Multiple Forms of Choline Acetyltransferase in Several Species Demonstrated by Isoelectric Focusing

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1. The behaviour of choline acetyltransferase from pigeon, guinea-pig, rat and cat brain on isoelectric focusing was studied. 2. Choline acetyltransferase from pigeon and guinea-pig brain showed single peaks with isoelectric points at pH 6.6 and 6.8 respectively. Only one molecular form of the enzyme was therefore detected in these species. 3. Three peaks of choline acetyltransferase activities with isoelectric points 7.3–7.6, 7.7–7.9 and 8.3 were obtained with enzyme preparations from rat brain. 4. The separate identities of each of the three forms were confirmed by refocusing. 5. Choline acetyltransferase activity from a high-speed supernatant of rat brain homogenate was distributed similarly to a partially purified enzyme preparation from rat brain in the isoelectric gradient. 6. The enzyme activities from cat brain were separated into two distinct peaks with isoelectric points 7.0 and 8.4, and a possible third peak with isoelectric point 7.6. 7. The two main peaks showed considerable differences in stability on storage, and their identities were confirmed by refocusing. 8. The distribution of the enzyme activities was unaltered by isoelectric focusing in the presence of 3M-urea. 9. The apparent K_m for choline of choline acetyltransferase from rat, cat and guinea-pig brain was 0.8 mM, whereas for the pigeon enzyme it was 0.4 mM.

Choline acetyltransferase is an important enzyme in nervous tissue since it catalyses the synthesis of the transmitter substance acetylcholine. Previous work has shown very little heterogeneity between choline acetyltransferase molecules from different avian and mammalian species with regard to their substrate affinities (Berry & Whittaker, 1959; Morris & Tuček, 1966), inhibition by styrylpyridine analogues (White & Cavallito, 1970) and molecular weights (Bull *et al.*, 1964). There is, however, a striking difference in the membrane affinities between choline acetyltransferase molecules from different species (Fonnum, 1967, 1968) owing to the differences in the surface charge of the enzyme molecules (Fonnum, 1970). This charge may be of importance for the storage and synthesis of the cholinergic transmitter. The present paper gives further information on the charge of choline acetyltransferase molecules from different species investigated by isoelectric focusing. Particular attention was paid to the possibility of detecting multiple molecular forms of the enzyme by this technique. Part of this work has been presented in preliminary forms (Fonnum & Malthe-Sørensen, 1972; Malthe-Sørensen & Fonnum, 1971a,b).

Methods

Chemicals

Ampholine carrier ampholytes of various pH ranges and LKB 8101 column were obtained from

LKB Producter, Stockholm, Sweden. [$1\text{-}^{14}\text{C}$]Acetyl-CoA (specific radioactivity 58.3 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A.

Preparation of brain extracts

The animals were killed by a sharp blow on the neck and the cerebra were immediately removed and placed on ice. The cerebra were homogenized in 10 vol. of 10 mM-sodium phosphate buffer, pH 7.4, with a Potter-Elvehjem homogenizer rotating at 1700 rev./min. The phosphate buffer concentration was then raised to 25 mM by the addition of 0.5 M-sodium phosphate buffer, pH 7.4, to minimize adsorption of enzyme to membranes and the homogenate was centrifuged in a Spinco Ultracentrifuge at 100 000g for 1 h. The supernatant was either used directly for isoelectric focusing or for further enzyme purification.

Purification of choline acetyltransferase

The brain extract was purified essentially as described by Fonnum (1970). The pH of the extract was adjusted to pH 4.5 by dropwise addition of 50% (v/v) acetic acid under stirring. After 30 min (rat and cat) or 60 min (pigeon and guinea pig) in an ice bath, the enzyme solutions were centrifuged at 200 000g for

20 min. The pellet was discarded and the pH of the supernatant readjusted to pH 6.0 with 1M-NaOH. Any precipitate then formed was discarded after centrifugation. The clear supernatant was subjected to fractional $(\text{NH}_4)_2\text{SO}_4$ precipitation at pH 6.0 and the fractions were collected by centrifugation at 20000g for 15 min. The choline acetyltransferase activity was recovered in the 40–60%-satd. $(\text{NH}_4)_2\text{SO}_4$ fraction. The pellet was dissolved in 10mM-sodium phosphate buffer, pH 7.4 (0.5 ml/g original wet wt. of brain tissue). This fraction was usually used for isoelectric focusing. In some cases the fraction was dialysed against 10mM-sodium phosphate buffer to remove $(\text{NH}_4)_2\text{SO}_4$ before isoelectric focusing.

