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An asymmetric form of muscle acetylcholinesterase contains three subunit types and two enzymic activities in one molecule

(chick muscle/pseudocholinesterase/monoclonal antibodies)

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ABSTRACT **We have purified completely the principal asymmetric ("heavy") form of acetylcholinesterase (AcChoEase; EC 3.1.1.7) from chick muscle (i.e., the synaptic form in the twitch muscle fibers) by using a monoclonal antibody that recognizes AcChoEase but not pseudocholinesterase (ChoEase; cholinesterase, EC 3.1.1.8). The purified protein exhibits catalytic and inhibition properties characteristic of AcChoEase and ChoEase and contains three distinct subunits of apparent sizes 110 kDa, 72 kDa, and 58 kDa in the ratio 2:2:1. The discovery of an AcChoEase/ChoEase hybrid asymmetric form has been further supported by (*i*) the identification of active site properties of AcChoEase in the 110-kDa subunit and of ChoEase in the 72-kDa subunit, (*ii*) the purification or precipitation of both activities together by, also, a ChoEase-specific monoclonal antibody, and (*iii*) evidence that all subunits are bound in the asymmetric forms by disulfide bonds. The 58-kDa subunit is the only one that is sensitive to digestion with purified collagenase; it carries the collagenous "tail" of the asymmetric form. A model is proposed for this form of AcChoEase.**

In vertebrate skeletal muscles, acetylcholinesterase (AcChoEase; EC 3.1.1.7) occurs at the synapses as a very large, dimensionally asymmetric molecule, having a sedimentation constant of about 16 S in mammals and 20 S in birds (1–8). An apparently analogous form in eel electric organ contains 12 identical catalytic subunits, linked by disulfides to collagenous structural subunits, a structure that has been designated "A_{12}" (7, 8). In *Torpedo* electric organ the structure of the catalytic subunit (65 kDa) has been established by cDNA cloning (9, 10); the asymmetric AcChoEase there has a structure generally similar to that of eel (11). From muscles, the asymmetric form of AcChoEase has hitherto not been purified from any species. In detergent/salt extracts of the muscles of mammals and birds, however, an asymmetric ("heavy") form of AcChoEase is present and has properties suggesting that it, also, has the A_{12} structure (8, 12–14).

There are clues, however, that could indicate that the situation generally is not so simple as this. In fish electric organ, mammalian skeletal muscle, and other cholinergic tissues, other forms of AcChoEase exist that are smaller and globular, and these contain either hydrophobic or hydrophilic AcChoEase catalytic subunits (8); at least two types of catalytic subunit are present in *Torpedo* electric organ, distinguishable by peptide mapping or immunochemically (11, 15), and multiple mRNA species for this subunit have been detected there (9, 16). Further, a cholinesterase of different specificity, termed pseudocholinesterase (ChoEase; cholinesterase, EC 3.1.1.8) or butyrylcholinesterase [for its preferred substrate when from mammalian—although not avian (13)—sources], exists in skeletal and cardiac muscles (8) but not in *Torpedo* electric organ (17). Comparison of the complete amino acid sequences of human ChoEase (18) and *Torpedo* AcChoEase catalytic subunits (9, 10) plus the absence of cross-reactivity between those two enzymes from a given species in the antibodies so far described (8, 17, 19–23) suggest independent genes for the two catalytic subunits. Yet ChoEase exists in precisely the same set of molecular forms as AcChoEase (7, 8, 12, 13, 24, 25) and, at least in chicken muscles (24), they appear to be coregulated, although different behavior has been found in the rat (22, 26).We report now that the asymmetric AcChoEase of chick muscle contains, in fact, predominantly a hybrid of AcChoEase and ChoEase catalytic subunits, attached by way of disulfides to a common collagenous tail.

MATERIALS AND METHODS

Purification of AcChoEase. The AcChoEase-specific monoclonal antibody (mAb) ACB-2 (23) was purified from hybridoma medium by protein A-Sepharose chromatography (27) and covalently attached to CNBr-activated Sepharose 4B (23). The 1-day-old chick muscle was homogenized (1:10, wt/vol) in 10 mM Hepes, pH 7.5/0.5% Triton X-100/5 mM EGTA/5 mM EDTA/5 mM *N*-ethylmaleimide/2 mM benzamidine/1 mg of bacitracin per ml (low-salt/Triton buffer). After a 30,000 × *g* centrifugation, the supernatant was reserved as the low-salt extract, and the pellet was extracted with low-salt/Triton buffer (1:10, wt/vol) containing 1 M NaCl and centrifuged at 100,000 × *g* for 1 hr. The supernatant was recirculated through the ACB-2-Sepharose column for 15 hr, washed extensively with 10 mM Hepes, pH 7.5/2 M NaCl/0.01% Triton X-100, then washed with 50 mM glycine·NaOH, pH 9.0/0.01% Triton X-100, and eluted with 50 mM glycine·NaOH, pH 11.0/0.5 M NaCl/0.01% Triton X-100. The eluate was immediately neutralized by adding one-quarter of its volume of 1 M Tris·HCl (pH 7.5). Fractions containing the peak AcChoEase activity were pooled and passed through a heparin-agarose (28) column, which was preequilibrated and subsequently washed extensively with 10 mM Hepes, pH 7.5/0.4 M NaCl/0.01% Triton X-100, and finally eluted with the same buffer containing 1 M NaCl. All procedures were performed at 4°C.

