Multiple Isoelectric and Molecular Weight Variants of Choline Acetyltransferase

The existence of isoelectric variants of bovine brain and human placental choline acetyltransferase was confirmed by chromatofocusing. The identification of molecular weight variants (bovine brain, \( M_r = 68,000 \) and 63,000; human placental, \( M_r = 66,000 \) and 64,000) was also demonstrated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by "Western blotting" and immunochemical visualization of choline acetyltransferase with monoclonal antibodies. No correlation between the isoelectric variants and molecular weight variants could be observed; however, in the case of the bovine brain enzyme the more alkaline isoelectric variant was enriched in the higher molecular weight form of the enzyme.

Treatment of the bovine brain enzyme with *Staphylococcus aureus* V8 protease resulted in the conversion of the \( M_r = 68,000 \) form of the enzyme to the \( M_r = 63,000 \) form. During this conversion no change in the enzyme activity was observed demonstrating that the \( M_r = 63,000 \) form of the enzyme and probably also the \( M_r = 68,000 \) form of the enzyme are active.

Preparation of the bovine brain enzyme in the presence of proteolytic enzyme inhibitors yielded a new higher molecular weight form of the enzyme, \( M_r = 73,000 \), which was enzymatically active. The \( M_r = 73,000 \) form of the enzyme exhibited a single isoelectric form when analyzed by chromatofocusing. These results suggest that the \( M_r = 73,000 \) form of the enzyme represents the native form, and that other molecular weight and isoelectric variants may arise by proteolysis.

Choline acetyltransferase (acetyl-CoA:choline O-acetyltransferase, EC 2.3.1.6) catalyzes the reversible formation of the neurotransmitter acetylcholine from acetyl-CoA and choline. The existence of multiple forms of the enzyme has been reported by a number of laboratories. Isoelectric focusing has been used to identify three choline acetyltransferase variants from rat brain (1, 2), monkey brain (3), mouse brain (4), bovine brain (4), and human brain (5), and two or possibly three isoelectric forms from cat brain (1). The nature of these isoelectric variants of choline acetyltransferase has been the subject of debate. White and Wu (5) observed a change in the distribution of isoelectric variants upon refocusing and suggested that enzyme aggregation may give rise to different apparent isoelectric forms. A similar conclusion was reached by Froissart et al. (6) in their studies of the human placental and rat brain enzyme. In contrast, Malthe-Sorensen and Fonnun (1) found no significant differences in the distribution of isoelectric forms of the rat brain enzyme using preparations of different purity and by refocusing. Atterwill and Prince (2) found that in the developing rat brain the most basic isoelectric variant of choline acetyltransferase was decreased when compared to enzyme derived from mature and 7-day-old rats.

Ion exchange chromatography has also been reported to separate different forms of choline acetyltransferase from rat brain (7-9) and bovine brain (8), although the nature of these forms has not been established.

Most recently the existence of multiple molecular weight variants of choline acetyltransferase has been reported. Dietz and Salvaterra (10) observed three molecular weight variants in their homogeneous rat brain choline acetyltransferase preparation all having molecular weights close to 67,000, while Cozarri and Hartman (11) found two molecular weight forms in their homogeneous bovine brain choline acetyltransferase preparation, \( M_r = 76,000 \) and 72,000. Using monoclonal antibodies to bovine choline acetyltransferase, Levey et al. (12) also identified two molecular weight variants (\( M_r = 70,000 \) and 68,000) and in addition, identified the same two molecular weight variants for the human placental enzyme (13).

In this paper a study has been conducted of the relationship of the isoelectric and molecular weight variants of bovine brain and human placental choline acetyltransferase. In addition, the possible generation of these choline acetyltransferase variants by proteolysis has been investigated.