Isoelectric focusing

Isoelectric focusing was carried out in a 110 ml electrofocusing column (LKB 8101) and with 1% solution of synthetic carrier ampholytes (Ampholine, LKB) with isoelectric points in the ranges pH 6–9 or pH 3–10. Some experiments were also run with 2% Ampholine. The column was cooled with circulating ethanol at 0°C. The bottom of the column was layered with 10 ml of anode solution (12 g of sucrose and 0.2 ml of conc. phosphoric acid dissolved in 14 ml of water). A discontinuous sucrose density gradient with carrier ampholyte was prepared according to the manufacturer's instructions.

On top of the column was layered the cathode solution (0.2 ml of ethylenediamine + 10 ml of water). The choline acetyltransferase preparation was usually mixed with fractions 8–20 in the sucrose gradient. The fractions were 4.6 ml. The electrofocusing was done at a constant load of 0.5 W for the pH 3–10 gradient and 0.75 W for the pH 6–9 gradient. Constant current was usually obtained after 36 h but the electrofocusing was continued for 46 h. The column was drained slowly through the bottom tubing and 2.5 ml fractions were collected. The fractions collected for refocusing were concentrated and dialysed by ultrafiltration in collodion bags against 10mM-sodium phosphate buffer, pH 7.4, containing 1 mM-EDTA and 5% (w/v) sucrose.

Examination of fractions

The pH of each fraction was determined at 4°–6°C with a Radiometer 22 pH-meter. Each fraction was then examined for choline acetyltransferase activity, protein content and in some cases for distribution of haemoglobin. Choline acetyltransferase activity in the fractions was determined at 37°C as described by Fonnum (1969). To 2 μ l of enzyme was added 5 μ l of an incubation mixture containing (final concns.):

8 mM-choline, 200 mM-NaCl, 0.12 mM-[1- 14 C]acetyl-CoA, 4 mM-EDTA, 17 mM-sodium phosphate buffer and 0.06 mM-physostigmine. Incubation time was 5–15 min depending on the enzyme activity. It was found unnecessary to dialyse the enzyme preparation before assay. Sucrose, ampholytes or 1 M-urea had very little effect on the determination of choline acetyltransferase. Protein was determined by measuring the E_{280} (Warburg & Christian, 1941) in a Beckman DB spectrophotometer. Haemoglobin was determined by measuring the E_{420} (Raw *et al.*, 1958). When the enzyme activities were recovered in single peaks (Fig. 1) the isoelectric points were determined by interpolation of the pH and enzyme activities of the fractions on both sides of the peak. When the enzyme activities were obtained in several peaks in one run (Figs. 2 and 3), only the observed pH value of the peak is given.

Results

The enzymes were partially purified before isoelectric focusing to minimize the amount of protein loaded on the column. The acid treatment and $(\text{NH}_4)_2\text{SO}_4$ precipitation removed a considerable amount of protein, which otherwise was denatured and precipitated during the isoelectric focusing. Such a precipitate might disturb the distribution of the enzyme in the gradient. The two-step purification did not to our knowledge preferentially remove any form of choline acetyltransferase and it allowed more than 60% of the activity of the original brain extract to be put on the column. The distributions of the enzymes from the different species in the pH gradient were highly reproducible. The positions of the peaks of enzyme activity were independent of the amount of enzyme applied to the column and of the recovery of enzyme activities (Tables 1–4). To exclude the possibility that the distribution of the enzyme could be influenced by glass-wall effects and protein aggregation, the enzymes were also focused in the presence of 3 M-urea. This concentration is considered high enough to abolish such effects (O. Vesterberg, personal communication). Surprisingly, the enzyme was fairly stable to urea treatment and nearly full enzyme activity was recovered when it was assayed in the presence of 1 M-urea.

