Expression, purification and characterization of recombinant human choline acetyltransferase: phosphorylation of the enzyme regulates catalytic activity

Tomas DOBRANSKY*†, Wanda L. DAVIS*†, Gong-Hua XIAO*†¹ and R. Jane RYLETT*†²

*Department of Physiology, Medical Sciences Building, University of Western Ontario, London, Ontario, Canada, N6A 5C1, and †The John P. Robarts Research Institute, London, Ontario, Canada

Choline acetyltransferase synthesizes acetylcholine in cholinergic neurons and, in humans, may be produced in 82- and 69-kDa forms. In this study, recombinant choline acetyltransferase from baculovirus and bacterial expression systems was used to identify protein isoforms by two-dimensional SDS/PAGE and as substrate for protein kinases. Whereas hexa-histidine-tagged 82- and 69-kDa enzymes did not resolve as individual isoforms on two-dimensional gels, separation of wild-type choline acetyltransferase expressed in insect cells revealed at least nine isoforms for the 69-kDa enzyme and at least six isoforms for the 82-kDa enzyme. Non-phosphorylated wild-type choline acetyltransferase expressed in *Escherichia coli* yielded six (69 kDa) and four isoforms (82 kDa) respectively. Immunofluorescent labelling of insect cells expressing enzyme showed differential subcellular localization with the 69-kDa enzyme localized adjacent to plasma membrane and the 82-kDa enzyme being cytoplasmic at 24 h. By

64 h, the 69-kDa form was in cytoplasm and the 82-kDa form was only present in nucleus. Studies *in vitro* showed that recombinant 69-kDa enzyme was a substrate for protein kinase C (PKC), casein kinase II (CK2) and α -calcium/calmodulin-dependent protein kinase II (α -CaM kinase), but not for cAMP-dependent protein kinase (PKA); phosphorylation by PKC and CK2 enhanced enzyme activity. The 82-kDa enzyme was a substrate for PKC and CK2 but not for PKA or α -CaM kinase, with only PKC yielding increased enzyme activity. Dephosphorylation of both forms of enzyme by alkaline phosphatase decreased enzymic activity. These studies are of functional significance as they report for the first time that phosphorylation enhances choline acetyltransferase catalytic activity.

Key words: acetylcholine, cholinergic, protein isoform.

INTRODUCTION

Choline acetyltransferase (ChAT; EC 2.3.1.6) catalyses synthesis of the transmitter acetylcholine (ACh) in cholinergic neurons. While only cholinergic neurons express ChAT and use ACh to communicate neurochemical information in the nervous system, recent evidence indicates that ACh is also formed in non-neuronal cells in pro- and eukaryotes ranging from bacteria and primitive plants to epithelial and immune cells in human (for review see [1,2]). The physiological role of ACh in non-neuronal tissues is unclear, and may differ from the well-studied neurotransmitter function of ACh within peripheral and central nervous systems.

ChAT is encoded by a single gene for which there are multiple transcripts expressed by differential utilization and alternative splicing of three non-coding exons [3–5]. In human, three of the four transcripts identified all translate to the same 69-kDa protein (transcripts R, N1 and N2). The fourth transcript, M, has two translation-initiation sites and yields an 82-kDa enzyme in addition to the 69-kDa form. The 82-kDa form differs from 69-kDa ChAT in terms of a 118-residue N-terminal extension [3,5]. Mechanisms regulating production of the various transcripts and their physiological roles have not been elucidated, but they could vary in stability or translational efficiency or may be differentially expressed in response to trophic factors or hormones. Similarly, it has not been determined whether 82- and 69-

kDa ChAT have different physiological roles or are regulated by different cellular mechanisms. We observed recently that 82-kDa ChAT is localized to nucleus, whereas the 69-kDa enzyme is in the cytoplasm of living cells transiently expressing green-fluorescent-protein conjugates of the proteins [6].

To date, there have been three reports of post-translational modification of ChAT by protein kinases [7–9]. ChAT is a substrate for calcium-dependent protein kinases [9,10], and phosphorylation *in situ* in hippocampal nerve terminals can be modulated by altering cytosolic free calcium levels [7]. The role that phosphorylation plays in regulating ChAT function is unclear, although it has been suggested that increased phosphorylation may decrease binding of the enzyme to synaptic membranes and regulate subcellular compartmentation in the nerve terminal [8]. Functional studies in which inhibitors of serine/threonine phosphoprotein phosphatases were used to modulate phosphorylation of ChAT in nerve terminals indicate that phosphorylation may play a role in its partitioning between cytoplasm and membrane, and in regulation of ACh biosynthesis under some conditions [10]. Analysis of the primary structure of ChAT indicates that it has a number of potential phosphorylation sites that could be recognized by multiple protein kinases, including protein kinase A (cAMP-dependent protein kinase, PKA), protein kinase C (PKC), protein kinase G, α -calcium/calmodulin-dependent protein kinase II (α -CaM kinase), casein kinase II (CK2) and mitogen-activated protein kinase.

Abbreviations used: ACh, acetylcholine; CK2, casein kinase II; α -CaM kinase, α -calcium/calmodulin-dependent kinase II; ChAT, choline acetyltransferase; CTab, affinity-purified anti-ChAT antibody to C-terminal peptide; His6, hexa-histidine; NTab, affinity-purified anti-ChAT antibody to N-terminal peptide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; AEBSF, 4-(2-aminoethyl)benzenesulphonyl fluoride; TRITC, tetramethylrhodamine β -isothiocyanate.

¹ Present address: Department of Pharmacology, University of Louisville, Louisville, KY, U.S.A.

² To whom correspondence should be addressed, at the Department of Physiology (e-mail jrylett@physiology.uwo.ca).

In the present studies, recombinant 82- and 69-kDa human ChAT were prepared in baculovirus and bacterial expression systems to facilitate comparative studies of these two forms of enzyme. Affinity-purified antibodies recognizing either the C-termini of both 82- and 69-kDa ChAT or only the N-terminus of the 82-kDa enzyme were generated and used as reagents for immunoblotting, immunohistochemistry and immunoprecipitation. Insect cells expressing recombinant enzymes revealed different subcellular distribution for 82- and 69-kDa forms. Proteins were analysed by two-dimensional gel electrophoresis to reveal different isoforms present in each, and were tested as substrates for a panel of protein kinases. Importantly, we report for the first time that phosphorylation of human ChAT resulted in enhanced catalytic activity.

