CHOLINE ACETYLTRANSFERASE AGGREGATES FROM HUMAN PLACENTA AND RAT BRAIN

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1. Introduction

Choline acetyltransferase (EC 2.3.1.6., ChAcT) is the enzyme which synthesizes acetylcholine (ACh) from choline and acetyl-CoA. Animal species may have one or more forms of ChAcT showing different pH values, ranging from pI 5–8.5 [1–4]. In rat [4] and human [2] brains the presence of an acidic form of ChAcT (pI <7.1 and 5.5, respectively) was thought of being artifactual and possibly due to binding of the enzyme with other proteins. Moreover, in the rat brain [4], three basic forms of ChAcT were shown to have different affinities for membranes depending on the ionic concentration of the medium and/or pH.

ChAcT has been purified from different animal sources in several laboratories [5–14] and some authors have claimed to have obtained a monospecific antibody to their purified preparation [15,16]. There is however much debate concerning the validity of such antibodies [17,18] and the enzymatic forms of ChAcT [11]. Two bands of proteins have been obtained on SDS–polyacrylamide gels, [11,12,19] one of which is not pure enzyme in the opinion of the same authors. Others [16,20] have found several bands on SDS–gels and have explained this result as due to different subunits and/or polymers of ChAcT.

Our group is presently involved in the purification of ChAcT from human placenta (preliminary reports have already appeared [21,22]) and in the course of this study we suspected that the enzyme might form homo-aggregates or aggregates with other proteins. For this purpose different forms of ChAcT were analyzed by isoelectric focusing of the native and of a partially-purified enzyme preparation.

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2. Materials and methods

Human placentas at term, obtained from the maternity ward of Strasbourg City Hospital, were placed, after expulsion, at 4°C, and kept at this temperature until homogenization (1–2 h, maximum time interval). Placenta fragments, excised with scissors, were homogenized in 5 vol. 10 mM Tris–HCl buffer, containing 10 mM EDTA and 0.2 mM dithiothreitol (pH 8.2) (buffer A), centrifuged (105 000 × *g*, 60 min) and the supernatant (HSS, high speed supernatant) collected.

Samples for isoelectric focusing were either simply dialysed (at least 4 h) against buffer A (pH 8.2) or first concentrated by ultrafiltration in a dialysis tube under vacuum and then dialysed. This treatment is rather mild and does not produce any measurable loss of enzyme activity.

An horizontal 'thick layer' isoelectric focusing apparatus (LKB-2117 Multiphor) was used for preparative isoelectric focusing. Ultrodex (LKB) was the supporting medium and the pH gradient was established between pH 6 and pH 10 with Ampholines (LKB). After prefocusing (3 h, 8 W, c.c.) 3–5 ml of the sample were applied in the basic region of the pH gradient (around pH 8.7), and the isoelectric focusing was carried out for 18 h, at 8 W, c.c.

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The gel fractions from different regions of the gradient were collected with a spatula into conical tubes, and the enzyme activity, eluted with buffer A was measured using a modification of Fonnum's [23] method [24].

Proteins were analysed by the Lowry method [25]. It must be emphasized that the thick-layer technique is thought to avoid a source of artifacts present when vertical isoelectric focusing in sucrose medium is used, that is floculation of proteins and sedimentation by gravity in pH areas different from the pI of the protein.

Partial purification of the enzyme was performed as follows: the homogenate was slowly acidified with citric acid 0.1 M to pH 5.1, centrifuged (20 000 × *g*, 60 min) and the pellet resuspended in buffer A containing NaCl 0.3 M. After centrifugation (20 000 × *g*, 30 min) the enzyme contained in the supernatant was precipitated by ammonium sulfate fractionation (30–60% saturation). The pellet, dissolved in a minimum volume of buffer A, was dialyzed and either added on top of a DEAE-cellulose column (25 cm × 3 cm/g protein loaded; 25 ml/h) or electrofocused. The run through of the DEAE column which contained the enzyme activity was either electrofocused as such (3 mg protein/ml) or concentrated by vacuum dialysis (21 mg/ml) and then electrofocused.

