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PREPARATION AND CHARACTERIZATION OF TWO ISOZYMES OF CHOLINE ACETYLTRANSFERASE FROM SQUID HEAD GANGLIA

II. SELF-ASSOCIATION, MOLECULAR WEIGHT DETERMINATIONS, AND STUDIES WITH INACTIVATING ANTISERA

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Summary

The two isozymes of choline acetyltransferase (Acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) from head ganglia of *Loligo pealei* have been examined by polyacrylamide gel electrophoresis, gel chromatography, and equilibrium sedimentation in the ultracentrifuge. Inactivating antisera, prepared to both native and dithiothreitol-treated isozymes 1 and 2 of squid choline acetyltransferase, were used to demonstrate the immunologic identity of isozymes 1 and 2. Each isozyme appeared to contain two non-identical catalytically active subunits, with molecular weights of approx. 37 000 and 56 000. A staining method was developed to visualize choline acetyltransferase activity in acrylamide gels. The method is based on the formation of a precipitate of manganese ferrocyanide at sites where free coenzyme A is released. By this method, and by analysis of gel slices, it was found that each of the isozymes can form aggregates of several different sizes. The formation of immune precipitates with the aggregates showed the identity of the multiple bands of enzyme protein resolved on disc gel electrophoresis. Isozyme 1 was most active as a small aggregate, whereas isozyme 2 was most active as a large aggregate. Both chromatography on Sephadex G-200 and isoelectric focusing yielded a number of active species with molecular weights ranging from 35 000 to 300 000. In addition, we demonstrated the dissociation of enzyme protein in the presence of $1.0 \cdot 10^{-2}$ M dithiothrei-

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The abbreviations used in this paper are as follows: hydroxyapatite 1, for hydroxyapatite-purified isozyme 1 from *L. pealei*; hydroxyapatite 2, for hydroxyapatite-purified isozyme 2 from *L. pealei*; hydroxyapatite 1 M, for hydroxyapatite-purified isozyme 1 purified from *L. opalescens*; SDS for sodium dodecyl sulfate; Ab, for antiserum; (N), for native; (D) for dithiothreitol-treated.

tol, the formation of multiple precipitin bands by aged enzyme, and the identity of the different isoelectric fractions of each of the isozymes.

Introduction

Choline acetyltransferase (Acetyl-CoA:choline *O*-acetyltransferase, EC 2.3.1.6) purified from squid head ganglia has been separated into two isozymes which differ in their stabilities and in their activation by salts. Each isozyme separates into at least three active forms on isoelectric focusing [1,2]. Experiments performed by other workers on choline acetyltransferases isolated from various species, have lead to results that are difficult to interpret and/or suggest the existence of multiple forms [3—6]. It is not clear whether these multiple forms are isozymes, aggregates or conformers. The results of the experiments reported here show that each isozyme of squid choline acetyltransferase is capable of generating a family of active and inactive aggregates which occupy a large molecular weight range, and which behave somewhat differently for each of the isozymes isolated.

Several laboratories have attempted to investigate the subunit composition of choline acetyltransferase. In 1968, Potter et al. [7] reported the size of bovine brain choline acetyltransferase to be 69 000 daltons, whereas, Chao and Wolfgram [8], in 1973, reported it to be composed of two non-identical subunits of 51 000 and 69 000 daltons. Husain and Mautner [9], working with squid head ganglia from *Loligo opalescens*, reported that squid choline acetyltransferase contained only one subunit of apparent molecular weight of 55 000. In this paper we report that squid choline acetyltransferase is composed of two non-identical "subunits" (molecular weights 37 000 and 56 000), although certain fractions containing only one "subunit" can be isolated from each isozyme.

Several attempts have been made to develop histochemical stains for localizing choline acetyltransferase in tissue slices [10—14]. In order to compare the distribution of activity in gels of different isozymes, and in order to study the aggregation of the isozymes, we decided to adapt the method of Feigenson and Barrnett [14] to staining in gels. By means of this staining method we were able to show distinct, multiple, active forms to be present in both isozymes of choline acetyltransferase isolated from *Loligo pealei*. We were also able to show that the active forms of the two isozymes are resolved differently on gel electrophoresis at pH 8.4.

Antisera specific for choline acetyltransferase were prepared in order to provide an additional means of comparing the isozymes and their aggregated forms. Antisera to the enzyme from mammalian sources have been prepared by Eng et al. [15], by McGeer et al. [16], by Rossier et al. [17,18], and by Shuster and O'Toole [19]. Such antisera could also be used to localize the enzyme in situ, in squid ganglia as has been done in bovine brain by Eng et al. [15], and in beef spinal cord and rat cortex by McGeer et al. [16].

Materials

L. pealei (Native Atlantic squid) were purchased from The Woods Hole Ma-

rine Biological Laboratory (Woods Hole, Mass.) or Abramo Fish Co. (Boston, Mass.). *L. opalescens*, caught in the Pacific near Monterey were purchased from Globe Fish Co. (Boston, Mass.). Rabbits were purchased from the Gloucester Rabbit Farm (Gloucester, Mass.).

The resins used for ion-exchange chromatography were obtained from the following companies: carboxymethyl-cellulose and cellulose phosphate, Whatman, W. and R. Balston, Ltd. (England); AG 1-X8 (chloride form) and hydroxy apatite, Bio-Rad Labs. (Richmond, Calif.). Sephadex was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

[1-^{14}C] Acetyl-coenzyme A (approx. 50 Ci/mol) and Aquasol, universal liquid scintillation counting cocktail, were purchased from New England Nuclear (Boston, Mass.).

The lithium salt of acetyl-coenzyme A was obtained from P-L Biochemicals (Milwaukee, Wisc.). dithiothreitol, choline and carnitine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Ampholines were purchased from LKB Produckter (Sweden). Bovine serum albumin (Cohn Fraction V) was obtained from Nutritional Biochemical Co. (Cleveland, Ohio). Neostigmine bromide (prostigmine) was purchased from Hoffman-La Roche (Nutley, N.J.). Collodion bags and Collodion Bag Apparatus were obtained from Schleicher and Schuell, Inc. (Keene, N.H.). Protein standards were obtained from Sigma Co. (St. Louis, Mo.). All other chemicals were purchased from Fisher Scientific Co. (Boston, Mass.).

Methods

The assay employed to measure choline acetyltransferase activity was essentially that of Schrier and Shuster [20]. For quantitation, the enzyme was usually such that a 0.1 ml aliquot contained enough activity (0.0003 unit) * to convert approx. 10% (1200 cpm) of the trace-labelled acetyl-coenzyme A to acetylcholine during a 10 min assay. However, even under conditions where up to 25% of the substrate was consumed, the reaction was essentially linear with time for at least 40 min. Full activity was maintained both before and during assay by the use of a solution containing 1 mg/ml bovine serum albumin, 10^{-3} M EDTA and 10^{-4} M dithiothreitol as diluent. To the 0.1 ml aliquot of enzyme, a 0.1 ml aliquot of a substrate mixture containing the following reagents was added: $3.0 \cdot 10^{-4}$ M acetyl-coenzyme A (the lithium salt, trace labelled with 0.01 μCi [1-^{14}C]acetyl-coenzyme A/0.1 ml), 0.10 M choline chloride, 0.05 M potassium phosphate buffer, pH 7.0, $1.0 \cdot 10^{-4}$ M neostigmine bromide, $5.0 \cdot 10^{-4}$ M EDTA, $5.0 \cdot 10^{-5}$ M dithiothreitol, 0.55 mg/ml bovine serum albumin, and 0.30 M NaCl. Incubation was at 32°C, and the reaction was stopped by immersion of the tubes containing the incubation mixture in an ice/water bath.

Preparation of Sephadex G-200 gel. Sephadex G-200 was swollen for 5 h in a boiling water bath. It then was equilibrated with 0.01 M potassium buffer, pH 7.0, containing 0.1 M NaCl. Cytochrome *c*, ovalbumin, bovine serum albumin,

* One unit of choline acetyltransferase activity is that amount of enzyme capable of producing 1 μmol of acetylcholine per min.

and γ-globulin were used to calibrate each Sephadex G-200 column, and the void volume of each column was determined with 0.02% blue dextran. All standards were applied in eluting buffer containing 5% sucrose. Calibration curves for each of the two columns used were obtained by plotting the relative elution volumes of the standards versus the logarithms of the molecular weights of the standards. Both of these curves were essentially linear, and were used to determine the molecular weight scales superimposed on all graphs of data derived from gel filtration experiments. Sephadex columns were stored in 0.02% NaN_3.

Enzyme preparation. Enzyme was purified according to the method of Polsky and Shuster [2]. The hydroxyapatite-purified preparation from *L. opalescens* was called hydroxyapatite 1M, and had specific activity 9.1 units/mg protein. Isoenzymes hydroxyapatite 1 & 2, isolated from *L. pealei* were also purified on hydroxyapatite, and had specific activities of 10 and 30 units/mg, respectively.

Both isozymes from *L. pealei*, and isozyme 1 M from *L. opalescens* were subjected to isoelectric focusing. Each isozyme was separated into three active isoelectric fractions called, A, B, and C in order of their increasing isoelectric points.