EXPERIMENTAL

Preparation and purification of baculovirus-expressed recombinant human ChAT

Wild-type and hexa-histidine (His6) epitope-tagged recombinant 82- and 69-kDa human ChAT were produced in High-5 insect cells using a baculovirus expression system. M- and N1-ChAT cDNAs were obtained as a generous gift from Dr H. Misawa (Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan). To produce 82-kDa ChAT, the M-ChAT cDNA was used as a template for the preparation of the mutant cDNA M119A in which the second translation-initiation site (ATG, Met-119) was changed to Ala (GCG) by PCR-mediated site-directed mutagenesis; the resulting mutant cDNA encodes only the 82-kDa form of ChAT, whereas wild-type M-ChAT is translated to both 82- and 69-kDa ChAT [6]. For His6-tagged proteins, cDNA inserts were modified by PCR to contain flanking 5' *EcoRI* sites and 3' *SalI* restriction-endonuclease sites to facilitate ligation into the insect expression vector pBlueBacHis (Invitrogen). The translation start codons were removed during the addition of the 5' *EcoRI* sites to allow translation to begin at the N-terminal His6-epitope tag, and result in the production of an epitope-tagged ChAT fusion protein; the 3' *SalI* sites were located after the stop codon of the ChAT cDNAs. For wild-type proteins (non-His6-tagged proteins) cDNA inserts encoding either 69- or 82-kDa ChAT were cut out of other vectors at 5' *KpnI* and 3' *SalI* sites and ligated into the *KpnI/XhoI* sites of pBlueBac4.5. Recombinant viruses for both 82- and 69-kDa ChAT were prepared from these plasmids (4 µg) and linearized Bac-N-Blue DNA (0.5 µg; Invitrogen) in High-5 cells using the liposome transfection method (Invitrogen). Following plaque purification and confirmation of the presence of the cDNA insert from viral genomic DNA by PCR, high-titre viral stocks were prepared and stored at 4 °C for use in cell infections.

High-5 cells were grown in Grace's media supplemented with yeastolate (1.7 g/500 ml; Gibco), lactalbumin hydrolysate solution (1.7 g/500 ml; Gibco), 8% fetal bovine serum (Hyclone) and gentamicin (50 µg/ml; Gibco). Infections with recombinant baculovirus were made in monolayers of cells at the required multiplicity of infection. At 40 h after infection, High-5 cells were washed twice with PBS, scraped into PBS, then pelleted by centrifugation at 700 g for 5 min at 4 °C. Cells were lysed by incubation for 30 min at 4 °C in 5 vols of lysis buffer [20 mM Tris/HCl, pH 8.0/150 mM NaCl/2.5 mM β-mercaptoethanol/10% ethanol/2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF)/2 µg/ml pepstatin/2 µg/ml leupeptin/200 µg/ml aprotinin] with 0.5% of Triton X-100. Lysates were centrifuged at 12000 g for 15 min at 4 °C with the resulting supernatant used for further protein purification.

Purification of ChAT-His6 fusion protein

ChAT-His6 proteins were purified by a two-step purification protocol with DEAE-Sepharose chromatography (Pharmacia) followed by Talon resin metal-affinity chromatography (Clontech). Supernatants from cell lysates were diluted 1:3 with buffer A (20 mM Tris/HCl, pH 7.0/150 mM NaCl/10% ethanol/2.5 mM β-mercaptoethanol/0.5 mM AEBSF/2 µg/ml pepstatin/2 µg/ml leupeptin/20 µg/ml aprotinin) and applied to a DEAE-Sepharose column (50 mg of protein/ml of resin) at 1 ml/min. The flow-through from this column was used for the Talon metal-affinity purification step. The affinity resin was washed twice with 10 vols of buffer B (20 mM Tris/HCl, pH 8.0/150 mM NaCl), then pre-equilibrated with active fraction eluted from DEAE-Sepharose column which had been diluted 1:1 with buffer B (20 mg of total protein in active fractions/ml of resin) for 2 h at 4 °C with gentle agitation. The suspension was loaded into a chromatography column, washed with 5 column-bed vols of buffer B and 10 column vols of buffer B containing 5 mM imidazole. ChAT was then eluted with 7 column vols of buffer B containing 50 mM imidazole.

Fractionation of wild-type ChAT on DEAE-Sepharose

Wild-type 82- and 69-kDa ChAT were partially purified by fractionation on DEAE-Sepharose with an NaCl gradient. Supernatants from lysed cells were dialysed against three changes of 20 vols of buffer C (20 mM Tris/HCl, pH 8.8/10 mM NaCl/2 mM β-mercaptoethanol/0.5 mM AEBSF/2 µg/ml pepstatin/2 µg/ml leupeptin/20 µg/ml aprotinin) at 4 °C. Dialysed samples were loaded on to a DEAE-Sepharose column that had been equilibrated with buffer C. Fractions of 10 ml each were collected during stepwise elution of the column with 50, 100, 200, 300 and 1000 mM NaCl added to buffer C.

