Choline Acetyltransferase from the Electric Organ of *Electrophorus electricus* (L.) – Physicochemical Characterization and Immunochemical Identification

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It is well known that the regulation of choline acetyltransferase (ChAT) activity, under physiological conditions, is important for the development and neuronal activities of cholinergic systems. The purification of ChAT has been obtained from many sources such as electric organs of fishes, *Drosophila melonogaster*, and mammals. We have prepared choline acetyltransferase from a pool of supernatants obtained by differential centrifugation of electric organ homogenates from **Electrophorus electricus** (L.) in Tris-phosphate buffer, 0.05 M, pH 7.6. The first step of the enzyme purification was performed by ammonium sulfate precipitation at 40% and 80%. The precipitate at 80% was solubilized with sodium-phosphate buffer 0.05 M, pH 7.6, dialyzed, chromatographed on DEAE-52 column and the active fraction submitted to FPLC system columns (Mono-Q: ion exchange – Superose-12: gel filtration). ChAT activity from the eluates was estimated by Fonnun's method [Fonnun, 1975], with Acetyl-Coenzyme A tritium labelled ([^{3}H]AcCoA) as substrate, and the synthesis of ^{3}HACh formed was measured. The peak from gel filtration showed a relative molecular mass of 80 kDa with highest activity in the order of 77,42 nmoles ACh/min/mg protein. This fraction was analyzed by SDS-PAGE and a band of 42 kDa was detected with Coomassie blue stain, indicating that the enzyme is formed by two subunits. Employing an antibody, the presence of ChAT was confirmed with the Western blotting technique. Isoelectrofocusing analysis demonstrated two isoforms with pI of 6,49 and 6,56, respectively.

Introduction

Choline acetyltransferase (ChAT; EC 2.3.1.6) is the enzyme that catalyzes the biosynthesis of the neurotransmitter acetylcholine (ACh) and was initially described by Nachmansohn and Machado (1943). The scarcity of rich sources of the enzyme and its relative instability during the purification process have delayed its isolation and many of the characteristics of this enzyme still remain obscure (Salvaterra *et al.,* 1982). ACh was the first neurotransmitter to be described (Loewi, 1921) and plays a central role in fundamental processes such as learning, memory, sleep (Bartus and Beer., 1982; Aigner and Mishkin, 1986) and muscle contraction in mammals. Evidence has been presented by many authors indicating that choline acetyltransferase is related to many kinds of pathological diseases such as Alzheimer, Myasthenia Gravis and multiple sclerosis (Rossier, 1977; Molenar *et al.,* 1981; Kato, 1989). Previous work has shown little heterogeneity between choline acetyltransferase obtained from different animal species such as *Drosophila melanogaster*, pigeon, *Torpedo* and mammals (Malthe-Sørenssen and Fonnun, 1972; Salvaterra *et al.,* 1982; Massarelli *et al.,* 1988). Although ChAT has been described as a cytosolic protein (Mautner, 1977; 1986) its presence was observed also in membrane fractions (Schmidt and Rylett, 1993) and associated with the biosynthesis, packing and storage of vesicular ACh (McCaman

Abbreviations: ChAT, Choline acetyltransferase; ACh, Acetylcholine; NaEDTA, Tetrasodium ethylene-diaminetetraacetate; TBS, Tris-buffered saline; PBS, Phosphate-buffered saline; FPLC, Fast performance liquid chromatography; DTT, Dithiothreitol; PMSF; Phenylmethylsulfonyl Fluoride; AcCoA, Acetyl coenzyme A.

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et al., 1965). Purification methods have been improved by development of specific antibodies to choline acetyltransferase, the antiserum allowing a more detailed analysis of the enzyme (Bruce and Hersh, 1985).

We have been studying the ChAT from the electric organ of *Electrophorus electricus* (L.), an important model for the study of bioelectrogenic events (Keynes and Martins-Ferreira, 1953) and of the nicotinic receptor for acetylcholine. The electric organ has cholinergic neurons that innervate electrocytes growing from myotubes and have about 1,000 fold more ACh receptors than muscle cells (Whittaker, 1987). An analogy between the electric organ and muscles has also been determined in studies of important proteins of the energetic metabolism, such as creatine phosphokinase (Hazan-Carneiro and Hasson-Voloch, 1983) and of cytoskeletal proteins, such as desmin (Costa *et al.*, 1986; 1988).