Purification of ChoEase by using its specific mAb, 7D-11, was performed similarly except that the above-described low-salt/Triton extract, or adult chicken serum diluted 1:5 with low-salt/Triton buffer, was applied and recirculated through the 7D-11-Sepharose column. The column was

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Abbreviations: AcChoEase, acetylcholinesterase; ChoEase, pseudocholinesterase; mAb, monoclonal antibody(ies); iPr_2P-F, diisopropyl fluorophosphate; iso-OMPA, tetraisopropylpyrophosphoramide; BW 284c51, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide.

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Table 1. Immunopurification of asymmetric AcChoEase from chick muscle (100 g)

Stage	Volume, ml	AcChoEase, units	Protein, mg	Units/mg	Purification factor	Recovery, %
1 M NaCl extract	760	337	1330	0.25	1	100
Immunoaffinity eluate	25	234	0.26	908	3633	69
Heparin-agarose eluate	8.6	189	0.17	1132	4531	56

equilibrated, washed, and eluted as described above, except that the 2 M NaCl-containing buffer was omitted.

Diisopropyl Fluorophosphate (iPr_2P-F) Treatments. Samples were incubated for 15 min at room temperature with 0.1 mM [^{3}H]iPr_2P-F (Amersham), precipitated with 12% trichloroacetic acid (Cl_3CCOOH), separated by $NaDodSO_4$/PAGE, and processed for fluorography. [^{3}H]iPr_2P-F binding was quantitated after a similar incubation and precipitation except that the precipitate was bound to a Whatman GF/B filter, washed with 20 ml of 8% Cl_3CCOOH, dried, and assayed for radioactivity after addition of 4 ml of Hydrofluor (National Diagnostics, Somerville, NJ). Control filters gave a background of about 100 cpm.

Digestion with Collagenase. Asymmetric AcChoEase was incubated for 15 hr at 37°C in the presence of highly purified collagenase (Worthington; clostridiopeptidase A). A preliminary study established the optimum concentration of collagenase as 1 μg/50 μl in the presence of 5 mM $CaCl_2$, to completely remove the collagen-like tail of the asymmetric form.

Enzyme Assays and Size Determination. AcChoEase activity, relative to protein content (29), was measured either radiometrically (23) with 0.75 mM [^{3}H]acetylcholine (Amersham) as the substrate or by using 0.75 mM acetylthiocholine as the substrate (24), in each case treating (24) with 0.1 mM tetraisopropylpyrophosphoramide (iso-OMPA). ChoEase activity was measured (24) by using 0.75 mM butyrylthiocholine in the presence of 0.05 mM 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284c51). Molecular forms were separated by 5–20% sucrose gradients, centrifuged at 36,000 rpm in a Beckman SW40 rotor (18 hr, 4°C), and calibrated with marker proteins (24).

Production of mAb. Immunization, production of mAb, and screening for anti-AcChoEase antibodies were as described in detail (23), but the purified asymmetric AcChoEase was used as the immunogen (30 μg per injection). For the assays, extracts containing asymmetric AcChoEase were prepared by homogenization of 1-day-old chick muscle (1:10, wt/vol) in 10 mM Hepes, pH 7.5/1 M NaCl/0.5% Triton X-100, with centrifugation at 30,000 × *g* for 30 min; immunoassay was in antibody-coated wells as described (23). Of 10 mAb identified in the screen using asymmetric AcChoEase, 1 (7D-11) was similarly positive; this was also true in a second screen using adult chicken serum in place of the muscle extract and using the binding of ChoEase activity as the test. This clone was expanded and recloned twice in soft agarose (23). The antibody protein was purified (27) as above; its properties will be described elsewhere.