Preparation and DEAE-Sepharose fractionation of bacterially expressed recombinant human ChAT

Recombinant 82- and 69-kDa ChAT were also produced in a bacterial expression system to obtain non-phosphorylated proteins. Consequently, cDNA inserts for both 82- and 69-kDa human ChAT were ligated into 5' *BamHI* and 3' *NcoI* sites of the bacterial protein expression vector pET3d (Novagen) following addition of these restriction sites by PCR. Plasmids were expressed in and isolated from DH5α cells, then used to transform BL21(DE3) bacterial cells by electroporation. Individual colonies were used to inoculate 2-ml cultures of Luria-Bertani medium containing 100 µg/ml ampicillin and grown to an A_{600} value of 0.6. This culture was used to inoculate Luria-Bertani/ampicillin medium (100–500 ml), which was grown to an A_{600} value of 0.6 before addition of 0.4 mM isopropyl β-D-thiogalactoside to induce protein formation. After 3 h of incubation, bacterial cells were harvested by centrifugation at 5000 g for 5 min at 4 °C. Cells were resuspended in 0.25 culture vol. of cold 20 mM Tris/HCl, pH 8.0, and recentrifuged. Cell pellets were sonicated in 5 vols of buffer D (buffer C supplemented with 1 mM EDTA) on ice (3 × 30 s), then centrifuged at 5000 g for 5 min. The resulting pellets were washed twice with 3 vols of buffer D and once with 1 vol of 6 M urea (this wash contained a substantial amount of ChAT activity). Supernatants were combined and centrifuged at 15000 g for 30 min, then dialysed against three changes of 20 vols of buffer D overnight. Dialysed samples were loaded on to a DEAE-Sepharose column equilibrated with buffer D. Fractions containing different forms of ChAT were

collected during stepwise elution with 50, 100, 200, 300 and 1000 mM NaCl added to buffer D.

Preparation of anti-ChAT antibodies

Polyclonal antibodies to two human ChAT peptides (GeneMed Synthesis) were prepared in rabbits. The first antibody (affinity-purified anti-ChAT antibody to C-terminal peptide, CTab) was raised against the sequence CEKATRPSQGHQP [5], located at the C-termini of both 82- and 69-kDa ChAT, and recognized both forms of enzyme. The second antibody (affinity-purified anti-ChAT antibody to N-terminal peptide; NTab) was raised to the sequence CAEAAEPRRAGPH that is present in the N-terminal extension of 82-kDa ChAT (residues 87–98), and recognizes only that form of the enzyme. Both peptide immunogens were conjugated to maleimide-activated keyhole limpet haemocyanin at the N-terminal cysteine residue of the peptide. Specific immunoglobulins were purified from both antibody preparations by affinity chromatography on NHS-Sepharose columns to which the peptides used as immunogen had been coupled (4 mg/ml of resin) according to the manufacturer's instructions (Pharmacia). Antisera (25 ml) were loaded on to affinity columns (2.5 ml) pre-equilibrated with PBS at a rate of 0.5 ml/min. Following loading, columns were washed with 10 vols of PBS containing 500 mM NaCl and 0.1% Nonidet P-40. Immunoglobulins were eluted with 100 mM glycine/HCl, pH 2.7. A volume of 1 ml of immunoaffinity-purified antibody represents 2.5 ml of crude serum.

One- and two-dimensional PAGE

One-dimensional SDS/PAGE was performed on 12% gels according to the method of Laemmli [11]. Two-dimensional SDS/PAGE was performed by the method of Garrels [12], with isoelectric focusing gels containing 2% ampholines pH 3.5–10 or pH 5–7 (80% of 5–7 and 20% of 3.5–10; Pharmacia), and the second dimension run on 10% separating gels. To determine the pI values of different isoelectric forms of ChAT, samples were analysed by co-migration with 12 μ l ($\approx$ 7.2- μ g protein content) of two-dimensional SDS/PAGE standards (Bio-Rad) composed of: soya bean trypsin inhibitor, pI 4.5; bovine muscle actin, pIs 5.0 and 5.1; BSA, pIs 5.4, 5.5 and 5.6; bovine carbonic anhydrase, pIs 5.9 and 6.0; hen egg white conalbumin, pIs 6.0, 6.3 and 6.6; and equine myoglobin, pI 7.0. In some cases for ChAT pI determinations, carbamylated protein standards were used (Pharmacia) with carbonic anhydrase, pI range 4.8–6.7, and glyceraldehyde-3-phosphate dehydrogenase, pI range 4.7–8.3. After electrophoresis, proteins were either stained with Coomassie Brilliant Blue or transferred to nitrocellulose for immunoblotting. Nitrocellulose membranes were stained after protein transfer with 0.02% Ponceau-S in 1% acetic acid solution to visualize transferred proteins and standards.

Western-blot analysis

Proteins from one-dimensional SDS/PAGE gels were transferred to nitrocellulose membranes in a semi-dry electroblotting apparatus using transfer buffer (48 mM Tris/39 mM glycine) containing 20% methanol. Membranes were saturated with 8% non-fat milk powder in PBS and probed with anti-ChAT antibodies CTab (1:2000) or NTab (1:500) for 1 h at room temperature. Membranes were washed with PBS containing 0.5% Triton X-100, then bound antibodies were detected with peroxidase-coupled secondary antibodies and ECL (Amersham).

Phosphorylation of ChAT *in vivo*

32 P Labelling of recombinant 82- and 69-kDa ChAT expressed in High-5 cells was performed by preincubating cells for 3 h with 0.2 mCi of 32 PO $_4^{3-}$ in 500 μ l of phosphate-free culture media (MEM, Sigma) on 35-mm culture dishes. After treatment, crude cell extracts were prepared and ChAT was fractionated on DEAE-Sepharose as described above.

Phosphorylation reactions *in vitro*

Partially purified 82- or 69-kDa recombinant ChAT from *Escherichia coli* (flow through after DEAE-Sepharose fractionation step; 10 μ g of total protein) was incubated at 30 °C for 15 min with PKA (10 m-units), PKC (0.4 m-unit), CK2 (1 m-unit) or α -CaM kinase (5 m-units). Incubation reactions also included 5 μ Ci of [γ - 32 P]ATP in 50 μ l of phosphorylation buffer comprised of: 50 mM Tris/HCl, pH 7.0, 20 mM MgCl $_2$, 50 mM KCl, 1 mM EGTA, 1 mM dithiothreitol and 25 μ M ATP for PKA; 50 mM Hepes, pH 7.4, 20 mM MgCl $_2$, 1.5 mM CaCl $_2$, 0.1 mg/ml BSA, 0.05 mg/ml phosphatidylserine, 10 μ g/ml 1,2-dioleoyl-*sn*-glycerol and 10 μ M ATP for PKC; 50 mM Tris/HCl, pH 7.0, 10 mM MgCl $_2$, 80 mM KCl, 1 mM dithiothreitol and 10 μ M ATP for CK2; and 50 mM Tris/HCl, pH 7.2, 0.4 mM dithiothreitol, 0.5 mM CaCl $_2$, 5 mM MgCl $_2$, 1 μ M calmodulin and 100 μ M ATP for α -CaM kinase. Reactions were stopped by the addition of sample buffer. Following electrophoresis, proteins were transferred from gels to nitrocellulose membranes, which were first exposed to film (Kodak X-OMAT or XAR-5) to identify phosphoproteins, and then immunoblotted with CTab to co-localize phosphorylated forms of ChAT with immuno-reactive ChAT isoforms.