Previous work made in our laboratory by Soares and Hassón-Voloch (1972) demonstrated the effects of pH and different cations on the acetylcholine synthesis. The present paper describes the purification and the physicochemical characterization of choline acetyltransferase from the main electric organ of *Electrophorus electricus* (L.) as well as its immunological identification.

Materials and Methods

Specimens of *Electrophorus electricus* (L.) were provided to us by Museu Emílio Goeldi, Belém do Pará – Brasil. Trizma, DTT, PMSF, $MgSO_4$, AcCoA, β-mercaptoethanol, bovine serum albumin, DEAE-52 (cellulose anion exchange), were purchased from Sigma Chemical Company (USA); [^{3}H]AcCoA (4.0 Ci/mmol) and ECL kit from Amersham (England). The FPLC system and Ampholine carrier ampholytes were obtained from Pharmacia (Sweden). The polyclonal antibody to ChAT was obtained from Chemicon Co. (USA). All other chemicals were of analytical grade. Water used in this study was purified by a NANOpure system (Barnstead).

Procedures for isolation and solubilization of ChAT

A specimen of *Electrophorus electricus* (L.) was anesthetized with ice-cold water containing 2.0% urethane and killed. A piece of the main electric organ was excised and homogenized in sucrose 0.25 M containing 1 mM DTT, 1 mM NaEDTA, 1 mM $MgSO_4$, 1 mM β-mercaptoethanol and 1 mM PMSF a protease inhibitor and adjusted to pH 7.6 (1 part of tissue to 3 parts of the extraction solution) in a Virtis apparatus and fractionated using the method of Somló *et al.* (1977) modified. Briefly, the homogenate (E_0) was gently stirred overnight at 4 °C and then centrifuged at 5,000×*g*, for 20 min, in a Sorvall refrigerated centrifuge. The supernatant (S_0) was kept frozen for later utilization. The precipitate (P_0) was resuspended in Tris-phosphate buffer 0.05 M containing 1 mM DTT, 1 mM NaEDTA, 1 mM $MgSO_4$, 1 mM PMSF, 1 mM β-mercaptoethanol adjusted to pH 7.6 and centrifuged at 5,000×*g*. The supernatant (S_1) was then centrifuged at 100,000×*g*, for 2h. The supernatants S_2 and S_0 were joined together and utilized for ChAT purification procedure. Initially the mixed supernatants were submitted to a precipitation with 40% ammonium sulfate (w/v) followed by centrifugation at 20,000×*g*, at 4 °C, for 30 min. The supernatant from this centrifugation was then precipitated with 80% ammonium sulfate (w/v) and centrifuged at 20,000×*g*, at 4 °C, for 20 min. The resulting precipitate (P_{80}) was solubilized in phosphate buffer 0.05 M, pH 7.6, containing inhibitor of proteolysis (PMSF), and dialyzed in the same buffer, for 8 hours, with three changes of the dialyzing buffer (Rossier *et al.*, 1973). The P_{80} sample was submitted to chromatography on a DEAE-52 column and to FPLC system columns, Mono-Q and Superose-12, successively.

Protein assay

Protein concentration was determined by the Folin-phenol method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Anion-exchange chromatography and FPLC system

DEAE-52 column

The dialyzed 80% ammonium sulfate precipitate was submitted to an anion exchange chromatography in a DEAE -52 column as described below. Elution was started with 50 ml of 0.05 M sodium phosphate buffer, containing 1 mM DTT,

1 mM PMSF, 1 mM NaEDTA, 1 mM $MgSO_4$, 1 mM β-mercaptoethanol adjusted to pH 7.6 (elution buffer), and followed by a 0–0.5 M linear NaCl gradient in the same buffer (total volume 250 ml). Fractions of 3 ml were collected and samples of 100 µl of each fraction were used for protein determination. Protein peaks were separated for enzyme assay by the radiochemical method of Fonnun (1975).