3. Results

When samples of dialysed HSS from human placenta were electrofocused, (11.8 mg/ml) ChAcT activity was detected, only as a single peak, in a region between pH 7 and 9 with a maximum around pH 8.5 (fig.1A). In contrast to this, when a concentrated solution of the same sample (85 mg/ml) was electrofocused under identical conditions only 27% of the activity was found in the same pH region (basic pI forms), while most of the activity (58%) was found around pH 6.0 (acid pI form) (fig.1B). The results were identical

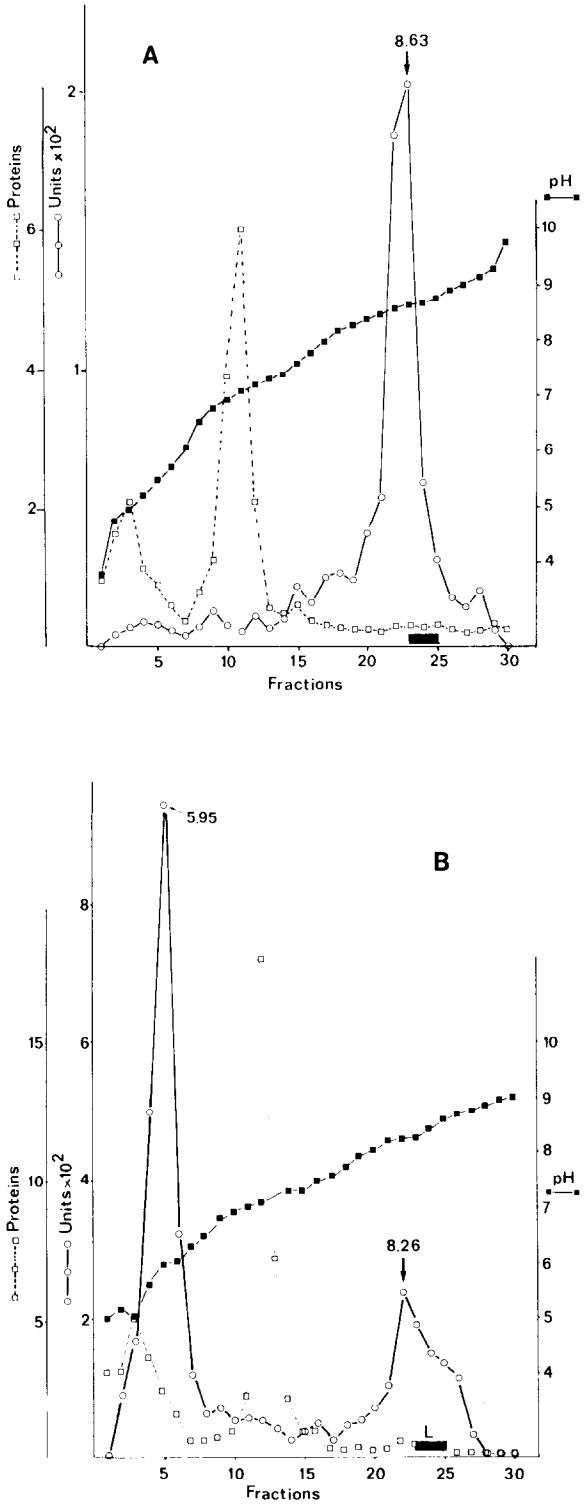


Fig.1. Isoelectric focusing pattern of human placenta ChAcT. (A) HSS (11.8 mg protein/ml) dialysed. (B) HSS concentrated by vacuum dialysis (85 mg protein/ml) dialysed. Each sample (5 ml) was loaded at the L region of the pH gradient. Hemoglobin focused at pH 7–7.8.

whether samples were concentrated by ultrafiltration or by ammonium sulfate precipitation (30–60% saturation).

As shown in fig.1B the peak of ChAcT activity found in the basic region after protein concentration seems to include several forms of enzyme activity at different pI. However, for sake of simplicity all basic forms will be here considered together and a detailed analysis of the basic forms will be reported elsewhere.

Regardless of the protein concentration, the bulk of soluble proteins were separated by isoelectrofocusing into two peaks, one at pH <6 and the other, containing hemoglobin at pH 6–7. At pH >7 the amount of proteins was comparatively negligible (see fig.1,2).

The acidic pI form of ChAcT, eluted from electrofocusing gel, was re-electrofocused at high (0.045 mg/ml) as well as low (0.015 mg/ml) protein concentration. In the first case only the acidic pI form was again obtained, while in the second case, 20% of the activity remained at acid pH and the rest reverted to the basic pI forms (table 1). Thus the formation and disappearance of the acidic pI form is (at least partially) a reversible concentration-dependent process.

Conversely when the basic pI region of ChAcT activity was eluted from the electrofocusing gel and re-electrofocused after concentration (0.250 mg/ml), only basic pI forms were obtained.