For determination of the effects of phosphorylation on ChAT enzymic activity, phosphorylation reactions were carried out for 10 min under conditions as described above. Then ChAT activity was measured immediately at 37 °C in an additional 20-min incubation.

Dephosphorylation and assay of ChAT activity

Fractions eluted from DEAE-Sepharose with 200 mM NaCl containing constitutively phosphorylated 82- and 69-kDa ChAT from High-5 cells were used to study effects of dephosphorylation on enzyme activity. Samples (about 5 μ g of protein) were incubated in Tris/HCl, pH 8.0, with 2 mM AEBSF and 0.5% Triton X-100 for times up to 30 min at 37 °C with 0.5 unit of alkaline phosphatase. Separate controls were performed at each time point. ChAT activity was measured immediately at 37 °C in an additional 20-min incubation, and samples were retained for ChAT immunoblotting to determine that enzyme protein was not lost during the experimental manipulations.

ChAT activity was measured radioenzymically by a modified method of Fonnum [13] as published previously [14].

Immunohistochemistry and confocal microscopy

High-5 cells expressing either wild-type 82-kDa (M119A mutant) or 69-kDa forms of ChAT were washed three times with PBS, then fixed with 4% paraformaldehyde in PBS for 15 min at 4 °C. Following four washes with PBS, fixed cells were incubated for 30 min at room temperature in PBS containing 1% BSA. Cells were incubated subsequently for 1 h with CTab (1:500) or NTab (1:200) in PBS containing 1% BSA. After washing three times for 5 min with PBS, cells were incubated for 45 min with a secondary tetramethylrhodamine β -isothiocyanate (TRITC)-

coupled AffiniPure donkey anti-rabbit IgG (Jackson Immuno-Research Laboratories) at a 1:50 dilution. Preparations were observed with a confocal laser scanning microscope (Zeiss LSM 510).

RESULTS

Expression of recombinant human 82- and 69-kDa ChAT in High-5 cells using a baculovirus expression system

High-titre recombinant baculoviruses containing cDNA inserts encoding 82- and 69-kDa ChAT were constructed and used to infect High-5 insect cells; both wild-type and His6-tagged proteins were produced under control of the polyhedrin promoter. Maximal ChAT protein expression and enzyme specific activity were observed in crude cell extracts at 40 h post-infection, with these measures stable up to 60 h after infection (results not shown).

Purification and analysis of 82- and 69-kDa ChAT-His6

Figure 1 illustrates protein profiles on one-dimensional SDS/PAGE for 82- and 69-kDa ChAT-His6 in cell homogenates and at each purification step. This protocol yielded enzyme that was purified essentially to homogeneity. Immunoblots shown in Figure 1(B) demonstrate the specificity of the CTab anti-ChAT antibody, particularly in the case of the crude cell lysate and after the first purification step (Figure 1, lanes 1, 2, 4 and 5). A summary of the purification of His6-tagged recombinant human ChAT is presented in Table 1; comparable purification and specific activity were obtained for both forms of the enzyme. The specific activity of 69-kDa ChAT-His6 in crude extracts of High-5 cells was 425–570-fold greater than the activity found in homogenates of human placenta [15,16].

Figure 1 Purification of 82- and 69-kDa ChAT-His6

Proteins corresponding to each purification step were run on SDS/PAGE under reducing conditions. In (A) the gel was stained with Coomassie Brilliant Blue, and (B) represents the corresponding Western blot probed with CTab anti-ChAT antibody (see the Experimental section). Lane M, marker proteins (Pharmacia) from top to bottom: phosphorylase b (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa); Lanes 1–3, recombinant human 82-kDa ChAT-His6; lanes 4–6, recombinant human 69-kDa ChAT-His6; lanes 1 and 4, crude cell extract (30 μ g of protein); lanes 2 and 5, fraction from DEAE-Sepharose purification step (30 μ g of protein); lanes 3 and 6, Talon affinity column-purified fraction (3 μ g of protein for Coomassie Brilliant Blue stain; 0.5 μ g of protein for Western blot).

The DEAE-Sepharose chromatography loading buffer contained 10% ethanol. When the enzyme was purified in the absence of ethanol, a second peak of ChAT activity (17 and 20% of the total activity of 82- and 69-kDa ChAT, respectively) was eluted with 250 mM NaCl in buffer A (results not shown). This suggests that a fraction of both forms of the enzyme interacted ionically either with the column medium or with proteins retained on the column when the sample was loaded. As the column flow-through fraction was to be used for the second purification step, ethanol was included in the loading buffer to minimize ChAT bound to the column and to achieve greater recovery of enzyme in this fraction. Inclusion of ethanol in the elution buffer during ChAT purification by ion-exchange chromatography has been described previously [17].

Multiple isoforms for 82- and 69-kDa ChAT-His6 were revealed by two-dimensional SDS/PAGE

As observed with many other proteins, both 82- and 69-kDa human ChAT-His6 are comprised of multiple isoforms when the proteins were subjected to two-dimensional SDS/PAGE (Figure 2). Both forms of His6-tagged enzyme appeared as a broad smear over a pH range from 7.7 to 9.8. Also, as shown in Figure 2(A), preparations of 69-kDa ChAT-His6 contained minor protein contaminants at roughly the same apparent molecular mass that were not detected on the immunoblot with anti-ChAT antibody (Figure 2B).