Mono-Q column

The peak with the highest ChAT activity obtained from DEAE-52 column was submitted to a second anion-exchange chromatography, this time in the FPLC system. The Mono-Q column was equilibrated in elution buffer, containing protease inhibitor (PMSF). The sample (5.2 mg of protein) was applied to the column and submitted to a linear gradient of NaCl until a concentration 0.25 M was reached (total volume of 50 ml) at a flow rate of 1.0 ml/min. Fractions of 1 ml were collected. Samples (100 µl) of each fraction were analyzed to obtain the protein profile, and the peaks eluted from this column were submitted to ChAT activity assays.

Superose-12 column

The peak with the highest ChAT activity from Mono-Q column was then concentrated and loaded onto a gel filtration column in FPLC system. Proteins were eluted with elution buffer (total volume of 50 ml), containing PMSF, at a flow rate of 0.3 ml/min. The peaks emerging from this procedure had their protein profile analyzed, and were assayed for enzyme activity and had their relative molecular mass estimated after column calibration with protein standards.

Enzyme assay

Assay for choline acetyltransferase activity was made by the radiochemical method of Fonnun (1975), with [^{3}H]AcCoA as substrate. The substrate mixture contained: 64 mM sodium phosphate buffer, pH 7.6; 384 mM NaCl; 10 mM choline chloride; 25 mM NaEDTA; 10 mM eserine sulfate and 12 µM AcCoA. To the assay tube containing 10 µl of substrate mixture, 20 µl [^{3}H]AcCoA (0.1 µCi) was added plus 20 µg of the protein from the fraction being analyzed, at intervals of 30 seconds, at 37 ^{0}C, for 15 minutes. The reaction was stopped by addition of 50 mM NaEDTA, in 2 ml of Kalignost solution (5 mg/ml tetraphenylborate in acetonitrile). The synthesis of [^{3}H]ACh formed was measured in a liquid scintillation Packard apparatus (Model 1600 TR) and the activities are expressed as nmoles ACh formed per minute, per mg of protein. Each assay was made in triplicate. The catalytic reaction is indicated below:

$$\text{Choline} + \text{Acetyl CoA} \xrightarrow{\text{ChAT}} \text{Acetylcholine} + \text{Coenzyme A}$$

Electrophoresis analysis

The SDS-PAGE analysis was performed by the method of Laemmli (1970) with a mini-gel apparatus (PROTEAN II; Bio Rad) at 15mA, 200 volts for 2 hours. The running gels contained 10% acrylamide and 0.07% Bis (N,N'-methylene-bis-acrylamide). Samples were prepared by incubating 20 µg of protein in 30 µl of sample buffer. Protein bands were detected by Coomassie blue staining method. Sigma SDS molecular weight marker kit was used as protein standard.

Isoelectric focusing and two-dimensional SDS-PAGE

Isoelectric focusing was carried out by the method of O'Farrel (1975). Isoelectrofocusing gels (130 x 2.0 mm) contained 7% acrylamide, 0.3% Bis, 9.5 M urea and a 2% solution of synthetic carrier ampholytes with isoelectric points ranging from pH 3.5 -10.0 and 5.0–8.0 (1:4 v/v). Each gel was initially covered with a small volume of 8 M urea and water, both being replaced by lysis buffer after 1:30 h interval. For pre-focusing we used a solution of 0.02 M NaOH in the cathode and a solution of 0.01 M H_3PO_4 in the anode. The pre-focusing of ampholytes was done in the following conditions: 15 min at 200 watts; 30 min at 300 watts; 30 min at 400 watts, sequentially. Twenty µl of the concentrated enzyme obtained from the Superose-12 column was applied to the gel. The electrofocusing was carried out in constant load at 400 watts, for 16 hours plus 800 watts for 2 hours. The gels were kept in equilibrium buffer (10% glycerol, 5% β-mecaptoethanol, 2.3% SDS and 62.5 mM Tris-HCl buffer, pH 6.8) for 1 hour. The

gel used for pH gradient determination was frozen and the pH gradient was determined by an LKB contact electrode. The electrofocused gel containing the choline acetyltransferase was submitted to 2D SDS-PAGE (O'Farrel, 1975).