**EXPERIMENTAL PROCEDURES**

Assay of Choline Acetyltransferase Activity—Choline acetyltransferase activity was measured by the general procedure of Fonnum (14). Reaction mixtures contained 50 mM sodium phosphate buffer, pH 7.4, 150 mM sodium chloride, 10 mM EDTA, 0.1 mM eserine, 0.2 mM \( [3H] \) acetyl-CoA (specific activity, 1.2 \( \times 10^{6} \) cpm/nmol), 5 mM choline, and enzyme in a final volume of 40 \( \mu \)l. After incubation of the reaction mixture for 15-60 min at 37°C, the reaction was terminated by adding a 25-\( \mu \)l aliquot to a scintillation vial containing 7 ml of 10 mM sodium phosphate buffer, pH 7.0, 2.4 ml of scintillation fluid (4 gms 2,5-diphenyloxazole and 0.1 g of P-bis[methylstyril] benzene/liter of toluene), and 0.55 ml of tetraphenylboron in acetonitrile (10 mg/ml). After gentle shaking the solution was counted. Control samples were run in which boiled enzyme replaced native enzyme or in which choline was omitted from the reaction mixture. 1 unit is defined as the amount of enzyme producing 1 \( \mu \)mol of...
acetylcholine/min under the above assay conditions.

Purification of Choline Acetyltransferase—All purification steps were performed at 4 °C. Choline acetyltransferase from frozen bovine caudate nucleus was extracted by homogenization in 2.5 ml/g of tissue of 25 mM sodium phosphate buffer, pH 7.4, using a Teflon homogenizer with 20 up and down strokes at 1300 rpm. The initial homogenate was mixed with an equal volume of 10-fold concentrated 40-60% ammonium sulfate solution and homogenized a second time. After centrifugation at 20,000 × g for 1 h, the supernatant was adjusted to pH 4.5 with 50% acetic acid over a period of 45 min. The solution was stirred for 50 min, and then centrifuged as described above. The supernatant was adjusted to pH 6 by the addition of 1 M NaOH and then fractionated with ammonium sulfate. The precipitate obtained from a 40-60% ammonium sulfate fraction was redissolved in a minimal volume of citrate/phosphate buffer. (10 mM disodium phosphate neutralized to pH 7.2 with citric acid and containing 0.1 mM EDTA and 0.1 mM dithiothreitol) and either dialyzed overnight against this buffer or passed over a Sephadex G-25 column equilibrated with the citrate/phosphate buffer. This procedure resulted in a 10-fold purification of the enzyme in 78% yield. The specific activity of enzyme preparations ranged from 15 to 21 milliunits/mg of protein.

Human placental choline acetyltransferase was prepared as previously described (15) through the acid/ammonium sulfate fractionation step and dialyzed against the citrate/phosphate buffer before use. The specific activity of the human placental enzyme preparations ranged from 5 to 7 milliunits/mg of protein.

Chromatofocusing—Choline acetyltransferase dialyzed against citrate/phosphate buffer was used directly for chromatofocusing on columns of 25-40 ml of gel volume. Gradient runs were performed over the pH range of 9.4 to 6 according to the manufacturers specifications, except that 15% glycerol and 0.05 mM dithiothreitol were added to all buffers.

Immunoimmunoprecipitation of Choline Acetyltransferase—Choline acetyltransferase was immunoprecipitated by first forming a soluble antigen-antibody complex with monoclonal anti-choline acetyltransferase antibody followed by secondary complex formation with rabbit anti-rat antibody, and precipitation of the total complex with Pansorbin. The immunoprecipitates were dissolved in SDS buffer (125 mM Tris-hydrochloride, pH 6.8, 20% glycerol, 4.5% SDS, and 1.0 M 2-mercaptoethanol). Gel Electrophoresis—SDS-PAGE was conducted on 10% polyacrylamide slab gels according to the method of Laemmli (17).

Western Blotting—SDS-polyacrylamide gels were electroblotted onto nitrocellulose paper using a Bio-Rad Trans-Blot Cell Apparatus. An electroblotting buffer consisting of 190 mM Tris, 10 mM glycine, pH 9.4, was used to insure efficient transfer of the isoelectric variants of choline acetyltransferase exhibiting a pH greater than 8.

Immunoochemo Visualizaion of Choline Acetyltransferase—Choline acetyltransferase was visualized on nitrocellulose paper by forming a soluble antigen-antibody complex with monoclonal anti-choline acetyltransferase antibody 12 or 16 followed by binding of horseradish peroxidase conjugated rabbit anti-rat IgG and incubation was continued for 30 min. Pansorbin (2 mg) was prewashed with 10 mM citrate/phosphate buffer, pH 7.0, containing 0.1 mM EDTA, 7% glycerol, and 0.5% Nonidet P-40. The mixture was incubated for 15 min at room temperature. The immune complex was centrifuged in an Eppendorf centrifuge for 5 min and washed three times with 100 mM borate buffer, pH 8.3, containing 75 mM sodium chloride and 0.5% Nonidet P-40. Control samples contained rat IgG in place of monoclonal antibody. The immunoprecipitates were dissolved in SDS buffer (125 mM Tris-hydrochloride, pH 6.8, 20% glycerol, 4.5% SDS, and 1.0 M 2-mercaptoethanol).