DEAE-Sepharose fractionation and analysis of wild-type 82- and 69-kDa ChAT expressed in High-5 cells and in *E. coli*

Since ChAT-His6 proteins appeared as a relatively continuous smear on two-dimensional SDS/PAGE gels and immunoblots, we addressed the issue of whether the positively charged His6-epitope tag contributed to this pI distribution profile. In the first instance, recombinant viruses were prepared which encoded an enterokinase cleavage site between the N-terminal His6 tag and the ligation site for the 5'-end of the cDNA of interest. This yielded recombinant proteins from which the epitope tag could potentially be cleaved during incubation with enterokinase. In the case of both 82- and 69-kDa ChAT-His6, this was not feasible as ChAT protein was not stable during the prolonged (16-h) enterokinase cleavage step. Modification of this protocol with considerably shorter enterokinase treatment (4 h) also did not yield adequate amounts of functional ChAT (results not shown).

As an alternative strategy to obtain non-His6-tagged ChAT, we prepared high-titre recombinant baculovirus expressing wild-type 82- and 69-kDa human ChAT. Upon comparison of specific activities of wild-type and His6-tagged enzymes, it was determined that the N-terminal His6 tag significantly modified expression levels of the recombinant proteins. Specific activities of both 82- and 69-kDa ChAT-His6 were only about 50% of that measured for wild-type enzymes (Tables 1 and 2). Also by comparison, specific activity of both 82- and 69-kDa wild-type ChAT was found to be higher in crude extracts of *E. coli* expressing the enzyme compared with that measured in High-5 cell homogenates (Table 2). Specific activity of 69-kDa ChAT in crude extracts of *E. coli* was 4200–5630-fold greater than reported for human placental extracts [15,16].

Recombinant wild-type enzymes produced in High-5 cells and *E. coli* were partially purified by fractionation on DEAE-Sepharose; further purification was not carried out at this time as the enzyme preparations available allowed the questions posed to be addressed adequately. As shown in Table 2, wild-type 82-kDa ChAT produced in High-5 cells was detected

Table 1 Purification of 82- and 69-kDa ChAT-His6

His6-epitope-tagged ChAT was purified from baculovirus-infected High-5 cells expressing 82- and 69-kDa forms of the enzyme. The data are based on a starting preparation of 1.0 g (wet weight) of cells. Enzyme activity: 1 unit of enzyme catalyses the formation of 1 μ mol of ACh/min at 37 °C.

Purification step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
82-kDa ChAT					
Cell extract	97.3	133.2	0.7	100	1
DEAE-Sepharose	84.1	48.8	1.7	86.4	2.4
Talon column	73.7	1.6	46.1	75.7	65.9
69-kDa ChAT					
Cell extract	122.8	144.8	0.9	100	1
DEAE-Sepharose	109.7	49.4	2.2	89.3	2.4
Talon column	93.9	2.1	44.7	76.5	49.7

predominantly in the column flow-through with about 28 % of enzyme activity eluted with 200 mM NaCl. Interestingly, while this salt fraction contained substantial enzyme activity, it was represented by only a weak band on immunoblots (Figure 3A). Densitometric analysis of Western blots, in which band intensity was normalized to total protein content of crude cell extracts and fractions eluted with 200 mM NaCl, revealed that the latter represents only 4 % of total ChAT protein in crude extracts, but comprises 28 % of total enzyme activity. By comparison, a different elution profile emerged when wild-type 82-kDa ChAT expressed in *E. coli* was fractionated on DEAE-Sepharose; again, most enzyme activity was recovered in the column flow-through, with minor amounts in the low-salt fractions and no enzyme activity in the 200 mM salt fraction (Table 2). On Western blotting, immunoreactive bands at 82 kDa were observed for only the flow-through fraction and none of the salt fractions (Figure 3E). Interestingly, weak immunopositive bands with apparent molecular masses of about 47 and 30 kDa were detected in the 50 and 100 mM NaCl elution samples corresponding to a measurable amount of enzyme activity (results not shown). These bands could represent degradation products formed during fractionation; catalytically active truncated forms of rat ChAT produced by proteolytic treatment of enzyme have been described previously [18].

Table 2 Fractionation of wild-type ChAT on DEAE-Sepharose

Crude extracts of High-5 cells (1 g wet weight) expressing 82- or 69-kDa ChAT, or *E. coli* (1-g bacterial pellet) expressing 82- or 69-kDa enzyme were fractionated on a DEAE-Sepharose column using a stepwise elution with NaCl. Enzyme activity: 1 unit of enzyme catalyses the formation of 1 μ mol of ACh/min at 37 °C.

Sample	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)
82-kDa ChAT (High-5 cells)				
Crude extract	78.4	52	1.5	100
DEAE-Sepharose flow-through	49.7	27.2	1.8	63.4
[NaCl]				
50 mM	—	4.1	—	—
100 mM	—	6.6	—	—
200 mM	21.6	7.2	3	27.6
300 mM	—	0.9	—	—
1 M	—	0.4	—	—
69 kDa ChAT (High-5 cells)				
Crude extract	93.8	58.2	1.6	100
DEAE-Sepharose flow-through	56.8	30.8	1.8	60.6
[NaCl]				
50 mM	12	5.2	2.3	12.8
100 mM	6.7	7.1	0.9	7.1
200 mM	13.9	11.9	1.2	14.8
300 mM	—	0.7	—	—
1 M	—	0.3	—	—
82 kDa ChAT (<i>E. coli</i>)				
Crude extract	46.3	18.6	2.5	100
DEAE-Sepharose flow-through	35.6	10.8	3.3	77
[NaCl]				
50 mM	4	4.8	0.8	8.6
100 mM	3.3	8.4	0.4	7.1
200 mM	—	9.8	—	—
300 mM	—	0.4	—	—
1 M	—	0.1	—	—
69-kDa ChAT (<i>E. coli</i>)				
Crude extract	335	21	15.9	100
DEAE-Sepharose flow-through	201	10	20.1	60
[NaCl]				
50 mM	35	2.3	15.2	10.4
100 mM	28	1.7	16.5	8.4
200 mM	58	13.2	4.4	17.3
300 mM	—	1.5	—	—
1 M	—	0.2	—	—

Figure 2 Two-dimensional profile of purified ChAT-His6

Recombinant 82- and 69-kDa ChAT-His6 were purified as described in the Experimental section, then analysed by two-dimensional gel electrophoresis. Proteins were separated in the first dimension (isoelectric focusing, IEF) using a pH gradient 3.5–10, then separated in the second dimension on a 10% polyacrylamide/SDS slab gel. Proteins were stained with Coomassie Brilliant Blue (A), or transferred to nitrocellulose membranes for Western immunoblotting (B). Western blots were probed with CTab anti-ChAT antibody. The upper gels show the pattern for 82-kDa ChAT-His6, and the lower gels show the pattern for 69-kDa ChAT-His6.

