

Collagen-tailed and hydrophobic components of acetylcholinesterase in *Torpedo marmorata* electric organ

(solubility/ionic strength/detergents/teololysis/reduction)

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ABSTRACT We have distinguished three fractions of acetylcholinesterase (AcChoE; acetylcholine acetylhydrolase, EC 3.1.1.7) from *Torpedo marmorata* electric organs, according to their solubilization characteristics. The low-salt-aggregating collagen-tailed forms are soluble in high-salt buffers; their hydrodynamic properties are not modified in the presence of detergents. They constitute the A fraction, which amounts to about a third of the tissue's AcChoE activity. The low-salt-soluble (LSS) and detergent-soluble (DS) fractions are not sensitive to ionic strength and collagenase. In the presence of nonionic detergents or bile salts, both fractions behave as a monodisperse "6.3S" form, the properties of which have been investigated mostly in the case of Triton X-100. Disulfide bond reduction dissociates the detergent form into a smaller "5S" form. These two forms are thought to be, respectively, detergent-associated dimers and monomers. In the absence of detergent, the LSS fraction is polydisperse: it contains a major 8S component, 11S and 14S components, and faster-sedimenting aggregates, which appear to represent dimers, tetramers, and higher polymers. The heterogeneity of the 8S component in gel filtration suggests that it also contains variable noncatalytic elements. Upon removal of the detergent the DS fraction forms ill-defined aggregates. Trypsin induces quaternary rearrangements of part of the 8S component into 11S and 14S components, which are still convertible into the detergent form; therefore trypsin probably digests noncatalytic elements. Pronase and proteinase K, on the other hand, convert the enzyme into a dimeric form, G₂, that does not interact with detergents, probably by cleaving a minor fragment of the subunit that is involved in hydrophobic interactions.

Studies of acetylcholinesterase (AcChoE; acetylcholine acetylhydrolase, EC 3.1.1.7) from *Electrophorus* electric organs have led to the general agreement that the collagen-tailed forms that may be isolated from this tissue are not directly bound to the electroplaque membrane, but are associated with the basal lamina, through ionic interactions. These asymmetric forms are assemblies of one, two, and three catalytic tetramers (respectively called A₄, A₈, and A₁₂) with a collagen-like rod-shaped structural element (1–5). These forms do not interact with nonionic detergents, but aggregate, under physiological ionic conditions, in the presence of polyanions such as chondroitin sulfate (6). We have recently shown that some of the molecular forms of AcChoE from mammalian and avian tissues (7–9) are structurally equivalent to the *Electrophorus* collagen-tailed molecules.

The collagen-tailed A forms from *Electrophorus* may be degraded, by proteolytic treatment or sonication, into enzymically active tetramers, dimers, and monomers. These globular forms, respectively called G₄, G₂, and G₁, do not appear to exist naturally in significant quantities in the electric organ. The predominant lytic form G₄ has been shown not to bind any detectable amount of the detergent Triton X-100 near its critical micellar concentration (10).

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In contrast, a fraction of AcChoE from higher vertebrates is clearly membrane bound, and the hydrophobic properties of the human erythrocyte enzyme, for example, have been particularly well characterized by Brodbeck and colleagues (11–13). In the present paper we report that *Torpedo* electric organs, in addition to the collagen-tailed A forms (14, 15), contain a large proportion of hydrophobic AcChoE, which may be equivalent to some of the globular enzyme forms encountered in higher vertebrates.

MATERIALS AND METHODS

The analytical methods used in this work have already been described (2, 6). AcChoE was assayed with the Ellman method (16) as described (2). Trypsin (bovine pancreas) was from Koch-Light Laboratories (Colnbrook, Bucks, England), proteinase K from Boehringer Mannheim, and Pronase (B grade) from Calbiochem.