RESULTS

Immunopurification of Muscle Asymmetric AcChoEase. The mAb ACB-2, previously shown to bind specifically to the catalytic subunit of chicken brain AcChoEase but not to chicken muscle or serum ChoEase (23), was used. By applying an extract of chick muscle to a column of immobilized ACB-2, followed by chromatography on a heparin-agarose column (28), we obtained an enrichment for AcChoEase activity of >4500-fold from the original crude extract (Table 1) and achieved a final specific activity of >1100 units/mg of protein. The purified asymmetric enzyme had in each preparation a final specific activity on [^{3}H]-acetylcholine of approximately one-half that found for the completely purified globular AcChoEase from chicken brain [which was ≈2200 units/mg of protein, as reported (23, 30)], suggesting that not all of the protein contained in the asymmetric form is the AcChoEase catalytic unit.

Substrate Specificity and Sensitivity to Inhibitors. The purified muscle enzyme showed enzymic activity for AcChoEase and for ChoEase (Table 2). About 95% of acetylthiocholine hydrolysis was inhibited by incubation with 0.05 mM BW 284c51, indicating that the great majority of the enzyme activity was AcChoEase. However, of the order of 5% of the total enzymic activity reproducibly persisted in the presence of 0.05 mM BW 284c51 or likewise was exhibited on butyrylthiocholine as substrate or was abolished by 0.1 mM iso-OMPA, indicating that some ChoEase activity had been copurified.

Sedimentation of the Asymmetric AcChoEase. The molecular forms of the purified enzyme showed peaks only at 15 S and 20 S when analyzed by sucrose density gradient sedimentation, confirming that the asymmetric forms were the only species in the sample (Fig. 1*A*). These forms were further shown to contain collagen-like structures by showing the predicted (31) aggregation in the absence of NaCl (as seen on sucrose density gradients; data not shown) and by exhibiting a sedimentation shift of 1–2 S [diagnostic of the change to a less asymmetric shape (31)] after treatment with purified collagenase (Fig. 1*B*). Further, the collagenase-digested forms could be completely converted to a single 11.5S form when the gradients were run under reducing conditions (10 mM dithiothreitol), indicating that disulfide bonds hold the tailed structure together (Fig. 1*B*). Prior to the collagenase treatment, the dithiothreitol addition had no effect on the profile of Fig. 1*A* (data not shown). Interestingly, when fractions from the two extremes of the 11.5S peak were analyzed for their ability to hydrolyze acetylthiocholine and butyrylthiocholine we found no difference in their activity ratio, suggesting that the 11.5S peak contains a homogeneous population of oligomeric molecules.

Subunit Analysis of the Asymmetric AcChoEase. The purified enzyme showed three distinct subunits when separated by $NaDodSO_4$/PAGE under reducing conditions (Fig. 2, lane A). A subunit migrating at 110 kDa (more strongly staining with Coomassie blue than with silver, lane B) had a similar mobility to the single catalytic subunit purified from

Table 2. Enzymic activities of purified AcChoEase

Substrate	% of activity on AcSCho
AcSCho	100
+ iso-OMPA (AcChoEase)	95
+ BW 284c51	4.0
BtSCho	4.5
+ BW 284c51 (ChoEase)	4.3
AcSCho + iso-OMPA + BW 284c51	0
AcSCho/BtSCho*	22

AcSCho, acetylthiocholine; BtSCho, butyrylthiocholine.

*Ratio of the activity (per ml) on acetylthiocholine to that on butyrylthiocholine (with no inhibitors used). This can be compared to a ratio for homogeneous AcChoEase from chicken brain (23) of 250:1 when measured on these two substrates or 150:1 when measured on [^{3}H]acetylcholine versus [^{3}H]butyrylcholine (30).

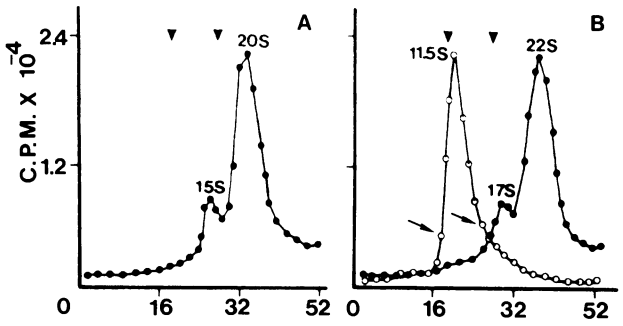

FIG. 1. Purified asymmetric AcChoEase. AcChoEase (0.07 unit) was sedimented as purified (*A*) or after digestion for 15 hr at 37°C with 1 μg of purified collagenase per 50 μl (*B*) (●) or after such digestion and treatment with 10 mM dithiothreitol and then 50 mM *N*-ethylmaleimide (*B*) (○). Activity: [^{3}H]acetylcholine (in cpm) hydrolyzed in 30 min by 30 μl from each fraction (*y* axis). *x* axis, fractions after sedimentation. Standards (arrowheads) are catalase (11.4 S) and β-galactosidase (16 S). Arrows mark the fractions taken (in several such gradients) for the ratio of AcChoEase to ChoEase: this ratio was 15.0 ± 0.98 (mean ± SEM; nine samples) in *both* of the marked fractions.

