ELECTROPHORUS ACETYLCHOLINESTERASE

Biochemical and Electron Microscope Characterization of Low Ionic Strength Aggregates

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ABSTRACT

The “tailed” molecules of Electrophorus (electric eel) acetylcholinesterase aggregate under conditions of low ionic strength. These aggregates have been studied by sedimentation analysis and high-resolution electron microscopy. They consist of bundles of at least half a dozen molecules, the tails of which are packed side by side, to form the core of the structure. Although aggregation is normally fully reversible, aggregates were irreversibly stabilized by methylene blue-sensitized photo-oxidation. This process was shown to consist of a singlet oxygen oxidation reaction and probably involves methionine or histidine residues. It did not modify the structural or hydrodynamic characteristics of the aggregates.

KEY WORDS acetylcholinesterase · aggregation · cross-linking · electron microscopy · photo-oxidation

Electrophorus acetylcholinesterase (EC 3.1.1.7.) exists in a number of molecular forms (16, 17, 23), all of which probably derive from the heaviest D form (18.5S = 1,150,000 daltons) (3, 5). This complex molecule consists of an asymmetric assembly of three tetrameric groups of globular catalytic subunits, linked by a rodlike “tail” (8, 22) formed by three elementary strands (7). Two other asymmetric forms of acetylcholinesterase exist which contain either one (form A, 9S) or two (form C, 14.2S) tetramers associated with one tail (3, 16). All of these forms can be disassembled further into individual tetramers (G form, 11.8S), dimers (G’ form, 7.7S), and monomers (G” form, 5.3S) (3, 4).

On the other hand, multimolecular aggregates are obtained under conditions of low ionic strength (11, 17). The formation of such multimolecular associations is of great interest, since similar interactions may be involved in the anchorage of acetylcholinesterase in the synapse (8). In this paper, we present a biochemical and electron microscope study of these aggregates. We propose that their formation relies upon side-to-side interactions of the rodlike tails.

MATERIALS AND METHODS

Preparation of Low Ionic Strength Aggregates

Acetylcholinesterase was purified from Electrophorus electric organs, by affinity chromatography, as described previously (15).

Aggregates were formed by dialyzing the purified, concentrated enzyme for 2 h against a low ionic strength buffer (MgCl₂, 0.04 M; Tris-HCl, pH 7, 0.01 M). Separation from nonaggregated enzyme was achieved by 5–20% (wt/vol) sucrose gradient centrifugation in the
same buffer (Beckman rotor SW 60, Beckman Instruments, Inc., Palo Alto, Calif.; 55,000 rpm, 1 h at 2°C). Fractions corresponding to apparent sedimentation constants of $70 \pm 20S$ were usually pooled and dialyzed against this buffer for microscope observation. In some cases, a protein-free extract (aggregating agent) from electric organ was used to promote aggregation of the purified preparations. It did not modify any of the aggregate characteristics, and will be fully discussed elsewhere.1

Acetylcholinesterase was assayed by the Ellman acetylthiocholine method (9) as described previously (15). Sedimentation coefficients were extrapolated from the values of standard proteins, *E. coli β*-galactosidase (16S), beef liver catalase (11.4S), and yeast alcohol dehydrogenase (7.4S). The sedimentation coefficient of the aggregates was determined by comparison with R17 phage (80S), a gift of Dr. M. Springer (Institut de Biologie Physico-Chimique, Paris). The Bio-Gel A 15 m column (Bio-Rad Laboratories, Richmond, Calif.) was calibrated as in previous studies (6, 7).

**Photo-Oxidation Procedure**

Photo-oxidation with methylene blue was performed at 0°C in normal daylight, in MgCl₂, 0.04 M; Tris-HCl, pH 7 or pH 8, 0.01 M.

**Electron Microscopy**

Thin carbon supporting films for high-resolution observations were prepared according to the technique of Fukami and Adashi (10). Since low protein concentrations were usually used (10-50 μg/ml), the spreading was facilitated by adding bacitracin (10-50 μg/ml) to the solution containing the enzyme (12). A 1% uranyl formate solution was used as negative stain according to the technique of Leberman (13).

Bright-field transmission electron microscope observations were made with a Philips EM 400 electron microscope with a 30-μm objective aperture and operating at 80 kV. The emission current was adjusted to 10 μA. To reduce the irradiation damage to the specimens, the micrographs were always exposed after focusing in another, nearby area. No through-focus series was made. The micrographs were taken at a direct magnification of 50,000 and subsequently printed with a pointed illumination enlarger device (Durst Variput, Durst Div., Beloit, Wis.).