On isoelectric focusing of the enzyme from pigeon brain the enzyme activity was recovered as a single peak (Fig. 1a) with its maximum at pH 6.6 (Table 1). The peak of choline acetyltransferase activity was sharper but the position of the peak was unchanged in the presence of 3 M-urea (Fig. 1b).

Similarly the enzyme from guinea-pig brain was also recovered as a single peak after isoelectric focusing, its isoelectric point at pH 6.8 (Fig. 1c; Table 2). Also in this case the peak of enzyme activity was

Fig. 1. Isoelectric focusing of partially purified choline acetyltransferase from pigeon and guinea-pig brain

Ampholine pH-gradient 3–10. ●, Choline acetyltransferase activity; △, E_{280} ; —, pH. Experimental details of the different runs are given in Tables 1 and 2. (a) Pigeon brain choline acetyltransferase, run 1 II; (b) pigeon brain choline acetyltransferase in 3M-urea, run 1 IV; (c) guinea-pig brain choline acetyltransferase, run 2 II; (d) guinea-pig brain choline acetyltransferase in 3M-urea, run 2 IV.

Table 1. Isoelectric focusing of pigeon choline acetyltransferase

The enzyme was partially purified as described in the Methods section. The focusing was done in the absence or presence of 3M-urea.

Run no.	pH ranges of gradient	Enzyme activity applied (μmol of acetylcholine/min)	Recovery (%)	Observed peak pH	Determined pI
1 I	3–10	0.45	48	6.60	6.60
1 II	3–10	0.94	82	6.50	6.49
1 III	3–10	2.70	62	6.65	6.62
1 IV	3–10 (urea)	0.71	77	6.60	6.60
1 V	5–9 (urea)	1.50	59	6.55	6.58

slightly sharper in urea, but the position of the peak was unaltered (Fig. 1d). Choline acetyltransferase from guinea-pig brain was slightly better separated from the main protein peak than was choline acetyltransferase from pigeon brain (Fig. 1). Choline acetyltransferase from rat brain behaved differently from the enzymes from pigeon and guinea-pig brain in that the enzyme activity was recovered over a much broader pH range. When a high-speed supernatant from rat brain homogenate was subjected to isoelectric focusing in a pH3–10 gradient two distinct peaks of enzyme activities with maxima at

pH7.4–7.8 and 8.3 (Fig. 2a) were detected. When the same enzyme preparation was focused in a pH6–9 gradient there were indications of three peaks at pH7.5, 7.9 and 8.4 (Fig. 2c). Multiple peaks of enzyme activity usually indicate the presence of closely related forms of the enzyme, enzyme–enzyme interactions or protein–enzyme interactions. Since the distributions of choline acetyltransferase activities were similar in 3M-urea (Table 3) the two latter explanations seem less likely. The identity of the two peaks from the pH3–10 gradient was established by isolating the most active fractions of each peak, dialysing them

Table 2. Isoelectric focusing of guinea-pig choline acetyltransferase

The enzyme was partially purified as described in the Methods section. The focusing was done in the absence or presence of 3 M-urea.

Run no.	pH ranges of gradient	Enzyme activity applied (μmol of acetylcholine/min)	Recovery (%)	Observed peak pH	Determined pI
2 I	3–10	0.33	52	6.65	6.74
2 II	3–10	1.10	90	6.85	6.87
2 III	3–10 (urea)	1.02	47	6.90	6.86
2 IV	3–10 (urea)	1.02	27	6.75	6.79

Fig. 2. Isoelectric focusing of choline acetyltransferase from rat brain extract

●, Choline acetyltransferase activity; Δ , E_{280} ; —, pH. Experimental details are given in Table 3. (a) Rat brain extract, pH gradient 3–10, run 3 VIII; the arrow indicates the maximum position of rat haemoglobin. (b) Refocusing of fractions from peaks a (●) and b (○) in (a) in pH gradient 6–9. Peak a, applied enzyme activity $0.021 \mu\text{mol}$ of acetylcholine/min, recovered enzyme activity 57%. Peak b, applied enzyme activity $0.020 \mu\text{mol}$ of acetylcholine/min, recovered enzyme activity 60%. The enzyme activity of each fraction is expressed as percentage of total recovered activity. (c) Rat brain extract, pH gradient 6–9, run 3 X. (d) Refocusing of fractions from peaks c (●) and d (○) in (c) in two separate columns. Peak c, applied activity $0.012 \mu\text{mol}$ of acetylcholine/min, recovered enzyme activity 83%. Peak d, applied activity $0.02 \mu\text{mol}$ of acetylcholine/min, recovered activity 65%. The enzyme activity of each fraction is expressed as percentage of total activity recovered.