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The recovery of enzyme activity was estimated by the method of Bradford (19). [3H]Acetyl-CoA was obtained from New England Nuclear. Acetyl-CoA was prepared by the method of Simon and Shemin (20). Choline chloride, eserine sulfate, phenylmethanesulfonfluoride, L-1-tosylamide-2-phenylthiol imidate ketone, Nα-p-tosyl-L-lysine chloromethyl ketone, leupeptin, and Clostridium perfringens neuraminidase (Type X) were obtained from Sigma. Staphylococcus aureus V8 protease and rabbit anti-rat IgG conjugated with horseradish peroxidase were obtained from Miles Laboratories Inc. Pansorbin was a product of Calbiochem-Behring. Rat IgG was obtained from Pel-Freez Biologicals, while wheat germ lectin immobilized to Sepharose was from P-L Biochemicals.

Polybuffer 94 exchanger and Polybuffer 96 were obtained from Pharmacia Fine Chemicals.

RESULTS

Isoelectric Variants—Isoelectric variants of bovine choline acetyltransferase have previously been identified by isoelectric focusing in liquid columns (1-4) or by isoelectric focusing in granulated gels (6). We have employed the method of chromatofocusing to investigate isoelectric forms of choline acetyltransferase. This method has the advantages of a larger sample load, shorter run times, and the lack of interference from precipitating proteins. Fig. 1 shows a typical elution profile of bovine choline acetyltransferase over the pH range of ~6.5-9.0. Three discernible peaks of choline acetyltransferase activity are apparent which elute at pH 8.2, 7.8, and 7.4. In order to rule out artifacts during the chromatofocusing run, the pH 8.2 and 7.4 forms were pooled together and the pH 7.8 form was pooled separately. After concentration by precipitation with 80% ammonium sulfate and dialysis, samples were resubjected to chromatofocusing. As shown in Fig. 2, the rechromatographed fractions exhibited similar isoelectric variants to those obtained initially. The pH 8.2 and 7.8 variants eluted at pH values of 8.15 and 7.7, respectively, while the pH 7.4 variant eluted at pH 6.95. This apparent shift in the elution of the pH 7.4 variant is probably due to poor resolution of the pH 7.8 and 7.4 forms in the initial chromatofocusing experiment which resulted in a higher estimate of the actual pH of elution of the more acidic enzyme form.

![Fig. 1. Elution profile of bovine brain choline acetyltransferase (ChAT) from a chromatofocusing column. Bovine brain choline acetyltransferase (~400 milliunits) purified as described under "Experimental Procedures" was chromatographed on a 30-ml column of Polybuffer 94 exchanger initially equilibrated with 5 mM ethanalamine buffer, pH 9.4, containing 5% glycerol and 0.05 mM dithiothreitol. A pH gradient was generated by eluting with Polybuffer 94 diluted 1/10 and neutralized to pH 6. The Polybuffer contained 5% glycerol and 0.05 mM dithiothreitol. Fractions of 3.0 ml were collected and every other fraction was assayed for enzyme activity. The recovery of enzyme activity was ~85%. Enzyme activity: ○, pH.](http://www.jbc.org/)

1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N’-N’-tetraacetic acid; 66K form represents, for example, a form of Mr, 66,000.)
ferae—Recent studies have shown the existence of two molecular weight forms of human placental choline acetyltransferase (11, 13). It was therefore of interest to make a comparison of these different choline acetyltransferase forms with the human placental enzyme. Fig. 4 shows the chromatofocusing profile of the human placental enzyme. Three forms of the enzyme are observed eluting at pH 8.6, 8.0, and 7.4. Immunoprecipitation of the fractions from the chromatofocusing column, followed by SDS-PAGE, transfer to nitrocellulose, and visualization by antibody staining yielded the results shown in Fig. 5. In the case of the human placental enzyme, the major choline acetyltransferase form exhibits M₆₄, 64,000, with only a small amount of a 66K form detectable. As can be seen in Fig. 5, the low molecular weight variant is seen in all of the isoelectric variants, with the high molecular weight variant being observed only in the pH 8.6 form of the enzyme. Thus, although the ratio of the three placentale enzyme forms obtained from chromatofocusing are similar to the three bovine enzyme forms, the major molecular weight variant in the placental preparation is the 64K form. This is in contrast to bovine brain in which both the 68K and 69K forms are major enzyme species.