chicken brain extracts (23, 30) and was the only one that was recognized on immunoblots by one of the mAb (ACB-1) specific (23) for the AcChoEase catalytic subunit (Fig. 2, lane H). Two other subunits, at 72 kDa and 58 kDa, were clearly detectable in the dye- or silver-stained gels. The relative contents of the three subunits, estimated by scanning densitometry (corrected for the relative molecular weight values) in gels stained with Coomassie blue (Fig. 2, lane A), were 42%:41%:17% (from highest to lowest molecular weight, respectively), approximating to a ratio of 2:2:1.

The asymmetric AcChoEase was also allowed to react with [^{3}H]iPr$_2$*P*-F and then was analyzed by NaDodSO$_4$/PAGE and autoradiography. Two of the subunits (110 kDa and 72 kDa) were reactive (Fig. 2, lane C), in contrast to the single subunit that can be [^{3}H]iPr$_2$*P*-F-labeled in AcChoEase purified from chicken brain (23, 30). Pretreatment of the sample for 30 min with 0.05 mM BW 284c51 (Fig. 2, lane D) or with 0.1 mM iso-OMPA (Fig. 2, lane E), prior to the [^{3}H]iPr$_2$*P*-F treatment, prevented the labeling of the respective 110-kDa and 72-kDa subunits, indicating that the 110-kDa subunit contains the AcChoEase active site and the 72-kDa subunit contains the ChoEase active site. Since there was twice the binding of [^{3}H]iPr$_2$*P*-F (per unit of AcChoEase) to the purified muscle AcChoEase compared with the purified AcChoEase from 1-day chick brain (23) (Table 3), and since the brain AcChoEase has been shown to contain only a single type of subunit (≈110 kDa) by NaDodSO$_4$/PAGE (23, 30), the two reactive subunit types of the asymmetric enzyme show a 1:1 ratio of iPr$_2$*P*-F binding.

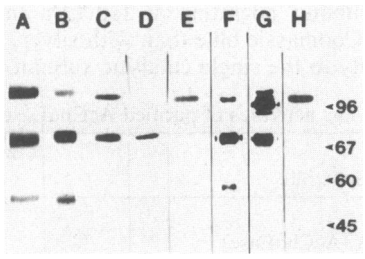

FIG. 2. NaDodSO$_4$/PAGE (23) of the purified, reduced asymmetric AcChoEase. Stained with Coomassie blue (lane A; 10 μg) or with silver (lanes B, F, and G; 0.5 μg). Lanes C–E, fluorographs of asymmetric AcChoEase treated with 0.1 mM [^{3}H]iPr$_2$*P*-F (lane C) or of similar samples treated with 0.05 mM BW 284c51 (lane D) or with 0.01 mM iso-OMPA (lane E) prior to and during the [^{3}H]iPr$_2$*P*-F treatment. The AcChoEase was also incubated for 15 hr at 37°C in the absence (lane F) or presence (lane G) of 1 μg of purified collagenase per 50 μl. Lane H, immunoblot [method as in (23)] of the asymmetric AcChoEase, using mAb ACB-1. Arrowheads show positions of markers (right).

Only one of the subunits (58 kDa) was found sensitive to collagenase digestion when analyzed by NaDodSO$_4$/PAGE (Fig. 2, lanes F and G). Although the collagenase itself migrated as a composite of three bands near the 96-kDa region (Fig. 2, lane G), the 110-kDa subunit of the enzyme was clearly distinguishable; it did not decrease in intensity as the concentration of collagenase was increased (data not shown).

When analyzed by NaDodSO$_4$/PAGE in a 5–12% gradient gel under nonreducing conditions (in the presence of 0.2 M *N*-ethylmaleimide), the subunit bands were entirely absent and all of the protein remained at the interface between the stacking and the running gels (data not shown). This suggested that all of the subunits are linked together by disulfide bonds. This was confirmed by examining the forms in FPLC separation when denatured, instead, by 8 M urea at 50°C (Fig. 3*A*). Purified asymmetric [^{3}H]iPr$_2$*P*-F labeled AcChoEase (unreduced) was virtually all eluted just after the void volume; a very small amount of tetramer appeared (perhaps by artefact) but no dimers or monomers were released by the urea. In contrast, from a parallel sample that had been reduced with 100 mM dithiothreitol, all of the [^{3}H]iPr$_2$*P*-F-labeled protein was eluted beyond an aldolase marker (158 kDa). This was in two peaks that correspond to the 110-kDa and the 72-kDa subunits.