Immunoblotting analysis

Proteins were first separated by SDS-PAGE analysis and then transferred to nitrocellulose membrane (0.45 μm, from Schleicher & Schuell BA 85) by the method of Towbin *et al.* (1979) modified by Moura Neto *et al.* (1985). The nitrocellulose membrane was incubated in phosphate-buffered saline or Tris-buffered saline (PBS or TBS; 100 mM phosphate or Tris, pH 7.4, 100 mM NaCl) containing non-fat milk (5% w/v), at room temperature, for 1 h. The membrane was sequentially incubated with antibody against ChAT (1:500 v/v) in PBS at 4 °C overnight and with a secondary antibody conjugated to horseradish peroxidase (HRP), at room temperature, for 1 h (1:10000 v/v). An ECL kit (Amersham) was used for detection with Kodak film (X-OMAT).

Results

Purification steps

Extraction of cytosolic ChAT

Total electric organ homogenate, with ChAT activity, was submitted to differential centrifugation as described in Materials and Methods. More than 95% of the ChAT activity was found in the supernatant and removed from it by precipitation with 80% ammonium sulfate (P_{80}). These results suggested that the enzyme is weakly or not at all associated with membrane. The enzymatic activity was estimated in both the homogenate and the P_{80} fraction yielding 0.55 and 3.28 nmoles ACh/min/mg of protein, respectively (Table I). The dialyzed P_{80} fraction was then loaded onto a DEAE-52 column (anion exchange).

DEAE-52 column

The fractions obtained from the ion-exchange column were submitted to protein profile analysis and enzymatic activity estimation. The first peak yielded an enzymatic activity of 23.57 nmoles ACh/min/mg protein (Fig. 1), the highest among an eluted fraction.

Mono-Q column

The first peak obtained from DEAE-52 column, which yielded the highest enzymatic activity, was then passed in a Mono-Q column at FPLC system and the peaks eluted from this column were submitted to protein and ChAT assays. We observed that the second peak (eluted at 0.06 M of NaCl) had the highest activity, about 31.16 nmol ACh/min/mg protein (Fig. 2), and, thus, this peak was submitted to gel chromatography.

Superose-12 Column

The peaks from this column were assayed for enzymatic activity and protein concentration. We observed that the first peak yielded an enzymatic activity of 77.42 nmoles ACh/min/ mg protein (Fig. 3) and presented Mr 80 kDa (data not shown).

Table I. Summary of enzyme purification.

Purification step	Total Protein mg/vol	Specific activity *	Total units **	Recovery %	Purification (n-fold)
Homogenate	**5340.00**	**0.55**	**2937.00**	**100**	**1.0**
Precipited 80%	**780.00**	**3.28**	**2558.40**	**87.11**	**6.0**
Peak 1 DEAE-52	**5.20**	**23.57**	**122.56**	**4.17**	**43.0**
Peak 2 MONO-Q	**2.00**	**31.16**	**62.32**	**2.12**	**57.0**
Peak 1 SUPEROSE 12	**0.80**	**77.42**	**61.94**	**2.11**	**141.00**

*** nmol ACh · min^{-1} · mg protein^{-1}. ** Units of ACh produced.**

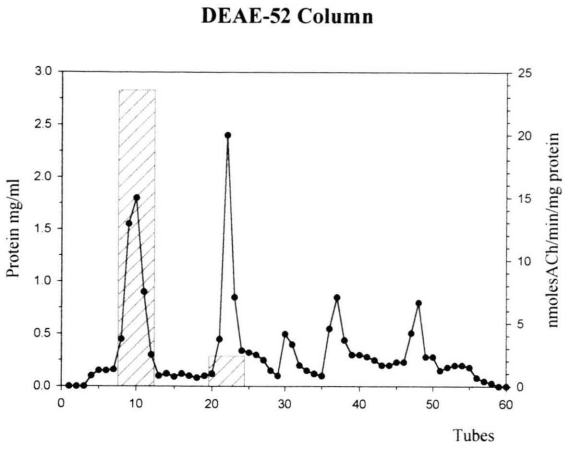

Fig. 1. Profiles of the protein concentration and ChAT activity of peaks eluted from ion exchange column (DEAE-52). Peaks were eluted as described in Materials and Methods. The first peak with highest acivity was eluted without salt addition. Samples of 100 µl from each peak were used for determination of protein concentration and enzymatic activity as described in materials and methods. Hatched areas indicate fractions with maximal ChAT activity.