Molecular Weight Choline Acetyltransferase Variants as Proteolytic Fragments—If the low molecular weight form of choline acetyltransferase was derived from proteolytic degradation of the high molecular form, one might be able to produce this conversion in vitro. In order to test this, a crude bovine brain homogenate was prepared and allowed to sit for 24 h at room temperature. Such a treatment, which could result in proteolysis by endogenous proteases, did not reveal any major changes in the size of the molecular weight variants; however, there did appear to be an increase in the amount of low molecular weight variant relative to the higher molecular weight form.

We next screened the effects of proteolytic enzymes on the distribution of the molecular weight choline acetyltransferase variants. As shown in Fig. 6, using low concentrations of S. aureus V8 protease a progressive decrease of the high molecular weight form of bovine choline acetyltransferase was observed with a concomitant increase in the low molecular weight variant. At higher concentrations of protease, total degradation of choline acetyltransferase was observed. During
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FIG. 3. Correlation of bovine brain choline acetyltransferase (ChAt) molecular weight variants and isoelectric variants. Aliquots of fractions from the chromatofocusing run shown in Fig. 1 were immunoprecipitated as described under “Experimental Procedures.” The immunoprecipitates were solubilized with SDS, and subjected to SDS-PAGE. Choline acetyltransferase was visualized after “Western blotting” as described under “Experimental Procedures.” The numbers in each gel lane designates the fraction from the chromatofocusing run shown in Fig. 1 which were immunoprecipitated. Fractions 32 and 33 correspond to the pH 8.2 variant, fractions 44-46 to the pH 7.8 variant, fractions 58, 60, and 62 to the pH 7.4 variant, and fraction 76 which contained little choline acetyltransferase activity served as a control. These fractions were chosen to minimize cross-contamination with other isoelectric variants. The protein bands staining nonspecifically were derived from the monoclonal antibody and Pansorbin as can be seen in the lane containing fraction 76 which had little choline acetyltransferase activity. The arrows designate the two choline acetyltransferase bands.

FIG. 4. Elution profile of human placental choline acetyltransferase (ChAt) from a chromatofocusing column. Human placental choline acetyltransferase (~50 milliunits) was subjected to chromatofocusing as described in the legend to Fig. 1. □, enzyme activity; ●, pH.