Molecular Mass Estimates of the AcChoEase Molecular Forms. The size of the major native form (20 S) was measured by gel permeation chromatography in the absence of detergents (Fig. 3*B*). The asymmetry of the enzyme was diminished by first digesting it with purified collagenase to remove the tail, which produced a molecular form sedimenting at ≈22 S (see Fig. 1*B*). This form showed a Stokes radius of 9.6 nm or an approximate molecular mass (if now globular) of 960 kDa. Additionally, when the 22S form was completely converted to an 11.5S form by dithiothreitol reduction after collagenase treatment, a Stokes radius of 6.4 nm was measured, or molecular mass 390 kDa (Fig. 3*B*). We found no change (data not shown) in the measured Stokes radius for either the 22S form or the 11.5S form when these samples were run similarly except that 0.5% Triton X-100 was now present (and the same was true for the sedimentation coefficient), indicating, as suggested likewise for the *Torpedo* (32) and muscle (7, 11) asymmetric forms, that their catalytic subunits do not contain detergent-binding structures. This lack of Triton X-100 binding justifies the estimation of molecular mass (approximately) by hydrodynamic methods.

Identification of the 72-kDa Subunit as ChoEase by a mAb. mAb were raised in mice immunized with the purified asymmetric AcChoEase and were tested for their cross-reactivity with ChoEase. Although 10 different mAb were found that would precipitate the chicken brain AcChoEase (to be described elsewhere), one mAb, 7D-11, immunoprecipitated AcChoEase only in the form of the purified asymmetric enzyme or of its 15S and 20S components when separated on a sucrose density gradient. It did not immunoprecipitate any of the large amount of AcChoEase activity in

Table 3. [^{3}H]iPr$_2$*P*-F reaction (inhibitors absent)

Purified AcChoEase	Source	Reaction units	[^{3}H]iPr$_2$*P*-F bound, cpm	cpm/unit of AcChoEase*
Brain	Globular	1.98	6038	3049
Muscle	Asymmetric	1.08	7003	6484

*Activity was assayed with 0.1 mM iso-OMPA treatment (24).

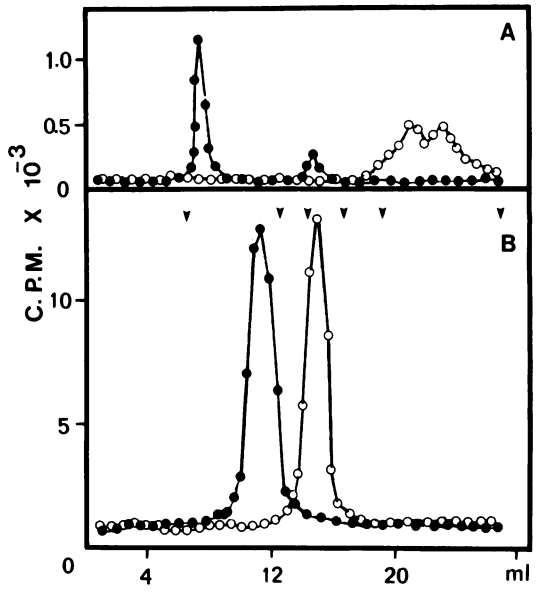

FIG. 3. Gel permeation chromatography of products. (*A*, in urea) Purified asymmetric AcChoEase (3 μg) was labeled with 0.1 mM [^{3}H]iPr$_2$*P*-F, precipitated by Cl_3CCOOH (12%), washed with acetone (four times), and denatured with 8 M urea at 50°C (30 min) in the presence (○) or absence (●) of 100 mM dithiothreitol. The samples were applied to a Superose 6 (Pharmacia) FPLC column, equilibrated in and eluted (0.5 ml/min) by 0.1 M citrate, pH 5.5/0.5 M NaCl/2 M urea. To the reduced sample, 0.2 M *N*-ethylmaleimide was added (15 min, 23°C) prior to the chromatography. (*B*, in water) Purified asymmetric AcChoEase (detergent-free) was in 10 mM Hepes, pH 7.5/0.5 M NaCl (used also for equilibration and elution of the column). It was digested for 15 hr at 37°C with 1 μg of purified collagenase per 50 μl and was then used alone (●) or after reduction with 10 mM dithiothreitol and alkylation (as above) with 50 mM *N*-ethylmaleimide (○). The radioactivity shown was measured in the AcChoEase assay (as in Fig. 1) (*A*) or was due to the [^{3}H]iPr$_2$*P*-F reaction (*B*). Molecular markers (arrowheads, from left to right in *B* and at almost coincident positions in *A*): blue dextran (V_o), thyroglobulin (670 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), and tryptophane (V_t). Plots (not shown) of marker Stokes radius vs. $(-\log K_d)^{1/2}$ (defined and plotted in ref. 33) or of the logarithm of molecular mass vs. K_d were linear and were used for the enzyme parameter estimations.

the low-salt extract of chick muscle or in a detergent extract (23) of chicken brain. In addition, 7D-11 quantitatively immunoprecipitated the ChoEase activity in the same muscle extract and also the ChoEase activity of adult chicken serum. These data would suggest that 7D-11 is directed against an epitope within the asymmetric form that is not present on the subunit containing the catalytic activity for AcChoEase but is present on the subunit showing ChoEase properties.