AcChoE was extracted in successive fractions from *Torpedo* electric organs: The low-salt-soluble fraction was first solubilized in 50 mM MgCl₂/10 mM Tris-HCl, pH 7. Then either the A fraction or the detergent-soluble (DS) fraction was solubilized in 500 mM MgCl₂/10 mM Tris-HCl, pH 7, or 1% Triton X-100/50 mM MgCl₂/10 mM Tris-HCl, pH 7, in indifferent order. Bacitracin (0.1 mg/ml) was usually included in the sucrose gradient and gel filtration buffers, because it was found to stabilize the AcChoE activity, especially at low enzyme concentration. It did not by itself modify the sedimentation or elution patterns. Severe losses of activity occurred when extracts were stored in the presence of Triton X-100, especially when frozen.

RESULTS

Low-Salt, High-Salt, and Detergent-Soluble Components of AcChoE. By homogenizing the electric organs in various media, we have been able to distinguish three different pools of enzyme of roughly equivalent activity, which may be solubilized, respectively, in low-salt buffer (low-salt-soluble, or LSS, fraction), in the presence of detergent (detergent-soluble, or DS, fraction), and in high salt. This last fraction contains the previously characterized asymmetric forms (14, 15), and is therefore designated the A fraction. All these fractions contain "true" acetylcholinesterase, as indicated by substrate and inhibitor specificity (17), and it must be noted that their catalytic activity was not modified by the nonionic detergent Triton X-100, although this detergent profoundly affects the solubility and molecular state of the LSS and DS fractions. In a preliminary analysis by sodium dodecyl sulfate electrophoresis of partially purified enzyme labeled with [³H]diisopropyl fluorophosphate, we observed that the catalytic subunits of the three fractions have a mass of about 80,000 daltons and are associated

Abbreviations: AcChoE, acetylcholinesterase; LSS, low-salt-soluble; DS, detergent-soluble.

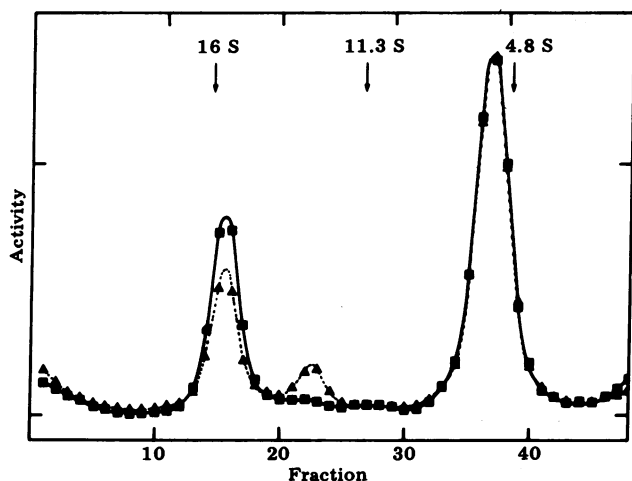


FIG. 1. Sedimentation pattern of AcChoE from electric organs of adult and newborn *Torpedo*, in detergent/saline medium. AcChoE was solubilized quantitatively by homogenizing 2-g samples of the electric organs with 9 vol of 1 M NaCl/500 mM MgCl₂/1% Triton X-100/10 mM Tris-HCl, pH 7/0.1 mg of bacitracin per ml, using a Polytron homogenizer. Aliquots of the low-speed supernatant extracts were centrifuged in 5–20% (wt/vol) sucrose gradients, in 1 M NaCl/50 mM MgCl₂/1% Triton X-100/10 mM Tris-HCl, pH 7/0.1 mg of bacitracin per ml, using a Beckman SW 41 rotor at 40,000 rpm, for 18½ hr, at 4°C. One milliliter of newborn (■) and adult (▲) *Torpedo* extracts hydrolyzed, respectively, 22.5 and 21 μmol of acetylthiocholine per min. The positions of β-galactosidase (16 S), catalase (11.3 S), and alcohol dehydrogenase (4.8 S) are shown by arrows. AcChoE activity is plotted on an arbitrary scale, as a function of fraction number, from the bottom of the tube.

in dimers by disulfide bonds, as reported previously in the case of the asymmetric and lytic *Torpedo* enzymes (14).