Discontinous (disc) polyacrylamide gel electrophoresis. Disc gel electrophoresis was conducted in Tris/glycine buffer, pH 8.4, in accordance with the procedure of Davis [21]. 2.0 ml of 7.5% acrylamide containing 0.2% bis-acrylamide was overlaid with 0.5 ml of a 2.5% acrylamide stacking gel containing 0.65% bis-acrylamide, and both were polymerized with $(NH_4)_2S_2O_8$. The gels were made up in a 10% sucrose solution and were overlaid with stacking gel buffer containing 0.05 M dithiothreitol and 10% sucrose 90 min prior to the layering on of the enzyme. Directly before sample addition, all gel surfaces were thoroughly rinsed. Gels were cast in tubes of either 5.5 or 6.0 mm internal diameter, and were either 75 or 62 mm in length, respectively. The samples were in 0.01 M potassium phosphate buffer, containing 20—60% sucrose, and which had been adjusted to contain the same concentration of Tris/glycine buffer as was present in the running buffer. 5 μl of 0.05% bromphenol blue in 0.005 M NaOH was added to each sample to mark the front. All gels were measured and the positions of the fronts were recorded immediately upon removal of the gels from the tubes. In all experiments the R_F values of the protein bands were calculated relative to bromphenol blue migration. Gels were subjected to a current of 4—5 mA/gel at 0°C. Immediately upon cessation of electrophoresis, the gels were removed from the tubes by rimming them with a hypodermic needle through which distilled water was flowing. Gels to be stained for enzyme activity were placed in 0.3 M potassium phosphate, pH 6.0, containing 10^{-3} M EDTA and 10^{-4} M dithiothreitol (for the rest of the gel staining procedure, see gel staining section of Results).

A modification of the Coomassie blue staining method of Weber and Osborne [22] was used to enhance staining sensitivity (Brawerman, G., personal communication). The stain consisted of 0.005% Coomassie Brilliant Blue, and 0.005% copper sulfate dissolved in a mixture of methanol, water and glacial acetic acid in a 5 : 5 : 1 (v/v) ratio. Gels were allowed to stand in small tubes containing 5 ml of this solution overnight. Destaining was accomplished by placing the gels in a solution of 5% methanol and 7.5% glacial acetic acid in dis-

tilled water overnight. When gels were to be assayed to localize enzymatic activity, they were sliced into 0.8 mm slices with a slicer fashioned by threading a series of 80 double-edged stainless steel razor blades, separated by number 8 burrs, on three 3-inch screws (Brawerman, G., personal communication). Gels were semi-frozen in test tubes immersed in an acetone and solid CO_2 bath prior to slicing. Each slice was extracted overnight into 1.0 ml of 20% sucrose solution containing 10^{-3} M EDTA and 10^{-3} M dithiothreitol.

After gels had been appropriately stained, they were scanned in a Gilford recording spectrophotometer modified for gel scanning at a wavelength of 650 nm. When enzyme was to be eluted for assay from sliced gels, the gels were sliced into 0.8 mm slices by the use of the gel-slicer described above. Each slice was extracted overnight into 1.0 ml of a 20% sucrose (by weight) solution containing $1.0 \cdot 10^{-3}$ M EDTA and $1.0 \cdot 10^{-3}$ M dithiothreitol.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS). Polyacrylamide gel electrophoresis was conducted in SDS and the resulting gels were stained exactly as specified by Weber and Osborne [22]. Samples were routinely dialyzed overnight against 0.01 M sodium phosphate buffer, pH 7.0, containing 1% SDS, 1% mercaptoethanol, and 8 M urea, and then heated in the same buffer at 60°C for 1 h. It was found necessary to heat the enzyme solutions during denaturation in SDS, mercaptoethanol and urea, in the manner described, in order to eliminate spurious heavy molecular weight bands that appeared in all samples not so treated.

After the initial dialysis and heating step had been completed, a second overnight dialysis was performed against 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1% SDS & 1% mercaptoethanol. The sample was then applied to the gel surface in 0.2—0.8 ml of the second buffer described. Before being applied to the gel surface, the sample was adjusted to contain 30% glycerol or 5—20% sucrose and 3% mercaptoethanol. 5 μl of 0.05% Bromphenol Blue in 0.005 M NaOH was added to each sample to mark the front. The gels contained 0.2% SDS while the phosphate running buffer contained 0.1% SDS. The gels also contained 10% acrylamide and 0.5% bis-acrylamide, and electrophoresis was conducted at 8 mA/gel tube for 3—4 h at room temperature, and was terminated when the dye fronts had migrated 1/2 to 3/4 of the way down the gels.

Myoglobin and/or ovalbumin were often included in samples as internal standards. The approximate molecular weights of all bands were calculated with regard to both the fronts and to protein standards.

Preparation of antisera. Rabbits were immunized by intradermal administration of 0.1—0.6 mg of protein in a 1 : 1 emulsion of isotonic saline and Freund's complete adjuvant. These injections were followed by weekly intravenous injections of enzyme alone for 2 successive weeks. The rabbits were bled from the ear vein 1 week after the last of the intravenous injections. The antisera prepared to the two native (N) isozymes purified from *L. pealei* were called Ab 1 (N) and Ab 2 (N), referring to their specificities for isozyme 1 (hydroxyapatite 1) and isozyme 2 (hydroxyapatite 2), respectively. Booster injections (about 0.1 mg) were then administered intradermally to the same rabbits for 3 successive weeks. The enzyme that was used for these booster injections had been eluted from polyacrylamide gel slices corresponding in position to R_F values of between 0.14 and 0.19, and had been stored at 2—4°C for 2 weeks in

10^{-2} M dithiothreitol prior to the commencement of the series of booster injections. The animals were bled 1 week after the last injection in the series of boosts. The resulting antisera were Ab 1 (D) and Ab 2 (D) prepared to dithiothreitol-treated (D) isozymes 1 and 2, respectively. In order to eliminate any spurious acetyltransferase activity, all sera were heated to 56°C for 30 min prior to use.

Ouchterlony double diffusion plates. Ouchterlony double diffusion plates [23] were prepared on 3 inches by 1 inch glass slides. Each slide was covered with 3 ml of solution containing 0.15 M NaCl, 0.01 M Tris · HCl buffer, pH 7.4, 0.01 M EDTA and 0.8% agarose in distilled water.

Titration of enzymatic activity. Titrations of enzymatic activity were performed with each of the antisera against both the homologous and the heterologous antigens. The enzyme to be titrated was diluted into control serum (from un-immunized rabbits) so that 10 µl contained approx. 0.0007 unit of activity. A 10 µl aliquot of that dilution was then added to 40 µl of a 1 mg/ml bovine serum albumin solution containing $1.0 \cdot 10^{-3}$M EDTA and $1.0 \cdot 10^{-3}$ M dithiothreitol. Amounts of antiserum ranging from 0.01 to 50.0 µl were added to this solution, and the volume was then adjusted to 100 µl by the addition of control serum to bring the final concentration of serum up to 50%. These samples were incubated at 2—4°C overnight to allow inactivation to occur. The following day, the activity remaining in the tubes was assayed as described in Methods. Control serum caused no inactivation of squid choline acetyltransferase, even at concentrations as high as 80% for 24 h at 2—4°C.

Results

Manganese ferrocyanide staining. When purified squid choline acetyltransferase was run on disc gel electrophoresis, multiple bands of both protein and of enzymatic activity were resolved, as determined from Coomassie blue staining in conjunction with slicing, elution, and assay done on companion gels. We developed a staining method specific for staining choline acetyltransferase activity in situ on gels in order to visualize more easily enzymatic activity in gels. The method is based on the procedure used by Feigenson and Barrnett [14] for localization of choline acetyltransferase at myoneural junctions. The reaction sequence begins with the reduction of the ferricyanide radical to ferrocyanide by the free coenzyme A released by enzymatic action. Incorporation of manganese chloride into the incubation mixture results in the precipitation of insoluble, white manganese ferrocyanide during the enzymatic reaction. Since the reaction product is formed directly from the sulfhydryl group of coenzyme A liberated during the reaction, only a minimal amount of diffusion of product can occur before reduction and precipitation can take place. In polyacrylamide gels, the white manganese ferrocyanide can easily be photographed and/or scanned in a spectrophotometer; it may also be converted into insoluble, blue ferric ferrocyanide, as in the Prussian blue staining reaction, by addition of $FeCl_3$.

Staining procedure. All operations prior to the actual enzymatic reaction were performed at 0—4°C. For the staining reaction to proceed, it was necessary to equilibrate the gels with 0.025 M potassium phosphate buffer, pH 6.0, prior to reaction.

In order to achieve the correct internal pH in the gels, a series of four changes (50 ml each) of potassium phosphate buffer, pH 6.0, was employed. Each gel was first placed in 0.3 M buffer for 1 h, followed by 0.1 M buffer for another hour, followed by 0.025 M buffer. The gels were allowed to equilibrate in the 0.025 M buffer overnight. The next day, the buffer was changed to 0.025 M potassium phosphate without either EDTA or dithiothreitol in it, and the gels were kept in that buffer for an additional 4 h (if EDTA is included in the buffer, or the reaction mixture, the reaction is impeded since the EDTA chelates the metal ions necessary for reaction to proceed; if dithiothreitol is included, it reacts as a competitive substrate).