**RESULTS**

**The Structure of Low Ionic Strength Aggregates**

Reversible aggregation of acetylcholinesterase under low-salt conditions has been well documented, mainly with crude extracts of electric organs or incompletely purified preparations (11). This phenomenon was also found to occur when the enzyme was purified by affinity chromatography. After dialysis against low ionic strength buffer, the preparation was subjected to sucrose gradient analysis in the same medium (Fig. 1). Most of the activity migrated as a broad peak, the mean sedimentation coefficient of which was 70S.

A small fraction of acetylcholinesterase activity migrated more slowly and contained the globular form G present in our sample. The 70S aggregates were stable at low ionic strength and, when filtered on a Bio-Gel A 50 m column, were eluted in the void volume; their Stokes radii must thus be >30 nm. From these physical chemical data, the minimal mass was calculated to be $7 \times 10^8$ daltons.

The aggregates were dissociated in high ionic strength concentration saline buffer (NaCl, 1 M;

![Figure 1](https://example.com/figure1.png)

**Figure 1** Low ionic strength aggregation of acetylcholinesterase. Centrifugation in a 5-20% low ionic strength sucrose gradient (MgCl₂, 0.04 M; Tris-HCl, pH 7, 0.01 M) with a Beckman SW 60 rotor, 55,000 rpm, for 1 h at 2°C. Sedimentation coefficients were obtained by comparison with *E. coli β*-galactosidase (16S) and phage R17 (80S). Acetylcholinesterase activity is plotted on an arbitrary scale. Most of the activity appears as a heterogeneous peak of aggregates (70 ± 20S). The unaggregated tetrameric molecules (G, 11.8S) formed a distinct peak.

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MgCl₂, 0.05 M; Tris-HCl, pH 7, 0.01 M). Analysis of the dissociated molecules by sucrose gradient centrifugation in a high ionic strength medium demonstrated that the aggregates contained all three asymmetric molecular forms (Fig. 2). Further analysis of fractions corresponding, respectively, to the heavy (90S), medium (70S), and light (50S) portions of the broad aggregate peak revealed that the A, C, and D forms were present in identical proportions in all three fractions. This observation suggests that no segregation between the A, C, and D forms occurred during the formation of aggregates.

Observations of the low ionic strength aggregates by negative staining revealed a rather uniform population of structures in which bundles of filaments were linked to what appeared to be two groups of heads (Fig. 3). These groups were seen to consist of numerous globules, ~5 nm in diameter, corresponding to the subunits seen in all molecular forms of acetylcholinesterase. These globules usually appeared associated in clusters of various sizes or in tetramers, as in the “heads” of the asymmetric forms of acetylcholinesterase. In some well-splayed aggregates (Fig. 3c), it was possible to distinguish at least half a dozen filaments, the dimensions of which were comparable to those of the individual tails (2 nm wide and 30-50 nm long) and a few (two to five) clusters of globules in each group of heads. High ionic strength dissociation products of the aggregates, visualized by negative staining, showed exclusively asymmetric molecules resembling the native asymmetric forms. The complete absence of globular, tail-less forms emphasizes the role of the tails in the aggregation process.

We have also examined low ionic strength aggregates derived from purified A or D forms (Fig. 4). The main features of both kinds of aggregates were very similar; in particular, the number of visible filaments was rather constant (usually three or four filaments). However, the globular moieties derived from the A form were markedly smaller (4a and b). This correlated well with the fact that their mean sedimentation coefficient was 60S, compared with 72S for that obtained for the D form.

Stabilization of Aggregates by Photo-Oxidation

We observed that exposure to light in the presence of methylene blue stabilized the aggregates in such a way that they did not dissociate under high ionic strength conditions. This stabilization resulted from a dye-sensitized photo-oxidation (photodynamic effect) since it did not occur after extensive incubation with methylene blue in the dark (10⁻⁴ M methylene blue for 15 h at 4°C), or in the absence of oxygen (under continuous bubbling of argon).