The totality of AcChoE activity may be solubilized in a high-salt detergent buffer. As shown in Fig. 1, the sedimentation pattern obtained in the presence of 1% Triton X-100 reveals a prominent “6.3S” peak of activity, in addition to the previously characterized collagen-tailed forms. As will be shown later, this detergent form is derived from both LSS and DS fractions and probably represents a detergent-bound dimer of AcChoE catalytic subunits.

The solubilization of the *Torpedo* A forms depends upon

the salt concentration as indicated in Fig. 2: as observed by Lwebuga-Mukasa *et al.* (14), MgCl₂ is more efficient than NaCl, which solubilizes less than 20% of these forms at 1 M concentration. The proportion of the A₁₂, A₈, and A₄ forms (cf. Table 1) is independent of the solubilization yield, indicating that they possess identical solubilization characteristics.

When the salt concentration is lowered by dialysis, the *Torpedo* A forms reprecipitate. This does not occur after acetylation of the enzyme, suggesting that amino groups of these molecules are involved in their ionic interactions. The solubilization of these A forms was not modified by the presence of detergents; conversely, the salt concentration did not interfere with the solubilization of the DS AcChoE fraction. Thus the solubility of the A and DS fractions appears to depend upon entirely distinct interactions, respectively ionic and hydrophobic. This is illustrated by the parallelism of the solubilization curves obtained in the presence and absence of detergent, as a function of salt concentration (Fig. 2).

Sedimentation Analysis of the DS Fraction in the Presence and Absence of Detergent. The DS fraction of *Torpedo* AcChoE sediments as heterogeneous aggregates in the absence of detergent, but as a monodisperse species of approximately 6.3 S in the presence of Triton X-100 (Fig. 3). The minimal concentration of Triton X-100 that is required to obtain quantitative conversion into the “6.3S” detergent form is close to the critical micellar concentration (about 0.02%). We obtained similar effects in the presence of other nonionic detergents, or bile salts, but the apparent sedimentation of the detergent form varied, ranging from 5.5 S in the case of Brij 96 to 7 S in the case of sodium cholate (in 10 mM Tris-HCl sucrose gradient). This variability implies that the active species is a complex containing a significant amount of detergent. In agreement with this idea, we observed that its apparent sedimentation coefficient, obtained by comparison with the migration of standard proteins, was markedly lower in ²H₂O (5 S) than in H₂O (6.3 S) gradients (not shown), indicating a low density.

Centrifugation of DS AcChoE in cesium chloride showed that, in the presence of Triton X-100, the enzyme first formed a broad peak of low density but eventually equilibrated at the high density obtained for the LSS or A fraction in the absence of detergent (1.320 g/cm³). When analyzed in a detergent-free