The final concentrations in the substrate mixture used for the staining reaction were: 0.05 M potassium phosphate buffer, pH 6.0, 0.3 M NaCl, 0.1 M choline chloride $1 \cdot 10^{-3}$ M neostigmine bromide and $7.5 \cdot 10^{-4}$ M acetyl-coenzyme A. These were all dissolved in distilled water, containing no EDTA or dithiothreitol. 0.1 ml of 48 mM $MnCl_2$ was added per ml of reaction mixture and the solution was shaken well. 0.2 ml of 5 mM K_3 $[Fe(CN)_5]$ was added next, and the solution was again shaken. The resulting solution was light yellow-green and clear. Since a small amount of precipitation sometimes occurred in the solution during the first 0.5 h, the solution was allowed to stand for 0.5 h before use. 1.3 ml of this incubation mixture was used per 2 ml gel. Small test tubes (8 × 75 mm) were employed, so that this volume of solution was enough to cover the surfaces of the gels completely. The gels were allowed to remain in the incubation solution for 1.5 h prior to reaction, in order to allow the small molecules to diffuse into the gels. After the pre-incubation, the gel-containing tubes were placed at 32° C for 3 h. Within the first 0.5 h, a diffuse white precipitate began to form within the gel at the sites of enzyme action. When the reaction was terminated, the gels were removed from the incubation mixture, and placed in 100 ml of distilled water (containing no EDTA or dithiothreitol) in which they were kept overnight, in order to remove excess soluble reactants. The next day, the gels were photographed, and then scanned on a Gilford recording spectrophotometer at 650 nm. After scanning, the gels were sometimes stained by immersion in 0.15 M $FeCl_3$ for 18—48 h. The fact that the white precipitate was able to form blue ferric ferrocyanide was taken as a positive indication that the precipitate was the specific reaction product expected, and not merely protein that had precipitated non-specifically.

Before staining, the purified choline acetyltransferase to be stained was shown to be free of extraneous acylase or acetyltransferase activity. This was necessary, since the reaction sequence used for staining will localize such enzymes as well as choline acetyltransferase. Less than 1% of the label from the [1-^{14}C]acetyl-coenzyme A added as substrate, in either our normal assay mixture or in our staining mixture, was hydrolyzed as measured by the percent of label that became ether extractable upon exposure to enzyme for up to 3 h at 32° C.

The enzymatic activity in our purified isozyme preparations was protein dependent, choline dependent, time dependent, and heat labile. Squid choline acetyltransferase was one-third as active in the staining mixture as in the reaction mixture normally used for enzyme assay. The optimal amount of enzyme for good staining was 20—100 μg of protein containing 0.05—0.5 unit of activi-

ty. Other proteins, such as bovine serum albumin and myoglobin, showed no staining reaction (see Fig. 1, gels 4 and 5).

Trial staining. Trial staining experiments performed on hydroxyapatite 1 M showed that the manganese ferrocyanide precipitated at the same positions in the gel as would have been predicted on the basis of experiments in which the gels were sliced, eluted, and assayed.

Three identical gels of hydroxyapatite 1M were run on disc gel electrophoresis at pH 8.4. One was stained with Coomassie blue, one was stained with manganese ferrocyanide, and one was sliced, eluted, and assayed. The results of all three of these gels are shown in Fig. 2. Slicing, elution, and assay showed only the major peak of activity at an R_F of 0.25, whereas the manganese ferrocyanide stain revealed not only that peak, but shoulders of heavier, active, protein (R_F values of 0.10 and 0.15) as well. Since the enzyme used on this gel ran as a single band on polyacrylamide gel electrophoresis in SDS, and the heavier protein bands were immunologically identical with enzyme protein, the slight activity associated with the aggregated protein was expected.

The lower R_F enzyme protein bands typically appear to be more active relative to the other active bands when stained than when eluted and assayed. This finding can be explained on the basis of relative rates of diffusion of large versus small molecules. When gels are eluted and assayed the larger molecules typically elute poorly, in comparison to the lower molecular weight active aggregates. On the other hand, during the long equilibration periods necessary for staining of gels, the smaller more mobile, enzyme molecules are likely to diffuse out of the gel, thus making the lower molecular weight aggregates appear less active than they really are in comparison to the higher molecular weight en-

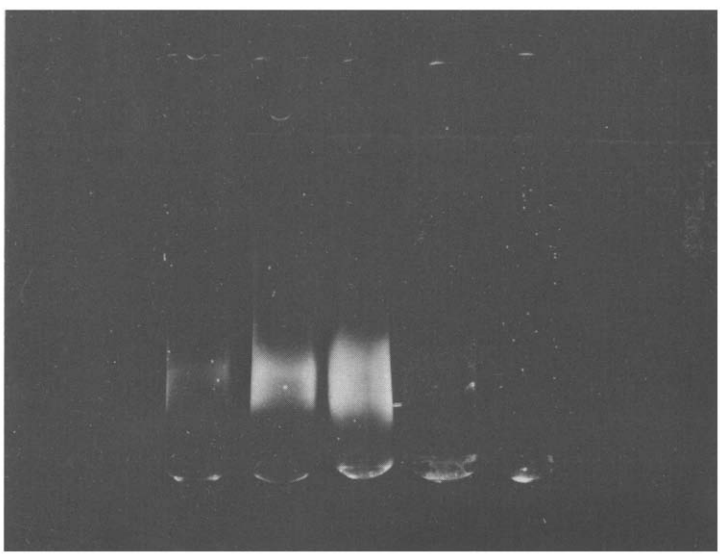

Fig. 1. Typical enzyme staining pattern for hydroxyapatite 1 M. The amounts of protein & activity loaded on gels were as follows: 1, 4 μg. 0.02 unit: 2, 20 μg, 0.11 unit; 3, 140 μg, 0.77 unit. Gels 4 and 5 received 14 and 140 μg of bovine serum albumin, respectively. The gels shown here were photographed with indirect lighting in order to accentuate the white manganese ferrocyanide precipitated at the sites of enzyme action. The tops of the gels are at the bottom of the picture.

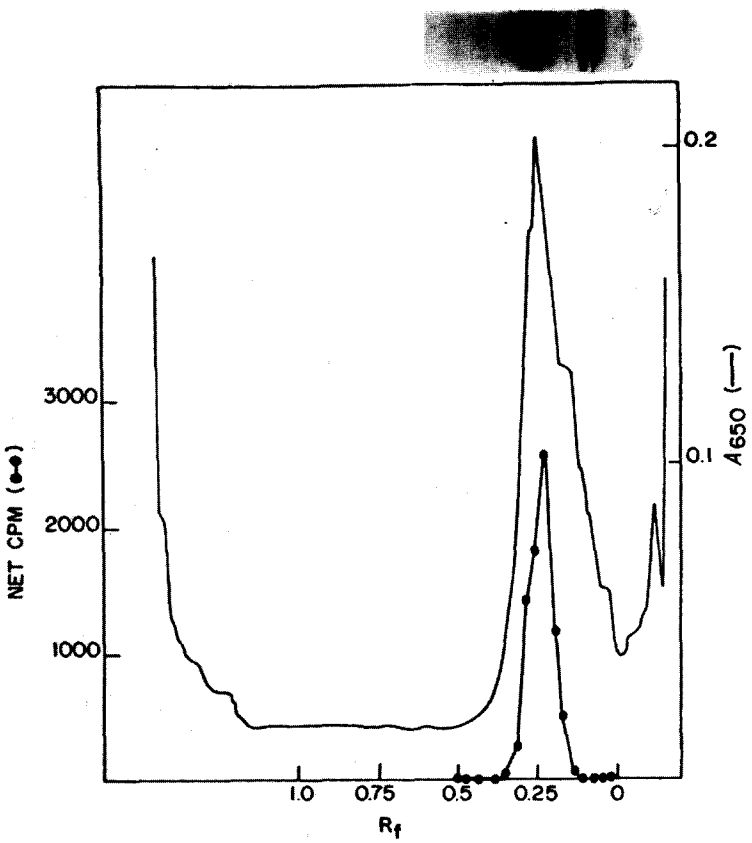

Fig. 2. Correlation of enzyme-staining pattern with activity elution profile for hydroxyapatite 1 M. The activity profile shown in this figure was determined by assaying slices of a gel that contained 250 μg (1.4 units) of enzyme. The enzyme-stained gel used to make the scan contained 31 μg (0.16 unit) of hydroxyapatite 1 M. The two are shown plotted on the same axes for ease of comparison. The gel shown in the insert contained 10 μg of purified choline acetyltransferase, and was stained with Coomassie blue.

zyme aggregates. Thus, manganese ferrocyanide staining is probably a more accurate method for localizing large enzyme molecules, whereas, elution and assay is probably more accurate for the smaller enzyme molecules. Since both procedures consistently agree qualitatively, and usually agree quantitatively, we felt that the multiple banding phenomena that we observed with each method for squid choline acetyltransferase were real.