A number of points indicate that the result of photo-oxidation was a covalent stabilization of the low ionic strength aggregates: (a) in low salt conditions, the characteristic sedimentation pattern of these aggregates was not modified; (b) the tetrameric G form, which does not aggregate, remained free; (c) acetylated asymmetric forms (treated with 2 × 10⁻² M acetic anhydride and progressively neutralized with NaOH within 10 min) did not aggregate in low salt and were not modified by photo-oxidation; and (d) in nonaggregating saline buffer, extensive photo-oxidation

![Figure 2 Sucrose gradient centrifugation of the aggregates dissociation products. 5-20% sucrose gradient in saline buffer (NaCl, 1 M; MgCl₂ 0.05 M; Tris-HCl, pH 7) with a Beckman SW 60 rotor, 36,000 rpm, for 16 h at 2°C. Sedimentation coefficients were obtained by comparison with the standard marker protein E. coli β-galactosidase (Z, 16S) and beef liver catalase (Cat, 11.4S). The dissociation of the 70S peak revealed that only the three asymmetric forms D, C, and A took part in the aggregates. Centrifugation of either the whole “70S” peak or heavy (90S), medium (70S), or light (50S) fraction yielded exactly identical patterns.](image-url)
FIrtrRE 3 Detail of individual low ionic strength aggregates. Most aggregates appeared as compact bundles of asymmetric molecules, with the tails in the center. Some were, however, more or less splayed, probably because of their mode of adsorption to the carbon film. In some aggregates the bundle of filaments was fanned out, allowing the visualization of several clusters of globules (arrows in b) or of numerous filaments (c). \( \times 300,000 \).

\( 10^{-4} \) M methylene blue for 15 h at 4°C did not modify the molecular properties of the individual asymmetric molecules.

Stabilized aggregates appeared identical to low ionic strength aggregates in electron micrographs (Fig. 5). In particular, the same number and the same relative disposition of the filaments were observed (Fig. 5b). The presence of free tetramers in one unfractionated sample (Fig. 5a) is in good agreement with the fact that these globular molecules do not undergo aggregation.

Stabilization of the aggregates was quantitative after 30 min of exposure to daylight with \( 10^{-4} \) M methylene blue. Identical results were obtained with acetylcholinesterase concentrations varying in the range of 5-500 \( \mu \)g/ml, in the presence or absence of bovine serum albumin (0.5 mg/ml).

We also investigated the influence of methylene blue concentrations (Fig. 6): with \( 10^{-5} \) M methylene blue under the conditions described, over 90% of aggregatable enzyme appeared in high ionic strength stable aggregates after 30 min; with \( 10^{-6}, 10^{-7}, \) and \( 10^{-8} \) M methylene blue, the proportions were respectively 70, 30, and <10%. It should be noted that incomplete cross-linking of the aggregates, e.g. with \( 10^{-7} \) M dye, resulted in high ionic strength aggregates with a lower sedimentation coefficient, confirming that low ionic strength aggregates are made up of more than two molecules (Fig. 6). Similar results, i.e. incomplete stabilization resulting in cross-linked aggregates of lower sedimentation coefficient, also occurred after prior photo-oxidation of acetylcholinesterase in high salt conditions, while this treatment did not modify low salt aggregation itself.

Reduction with dithioerythritol (DTE, 0.02 M; Tris-HCl, pH 8, 0.01 M, 1 h) followed by alkylation with excess iodoacetamide (IAA, 0.05 M; Tris-HCl, pH 8, 0.01 M, 1 h) did not dissociate the aggregates either under low salt conditions or, in the case of the cross-linked aggregates, under high salt conditions. It thus seemed that disulfide bonds were not responsible for the photodynamic stabilization. Indeed, the SH groups did not appear to play any role in these processes, since preliminary blocking of these groups by 5,5'-dithio-bis(2 nitrobenzoic) acid (DTNB, \( 10^{-4} \) M for 2 h at 0°C) or by IAA (\( 10^{-2} \) M for 2 h at 0°C) did not interfere with aggregation or with stabilization of the aggregates.

The photodynamic cross-linking reaction was abolished in the presence of sodium azide (\( 10^{-3} \) M) which is known to quench singlet oxygen (20), whereas the efficiency of the reaction was very markedly increased in \( D_{2}O \)-containing media, where the half-life of singlet oxygen is expected to be longer (19). For example, with a methylene blue concentration of \( 10^{-7} \) M, the yield of stabilized aggregates rose from 30% in an \( H_{2}O \) medium to 70% in 60% \( D_{2}O \), which is similar to the
Although both display a similar general structure (a compact bundle in which three or four filaments may be distinguished) (arrows in a), the aggregates derived from A molecules (a and b) exhibit a less bulky group of heads than those derived from D molecules (c and d). However, each kind of aggregate may well be composed of an identical number of asymmetric molecules (6-10). $\times 300,000$.