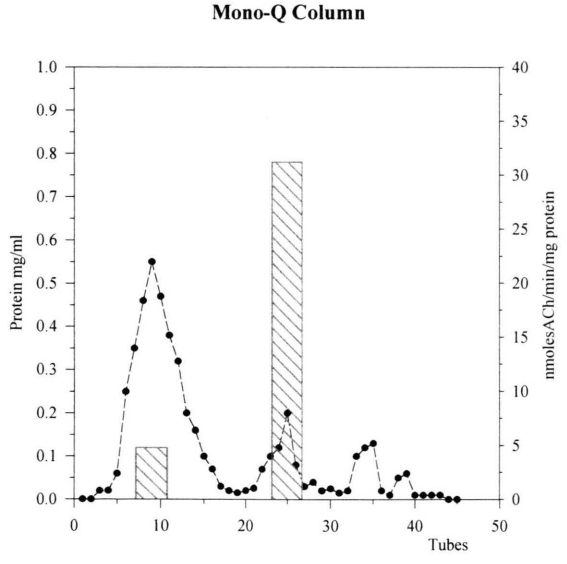

Fig. 2. Profiles of the protein concentration and ChAT activity of the fractions obtained from Mono-Q column in FPLC system. The column was equilibrated with elution buffer and submitted to a linear gradient of NaCl, as described in Materials and Methods. Peaks were eluted at a flow rate of 1.0 ml/min. Hatched areas indicate fractions with maximal ChAT activity.

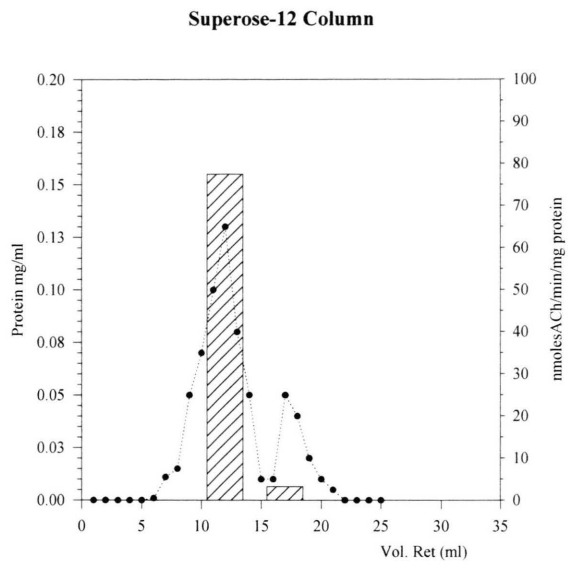

Fig. 3. Superose-12 column in FPLC system of the peak with highest enzymatic activity from Mono-Q column. Peaks were eluted with sodium phosphate buffer 0.05 M, with proteolysis inhibitor, pH 7.6; 0.3 ml/min. Samples of 100 µl were used for protein and enzymatic assay. Hatched areas indicate fractions with maximal enzymatic activity.

A summary of the purification steps is shown in Table I.

SDS-PAGE and immunoblotting analysis

SDS-PAGE analysis of samples from the first peak of DEAE-52, peak 1 from Superose-12 and peak 2 from Superose-12 columns can be seen in Fig. 4. The first peak from Superose-12 column that contained the majority of the enzymatic activity presented a single band of relative molecular mass 42,000; Mr 42 kDa (Fig. 4, lane 3) in SDS-PAGE. The 42 kDa band was recognized by anti-ChAT antibody in both peaks from DEAE and Superose-12 columns (Fig. 5). These data, together with those obtained from gel filtration analysis (Superose-12), indicate that *Electrophorus* ChAT is a dimer.