Antibody titration curves. The antisera prepared to each of the two isozymes were capable of inactivating 60—80% of the choline acetyltransferase activity (0.0007 unit) used for titration. The maximum amount of inactivation could not be increased by using the two antisera together. Titration curves for both the homologous and the heterologous isozymes are shown with Ab 1 (N) and Ab 2 (N) in Fig. 3 (A and B, respectively) and with Ab 1 (D) and Ab 2 (D) in Fig. 4 (A and B, respectively). Although the titer of inactivating antibody was approximately ten times lower in the antisera prepared to denatured, rather than native enzymes, Ab 1 (D) and Ab 2 (D) were still each capable of producing 70—80% inactivation. A comparison of the titration curves in Fig. 3 and

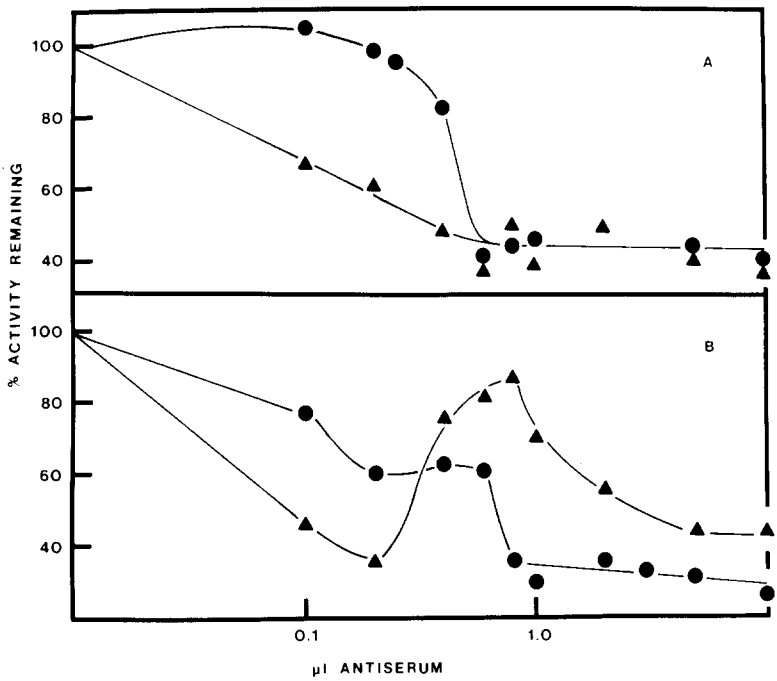

Fig. 3. Titration curves for antisera to native isozymes. All of the curves shown in this figure were obtained by using aliquots of enzyme containing 0.0007 unit of activity. The specific activities of the hydroxyapatite-purified isozymes were 14 and 10 units/mg for isozymes 1 (●) and 2 (▲), respectively. The curves in part A of the figure were obtained with Ab 1 (N), and those in part B of the figure were obtained with Ab 2 (N).

those in Fig. 4 for the antisera to native and denatured enzyme shows that both of the antisera prepared with native enzymes showed a greater specificity for isozyme 2, whereas the antisera to denatured enzyme could not distinguish between isozymes 1 and 2.

Ouchterlony precipitation of isozymes 1 and 2 and their isoelectric fractions. Fig. 5 shows the precipitin bands produced by double diffusion of fresh hydroxyapatite 1 and hydroxyapatite 2 with Ab 1 (N) and Ab (N). Each isozyme gave one predominant band with each antiserum. The precipitin bands were continuous, indicating identity of the antigenic determinants recognized by each antiserum. There was also some slight spurring, indicating the possibility of structural differences between the isozymes. This figure also shows that aged isozyme 1 gave multiple precipitin bands with Ab 1 (N).

Each of the isoelectric fractions of hydroxyapatite 1 (pre-A, A, B, and C) were precipitated on an Ouchterlony slide with Ab 1 (N), and the results are shown in Fig. 6. The pre-A fraction, which contained almost no enzymatic activity, gave a single, dense precipitin band that was continuous with the precipitin band in front of the well containing fraction A. That single band was continuous with all three bands that precipitated in front of the well containing fraction B. Such multiple bands of identity were typical of both isozymes, but were always most striking for isozyme 1 precipitated with Ab 1 (N) (left side of each slide). Much less protein was present in isoelectric fraction C, so the ex-

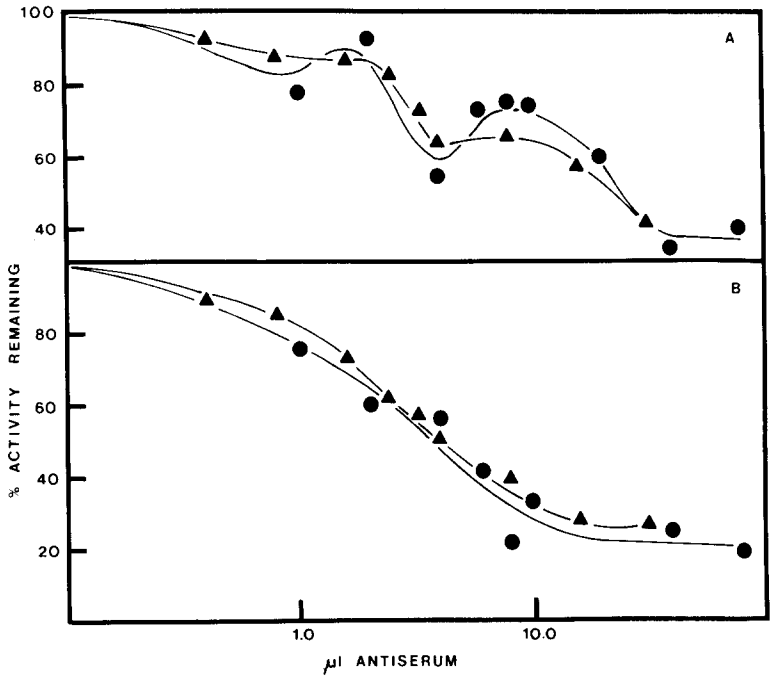

Fig. 4. Titration curves for antisera to dithiothreitol-treated isozymes. All of the curves have been normalized to 0.0004 unit of choline acetyltransferase so that their sharpest midpoints could be compared. The actual amount of enzyme activity added per assay was constant for each titration curve. The absolute amount of activity used for each curve never varied more than 3-fold from the value to which they have been normalized. Isozymes 1 and 2 are represented by the circles and triangles, respectively. The curves in part A of the figure were obtained with Ab 1 (D) and those in part B of the figure were obtained with Ab 2 (D).

tremely light precipitation seen with that fraction cannot necessarily be interpreted as a lack of reactivity. Immune precipitation of the isoelectric fractions of isozyme 2 with Ab 1 (N) showed precipitation with isoelectric fractions A and B, and very slightly with C; there was no precipitated pre-A isoelectric frac-

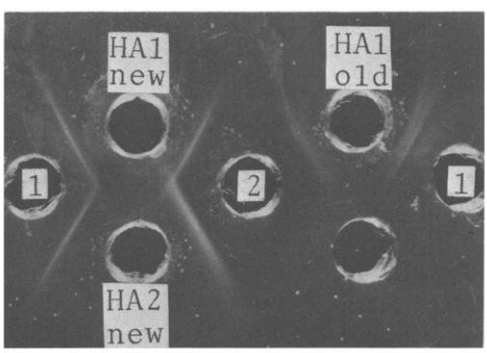

Fig. 5. Precipitation of isozymes 1 and 2 with the Ab 1 (N) and Ab 2 (N) antisera. The antigens and antisera were placed in the wells indicated on the photograph. 10 μl of Ab 1 (N) or 20 μl of Ab 2 (N) were used in the antiserum wells. Each isozyme was applied to the slide in a 10 μl aliquot of 0.01 M potassium phosphate buffer, pH 6.8, containing 60% sucrose. The amounts of protein applied in the enzyme aliquots were as follows: hydroxyapatite 1 new, 85 μg of freshly prepared hydroxyapatite 1; hydroxyapatite 1 old, 60 μg of aged hydroxyapatite 1; hydroxyapatite 2 new, 120 μg of freshly prepared hydroxyapatite 2.