Figure 3 One- and two-dimensional profiles of wild-type 82- and 69-kDa ChAT fractionated on DEAE-Sepharose

Crude cell extracts (CES) of High-5 cells and *E. coli* expressing recombinant ChAT were fractionated on DEAE-Sepharose, as described in the Experimental section, with column flow-through (FT) and 50, 100, 200, 300 and 1000 mM NaCl elution fractions collected for analysis. Protein (50 μ g) from each fraction was run on one-dimensional gels, then transferred to nitrocellulose membranes and probed with the CTab anti-ChAT antibody. (A and C) Distribution of 82- and 69-kDa ChAT proteins, respectively, in fractions from column fractionation for enzyme produced in High-5 cells. (E and G) represent distribution of 82- and 69-kDa ChAT proteins, respectively, in fractions from column fractionation for enzyme produced in bacteria. Column fractions that were determined to contain ChAT protein by immunoblot were used for further analysis of ChAT isoform profiles by two-dimensional gel electrophoresis, transferred to nitrocellulose and probed with CTab anti-ChAT antibodies. Proteins (100 μ g) were separated in the first dimension using a pH gradient of 3.5–10, except in the case (B) where a pH 5–7 gradient was used and the sample was concentrated with 500 μ g of protein loaded. (B and D) Isoform profiles present in the column fractions for 82- and 69-kDa ChAT, respectively, for enzyme produced in High-5 cells. (F and H) Isoform profiles present in the column fractions for 82- and 69-kDa ChAT, respectively, for enzyme produced in bacteria. The data are representative of at least five separate experiments.

DEAE-fractionation elution profiles for wild-type 69-kDa ChAT from both High-5 cells and *E. coli* were similar (Figures 3C and 3G), but differed substantially from that observed for the 82-kDa enzyme. ChAT activity was recovered predominantly in the column flow-through fraction, with small amounts of enzyme activity and immunoreactive protein contained in fractions eluted with up to 200 mM NaCl (Figure 3 and Table 2).

Multiple isoforms of both baculovirus- and bacterially produced wild-type human ChAT were resolved by two-dimensional SDS/PAGE, as illustrated in Figure 3, in contrast to the smears observed with His6-tagged enzymes. The 82-kDa form of ChAT produced in High-5 cells was represented by at least six isoforms detectable in the crude extract, with pI values of approx. 9.0, 8.7, 6.6, 6.5, 6.2 and 5.2 (Figure 3B). The flow-through from the DEAE-Sepharose column resolved as the first five isoforms, with isoform 6 being undetectable. The sixth isoform eluted from the column with 200 mM NaCl and, following a 5-fold concentration step, separated as seven isoforms with isoelectric points between 5.5 and 5.2 when a pH gradient of 5–7 was used in the first

dimension rather than a pH gradient of 3.5–10. *E. coli*-expressed 82-kDa ChAT was represented by only four isoforms with pI values of about 9.1, 8.6, 6.5 and 6.3 (Figure 3F). It is possible that the missing isoform with a pI of 6.6 is represented with isoform 3, but their separation was not clear. Importantly, the minor acidic isoform(s) (isoform 6) found in enzyme preparations from High-5 cells were not present in bacterially produced 82-kDa ChAT; one major difference between these two protein-expression systems is that, unlike insect cells, bacterially produced proteins do not undergo post-translational modification by conventional serine/threonine/tyrosine phosphorylation.

Separation of wild-type 69-kDa ChAT produced in High-5 cells by two-dimensional gel electrophoresis yielded nine isoforms with approx. pI values of 8.3, 8.0, 7.3, 7.0, 6.8, 6.7, 6.5, 5.1 and 4.9 (Figure 3D). By comparison, *E. coli*-expressed 69-kDa ChAT was represented by six isoforms with pI values centred around pHs of about 8.9, 8.3, 7.7, 6.8, 6.3 and 5.5 (some spots were elongated; Figure 3H). Similar to the finding with 82-kDa ChAT, the most acidic isoforms of 69-kDa ChAT observed with

Figure 4 Phosphorylation *in vivo* of 82- and 69-kDa ChAT

High-5 cells expressing wild-type 82- and 69-kDa ChAT were pre-labelled with [32 P]orthophosphoric acid. Subsequently, crude lysates of cells expressing 82-kDa ChAT, and the fraction of 69-kDa ChAT eluted from DEAE-Sephacrose with 200 mM NaCl, were analysed by two-dimensional PAGE. ChAT immunoblots using CTab (**A**) and autoradiographs (**B**) depicting ChAT protein distribution and 32 P incorporation are illustrated.

Figure 5 Phosphorylation *in vitro* of 82- and 69-kDa ChAT by multiple protein kinases

DEAE-Sephacrose column flow-through fractions of wild-type 82- and 69-kDa ChAT produced in *E. coli* were incubated with PKA, PKC, CK2 or α -CaM kinase under phosphorylating conditions to test the ability of the enzymes to serve as substrates for protein kinases. Enzymes were run on one-dimensional SDS/PAGE gels, transferred to nitrocellulose membranes and analysed by autoradiography (-80°C for 24–96 h) for incorporation of ^{32}P (**b**), then immunoblotted with CTab anti-ChAT antibody to allow co-localization with ChAT proteins (**a**). The data shown are representative of at least five separate experiments.

enzyme produced in High-5 cells (isoforms 8 and 9) did not appear in preparations of the bacterially expressed protein.

Importantly, phosphorylation experiments *in vivo* in High-5 cells expressing wild-type 82- and 69-kDa ChAT revealed that the most acidic isoforms of the enzyme incorporated [^{32}P]phosphate (Figure 4).