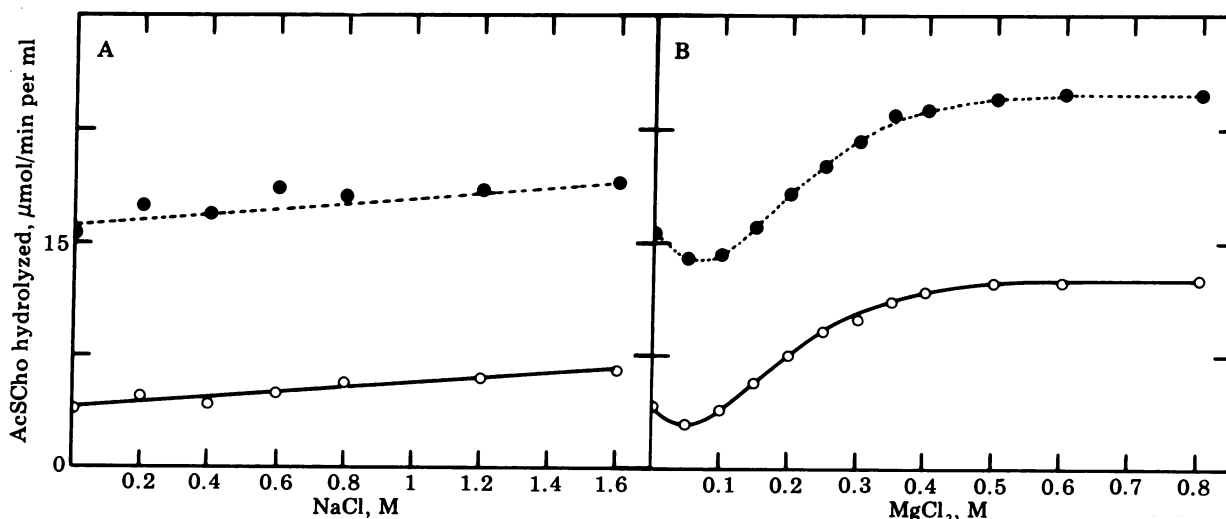


FIG. 2. Solubilization of the A, LSS, and DS AcChoE fractions, as a function of detergent and salt concentration. *Torpedo* electric organ was homogenized in 4 vol of 20 mM Tris-HCl, pH 7, and filtered through a nylon gauze to remove conjunctive fibers. The resulting suspension was diluted with an equal volume of NaCl (A) or MgCl₂ (B) solution, bringing the salt concentration to the indicated value, in the absence (O) or presence (●) of Triton X-100 (1% final concentration). After centrifugation (100,000 × g for 20 min), the supernatants were assayed for AcChoE activity. AcSCho, acetylthiocholine.

Table 1. Molecular forms of *Torpedo* acetylcholinesterase

	LSS fractions and trypsinolysis products (detergent sensitive)												
	A forms			8S					Lytic forms (detergent insensitive)			Triton X-100 complexes	
	A ₁₂	A ₈	A ₄	14S	11S	Large*	Medium*	Small* (or trypsin resistant)	G ₄	G ₂	G ₁	Un- reduced	Re- duced
Sedimentation coefficient (S)	17.4	13.4	9.1	14	11.1	8.3	8	7.8	10.9	7.1	5	6.3	4.5
Stokes radius, nm	17.2	16.2	13.1	10.7	8.7	8.8	7	5.6	8.1	5.9	4.6	8.1	4.6
Estimated molecular weight × 10 ⁻³	1200	870	480	600	390	290	225	180	355	170	95	—	—

The molecular weights are assumed to be proportional to the $R_e S$ product with the same proportionality coefficient as obtained for the *Electrophorus* enzymes (cf. ref. 3). The molecular weights of the detergent forms cannot be obtained in this manner because their partial specific volume is increased by the associated Triton X-100. Their apparent sedimentation coefficient varies with the density of the centrifugation medium.

* The parameters given refer to selected molecules from the 8S component, in order to indicate the range of values of its constituents, which have not been entirely resolved.

gradient, this detergent-depleted DS AcChoE formed aggregates, which were readily dissociated into the "6.3S" form upon addition of Triton X-100 (see Fig. 3).

Properties of the LSS Fraction: Interaction with Detergents. In the absence of detergent, the sedimentation pattern of the LSS fraction is usually poorly resolved. It shows, however, a major 8S component, minor 11S and 14S components, and heavier aggregates (Fig. 4). All these components are converted by Triton X-100 into the "6.3S" detergent form. After dissociation with Triton X-100 and removal of the detergent in CsCl isopycnic sedimentation, we obtained a similar pattern except that the heavy aggregates had disappeared.

The major 8S component was found to be heterogeneous in gel filtration chromatography, with Stokes radii ranging from about 6 to 10 nm. The different fractions from the column retained distinct elution properties in a second chromatography. Their molecular weights, as estimated from the data of Table 1, indicate that they contain at most one catalytic AcChoE dimer, probably associated with different structural elements that account for their heterogeneity, because they are all converted into indistinguishable "6.3S" molecules by detergents. The detergent-sensitive 11S component is similar to the lytic

G₄ enzyme in its sedimentation coefficient but differs significantly from it by a slightly larger Stokes radius (Table 1). The 14S and larger aggregates possibly correspond to hexamers and higher polymers.