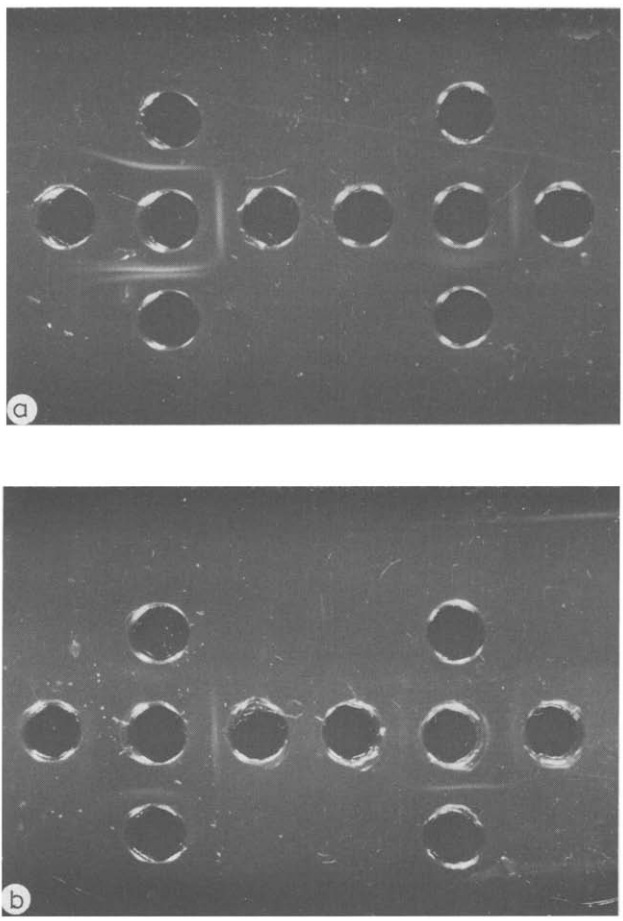

Fig. 6. Ouchterlony precipitation of isoelectric fractions of isozymes 1 and 2 with Ab 1 (N) and Ab 2 (N). Slide a: The center wells contained 10 μl of Ab 1 (N) and Ab 2 (N) on the left and the right sides of the slide, respectively. The outer wells contained, going clockwise from the top well, the following amounts of isoelectric fractions of hydroxyapatite 1: top, 0.0005 unit pre-A; right, 0.003 unit A; bottom, 0.003 unit B; left, 0.0003 unit C. Slide b: The center wells contained 10 μl of Ab 1 (N) and Ab 2 (N) on the left and the right sides of the slide, respectively. The outer wells contained, going in a clockwise direction from the top well, the following amounts of the isoelectric fractions of hydroxyapatite 2: top, 0.0011 unit A; right, 0.0012 unit A+B; bottom, 0.0008 unit B; left, 0.0001 unit C.

tion, and the multiple banding pattern was absent. The pattern of precipitation for isozymes 1 and 2 with Ab 2 (N) (right side of each slide) were similar to the pattern of precipitation observed for isozyme 2 with Ab 1 (N). That is, there was virtually no precipitation with the inactive pre-A fraction when antibody prepared to isozyme 2 was used for precipitation.

Manganese ferrocyanide staining of hydroxyapatite 1 and hydroxyapatite 2. Disc gel electrophoresis of squid choline acetyltransferase isolated from *L. pealei* clearly resolved multiple active forms of each of the isozymes. In addition, the distribution of the activity was different for isozymes 1 and 2.

Disc gel electrophoresis was performed on two gels of each isozyme, and the gels were stained either for activity or for protein. Scans of two manganese

ferrocyanide-stained gels and of two corresponding Coomassie blue-stained gels are presented in Fig. 7. Manganese ferrocyanide stain coincided with major protein bands, although the amount of activity in a particular band did not necessarily correlate directly with the amount of protein in that band. In the case of isozyme 1, the bulk of the activity was associated with fast-moving bands whereas the activity of isozyme 2 was predominantly associated with slow-moving bands. The most active forms of isozyme 1 were found at R_F values of 0.17 and 0.25; the most active form of isozyme 2 was found at an R_F of 0.07.

When choline acetyltransferase was eluted from gels rather than stained in situ the bulk of the enzyme activity eluted at R_F values of 0.17 and 0.25 in the case of isozyme 1, and at R_F values of 0.08 and 0.14 in the case of isozyme 2. The heaviest forms of choline acetyltransferase resolved on disc gel electrophoresis (R_F values 0.08 of isozyme 2 and 0.10 of isozyme 1) were the most labile and became almost inactive in enzyme that had been frozen or stored. This lability was shown both by staining and by elution and assay.

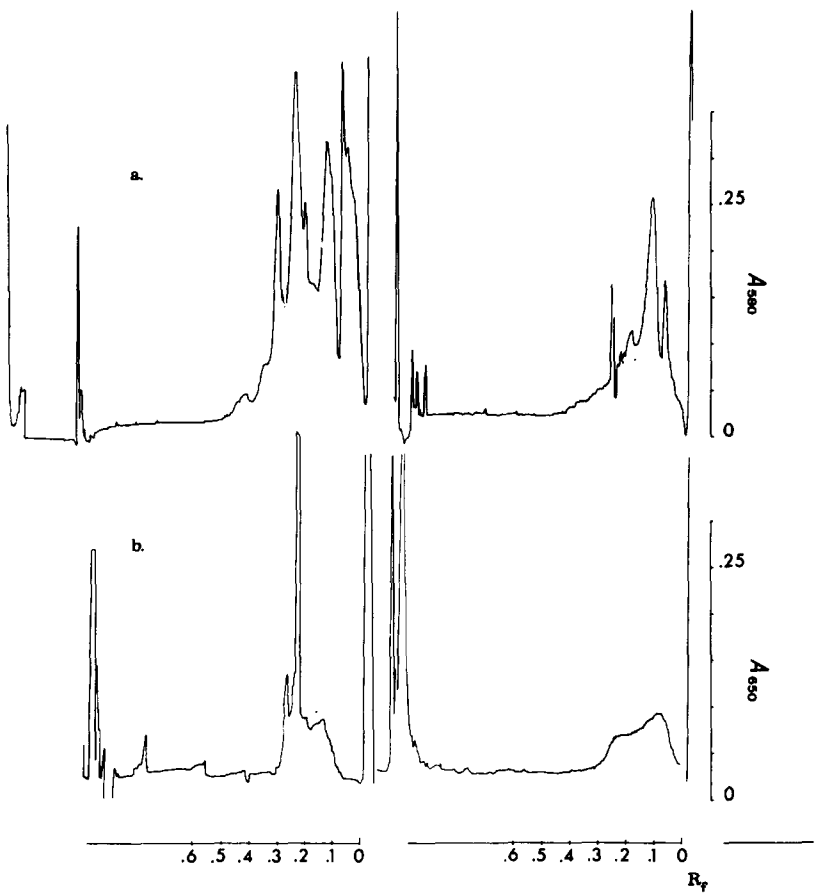

Fig. 7. Corresponding scans from gels stained for activity and for protein. The scans in the top line (a) of this figure are of Coomassie blue-stained gels. The scans shown in line (b) are for duplicate gels stained with manganese ferrocyanide. The gels that were scanned each contained 20 μg of enzyme protein. The gel scans on the left side of the figure are of hydroxyapatite 1 and those on the right are of hydroxyapatite 2.

The differences between the R_F values of the corresponding protein bands of isozymes 1 and 2 (i.e. R_F 0.08 versus 0.10, 0.14 versus 0.17 and 0.24 versus 0.27), were both reproducible and real. Electrophoresis of a sample containing both isozymes on one gel, resulted in the resolution of double peaks of activity, showing that the banding patterns of the two isozymes were non-superimposable.

Double diffusion experiments on eluates of polyacrylamide gel slices. In order to correlate the R_F values at which immune precipitation occurred with the R_F values at which the enzyme protein and activity were known to migrate on disc gel electrophoresis, double diffusion experiments were performed on eluates of polyacrylamide gel slices. In addition, the results of precipitation with the antisera prepared with native (N) and dithiothreitol-treated (D) enzyme were compared.

Precipitation with antisera to native enzyme. Hydroxyapatite 1 M (from *L. opalescens* and very similar to isozyme 1 [2]) was subjected to polyacrylamide gel electrophoresis at pH 8.4. After electrophoresis, the gel was sliced, and each slice was eluted into 100 µl of 20% sucrose. A diagram of the precipitation of every second slice with Ab 1 (N) or Ab 2 (N) is shown in Fig. 8a. Precipitation was most pronounced at R_F values 0.06—0.12, although precipitation was present through R_F 0.27. The multiple banding at R_F 0.06—0.12 should be noted.

Precipitation with antisera to dithiothreitol-treated enzyme. When the above experiments were performed using Ab 1 (D) or Ab 2 (D) in the center wells Fig. 8b), dense precipitation was observed only at R_F values 0.24—0.27, which exactly coincided with the position at which the activity of hydroxyapatite 1 M typically eluted from gels. These data show that antibody to dithiothreitol-

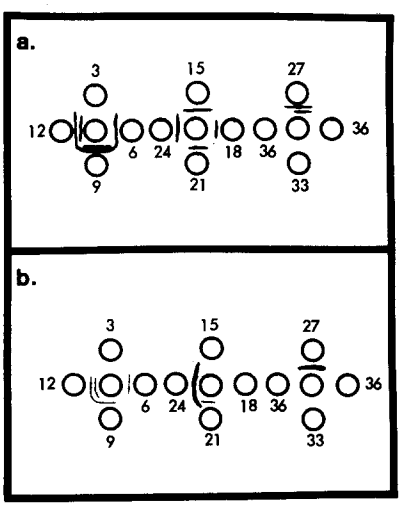

Fig. 8. Ouchterlony precipitation of hydroxyapatite 1 M fractions eluted from polyacrylamide gel. 250 µg hydroxyapatite 1 M was run on a gel which was then sliced and eluted as described in the text. 30 µl of each eluate was used to fill the outer wells of slide a and 40 µl of each eluate was used to fill the outer wells of slide b. Since the wells used were able to hold only 10 µl, the samples were applied in 3 or 4 separate applications. The number of each well corresponds to the R_F X 100 of the slice eluted. The center wells of slide a contained 10 µl of Ab 2 (N) and the center wells of slide b contained 40 µl of Ab 1 (D).

treated enzyme precipitated preferentially with the small enzyme molecules, as would be expected if dithiothreitol were causing dissociation. Exactly the opposite was true for Ab 1 (N) and Ab 2 (N), each of which was prepared to untreated enzyme and reacted preferentially with the large enzyme molecules.