Isoelectric focusing

The peak from Superose-12 column, with highest enzymatic activity and recognized by anti-ChAT antibody was concentrated and submitted to isoelectric point (pI) determination in first di-

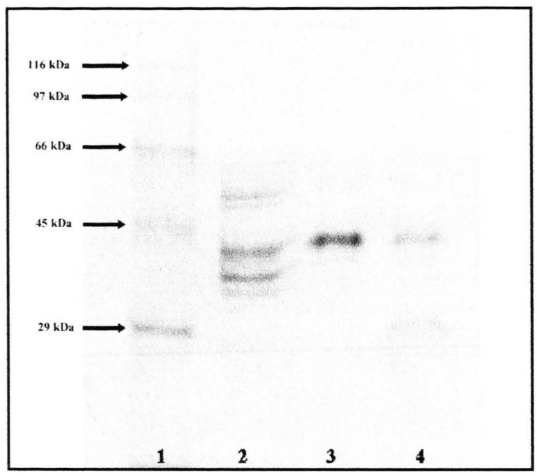

Fig. 4. SDS-PAGE gel (10%) used for Western blotting and immunoblotting, showing the protein profile at different purification stages. **1** – Molecular mass standards are indicated in the left margin; **2** – Peak 1 from DEAE-52 column; **3** – Peak 1 from Superose-12 column and **4** – Peak 2 from Superose-12. 20 μg of protein were used in lanes 2, 3 and 4. Protein bands were detected by Coomassie blue and transferred to nitrocellulose membrane of 0.45 μm.

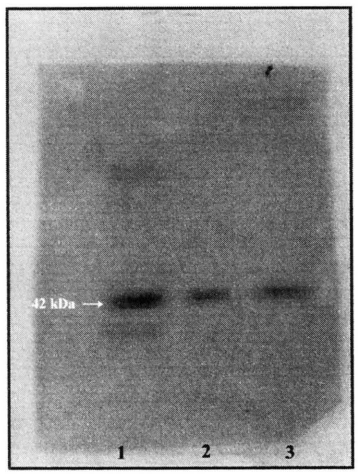

Fig. 5. Western Blot analysis of the first peak from DEAE-52 column (lane **1**) and the peaks 1 and 2 from Superose-12 column (lanes **2** and **3**, respectively). Proteins were incubated with anti-ChAT antibody (1:500) overnight and detected by ECL kit. The 42 kDa protein was detected.

mension gel electrophoresis as described in Materials Methods. The gel was also analyzed by SDS-PAGE in the second dimension for molecular weight determination. Two isoforms were found in this electrophoresis, with isoelectric points of 6.49 and 6.56 (Fig. 6), but with identical molecular mass.

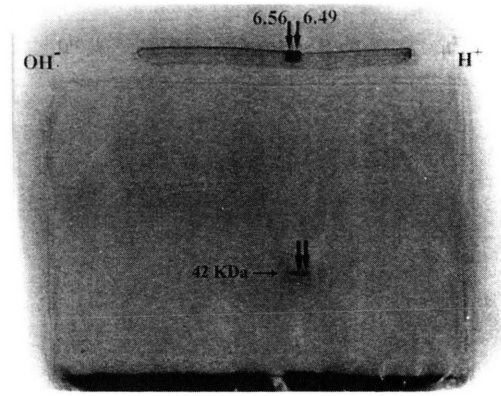

Fig. 6. Isoelectric focusing analysis 2D SDS-PAGE of peak 1 from Superose-12 column. The first dimension was performed as described in Materials and Methods and immediatly followed by 2D in SDS-PAGE, in a 10% gel, for molecular mass determination.

Discussion

Since the discovery of choline acetyltransferase (ChAT) in 1943, interest in its physiological role (Hersh and Wu, 1994), molecular properties (Malthe-Sørenssen and Fonnun, 1972; Polsky and Shuster, 1976), and its mechanism of enzymatic performance (Meyer, 1992) has increased. The molecular arrangement of this enzyme in the membrane and its role in regulation of acetylcholine biosynthesis have been the subject of recent studies (Eder-Colli *et al.*, 1986; Schmidt and Rylett, 1993).