The measured activity in the purified asymmetric form was much lower for ChoEase than for AcChoEase (see Table 2), and we deduced that the turnover number for chick muscle ChoEase was lower by a factor of ≈20 than that of chick muscle AcChoEase. To confirm this, we immunopurified ChoEase from a low-salt/Triton extract of the pectoral muscle of 1-day-old chicks by using mAb 7D-11 attached to Sepharose 4B. Analysis by NaDodSO$_4$/PAGE showed a single protein of 72 kDa (A, Fig. 4) that reacted with [^{3}H]iPr$_2$*P*-F (Fig. 4, lane B); the latter reaction could be prevented by preincubation for 15 min with 0.1 mM iso-OMPA (Fig. 4, lane C) but not with 0.05 mM BW 284c51 (Fig. 4, lane D). The purified protein had a specific activity (on butyrylthiocholine) for ChoEase of 150 units/mg of protein and had no detectable AcChoEase activity. The specific activity of ChoEase obtained for this preparation was lower by a factor of 15 than that (23, 30) for AcChoEase

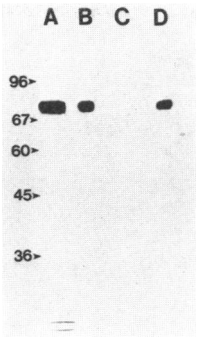

FIG. 4. NaDodSO$_4$/PAGE of reduced immunopurified muscle ChoEase. A single protein of 72 kDa is seen by silver staining (lane A). Adjacent are fluorographs from parallel samples treated with 0.1 mM [^{3}H]iPr$_2$*P*-F (lane B) or treated with 0.1 mM iso-OMPA (lane C) or 0.05 mM BW 284c51 (lane D) prior to and during the [^{3}H]iPr$_2$*P*-F treatment. Positions of markers (in kDa) are given on the left.

(on acetylthiocholine) as purified from chicken brain, a value that would explain the unusually low activity for ChoEase found in the purified asymmetric form. We have also immunopurified ChoEase from adult chicken serum and have found a similar single protein migrating near 72 kDa and exhibiting the same specific activity (150 units/mg) for ChoEase (data not shown).

DISCUSSION

The description here of a mAb (ACB-2) that quantitatively coprecipitates AcChoEase and ChoEase activities is at first sight surprising, since the other antibodies to either enzyme that have been described, both polyclonal and monoclonal (now considerable in number), are all specific for either AcChoEase or ChoEase (8, 15, 17, 19–23, 30, 34). However, the commonly drawn conclusion of an immunological and structural divergence of these two enzymes is not, in fact, compromised by the behavior of our mAb, ACB-2, since a range of evidence is presented above to show that this mAb recognizes only the AcChoEase catalytic subunit and that the ChoEase interaction occurs indirectly, simply because the two enzymes are covalently linked together in the asymmetric structure.

In fact, the asymmetric forms of AcChoEase, purified by immunoaffinity chromatography from immature chick muscle, show the active site characteristics, substrate specificity, and subunit properties of a molecule containing AcChoEase and ChoEase catalytic subunits. The alternative possibility, that these characteristics arise because a separate ChoEase molecule has been copurified with the AcChoEase, can be dismissed since (*i*) the asymmetric AcChoEase was purified by using an antibody that does not recognize ChoEase and (*ii*) mAb 7D-11 (which otherwise does not recognize AcChoEase) precipitates all of the AcChoEase along with the ChoEase in the purified asymmetric preparation. Indeed, mAb 7D-11 was raised by using asymmetric AcChoEase as the immunogen, and yet it recognizes (apart from that form) only ChoEase forms. (*iii*) Subunit analysis by NaDodSO$_4$/PAGE and gel permeation under nonreducing but strongly denaturing conditions (Fig. 3*A*) showed that all of the subunits present are linked together by disulfide bonds. It is difficult to explain these findings other than by a hybrid structure for the 20S form. The data of Table 3 and the protein densitometry indicate a 1:1 stoichiometry of AcChoEase and ChoEase subunits, whereas Fig. 2 (lane A) demonstrates an overall 4:1 ratio of the catalytic subunits (both types) to the collagen-like subunits.