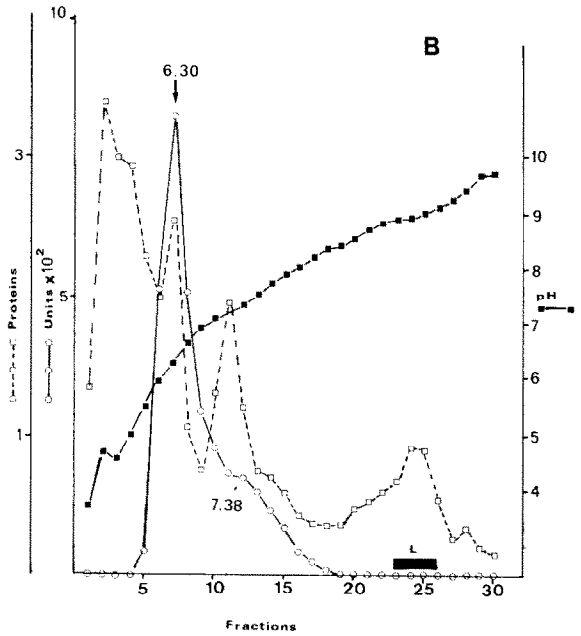
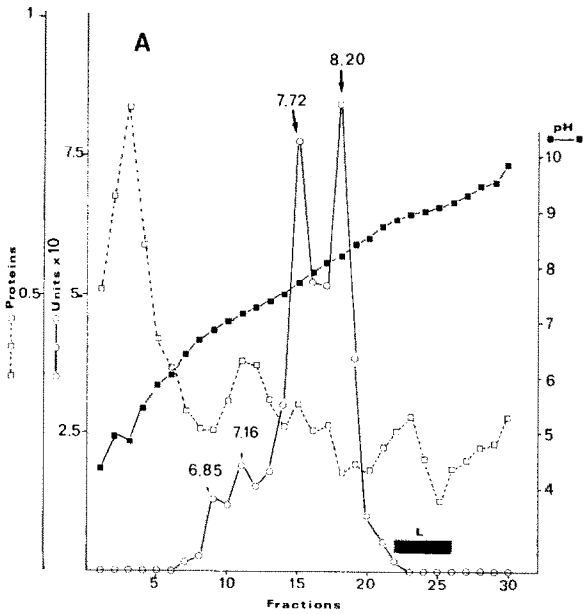


Fig.2. Isoelectric focusing pattern of rat brain ChAcT. (A) HSS (5 mg protein/ml) dialysed; (B) HSS concentrated by vacuum dialysis (30 mg protein/ml) dialysed. Each sample (5 ml) was loaded at the L region. Hemoglobin focused at pH 7–7.8.



This behaviour of ChAcT is not particular to human placenta, since (as shown in fig.2) rat brain ChAcT also shows only an acid pI form at high protein concentration (30 mg/ml) while only basic pI forms are detected at low protein concentration (5 mg/ml).

The presence of multiple forms of ChAcT at various protein concentrations could have been explained by artifactual interaction of the enzyme with ampholytes. This was excluded for the following reasons:

(i) The appearance of the acidic form is dependent upon the protein concentration and totally independent on the amount of protein added;

(ii) Since the enzyme is loaded at the basic pH region the interaction enzyme/basic ampholytes very unlikely can give an acidic form during migration [26];

(iii) Loading of diluted enzyme at the anode will give only a basic form.

4. Discussion

Our results indicate that the acidic pI form of ChAcT appears only after protein concentration and that it can, at least partially, be reverted to the basic forms by dilution. Moreover, the origin of this acidic form may be due to the formation of a complex, at high protein concentration, among ChAcT molecules or between ChAcT and other protein molecules. Such complexes are dissociated by dilution but not by isoelectric focusing.

A possible explanation for the formation of complexes between ChAcT and other proteins could be of charge interaction between the enzyme and strongly acidic proteins. Chromatography of human placental ChAcT on DEAE cellulose, at pH 7 as well as pH 8, shows that, as expected, the enzyme did not adsorb on the ion exchanger while acidic proteins were adsorbed. However, after concentration (to 21 mg/ml), the non-adsorbed material showed 44% of an acidic pI form (pH 6.0) in isoelectric focusing. Hence either proteins interacting with ChAcT are also present in the material non-adsorbed to DEAE or ChAcT forms homo-aggregates. It is difficult, at present, to state which of these possibilities is the most plausible.

Homo-aggregates of ChAcT have been found by several authors [8,15,27,28]. The present findings show that the formation of these complexes is strictly bound to the concentration of proteins. However during purification of the enzyme a progressively lower protein concentration will be sufficient to give 40% of an acidic enzymatic form (after HSS, 40 mg/ml; ammonium sulphate 30–60%, 25 mg/ml; DEAE-cellulose chromatography, 8.5 mg/ml).

These data give rise to the question: has the acidic enzymatic form a physiological meaning, in view of the fact that in vivo the concentration of soluble proteins should be ~60 mg/ml cytoplasmic fluid in the rat brain, or is it a simple artefact due to purification procedures?

The answer to this question will probably explain the reason for the differences observed, among different laboratories, in the preparations of purified enzyme and clarify the role of ChAcT in acetylcholine synthesis.

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