Dissociation of aggregated forms of hydroxyapatite 1 M. We have obtained some additional evidence for dissociation of isoelectric fraction B of isozyme hydroxyapatite 1 M stored in the presence of dithiothreitol. The protein banding patterns on disc gels of both fresh and aged preparations of isoelectric fraction B of hydroxyapatite 1 M (which can be resolved as a single band on SDS gel electrophoresis) are shown stained with Coomassie blue in Fig. 9.

When isoelectric fraction B was subjected to disc gel electrophoresis within 1 week of focusing, the protein banded as one major species (at an R_F of 0.15). Three minor bands were also visible on the gels. After the focused enzyme had been stored for 2 weeks in $1.0 \cdot 10^{-3}$ M dithiothreitol, the band at an R_F of 0.25 became a major, rather than a minor component.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate. In order to

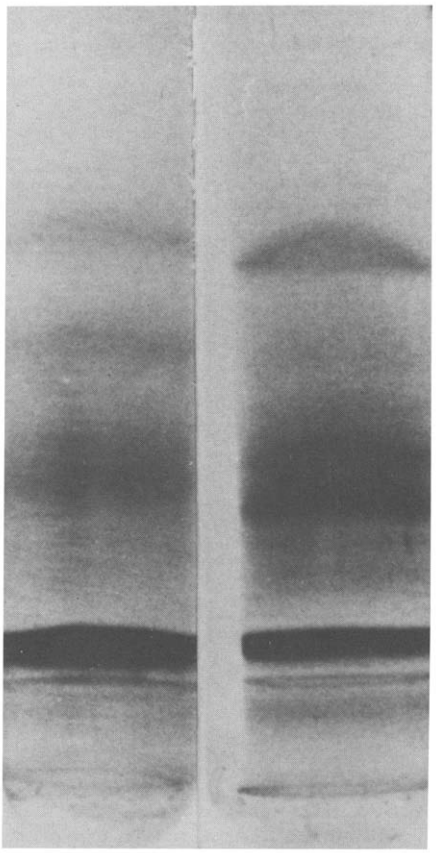

Fig. 9. Disc gel electrophoresis of isoelectric fraction B of isozyme 1 M. The tops of the gels in this figure are at the bottom of the tubes. The gel on the left contained 42 μg of freshly focused isoelectric fraction B of hydroxyapatite 1 M. The gel on the right contained 30 μg of the same preparation of isoelectric fraction B which had been aged for 2 weeks.

compare the subunit composition of the active forms of isozymes 1 and 2, enzyme that had been eluted from sliced gels was treated with SDS and mercaptoethanol as described in Methods, and was subjected to polyacrylamide gel electrophoresis in SDS. The eluates from both of the active bands of hydroxyapatite 1 and from all three active bands of hydroxyapatite 2 gave two protein bands with R_F values corresponding to molecular weights of 37 000 and 56 000 (see Fig. 10). The smaller species was a minor component of isozyme 2 (not visible in the photograph) and a major component of isozyme 1.

Similar patterns were obtained from polyacrylamide gel electrophoresis in SDS of isoelectric focusing fractions of the two isozymes. The main protein bands corresponded to molecular weights of 37 000 and 56 000, although, for focused enzyme, the low molecular weight component was a major component of only the high isoelectric point fraction (C) of both isozymes. Gels representative of A+B and of C of isozyme 2 are shown in Fig. 11. Gels of isozyme 1 are comparable, except that fraction A from hydroxyapatite 1 also showed an additional protein band (presumed to be a non-enzyme contaminant) at a position corresponding to a molecular weight of 42 000.

Gel electrophoresis in SDS of hydroxyapatite 1 M showed it to be composed of protein species with molecular weights of 42 000 and 53 000 (see Fig. 12), whereas isoelectric fraction B of hydroxyapatite 1 M ran as a single band, at a position corresponding to a molecular weight of 53 000, on polyacrylamide gel

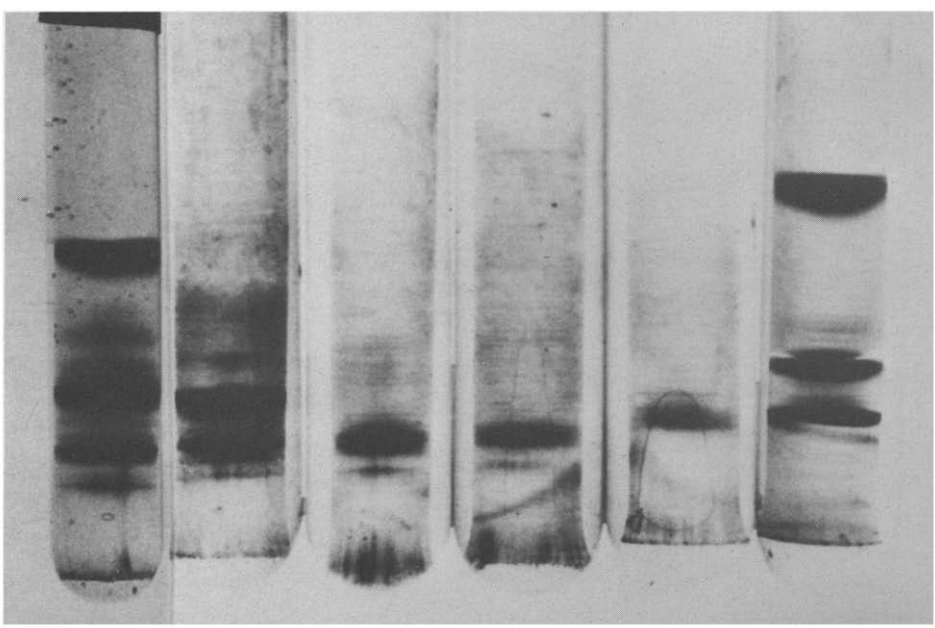

Fig. 10. Polyacrylamide gel electrophoresis in SDS of protein bands eluted from gels of hydroxyapatite 1 and hydroxyapatite 2. The gels in this figure are numbered 1—6, from left to right, and the tops of the gels are at the bottom of the picture. The eluates from R_F values of 0.17 and 0.25 of gel hydroxyapatite 1 were loaded on gel 1 and 2, respectively. Gels 3—5 contain protein eluted from a gel of hydroxyapatite 2 at R_F values of 0.08, 0.14, and 0.23, in that order. Gel number 6 contained only myoglobin, ovalbumin, and bovine serum albumin (30 μg each) as molecular weight markers. Gel number 1 also contained 30 μg of myoglobin as an internal molecular weight standard.

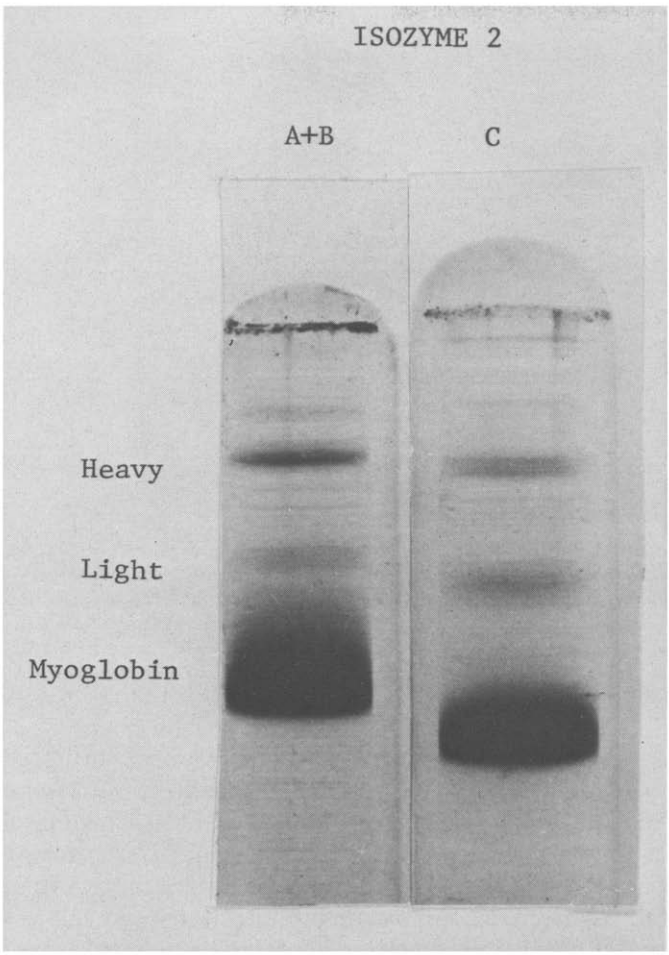

Fig. 11. Polyacrylamide gel electrophoresis in SDS of isoelectric fractions from isozyme 2. The tops of these gels are at the top of the figure. The gel on the left contained 0.05 unit of isoelectric fractions A and B (combined) of hydroxyaapatite 2. The gel on the right contained 0.08 unit of isoelectric fraction C. Both gels contained 10 μg of myoglobin.

electrophoresis in SDS. This result was in complete agreement with what would have been expected on the basis of the results that were obtained from polyacrylamide gel electrophoresis in SDS of isoelectric fractions isolated from isozymes 1 and 2 from *L. pealei.* No other isoelectric fraction of hydroxyapatite 1 M was examined for subunit composition.