These data, in addition to the estimates of molecular mass of the 22S collagenase-digested form (≈960 kDa) and of the 11.5S collagenase-digested, reduced form (≈390 kDa), allow us to propose a model for the structure of the native 20S asymmetric hybrid enzyme in young chick muscle (Fig. 5). In this model, there are an equal number of 72-kDa and 110-kDa subunits and one-third as many 58-kDa subunits. The assignment of the ChoEase subunits as internal to the

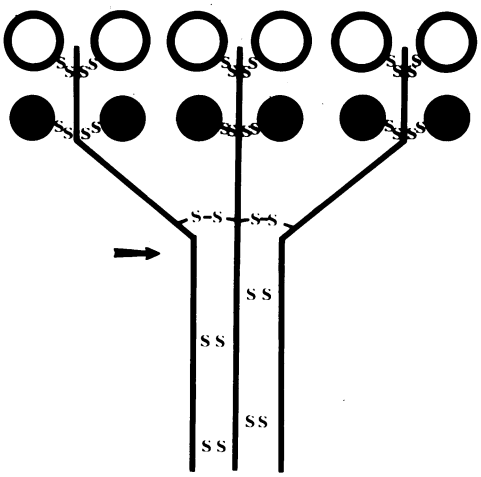

FIG. 5. Proposed model for the 20S asymmetric chick AcChoEase. Catalytic subunits are AcChoEase (○) or ChoEase (●). The lines represent the tail filaments, shown as containing a triple helical structure, contiguous with a noncollagenous terminal region within the same 58-kDa subunit. A minimum set of disulfide bridges (SS) is shown. The more internal arrangement suggested for the ChoEase has not been proven (see text). The arrow shows the site for collagenase digestion.

AcChoEase is arbitrary, but some evidence supporting this idea will be presented elsewhere, which shows that an anti-AcChoEase mAb cross-links several hybrid molecules together, whereas access therein of an anti-ChoEase mAb (7D-11) is more restricted and does not cross-link outside one asymmetric molecule. The calculated molecular mass of six AcChoEase subunits plus six ChoEase subunits would be 1092 kDa. Considering that these are estimated from mobilities of glycoproteins in $NaDodSO_4$/PAGE, this value agrees within experimental error with the mass estimated for the 22S form of this enzyme, where the hydrodynamic distortion due to the native asymmetry is largely removed by collagenase digestion (Fig. 1). In addition, the calculated value for a single tetramer (364 kDa) agrees within the error of the method with the estimated value of 390 kDa for the 11.5S form. Hence, the model, as before (7, 8), assigns 12 non-tail subunits to the heaviest asymmetric form, but it must be borne in mind that if the designation A_{12} (7, 8) is used for this structure, it does not distinguish the heterogeneity due to two different catalytic subunit types, as here, from that due to one catalytic and one noncatalytic type being present [as in *Torpedo* AcChoEase (11)] nor from the homogeneity of 12 AcChoEase catalytic units as found (8, 35) in *Electrophorus* asymmetric AcChoEase.

Also included in the model of Fig. 5 are possible sites for the disulfide linkages deduced to be present. The evidence of Fig. 3 shows that all of the catalytic subunits are linked to the asymmetric structure by disulfide bonds. However, we have not as yet determined whether or not there are also disulfide bridges between any of the catalytic subunits. The tail (in the 58-kDa subunit) is shown as containing a triple helix of collagen, as deduced for eel AcChoEase (33, 35).

If there were any structure present in the 20S form other than the one shown in which each tetramer contains one AcChoEase homodimer and one ChoEase homodimer (e.g., if random mixtures of the subunits or if homotetramers occur in the hybrid), then, since the mean activity ratio of 1:1 is maintained, it would have been possible to distinguish different forms at the two extremes of the 11.5S derivative peak in a sucrose gradient (Fig. 1*B*). This test could be applied because the ChoEase subunit has a 30% lower molecular mass than the AcChoEase subunit.

Asymmetric forms of AcChoEase in *Torpedo* also contain a noncatalytic subunit that is not collagen-like (11). It was, however, present at only one per tetramer and exhibited no iPr_2P-F reactivity (11). We have not found such a subunit in muscle AcChoEase.

In addition to the 1:1 hybrid, there is (as will be reported elsewhere) in the asymmetric species present in 1-day-old chick muscle about 20% of a 17S form having only ChoEase as the catalytic unit present—i.e., with 12 catalytic subunits each of molecular mass near 72 kDa. The corresponding A_{12} homogeneous AcChoEase is not more than 6% of the total asymmetric AcChoEase at 1 day. Recent investigations (K.W.K.T. and E.A.B., unpublished) of developing mammalian and chicken muscle support the generality of the occurrence of the hybrid asymmetric form, which changes during development to the homogeneous tailed forms.

