) Brief presentation of the story and present status of studies of the vertebrate cholinergic system. *Neuropsychopharmacology* **9**: 181–199
- Kawasima K, Fujii T (2003) The lymphocytic cholinergic system and its biological function. *Life Sci* **72**: 2101–2109
- Kong CF, Hilt D, Hersh LB (1989) Isolation of a genomic clone of human choline acetyltransferase. In *Proceedings of the International Symposium on 'Pharmacological Interventions on Central Cholinergic Mechanisms in Senile Dementia (Alzheimer's Disease)'*, Kewitz HT, T Bickel U (eds) Klin. Pharmakol., Berlin: Zuckschwerdt-Verl. Vol. 2, pp 15–19
- Kraner S, Laufenberg I, Strassburg HM, Sieb JP, Steinlein OK (2003) Congenital myasthenic syndrome with episodic apnea in patients homozygous for a CHAT missense mutation. *Arch Neurol* **60**: 761–763
- Leslie AG, Moody PC, Shaw WV (1988) Structure of chloramphenicol acetyltransferase at 1.75-Å resolution. *Proc Natl Acad Sci USA* **85**: 4133–4137
- Loewi O (1921) Über humorale Übertragbarkeit der Hirnenwicklung. *Pflügers Arch* **189**: 239–242
- Martinez-Murillo R, Villalba RM, Rodrigo J (1989) Electron microscopic localization of cholinergic terminals in the rat substantia nigra: an immunocytochemical study. *Neurosci Lett* **96**: 121–126
- Maselli RA, Chen D, Mo D, Bowe C, Fenton G, Wollmann RL (2003) Choline acetyltransferase mutations in myasthenic syndrome due to deficient acetylcholine resynthesis. *Muscle Nerve* **27**: 180–187
- Mattevi A, Obmolova G, Sokatch JR, Betzel C, Hol WG (1992) The refined crystal structure of *Pseudomonas putida* lipamide dehydrogenase complexed with NAD⁺ at 2.45 Å resolution. *Proteins* **13**: 336–351
- Mautner HG (1977) Choline acetyltransferase. *CRC Crit Rev Biochem* **4**: 341–370
- Nachmansohn D, Machado AL (1943) The formation of acetylcholine. A new enzyme choline acetylase. *J Neurophysiol* **6**: 397–403
- Nayal M, Di Cera E (1994) Predicting Ca(2+)-binding sites in proteins. *Proc Natl Acad Sci USA* **91**: 817–821
- Nayal M, Di Cera E (1996) Valence screening of water in protein crystals reveals potential Na⁺ binding sites. *J Mol Biol* **256**: 228–234
- Newton AC (1995) Protein kinase C: structure, function, and regulation. *J Biol Chem* **270**: 28495–28498
- Nicholls A, Sharp KA, Honig B (1991) Protein folding and association: insights from the interfacial and thermodynamic properties of hydrocarbons. *Proteins Struct Funct Genet* **11**: 281–296
- Oda Y (1999) Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system. *Pathol Int* **49**: 921–937
- Oda Y, Imai S, Nakanishi I, Ichikawa T, Deguchi T (1995) Immunohistochemical study on choline acetyltransferase in the spinal cord of patients with amyotrophic lateral sclerosis. *Pathol Int* **45**: 933–939
- Ohno K, Tsujino A, Brengman JM, Harper CM, Bajzer Z, Udd B, Beyring R, Robb S, Kirkham FJ, Engel AG (2001) Choline acetyltransferase mutations cause myasthenic syndrome associated with episodic apnea in humans. *Proc Natl Acad Sci USA* **98**: 2017–2022
- Otwinowski Z, Minor W (1997) Processing of x-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276**: 307–326
- Prado MA, Reis RA, Prado VF, de Mello MC, Gomez MV, de Mello FG (2002) Regulation of acetylcholine synthesis and storage. *Neurochem Int* **41**: 291–299
- Ramsay RR, Gandour RD, van der Leij FR (2001) Molecular enzymology of carnitine transfer and transport. *Biochim Biophys Acta* **1546**: 21–43
- Resendes MC, Dobransky T, Ferguson SS, Rylett RJ (1999) Nuclear localization of the 82-kDa form of human choline acetyltransferase. *J Biol Chem* **274**: 19417–19421
- Roskoski Jr R (1974) Choline acetyltransferase. Inhibition by thiol reagents. *J Biol Chem* **249**: 2156–2159
- Schmidt C, Abicht A, Krampfl K, Voss W, Stucka R, Mildner G, Petrova S, Schara U, Mortier W, Bufler J, Huebner A, Lochmuller H (2003) Congenital myasthenic syndrome due to a novel missense mutation in the gene encoding choline acetyltransferase. *Neuromuscular Disord* **13**: 245–251
- Shimojo M, Wu D, Hersh LB (1998) The cholinergic gene locus is coordinately regulated by protein kinase A II in PC12 cells. *J Neurochem* **71**: 1118–1126

- Tucek S (1990) The synthesis of acetylcholine: twenty years of progress. *Prog Brain Res* **84**: 467–477
- Wessler I, Kilbinger H, Bittinger F, Unger R, Kirkpatrick CJ (2003) The non-neuronal cholinergic system in humans: expression, function and pathophysiology. *Life Sci* **72**: 2055–2061
- Wu D, Ahmed SN, Lian W, Hersh LB (1995) Activation of rat choline acetyltransferase by limited proteolysis. *J Biol Chem* **270**: 19395–19401
- Wu D, Govindasamy L, Lian W, Gu Y, Kukar T, Agbandje-McKenna M, McKenna R (2003) Structure of human carnitine acetyltransferase. Molecular basis for fatty acyl transfer. *J Biol Chem* **278**: 13159–13165
- Wu D, Hersh LB (1995) Identification of an active site arginine in rat choline acetyltransferase by alanine scanning mutagenesis. *J Biol Chem* **270**: 29111–29116