Phosphorylation of ChAT by multiple protein kinases

Four protein kinases were tested for their ability to phosphorylate bacterially produced ChAT *in vitro*; DEAE-column flow-through fractions were used in these studies. Figure 5 illustrates on one-dimensional gels that 82-kDa ChAT served as a substrate for PKC and CK2, whereas 69-kDa ChAT was phosphorylated by PKC, CK2 and α -CaM kinase. Neither form of enzyme appeared to be a substrate for PKA under the experimental conditions tested.

Figure 6 Phosphorylation by some kinases stimulates ChAT activity

Bacterially expressed 82- (white bars) and 69- (black bars) kDa ChAT were incubated under phosphorylating conditions with four different protein kinases, followed by measurement of ChAT enzymic activity. Statistically significant increases in catalytic activity of 82-kDa ChAT were observed following incubation with PKC, and of 69-kDa ChAT after incubation with PKC and CK2. While 82-kDa ChAT was also phosphorylated by CK2 and 69-kDa ChAT by α -CaM kinase, neither of these treatments led to enhanced ChAT activity. Data are means $\pm$ S.E.M. for $n = 5$ separate experiments. * $P < 0.01$ by Student's *t* test.

Activity of human ChAT is enhanced by phosphorylation *in vitro*

In the present study, we began to address the functional role of phosphorylation of ChAT by protein kinases by determining whether phosphorylation altered enzyme catalytic activity. As illustrated in Figure 6, incubation of both 82- and 69-kDa ChAT produced in *E. coli* with PKC resulted in significantly increased enzyme activity by 57 ± 9 and $112 \pm 17\%$, respectively (means $\pm$ S.E.M.). CK2 did not alter activity of 82-kDa ChAT, but caused a small statistically significant stimulation of activity of the 69-kDa enzyme ($20 \pm 3\%$). Interestingly, even though α -CaM kinase phosphorylated 69-kDa ChAT, activity of the enzyme was not altered relative to the control. PKA did not phosphorylate either form of the enzyme, nor was the acetylating activity of ChAT different from controls following incubation with the kinase.

Figure 7 Dephosphorylation of ChAT decreases enzymic activity

Constitutively phosphorylated 82- and 69-kDa ChAT expressed in High-5 cells were used in these studies. Aliquots of each enzyme eluted from DEAE-Sepharose with 200 mM NaCl (50 μ g of protein per sample) were treated with alkaline phosphatase (0.5 units) for 1, 5, 10, 20 and 30 min at 30 °C. ChAT activity was measured in control samples (□) for comparison with those treated with alkaline phosphatase (■). Results are means $\pm$ S.E.M. for three separate experiments. Western immunoblots with CTab anti-ChAT antibody of control (C) and alkaline phosphatase-treated (S) samples were performed on samples at the times indicated (subscript numbers) to determine whether dephosphorylation resulted in loss of enzyme protein.

In other studies, we asked whether constitutive phosphorylation of ChAT was involved in regulating enzyme catalytic activity. Experimentally, both 82- and 69-kDa wild-type ChAT produced in High-5 cells were incubated for various times with alkaline phosphatase, then enzyme activity was measured. As illustrated in Figure 7, enzymic dephosphorylation of both forms of ChAT resulted in decreased specific activity. Immunoblots, with densitometric analysis (results not shown), demonstrated that this was not the result of loss of enzyme protein during incubation with alkaline phosphatase.

Subcellular localization of 82- and 69-kDa ChAT in insect cells by immunohistochemistry and confocal microscopy

Using an immunohistochemical approach, 82- and 69-kDa ChAT assume differential subcellular localizations when expressed in baculovirus-infected High-5 cells, as was noted previously in mammalian cells [6]. However, in contrast to mammalian cells, at 24 h after infection 82-kDa ChAT was present in cytoplasm and concentrated in a distinct ring around the nucleus of High-5 cells, and 69-kDa ChAT was localized to plasma membrane, often at points of contact of adjacent cells, as illustrated in Figure 8(A).

By 64 h after infection, ChAT proteins underwent redistribution within the High-5 cells to assume a distribution similar to that seen in mammalian cells expressing cDNAs for 82- and 69-kDa ChAT (Figure 8B). At this point, 69-kDa ChAT appeared in cytoplasm (Figure 8B, panel a) with 82-kDa ChAT localized to nucleus (Figure 8B, panel b). Use of the NTab anti-ChAT antibody produced staining in cells expressing 82-kDa ChAT, but no staining of cells producing 69-kDa ChAT (results not shown).

Polyclonal anti-ChAT antibody production

NTab anti-ChAT antibody revealed high specificity for 82-kDa ChAT and did not recognize 69-kDa ChAT (Figure 9A). When CTab antibody was used, both 82- and 69-kDa ChAT was recognized (Figure 9B). His6-tagged enzymes were detected on one-dimensional SDS/PAGE gels at slightly higher apparent molecular masses than were wild-type ChAT proteins by both CTab and NTab anti-ChAT antibodies (Figure 9). Both antibodies were capable of immunoprecipitating ChAT from crude extracts of bacteria and insect cells expressing the enzymes (results not shown).

Figure 8 Immunofluorescence labelling of High-5 cells with CTab anti-ChAT antibody detected with TRITC-conjugated secondary antibody

Cells were infected with recombinant baculovirus encoding 69-kDa ChAT (**a, c**) or 82-kDa ChAT (**b, d**) at 24 h (**A**) or 64 h (**B**) prior to fixation and staining of cells. (**a, b**) Immunofluorescence images; (**c, d**) corresponding differential interference contrast images.