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1. Hall, Z. W. (1973) *J. Neurobiol.* **4**, 343–361.
2. Jedrzejczyk, J., Silman, I., Lyles, J. M. & Barnard, E. A. (1981) *Biosci. Rep.* **1**, 45–51.
3. Lai, J., Jedrzejczyk, J., Pizzey, J. A., Green, D. & Barnard, E. A. (1986) *Nature (London)* **321**, 72–74.
4. Vigny, M., Koenig, J. & Rieger, F. (1976) *J. Neurochem.* **27**, 1347–1353.
5. Fernandez, H. L. (1981) *Neurochem. Res.* **6**, 1005–1017.
6. Younkin, S. G., Rosenstein, C., Collins, P. L. & Rosenberry, T. L. (1982) *J. Biol. Chem.* **257**, 13630–13637.
7. Bon, S., Vigny, M. & Massoulié, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2546–2550.
8. Massoulié, J. & Bon, S. (1982) *Annu. Rev. Neurosci.* **5**, 57–106.
9. Schumacher, M., Camp, S., Maulet, Y., Newton, M., MacPhee-Quigley, K., Taylor, S. S., Friedmann, T. & Taylor, P. (1986) *Nature (London)* **319**, 407–409.
10. Sikorav, J.-L., Krejci, E. & Massoulié, J. (1987) *EMBO J.* **6**, 1865–1873.
11. Lee, S. L., Heinemann, S. & Taylor, P. (1982) *J. Biol. Chem.* **257**, 12283–12291.
12. Allemand, P., Bon, S., Massoulié, J. & Vigny, M. (1981) *J. Neurochem.* **36**, 860–867.
13. Lyles, J. M., Silman, I., Di Giamberardino, L., Couraud, J. Y. & Barnard, E. A. (1982) *J. Neurochem.* **38**, 1007–1021.
14. Vigny, M., Bon, S., Massoulié, J. & Leterrier, F. (1978) *Eur. J. Biochem.* **85**, 317–323.
15. Doctor, B. P., Camp, S., Gentry, M. K., Taylor, S. S. & Taylor, P. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5767–5771.
16. Sikorav, J.-L., Vallette, F., Grassi, J. & Massoulié, J. (1985) *FEBS Lett.* **193**, 159–163.
17. Toutant, J. P., Massoulié, J. & Bon, S. (1985) *J. Neurochem.* **44**, 580–592.
18. Lockridge, O., Bartels, C. F., Vaughan, T. A., Wong, C. K., Norton, S. E. & Johnson, L. L. (1987) *J. Biol. Chem.* **262**, 549–557.
19. Brimijoin, S., Mintz, K. P. & Alley, M. C. (1983) *Mol. Pharmacol.* **24**, 513–520.
20. Marsh, D., Grassi, J., Vigny, M. & Massoulié, J. (1984) *J. Neurochem.* **43**, 204–213.
21. Koelle, G. B., Rickard, K. K. & Ruch, G. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 6012–6016.
22. Brimijoin, S., Rakonczay, Z. & Mintz, K. (1986) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **45**, 2960–2964.
23. Randall, W. R., Tsim, K. W. K., Lai, J. & Barnard, E. A. (1987) *Eur. J. Biochem.* **164**, 95–102.
24. Lyles, J. M., Silman, I. & Barnard, E. A. (1979) *J. Neurochem.* **33**, 727–738.
25. Vigny, M., Gisiger, V. & Massoulié, J. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2588–2592.
26. Berman, H. A., Decker, M. M. & Sangmee, J. (1987) *Dev. Biol.* **120**, 154–161.
27. Ey, P. L., Prowse, S. J. & Jenkin, C. R. (1978) *Immunochemistry* **15**, 429–436.
28. Brandan, E. & Inestrosa, N. C. (1984) *Biochem. J.* **221**, 415–422.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
30. Rotundo, R. L. (1984) *J. Biol. Chem.* **259**, 13186–13194.
31. Bon, S. & Massoulié, J. (1978) *Eur. J. Biochem.* **89**, 89–94.
32. Bon, S. & Massoulié, J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4464–4468.
33. Bon, S., Huet, M., Lemonnier, M., Rieger, F. & Massoulié, J. (1976) *Eur. J. Biochem.* **68**, 523–530.
34. Fambrough, D. M., Engel, A. G. & Rosenberry, T. L. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1078–1082.
35. Mays, C. & Rosenberry, T. L. (1981) *Biochemistry* **20**, 2810–2817.