Structural Equivalence of the Catalytic Units from the LSS and DS Fractions. In order to establish whether the distinct properties of the LSS and DS fractions result from an intrinsic difference in their catalytic AcChoE units or from their interaction with noncatalytic elements, we mixed 100- μ l aliquots of DS or LSS AcChoE with a 40-fold excess of either of the two diisopropyl fluorophosphate-inhibited fractions. The catalytic activity of the mixtures was essentially that of the small active aliquot (over 99%). The four combinations of DS and LSS mixtures were submitted to CsCl isopycnic equilibration, in the presence of Triton X-100, and samples from the high-density detergent-depleted peak were analyzed as shown in Figs. 3 and 4. The sedimentation patterns of activity obtained were characteristic of the diisopropyl fluorophosphate-inhibited fraction. Thus the DS catalytic units, when reassociated in the presence of an excess LSS extract, displayed a typical LSS-like sedimentation profile and vice versa. This implies that the recombination of the catalytic units upon removal of the detergent is directed by their protein environment, and suggests that the detergent forms are equivalent in terms of quaternary interactions regardless of their DS or LSS origin.

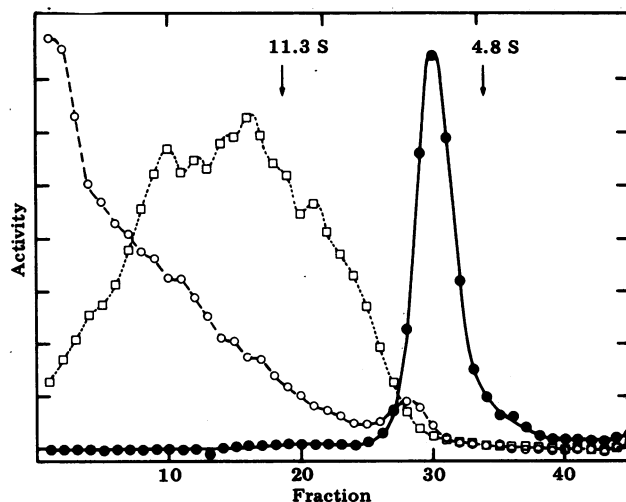


FIG. 3. Sedimentation pattern of the DS AcChoE fraction in the presence and absence of detergent. A sample of DS AcChoE was analyzed by sucrose gradient centrifugation in 40 mM MgCl₂/10 mM Tris-HCl, pH 7/0.1 mg of bacitracin per ml in a SW 41 rotor at 38,000 rpm for 18½ hr, at 4°C. ●, In the presence of 1% Triton X-100; ○, without detergent; □, after removal of detergent by equilibrium sedimentation in an isopycnic CsCl gradient.

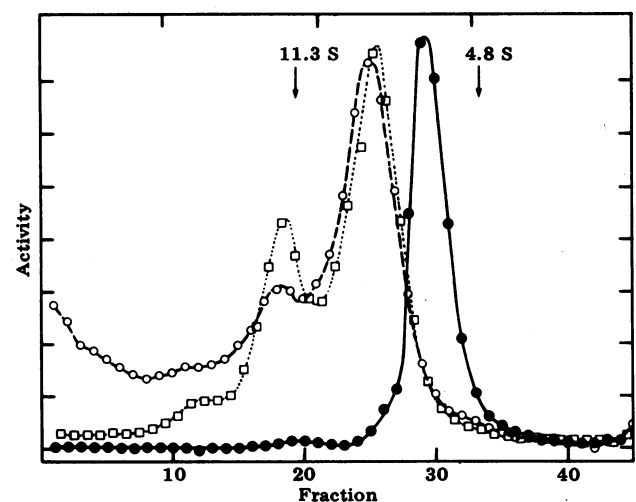


FIG. 4. Sedimentation pattern of the LSS AcChoE fraction in the presence and absence of detergent. The LSS AcChoE fraction was analyzed as indicated in Fig. 3; ○, without detergent; ●, in the presence of 1% Triton X-100; □, without detergent, after addition and subsequent removal of detergent in isopycnic CsCl equilibration.