In the molecular weight forms of choline acetyltransferase by endogenous proteolysis, we examined the effect of including proteolytic inhibitors in the initial extraction buffer. Using a combination of inhibitors which included EDTA, EGTA, phenylmethylsulfonyl fluoride, 1-1-tosylamido-2-phenylethyl chloromethyl ketone and N-p-tosyl-L-lysine chloromethyl ketone at 1.0 mM, and leupeptin at 0.1 mM we observed a new higher molecular weight (M, = 73,000) form of choline acetyltransferase, Fig. 7. It can be noted that the relative staining intensity of the 73K form of choline acetyltransferase is considerably weaker than that observed with the 68K or 63K enzyme forms. This could be due to a weaker interaction of the monoclonal choline acetyltransferase antibody with this form of the enzyme. When this enzyme form was subjected to chromatofocusing, a single peak emerged at a pH of ~ 8.5, Fig. 8. It is noteworthy to point out that in the standard purification procedure, the relative amounts of the M, = 68,000 and 63,000 variants of choline acetyltransferase are variable. Compare lanes 2 and 3 in Fig. 7. Preliminary attempts to convert the 73K form of choline acetyltransferase to either the 68K or 63K forms were unsuccessful using trypsin or S. aureus protease. Instead of seeing conversion among the molecular weight variants, the 73K form of the enzyme was degraded to unidentifiable fragments by S. aureus and was resistant to degradation by trypsin. The conditions used in the S. aureus digestion would have permitted detection of the 68K to 63K conversion. Thus it can be concluded that more than one proteolytic enzyme is responsible for the formation of the M, = 68,000 and 63,000 choline acetyltransferase variants. It has also been observed in preliminary experiments that omission of the acid step during enzyme purification gives rise to detectable amounts of the M, =
FIG. 6. Conversion of $M_r = 68,000$ variant of bovine brain choline acetyltransferase to the $M_r = 63,000$ variant by proteolysis. Bovine brain choline acetyltransferase (2 milliunits; 0.15 mg of protein) was digested with 8 ng of $S. aureus$ V8 protease at room temperature in 10 mM citrate/phosphate buffer, pH 7.0 containing 0.1 mM EDTA, 7% glycerol, and 0.5% Nonidet P-40. The reaction was terminated by boiling and then subject to SDS-PAGE. Immunochemical visualization of choline acetyltransferase after Western blotting was performed as described under "Experimental Procedures." Lane 1 shows undigested enzyme; lanes 2–4 are choline acetyltransferase digested for 5, 20, and 80 min, respectively; lanes 6–8 are antibody stain controls for the 0-, 5-, and 80-min digests in which rat IgG was substituted for monoclonal antibody. The arrows denote the $M_r = 68,000$ and 63,000 choline acetyltransferase variants. There is an apparent increase in staining intensity of the 63K band. Although this observation is not clearly understood, this apparent increase in staining may in part be due to the formation of a band of slightly greater than $M_r = 63,000$, and diffusion of the stain among these species. It is also possible that the 63K band exhibits a strong interaction with the monoclonal antibody and thus binds more antibody than does the 68K variant.

73,000 variant and an enrichment of the 68K form relative to the 63K form. These results suggest that either acid proteases, perhaps of lysosomal origin, are responsible for the proteolysis of choline acetyltransferase, or alternatively acid pH results in partial denaturation of the enzyme which makes it more susceptible to proteolytic digestion.

DISCUSSION

The objective of this study was to correlate the isoelectric and molecular weight variants of bovine brain and human placental choline acetyltransferase in order to establish the nature of these multiple enzyme forms. The use of chromatofocusing to separate isoelectric variants and monoclonal antibodies to identify molecular weight variants has made such a study feasible. In agreement with other studies (4, 11, 13) we have identified three isoelectric variants and two molecular weight variants of both bovine brain and human placental choline acetyltransferase. Since there are more isoelectric variants than molecular weight variants, no direct correlation would be expected. In fact, in the case of the bovine brain enzyme all three isoelectric variants of the enzyme contained both of the molecular weight variants yielding six putative forms of the enzyme.

The generation of an isoelectric variant of choline acetyltransferase $pI \sim 6.3$ has been suggested to arise from the binding of choline acetyltransferase to acidic proteins at high protein concentrations (>30 mg/ml) (6). In the present study the protein concentrations employed were in the range of 10–
form, or by the simultaneous conversion of the 73K form to both the 68K and 63K forms of the enzyme.

The generation of two lower molecular forms of choline acetyltransferase cannot account for the observed isoelectric variants per se, since both low molecular weight forms show three isoelectric forms. Instead, deamidation of glutamine or asparagine residues in the proteolytically cleaved forms of the enzyme could give rise to the observed isoelectric variants of the enzyme.

It is concluded that in the case of bovine brain choline acetyltransferase, and probably with choline acetyltransferase from other sources, the observed heterogeneity of the enzyme is not due to post-translational modification of the enzyme or to distinct genes for the enzyme, but rather is due to limited proteolysis of the enzyme during its preparation.

In the case of the human placental enzyme it appears that the lower molecular weight variant is predominant. This finding suggests that proteolysis of the human placental enzyme is more complete as compared to the bovine brain enzyme.

It is interesting to note that the best preparations of rat (10) and bovine (11) choline acetyltransferase which are apparently homogeneous contain two and three molecular weight forms of the enzyme, respectively. On the other hand, purification of the porcine brain enzyme gave a single species of Mᵣ = 67,000 (22, 23). Since these preparations were made in buffer lacking proteolytic inhibitors, they may have yielded partially degraded enzyme.

REFERENCES

Multiple isoelectric and molecular weight variants of choline acetyltransferase. *Artifact or real?*

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