and then refocusing them in two pH 6–9 gradients (Fig. 2b). When the fractions at pH 7.35–7.55 and pH 7.70–7.95 from the pH 6–9 gradient were isolated, dialysed and refocused in two new pH 6–9 gradients, the new peaks were well separated (Fig. 2d). Thus, all three peaks of rat choline acetyltransferase have been confirmed by refocusing. Results similar to those obtained in Figs. 2(a), 2(b) and 2(c) have also been

obtained with a partially purified enzyme preparation (Malthe-Sørensen & Fonnum, 1971b).

Table 3 summarizes the different runs with two different enzyme preparations from rat brain. The results show a high degree of reproducibility between the different runs and there are no apparent differences in the results obtained with brain extract or a partially purified enzyme preparation. The mole-

Table 3. Isoelectric focusing of rat brain choline acetyltransferase

The enzyme preparations were either a high-speed supernatant of brain homogenate or partially purified as described in the Methods section. The focusing was done in the absence or presence of 3 M-urea. The distribution of choline acetyltransferase activity in the different pH ranges is expressed as the percentage of the total enzyme activity found between pH 7.3 and 8.5. The enzyme activity found between pH 7.3 and 8.5 was 68–95% in the pH 3–10 gradient and 73–84% in the pH 6–9 gradient of the total enzyme activity recovered in the gradient.

Preparation of choline acetyltransferase	Run no.	pH ranges of gradient	Enzyme activity applied (μmol of acetylcholine/min)	Recovery (%)	pH of observed peaks	Distribution of choline acetyltransferase activity (%)		
						pH 7.3–7.9	pH 7.9–8.5	
Purified rat brain	3 I	3–10	1.40	78	7.45, 8.30	58	42	
	3 II	3–10	1.40	83	7.80, 8.40	37	63	
	3 III	3–10	0.83	39	7.65, 8.40	36	64	
	3 IV	3–10	0.96	71	7.70, 8.30	49	51	
Rat brain extract	3 V	6–9	0.72	69	7.65, 7.90, 8.20	24	25	pH 7.7–8.1
	3 VI	6–9	0.96	39	7.50, 7.80, 8.20	25	28	51
	3 VII	6–9 (urea)	0.96	44	7.50, 7.90, 8.15	27	26	47
						pH 7.3–7.7	pH 7.7–8.1	pH 8.1–8.5
	3 VIII	3–10	0.34	61	7.70, 8.40	42	58	
	3 IX	3–10	0.38	68	7.70, 8.50	36	64	
						pH 7.3–7.7	pH 7.7–8.1	pH 8.1–8.5
	3 X	6–9	0.55	81	7.50, 7.90, 8.40	25	30	45
	3 XI	6–9	2.90	67	7.55, 8.00, 8.45	29	33	38
						pH 7.3–7.7	pH 7.7–8.1	pH 8.1–8.5

cular form with isoelectric point 8.3 dominates in both preparations.

The positions of the peaks were unaltered when the focusing time was increased to 72h or when the Ampholine concentration was changed. Gel filtration of choline acetyltransferase on Sephadex G-150 before and after isoelectric focusing gave $K_{av} = 0.41$ (Laurent & Killander, 1964). In this experiment choline acetyltransferase was concentrated after isoelectric focusing by ultrafiltration against 10mm-sodium phosphate buffer containing 1mm-EDTA and 5% (w/v) sucrose. Although choline acetyltransferase after isoelectric focusing was very stable, there was a heavy loss of enzyme activity when we tried to subject the activity of single peaks to gel filtration directly after isoelectric focusing.