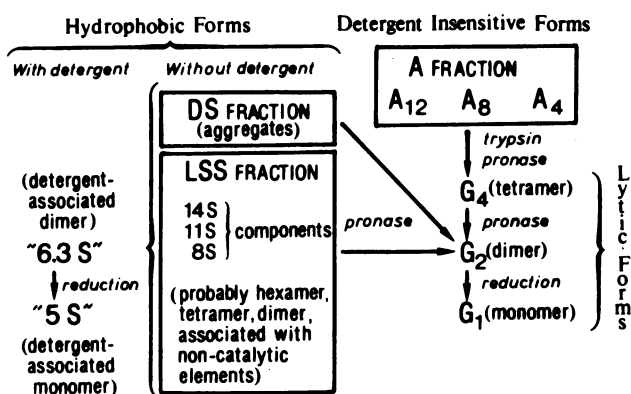


FIG. 5. Scheme of the hydrophobic, asymmetric, and lytic forms of AcChoE from *Torpedo marmorata* electric organ.

Effect of Proteolysis on the A, DS, and LSS Fractions. While collagenase modifies the *Torpedo* A forms, increasing their sedimentation coefficient and abolishing their low-salt aggregation properties, as described in the case of the *Electrophorus* and mammalian enzymes (8, 18), it does not modify the sedimentation pattern of the LSS or DS AcChoE, either in the presence or the absence of detergent.

Incubation of the heterogeneous 8S component isolated from the LSS fraction with trypsin induces a partial conversion into faster-sedimenting forms, mainly 11S, but also 14S and 16.5S (no such rearrangement occurred in control samples). A variable fraction of the enzyme (often 40%) sediments at about 7.8 S after prolonged trypsin treatment, and it appears less heterogeneous in gel filtration than the original 8S component. After the action of trypsin, all these AcChoE molecules remain detergent-sensitive.

Pronase and proteinase K (0.1 mg/ml, at 0°C or 20°C) degrade the A forms, as well as the LSS and DS enzyme, in the presence or absence of detergent, yielding a 7.1S form. This lytic G₂ form does not interact with detergents, as observed particularly in ²H₂O gradients, which differentiate it clearly from the detergent form (not shown). Although a lytic tetramer is also obtained as a transitory dissociation product of the A forms by these proteases, we were not able to observe this G₄ detergent-insensitive form at intermediate stages during the action of proteinase K on the probably tetrameric 11S form isolated from the LSS fraction, even at low protease concentration (0.1 μg/ml), at 0°C.

Effect of Disulfide Reduction on the DS and LSS Fractions. As in the case of the A forms, reduction of the LSS fraction with dithioerythritol did not produce any detectable change in its sedimentation pattern, in the absence of detergent (data not shown). After reduction, however, Triton X-100 partially dissociated the LSS and DS fractions into a slower-sedimenting "5S" form, which probably represents a detergent-bound monomer (Fig. 5). By combining Pronase digestion and reduction of the LSS fraction, we obtained a detergent-insensitive G₁ monomer. A severe inactivation occurred, however, during reduction, so that only a residual activity was found in the monomeric form.

DISCUSSION

Solubility Properties of the A, LSS, and DS Fractions of AcChoE: Association with Membranes. The solubility of AcChoE from *Torpedo* electric organs in different extraction media has led us to recognize three pools of enzyme, which contain roughly equivalent fractions of the total activity. One of them is readily solubilized even in low-salt aqueous buffers (LSS fraction), while the two others require either detergent

(DS fraction) or high salt (asymmetric, or A, fraction) to be solubilized.

The A fraction corresponds to the collagen-tailed forms of AcChoE, A₁₂, A₈, and A₄. In adult *Torpedo* the proportions of the three forms were approximately 65%, 30%, and 5%, but in newborn fish we observed only the A₁₂ form. The smaller A₈ and A₄ forms are therefore more likely degradation products than biosynthetic precursors of A₁₂. The three A forms, which have identical solubilization properties, appear to be involved in ionic interactions similar to those that are responsible for the low-salt aggregation of the collagen-tailed forms from other sources. However, the solubilization of the *Torpedo* A forms is limited in 1 M NaCl, and requires 0.5 M MgCl₂ to be complete, whereas the dissociation of aggregates is obtained with 0.3 M NaCl or 0.15 M MgCl₂ in the case of *Electrophorus* (6) or higher vertebrates (8).