Gel filtration on Sephadex G-200. Since native squid choline acetyltransferase had been shown to self-associate into multiple active forms, we felt it would be useful to find the molecular weights of these active forms. Also, because each isozyme of squid choline acetyltransferase had been shown to contain two non-identical subunits, we wanted to find out whether enzymatic activity was associated with either or both of the subunits.

Filtration on calibrated columns of Sephadex G-200 produced a range of multiple peaks of enzyme activity corresponding to molecular weights be-

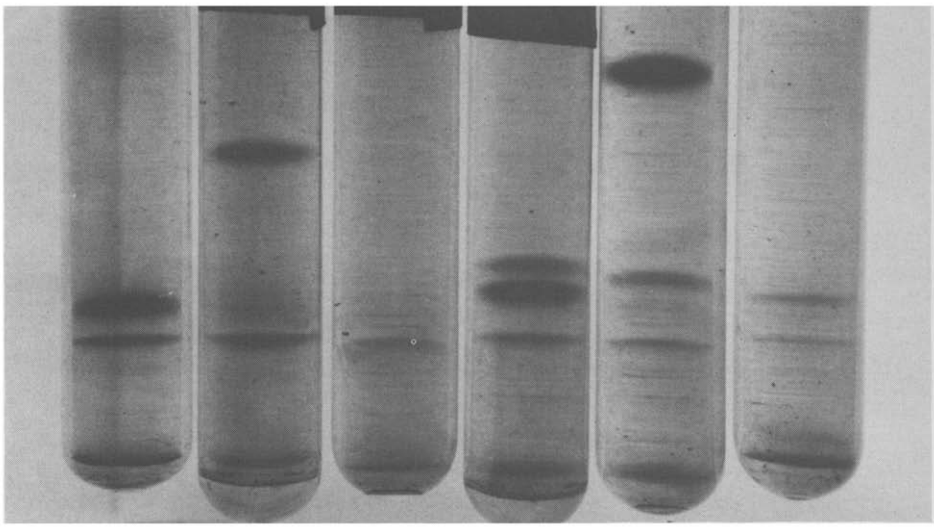

Fig. 12. Polyacrylamide gel electrophoresis in SDS of hydroxyapatite 1 M. The gels shown in this figure are numbered 1—6, from left to right, and the tops of the gels are in the bottoms of the tubes. Gels 1—3 contain hydroxyapatite 1 M, and gels 4—6 contain hydroxyapatite 1 M isoelectric fraction B. Each sample is shown with 15 μg of myoglobin, 15 μg of ovalbumin, or no marker, in that order, from left to right. Gels 1, 2, 4, and 5 contained 40 μg of enzyme protein each, and the other 2 gels were loaded with 20 μg of enzyme protein each.

tween 35 000 and 300 000. When the starting material was a 30—55% saturated ammonium sulfate fraction (prepared from *L. pealei)* the range of molecular weights was from 60 000 to 180 000, with a total recovery of 50%. Chromatography was also carried out on a 30—40% saturated ammonium sulfate precipitate (known from isoelectric focusing data to be enriched in fraction A) [2] and on a 45—55% saturated ammonium sulfate precipitate (enriched in fractions B and C). Each ammonium sulfate fraction contained enzyme species covering the whole range of molecular weights, although the average molecular weight of the 30—40% saturated ammonium sulfate fraction was higher than that of the 45—55% fraction.

Similar results were obtained when crude preparations of isoelectric fractions A and B prepared from a 30—55% saturated ammonium sulfate fraction of *L. pealei* were chromatographed on Sephadex G-200. The average molecular weight of fraction A was greater than that of fraction B, although each contained species that ranged from 35 000 to 300 000 in molecular weight (see Fig. 13). Since recoveries of enzymatic activity from Sephadex filtration were quite poor (less than 10% usually) for all but crude enzyme preparations, no experiments were performed on either of the isozymes alone, after purification.

Sedimentation equilibrium studies. Serial dilutions of several isoelectric fractions isolated from *L. pealei* were subjected to ultracentrifugation in 0.01 M potassium phosphate buffer, pH 6.8, containing 20% sucrose. The results of a typical sedimentation equilibrium experiment with isoelectric fraction B of isozyme 1 are shown in Fig. 14. The variation of the observed molecular weight distributions with loading concentration reflects the polydispersity of the sample, and suggests that self-association may be occurring. The range of average

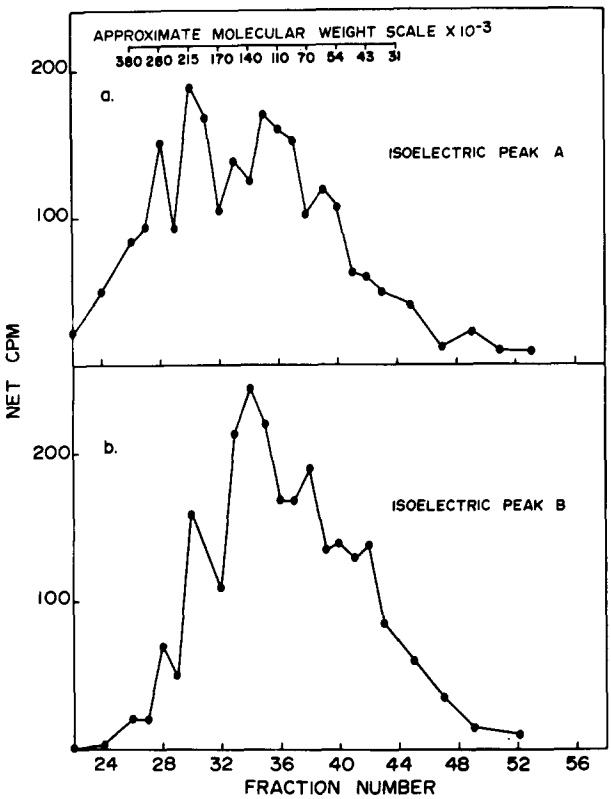

Fig. 13. Sephadex G-200 chromatography of isoelectric fractions A and B. The isoelectric fractions used in these experiments were prepared by isoelectric focusing of a 30—55% saturated ammonium sulfate fraction prepared from an extract of ganglia of *L. pealei*. After focusing, the isoelectric fractions were placed in dialysis tubing and concentrated overnight against solid sucrose. They were then dialyzed overnight against 0.01 M potassium phosphate buffer, pH 6.8, containing 0.1 M NaCl, 10^{-4} M dithiothreitol and 10% sucrose. 0.012 unit of A and 0.004 unit of B were applied to the column in separate experiments. The column used was 20 X 1.2 cm and had a void volume of 10.75 ml. 0.5-ml fractions were collected.

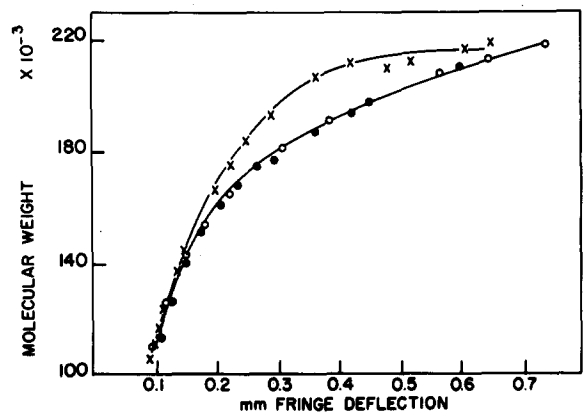

Fig. 14. Sedimentation equilibrium ultracentrifugation of isozyme 1. The graph shown is for isoelectric fraction A of hydroxyapatite 1. It shows weight average molecular weight as function of concentration plotted vs. mm fringe deflection as determined in a Spinco Model E analytical ultracentrifuge using a six-channel cell with a 30 mm centerpiece. The symbols used represents different loading concentrations: X, 3.1 mg/ml; •, 1.0 mg/ml; ○, 0.33 mg/ml. Centrifugation was performed in 0.01 M potassium phosphate buffer, pH 6.8, containing 20% sucrose at 4.5°C and a speed of 14 000 rev./min for 28 h.

molecular weights for isoelectric fraction A of isozyme 1 was from 70 000 to 235 000.

While the results for isozyme 2 were less definitive, they were consistent with the presence of a self-associating system with a higher equilibrium constant for association than isozyme 1. Preliminary results with isoelectric fraction A of isozyme 2 indicated the presence of molecules with molecular weights of up to 300 000.

Experiments were also performed on isoelectric fraction C of isozyme 1 after denaturation for 3 days in 6 M guanidine · HCl containing 1% mercaptoethanol. The molecular weights of the proteins contained in this fraction were determined to be 34 000 and 54 000.