DISCUSSION

We describe production and purification of recombinant human 82- and 69-kDa ChAT in baculovirus and bacterial expression systems, and characterization and phosphorylation of these two forms of the enzyme. New information includes: (i) both 82- and 69-kDa ChAT are comprised of multiple isoforms which differ and can be separated by two-dimensional SDS/PAGE, (ii) both forms of enzyme serve as substrates for multiple protein kinases, (iii) phosphorylation of 69-kDa ChAT by PKC and CK2, but not α -CaM kinase, and of 82-kDa ChAT by PKC, but not CK2, led to increased catalytic activity, whereas neither form of enzyme was phosphorylated by PKA, (iv) 82- and 69-kDa ChAT were distributed differentially in cells following their synthesis with subcellular compartmentation changing over time, and (v) polyclonal antibodies generated to peptides corresponding to a portion of the N-terminus of 82-kDa ChAT and C-termini of both 82- and 69-kDa ChAT proved useful for immunoblotting, immunoprecipitation and immunohistochemistry.

Figure 9 ChAT antibody specificity determination

Crude extracts of cellular proteins from High-5 cells expressing 82- and 69-kDa ChAT or ChAT-His6 were separated using one-dimensional SDS/PAGE. Proteins were transferred to nitrocellulose membranes and Western immunoblots were performed with (**A**) NTab anti-ChAT antibody, 1:500, then blots were stripped and reprobed with (**B**) CTab anti-ChAT antibody, 1:2000. Lanes 1, 69-kDa ChAT; lanes 2, 82-kDa ChAT; lanes 3, 69-kDa ChAT-His6; and lanes 4, 82-kDa ChAT-His6.

Recombinant ChAT proteins were produced efficiently in both High-5 cells and *E. coli*. In general, higher activities for both forms of ChAT were achieved in bacteria, with the yield of 69-kDa ChAT being greatest. Interestingly, inclusion of positively charged His6-epitope tags on the recombinant proteins decreased their yield, and also interfered with separation of protein isoforms by two-dimensional gel electrophoresis. While the epitope tag facilitated protein purification, partially purified wild-type ChAT was used in most studies to overcome problems with alteration of the charge on the proteins, and thus allow accurate determination of isoform pI values.

Using preparative isoelectric focusing to separate samples enriched in ChAT, it was reported previously that the enzyme was comprised of multiple isoforms [19–22]. However, distribution of ChAT in the resulting subfractions was determined by measuring enzymic activity rather than by documenting and comparing ChAT protein electrophoretic isoform patterns by immunoblotting, as ChAT antibodies were not generally available at that time. Measurement of ChAT activity would not distinguish and identify individual enzyme isoforms, but rather would give a relative distribution of enzyme according to fractionation condition. The present study comprises the first report of characterization of ChAT isoform patterns by two-dimensional gel electrophoresis. These data serve as an important baseline for identification and comparison of mechanisms, such as phosphorylation, which regulate the presence and/or abundance of individual enzyme isoforms.

Phosphorylation of cellular proteins by kinases is a common and important regulatory mechanism in intracellular signal transmission. Changes in the phosphorylation state of enzymes, for example, may control their biological function by altering catalytic activity and substrate specificity, or by stabilizing functional enzyme complexes. In the present study, we demonstrated that 82- and 69-kDa ChAT serve differentially as substrates for serine/threonine kinases that have significant roles in neuronal function, and in some cases, but not others, phosphorylation leads to enhanced catalytic activity for the enzymes. Whereas ChAT has consensus motifs for PKA, it was not

phosphorylated by this protein kinase in the present or a previous study [8]. Whereas phosphorylation of 69-kDa ChAT by PKC has been reported [8], this is the first demonstration that 82- and 69-kDa ChAT are substrates for PKC and CK2, and that 69-kDa but not 82-kDa ChAT is phosphorylated by α -CaM kinase. In the nervous system, PKC modulates neuronal plasticity and learning processes [23], with reduced levels of PKC and/or alterations in activities and distribution of isoenzymes correlating with degeneration of selective neurons during aging and Alzheimer's disease [24–26]. CK2 is also present in brain, and its activity appears to be altered in Alzheimer's disease [27]. α -CaM kinase is involved in Tau protein phosphorylation and formation of paired-helical filaments in Alzheimer's disease [28].

As these kinases recognize different phosphorylation consensus motifs, it is likely that multiple residues on ChAT can be phosphorylated. This has been demonstrated for other neurotransmitter-synthesizing enzymes, such as tyrosine hydroxylase, with regulation of phosphorylation events governed by a variety of biological processes (reviewed in [29]). It is important now to identify which amino acid residues in ChAT are phosphorylated, how phosphorylation is regulated by physiological events such as neuronal activity and endogenous factors including growth and trophic factors, and whether this is modified by pathology.

The functional significance of phosphorylation of ChAT has not been determined. It is clear from the present study that phosphorylation of the enzyme by some kinases, particularly PKC, leads to enhanced catalytic activity. This is the first investigation in which this parameter has been measured directly. In a previous study, using an indirect approach we determined that experimental conditions which decreased cytosolic free calcium levels in rat hippocampal nerve terminals and correlated with decreased phosphorylation of ChAT did not alter total synaptosomal ChAT activity [7]. The physiological consequence of enhanced catalytic activity for ChAT is unknown, but probably would result in increased ACh synthesis and the potential for increased neurotransmission.

Subcellular compartmentation of proteins can also be regulated by protein phosphorylation; alterations in charge on the protein brought about by addition or removal of phosphate groups could influence its ability to bind to subcellular membranes or organelles [8]. Phosphorylation can also regulate translocation of proteins bearing nuclear localization signals between cytoplasm and nucleus [30,31]. We reported recently that 69-kDa ChAT was distributed in cytoplasm of mammalian cells, whereas 82-kDa ChAT was localized to nucleus [6]. This observation was confirmed and extended in the present study where we observed a temporal shift in subcellular localization of the two forms of enzyme in High-5 cells. Regulatory events, including phosphorylation, which play a role in directing subcellular localization of ChAT, remain to be determined.

In summary, observations made in the present studies, including development of protein-isoform patterns for both 82- and 69-kDa ChAT and that human ChAT serves differentially as a substrate for multiple protein kinases, provide a basis for future functional investigations. Elucidation of aspects of cellular regulation of cholinergic neurotransmission awaits identification and mutagenesis of specific residues that serve as sites for phosphorylation of ChAT. Furthermore, extension of studies at the protein level to pathology of the nervous system, such as Alzheimer's disease, may reveal important clues for modification of neurochemical transmission.

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