In some experiments when we used an extract from rat caudate nucleus or hippocampus we obtained a fourth peak at about pH 6.5–7.0. Refocusing of this enzyme activity led to disappearance of this peak and the enzyme activity was redistributed in the pH range 7.5–8.3. The observed peak is probably a result of enzyme-protein interaction that is abolished on dialysis and refocusing.

Enzyme preparations from rat brain contain haemoglobin and the position of maximum extinction at 420nm was measured in some of these experiments to confirm the reproducibility. The main peak was always recovered in a fraction of pH value 7.05–7.25.

Choline acetyltransferase from cat brain was also distributed over a broad pH range after isoelectric focusing. Two distinct peaks of enzyme activity were observed in a pH 3–10 gradient with isoelectric points 7.0–7.2 and 8.3–8.5 and in addition a third peak or a shoulder at pH 7.5–7.7 (Fig. 3a). The two main peaks were identified by refocusing in a pH 6–9 gradient (Fig. 3d). Owing to the instability and low specific activity of cat choline acetyltransferase, such experiments were not performed with the third peak.

On focusing in urea there was a decrease of enzyme recovery, but the main peak at pH 8.5 was maintained (Fig. 3e).

On storage at 4°C there was a considerable loss of activity for the cat enzyme, and isoelectric focusing of such a preparation (Figs. 3b and 3c) showed that this loss was mainly due to the instability of the enzyme activity with isoelectric point 8.2–8.5. It is important to note that the activity in the two other peaks did not increase in absolute value when the activity in peak 3 was lost. There is thus no evidence of a transfer of enzyme activity from peak 3 to the other peaks.

The distribution of enzyme activity in different pH intervals was reproducible (Table 4) provided a fresh enzyme preparation was used. The main peak of enzyme activity was in all these experiments due to the isoenzyme of isoelectric point 8.3.

Fig. 3. Isoelectric focusing of partially purified choline acetyltransferase from cat brain

●, Choline acetyltransferase activities; Δ , E_{280} ; —, pH. Experimental details of the different runs are given in Table 4. (a) Freshly prepared enzyme, run 4 I; (b) enzyme preparation stored 2 weeks at 4°C, run 4 VI; (c) enzyme preparation stored 6 weeks at 4°C, run 4 VIII. (d) Refocusing of the most active fractions of peaks a and b in (a) in two separate columns. ●, Peak a, activity applied: 0.055 μ mol of acetylcholine/min, recovery 78%; $\blacktriangle$, peak b, activity applied: 0.050 μ mol of acetylcholine/min, recovery 68%. Enzyme activity of each fraction is expressed as percentage of total recovered. (e) Freshly prepared enzyme in the presence of 3 M-urea, run 4 V.

Kinetics

The apparent K_m values for choline of choline acetyltransferase from different species were obtained

Table 4. Isoelectric focusing of cat brain choline acetyltransferase

The enzyme was partially purified as described in the Methods section. The focusing was done in the absence or presence of 3 M-urea. For the determination of distribution of choline acetyltransferase activity in each pH range see Table 3.									
Run no.	pH ranges of gradient	Enzyme activity applied (μmol of acetylcholine/min)	Recovery (%)	pH of observed peaks	Distribution of activity (%)				
					pH 6.7-7.4	pH 7.4-8.1	pH 8.1-8.8		
Freshly prepared enzyme	4 I 3-10	1.31	74	7.0, 7.7, 8.4	25	31	44		
	4 II 3-10	0.54	29	7.0, 7.7* 8.2	17	35	48		
	4 III 3-10	0.50	40	7.0, 7.9* 8.5	23	24	53		
	4 IV 3-10	1.25	54	7.1, 7.7, 8.3	37	21	42		
	4 V 3-10 (urea)	1.32	40	7.2, 7.7* 8.5	18	33	49		
Enzyme stored 2 weeks at 4°C	4 VI 3-10	0.46	83	7.0, 7.6	28	32	38		
Enzyme stored 6 weeks at 4°C	4 VII 3-10	0.41	79	7.0	47	35	18		
	4 VIII 3-10	0.101	90	7.0	59	27	14		
* Shoulder, not peak.									