Discussion

The staining reaction described in this paper is quite useful for studying purified enzyme fractions with high specific activity, such as is typical of the choline acetyltransferases from invertebrates and bacteria. Unfortunately, because approx. 0.05 unit of activity is necessary for clear staining, this method would not be useful, unless modified, for the study of isozymes of mammalian choline acetyltransferases, whose intrinsic specific activities are low [7,8]. Before using this method for staining, one must be careful to remove both acylase and extraneous acetyltransferase activity from the preparation to be stained, because the presence of such enzymes will lead to spurious staining. When it is feasible to employ our method for staining of choline acetyltransferase on gels, its use makes the study of isozymes less tedious, less time consuming, and less expensive than slicing, elution, and assay of gel slices for localization of activity.

The two isozymes of choline acetyltransferase isolated from *L. pealei* were shown to be quite similar in structure by virtue of their almost complete cross reactions with the heterologous antisera (isozyme 1 with Ab 2 (N), and isozyme 2 with Ab 1 (N)). Some differences in their structures were also suggested by the slight spurring of the precipitin bands formed with both isozymes and either Ab 1 (N) or Ab 2 (N). Spurring was completely absent, however, when the different isoelectric forms of one single isozyme were precipitated next to one another. These results indicate that the different isoelectric forms of the same isozyme resemble one another more than the two isozymes resemble one another.

The three major protein bands that were isolated from choline acetyltransferase on polyacrylamide gel electrophoresis were all shown to precipitate with antiserum to choline acetyltransferase. The antisera prepared to native enzyme were prepared by immunization with aggregated enzyme protein eluted from gels at an R_F of 0.14—0.19. The antibodies formed, however, were able to precipitate material that migrated in polyacrylamide gels with R_F values of 0.10 and 0.25 as well. Thus, precipitation with antibody proved the identity of the three major protein bands of each isozyme.

The antiserum Ab 1 (N) contains antibodies to inactive choline acetyltransferase as well as to active enzyme, whereas Ab 2 (N) appears to contain only antibodies to active enzyme. Such findings could be predicted on the basis of the difference in the specific activities of two isozymes, and the fact that only

isozyme 1 has been shown to contain inactive enzyme protein. The antibody to inactive enzyme aggregates accounted for the multiple banding observed repeatedly with Ab 1 (N), especially with aged isozyme 1, with the inactive pre-A fraction isolated from isozyme 1 during isoelectric focusing, and with the highly aggregated material found to migrate at an R_F of 0.10 on disc gels.

Antisera Ab 2 (N) and Ab 1 (D) produced complex inactivation curves that could be separated into at least three different phases. Such curves can be explained in several ways; (1) reaction with a single antibody population was causing the enzyme protein to undergo alternate phases of inactivation and activation, depending on the size of the lattice of the antigen antibody precipitate; (2) several different populations of antibodies, at least one of which activated inactive protein by binding it into an antigen antibody precipitate, were present in the antisera, but at different titers so that their titration curves were not superimposable; (3) some combination of the above possibilities. Since antibodies have been found to activate other enzyme molecules such as ribonuclease and β-galactosidase [24,25], the presence of activating antibody in our antisera would not be unique. Reaction with antibody can cause profound conformational alterations in protein structure, either by inducing a new conformation, or by stabilizing one that already exists. In addition, the affinities of antibody for native and denatured proteins vary tremendously as do the affinities of antibodies for oxy- and deoxyhemoglobin [25]. Thus, it seems reasonable that an antigen that forms at least three different active aggregates, each of which appears to have a different specific activity [2], would present a complex titration curve. Indeed, the pattern of precipitation is known to be complex on the basis of Ouchterlony double diffusion experiments alone.

Singh et al. [26] have reported the isolation of two active fractions of choline acetyltransferase from human neostriatum by chromatography on phosphocellulose. The antiserum to fraction A was specific for this fraction, and did not cross react with fraction B. These data suggest that isozymes of choline acetyltransferase exist in mammalian species as well as in the squid.

Rossier et al. [17] have recently purified choline acetyltransferase from rat brain. The enzyme had a specific activity of 20 μmol/min per mg, and showed multiple bands upon gel electrophoresis. They used this enzyme to prepare antiserum specific for choline acetyltransferase [18]. Although the antiserum was capable of precipitating 100% of the choline acetyltransferase, it was not monospecific because it gave multiple bands on Ouchterlony double diffusion. Such multiple banding phenomena could be explained, in light of our data, by the presence in the antiserum of antibodies to inactive enzyme aggregates as well as to active enzyme.

In this paper, large aggregates of isozymes 1 and 1 M were shown to dissociate into smaller aggregates in the presence of dithiothreitol. The data obtained indicated a slow, but real, dissociation of enzyme protein in the presence of dithiothreitol. In addition, a preparation of isozyme 1 was also shown to perform like a self-associating system in the analytical ultracentrifuge.

Data regarding isozyme 2 are much less clear cut than in the case of isozyme 1, but our results are consistent with the hypothesis that isozyme 2 is a self-associating system with an extremely low equilibrium constant. Dissociation of aggregated (R_F 0.14) isozyme 2 protein has been shown indirectly. Ag-

gregates of isozyme 2 could be treated with 0.01 M dithiothreitol for 2 weeks and then used to elicit antibodies to isozyme 2 protein with an R_F of 0.24. From these data, and because isozyme 2 exists in multiple, active molecular forms, as well as because it seems to resemble isozyme 1 in so many ways, we conclude that isozyme 2 also self-associates.

It is possible that the degree of association of squid choline acetyltransferase is affected by the state of reduction of enzymic sulfhydryl groups. Sulfhydryl-protecting reagents cause association of other enzymes such as deoxycytidylate deaminase [27], fatty acid synthetase [28], and pyruvate kinase [29], and cause dissociation of DDT (the insecticide) dehydrochlorinase [30,31].

Chao and Wolfgram [32] reported that bovine brain enzyme aggregates on exposure to $(NH_4)_2SO_4$, and that much of the aggregation that they encountered during purification was an artifact resulting from the ammonium sulfate precipitation step in their purification. In contrast, we have observed that crude extracts of squid choline acetyltransferase contain considerable amounts of active aggregated enzyme protein, as shown by slicing, elution and assay of disc gels (Polsky, R. and Shuster, L., unpublished observations). Chao and Wolfgram [32] did not use any sulfhydryl reagents to protect their enzyme preparations, and, in fact, they reported that such reagents caused inactivation of their enzyme preparations. Thus mammalian choline acetyltransferase appears to differ significantly from squid choline acetyltransferase.

Squid choline acetyltransferase contains two non-identical "subunits", as shown by polyacrylamide gel electrophoresis in SDS, and sedimentation equilibrium centrifugation in guanidinium · HCl. The sizes of the "subunits" of the isozymes of choline acetyltransferase isolated from *L. pealei* were approx. 37 000 and 56 000 daltons.

The "subunits" of isozyme 1 M had molecular weights of 42 000 and 53 000; only the 53 000 mol. wt. "subunit" was present in isoelectric fraction B of isozyme 1 M. The choline acetyltransferase from bovine caudate nucleus has also been shown to contain two dissimilar "subunits" (of 51 000 and 69 000 daltons) [8].

From the results of our experiments, it did not appear that the low molecular weight species was present in any fixed proportion to the "larger subunit" in fractions that contained enzymatic activity. Yet the low molecular weight (37 000) protein was present in each isozyme after fractionation by chromatography on cellulose phosphate or isoelectric focusing or disc gel electrophoresis. In addition, some enzymatic activity was eluted at a volume corresponding to a molecular weight of 37 000 on Sephadex gel filtration. Thus the 37 000 dalton protein truly appeared to be a "subunit" of choline acetyltransferase. Although the heavier "subunit" alone might, it is possible, form active aggregated forms of the enzyme (as in the case of isoelectric fraction B of hydroxyapatite 1 M). Since both 'subunits" seemed to exhibit catalytic activity, it appears likely that the two are structurally related in some manner.

Our data is consistent with the hypothesis that the "subunits" which we have isolated are composed of still smaller subunits with a molecular weight of 18 000, and that these associate strongly even in SDS. Chao [33] has recently observed that bovine brain choline acetyltransferase has a molecular weight of 88 000 at alkaline pH, and a molecular weight of 60 000 at neutral pH as deter-

mined by ultracentrifugal techniques. He suggests, on the basis of chemical and enzymatic cleavage, and polyacrylamide gel electrophoresis in SDS, that the enzyme is composed of six identical subunits, each with a molecular weight of approx. 15 000. Chao also states that all conventional methods of dissociation of the subunits resulted in incomplete dissociation, and that polyacrylamide gel electrophoresis in SDS resulted in the resolution of multiple bands of protein with molecular weights ranging from 16 000 to 88 000.

Although the general patterns of behavior are quite similar for isozymes 1 and 2, they consistently differ in a number of specific aspects. It remains to be seen whether the basic structures of the subunits of isozymes 1 and 2 differ in a manner that would cause isozyme 1 to associate less strongly, to be less susceptible to salt activation, and to be less active when highly aggregated than isozyme 2.

Acknowledgements

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