The LSS and DS fractions of *Torpedo* AcChoE are not sensitive to ionic conditions, but, in contrast with the A forms, they both interact with nonionic detergents or bile salts, which convert them into a monodisperse "6.3S" detergent complex. We have found such hydrophobic AcChoE in a light density membrane fraction, distinct from the acetylcholine receptor-rich membranes (19–21), as well as in a synaptosomal fraction. The binding of the DS AcChoE fraction to membranes may occur either directly or through an interaction with hydrophobic proteins.

Molecular Structure of the LSS and DS Fractions: Nature of the Detergent Form. Because the catalytic subunits are associated as dimers by disulfide linkages, all components of the LSS and DS fractions must represent various combinations of such dimers. In particular, the major 8S component of the LSS fraction must contain a single catalytic dimer, as indicated by its mass. The heterogeneity of this component, which is particularly revealed by gel filtration, is probably due to the association of the dimers with different noncatalytic elements. The LSS fraction also contains components that appear to be composed of two (11S) or three (14S) dimers, and possibly noncatalytic elements. Collagenase does not modify the sedimentation pattern of the LSS fraction, indicating that the catalytic units are not associated with collagen-like structures.

All these different combinations are dissociated, in the presence of Triton X-100, and the AcChoE catalytic units form a monodisperse detergent complex, identical to that obtained by solubilization of the DS enzyme. The possibility of obtaining this "6.3S" detergent form from the dimeric 8S molecules suggests that it is a detergent-bound dimer. Its hydrodynamic properties and those of its reduced derivative are those that might be expected for detergent-bound dimer and monomer. The hydrophobic dimers from the LSS and DS AcChoE fractions were found to be interchangeable in their recombination with noncatalytic elements, upon removal of the detergent. The two fractions therefore represent different combinations of similar or identical AcChoE dimers, associated with soluble (LSS) or insoluble (DS) noncatalytic elements, and into higher polymers.

The different effects observed with proteases may be rationalized in a simple way, if we assume that Pronase and proteinase K produce detergent-insensitive dimers G₂ (or monomers G₁ after reduction) by cleaving such a hydrophobic peptide, whereas the action of trypsin, which induces quaternary rearrangements in the LSS fraction without suppressing the formation of the "6.3S" detergent complex, is restricted either to noncatalytic components or to a small, nonessential segment of the subunit. Because the collagen-tailed AcChoE molecules, as well as their trypsin degradation G₄ form, are not detergent-sensitive, they appear to lack the hypothetical hy-

drophobic peptide, which is therefore not required for their intersubunit interactions.

The lytic G₄ tetramer, obtained by treatment of the A forms by trypsin, Pronase, or proteinase K, cannot be distinguished from the detergent-sensitive 11S component of the LSS fraction by sedimentation analysis, but it has a slightly smaller Stokes radius and mass. It appears likely that the two types of 11S AcChoE are both tetramers and that their difference is due at least in part to the presence of a hydrophobic segment in the hydrophobic subunits of the LSS fraction.

Significance of the Collagen-Tailed and Hydrophobic AcChoE Fractions. Although hydrophobic AcChoE molecules have not been detected so far in *Electrophorus* electric organs, it is possible that they exist in this tissue in low proportions, as biosynthetic precursors of the A forms. The large proportion of the LSS and DS fractions in *Torpedo* suggest that this hydrophobic AcChoE may also play a functional part in the physiological inactivation of acetylcholine. In the tissues of higher vertebrates, the majority of AcChoE generally corresponds to globular forms. These forms occur as soluble variants, as well as hydrophobic components that possess the same solubility characteristics as the LSS and DS *Torpedo* fractions (22). *Torpedo* electric organs therefore represent an excellent experimental material for the study of tail-less forms of AcChoE and their hydrophobic interactions, as well as collagen-tailed forms and their ionic interactions.

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