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Table 5. *Apparent K_m values for choline of choline acetyltransferase from different species*

Enzyme preparations used were dialysed and concentrated fractions after isoelectric focusing. The incubation medium was as described in the Methods section except that the choline concentration was varied from 0.2 to 5 mM-choline. Enzyme activity used in each incubation was 0.002 μ mol of acetylcholine/min. The apparent K_m was determined from Lineweaver-Burk plots. The results are expressed as mean value and range. Numbers of experiments are given in parentheses.

Species	Apparent K_m values (mM)	
	Mean value	Range
Pigeon, activity peak pH 6.6	0.40	0.38–0.42 (4)
Guinea pig, activity peak pH 6.8	0.72	0.71–0.75 (2)
Rat, activity peak pH 7.4	0.76	0.72–0.84 (5)
Rat, activity peak pH 8.4	0.80	0.74–0.86 (6)
Cat, activity peak pH 7.0	0.78	0.78 (1)
Cat, activity peak pH 8.4	0.78	0.74–0.87 (4)

by Lineweaver-Burk plots. The apparent K_m values (Table 5) were similar for cat, guinea pig and two of the isoenzymes from rat; the value for pigeon was slightly lower than the others.

Discussion

The results show that a high degree of heterogeneity of choline acetyltransferase molecules within the same species and in different species can be detected by isoelectric focusing. The heterogeneity observed within a species is not due to artifacts caused by protein-enzyme, enzyme-enzyme or Ampholine-enzyme interactions. The distribution pattern of the enzyme would in the case of such interactions probably depend on several factors such as the degree of enzyme purification, the load of protein or enzyme on the gradient, the recovery of enzyme activity, the type and the concentration of Ampholine used. The distribution of enzyme activities were, however, highly reproducible and independent of the above-mentioned variables. In addition the distribution pattern of choline acetyltransferase activities was unaltered in the presence of 3 M-urea. This concentration is considered high enough (O. Vesterberg, personal communication) to abolish most protein-protein and protein-Ampholine interactions. Even more important is the fact that the three different forms of rat choline acetyltransferase and the two forms of cat choline acetyltransferase that we claim have all been confirmed by refocusing of the individual peaks. Any interactions would be expected to be abolished during the dialysis before the refocusing, since the K_{av} value of choline acetyltransferase obtained from gel filtration on Sephadex G-150 was the same before and after isoelectric focusing. If interactions had been important we would expect to see changes in the position of the peaks and to obtain

more than one peak on refocusing. This was never observed, even when refocusing was carried out with a different pH gradient (Figs. 2a and 2b, 3a and 3d).

Isoelectric focusing of choline acetyltransferase from a high-speed supernatant of rat brain homogenate and partially purified enzyme preparation gave similar distribution patterns. The multiple molecular forms were therefore not a product of the purification procedure such as deamidation. Also the purification procedure adopted did not preferentially isolate any of the different molecular forms (Table 3).

Previous work from our laboratory (Fonnum, 1967, 1968, 1970) has shown that choline acetyltransferase from different species can be divided into two groups. Choline acetyltransferases from pigeon and guinea-pig brain have weakly positive surface charges and therefore low affinities for membranes and cation-exchange resins. On the other hand choline acetyltransferases from rat and cat brain have strongly positive surface charges and high affinities for membranes and cation-exchange resins. There was a marked decrease in affinities at pH values below 6.5. These observations are in agreement with the present results in that choline acetyltransferase from pigeon and guinea-pig brain have markedly lower isoelectric points than the enzymes from rat and cat. Further, the decrease in binding below pH 6.5 indicates that there should be a large change in enzyme charge at this point.

The affinity for choline was the same for the different molecular forms of choline acetyltransferase. This means that the changes in amino acid composition of the different choline acetyltransferases responsible for the charge differences are localized far away from the active centre. This finding was not unexpected since White & Cavallito (1970) did not find any significant differences in effects of choline acetyltransferase inhibitors or any differences in substrate

affinity of choline acetyltransferase from such different sources as calf caudate nucleus and *Lactobacillus plantarum*. Some cross-contamination between the different multiple forms after isoelectric focusing is unavoidable and small differences in substrate affinities of the rat isoenzymes may therefore not have been detected.

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