The study of choline acetyltransferase is of particular interest, as this enzyme is a phenotypically specific marker of the cholinergic system (Waser *et al.*, 1989) and because it has been correlated with many physiopathological diseases. In this context, an understanding of the regulation of the ChAT activity becomes quite important. Some authors have reported a deficiency of ChAT activity in patients with Alzheimer diseases (Wurtman *et al.*, 1990) or Amyothrophic lateral sclerosis diseases (Kato, 1989). However, studies in skeletal muscles of patients suffering from Miasthenia Gravis disease demonstrated that the ChAT activity was increased (Molenaar *et al.*, 1981). In addition, there is increasing evidence that ChAT might

be regulated at the posttranslational level by compartimentation and/or covalent modifications, such as phosphorylation, as well as non covalent modifications (Hersh and Wu, 1994). Physicochemical aspects of electric organ ChAT were described in early studies made in our laboratory (Hasson-Voloch and Simas, 1969; Soares and Hasson-Voloch, 1972). In these studies, the effects of important cations, such as sodium, potassium, magnesium and calcium on ChAT activity were evaluated and the optimal pH for ACh synthesis was determined. It was observed that the monovalent cations Na^+ and K^+ have antagonistic effects, as Na^+ inhibits and K^+ activates the acetylation system. On the other hand, the divalent cation Mg^{2+} had no effect on the acetylation system while Ca^{++} either activated or inhibited the system at lower or increased concentrations, respectively. These results gave important contributions to a better understanding of mechanism of ACh biosynthesis from the electric organ of *E. electricus* (L.).

The extraction of choline acetyltransferase from the electric organ of *E. electricus* (L.) is advantageous in view of the lower tissue complexity of this organ, made up by a huge syncytium. Most of the problems encountered in ChAT purification procedures can be attributed mainly to the low amounts of the enzyme found in nervous tissues and to its natural tendency to become unstable during the purification process. As stated by Salvaterra *et al.* (1982), the amount of ChAT in relation to the whole protein content varies from 1 to 40 000 for *Drosophila melanogaster* heads to less than one part in several million for mammalian brain (Salvaterra *et al.,* 1982; Waser *et al.,* 1989). Owing to its extremely low concentration in some species and to its intrinsic instability, ChAT turned out to be a difficult protein to purify. This problem was solved when antibodies to ChAT were produced. To minimize these problems we utilized proteolysis inhibitor in all purification steps. The first chromatographic step, a DEAE-52 column, yields two peaks with enzymatic activity, the majority of which is concentrated in the first peak. After the enzyme assays of fractions from the Superose-12 column, we have obtained a fraction with 141 fold purification (P_1). The first peak from Superose-12 column presented a relative molecular mass in the range of 80 K and when analyzed by SDS-PAGE, this peak, which yielded the highest enzymatic activity, contained a major band of 42 K. These results indicate that the enzyme is formed by two identical subunits.

Our results were confirmed by immunoblotting with polyclonal anti-ChAT and are in agreement with results of Salvaterra *et al.* (1982), Hersh and Wu (1995) and Strauss *et al.* (1995). These authors studied the ChAT from different sources finding a molecular weight in the range of 37 to 68 kDa when analyzed by SDS-PAGE. On the isoelectric focusing of ChAT from *E. electricus* (L.) we observed two isoforms with isoelectric points of 6.49 and 6.56. Previous studies made by Malthe-Sørenssen and Fonnun (1972) have shown that choline acetyltransferase from different species can be divided into two groups, one with weakly positive charges and, therefore, low affinity for membranes and strongly charged cation-exchange resins; and another with high affinity for membranes and cation-exchange resins. In these context, ChAT from *E. electricus* (L.) should be placed in the first group, owing to its low isoelectric points when comparing with the enzymes from other species, such as cat and rat (Potter *et al.,* 1968). Further more, the decrease in binding to its substrate below pH 6.6 indicates that there should be a large change in enzyme charge at this point (Smith and Carroll, 1980; Massarelli *et al.,* 1988). Theses authors have analyzed the ChAT from mouse brain finding five isoforms ranging from pI 4.55 to 8.0 and measured that the affinity for choline was the same for different molecular forms of choline acetyltransferase.

The diversity of the results concerning some of the molecular characteristics of the enzyme may be derived from the great variety of purification, isolation and quantification methods used. On the other hand, the complexity of the different biological models used might explain the apparent divergence in the localization, storage and release of ACh.

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