DISCUSSION

When exposed to low ionic strength conditions, the asymmetrical forms of acetylcholinesterase from *Electrophorus* electric organs reversibly associate into fast-sedimenting aggregates (11, 17). This process is not correlated with any change in catalytic activity. In fact, the interactions responsible for aggregation seem to involve the tails rather than the catalytic subunits. As reported earlier, the globular forms underwent no aggregation and both gradient analysis and electron microscopy clearly demonstrated that only the tailed molecules participate in the formation of multimolecular assemblies. Moreover, it has been shown that chemical alterations of acetylcholinesterase which modify the tail structure also prevent aggregate formation at low ionic strength (21). It may therefore be assumed that the presence and the structural integrity of the rodlike tail are required for the aggregation of the asymmetric molecular species of acetylcholinesterase.

The sedimentation coefficient of the aggregates was $70 \pm 20S$. Aggregates which were prepared...
Figure 5 Photo-oxidized aggregates (saline buffer). (a) In a high ionic strength medium, the asymmetric forms of acetylcholinesterase have become irreversibly associated in structures which are indistinguishable from the low ionic strength aggregates (cf. Fig. 3). In this unpurified sample, tetramers representing the nonaggregating globular form G are visible (arrows). × 300,000. (b) After isolation by centrifugation, only the aggregates are visible. A remarkably well-preserved aggregate is outlined (arrows) displaying four filaments. × 300,000.

From the A (9S) form sedimented around 60S, whereas aggregates of the D (18.5S) form sedimented around 72S. Electron micrographs of such aggregates or of aggregates containing a mixture of A, C, and D forms showed remarkably uniform populations. These aggregates appeared as bundles of tailed molecules in which a small number of filaments, similar in length and width to individ-
ual tails, linked two groups of heads.

We observed a definite difference in the size of these groups between aggregates built up of A or D molecules. It seems, therefore, that the aggregates contain a small, perhaps constant, number of asymmetric molecules. A close look at the A-derived aggregates suggests that each group of heads consists of three to five tetramers, i.e., a total number of molecules of 6–10. This number is sufficient to account for the minimal molecular weight estimation obtained from the hydrodynamic parameters of the aggregates. Although up to eight filaments were detected in some splayed aggregates, their number generally appeared to be smaller. Whatever the exact number of aggregate molecules in these structures, the uniform appearance of the aggregates implies that there are stringent size limitations in their formation.

A methylene blue-sensitized photo-oxidation has been found to stabilize the aggregates without any visible structural modification, so that they no longer dissociate under high ionic strength conditions. This reaction is therefore thought to introduce covalent cross-links. The cross-links are not disulfide bonds as in dumbbells, and their chemical nature has not yet been determined. However, the inhibition of the cross-linking reaction by sodium azide and its stimulation in D$_2$O-containing media indicate that the reactive component is singlet oxygen. This is known to react primarily with methionine and histidine residues. Although disulfide groups may also react with singlet oxygen, reduction and SH-blocking experiments imply that they are not involved. The presence of hydroxyproline (1, 14, 21) and its three-stranded structure (7) suggest that the tail may bear some relationship to collagen, in which cross-links may occur through activation of histidine groups (2). It is therefore possible that the cross-links introduced in the photodynamic reaction involve histidine residues of the tails (18), and are similar to those in collagen.

Finally, we would like to stress that the low ionic strength aggregates are produced exclusively by the associated of tailed molecules. In this respect, the collagen-like tail plays an essential role in supramolecular assembly of the acetylcholinesterase. However, the tail does not seem to be involved in the regulation of catalytic activity of the enzyme. Rather, the collagen-like tail could be a structural device permitting the anchorage of the catalytic globular subunits at the synaptic level (8, 22). We have recently discovered that an acidic mucopolysaccharide, present in the electric organ, is required, as aggregating agent, for the multimeric tail-to-tail association of the enzyme. A description of this aspect will be soon published.

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Note Added in Proof: The recent findings of L. M. Marshall, J. R. Sanes, and U. J. McMahan (Proc. Natl. Acad. Sci. U. S. A. 74:3073–3077) and of U. J. McMahan, J. R. Sanes, and L. M. Marshall (1978. Nature (Lond.). 271:172–174) showing by histochemical methods that cholinesterase activity remains bound to the basal membrane of the synaptic folds of frog muscular endplates, while both nerve ending and muscle cell degenerated, appear to be readily explained on the basis of our present conclusions.

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