THE BINDING OF 18S ACETYLCHOLINESTERASE TO SPHINGOMYELIN AND THE ROLE OF THE COLLAGEN-LIKE TAIL

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Summary: The "native" forms of acetylcholinesterase (EC 3.1.1.7) from Electrophorus electricus have sedimentation coefficients of 18S, 14S, and 8.5S (1) and have been shown to possess a collagen-like tail structure thought to function in the immobilization of the enzyme on a membrane matrix. We report that collagenase treatment of the enzyme purified by affinity chromatography yields three products with sedimentation coefficients of 21.4S, 17.1S, and 11.8S. It is suggested that these species are tailless analogs of the 18S 14S, and 8.5S species, respectively.

The 18S acetylcholinesterase species is shown to bind to sphingomyelin at high (μ=1.0) or low (μ=0.1) ionic strength, but not to phosphatidylcholine. The failure of the corresponding tailless analog, the 21.4S species, to bind to sphingomyelin suggests that the sphingomyelin binding site or sites reside on the tail structure.

INTRODUCTION

Acetylcholinesterase (EC 3.1.1.7) has been isolated from several sources, the richest being the electric tissue of Electrophorus electricus (1). The enzyme isolated from this source exists in several different molecular species which can be identified by their sedimentation coefficients. The "native" forms as isolated by Dudai, et al. (2) and Massoulie, et al. (3) have sedimentation coefficients of 8.5S, 14S, and 18S and appear in electron micrographs (4,5) as clusters of 4, 8, and 12 subunits respectively, attached to a 50 nm collagen-like tail (6,7). The 18S and 14S forms aggregate at low ionic strength (8,9). A fourth species, a tailless tetramer, has a sedimentation coefficient of 11.4S and is thought to be a product of the proteolytic degradation of the "native" forms of the enzyme. The tailed forms may be converted into the 11.4S species without any loss of activity by the action of trypsin (3) or in the case of the enzyme from Torpedo californica, by collagenase (6,10). The same treatment also leads to solubilization of the enzyme from electric tissue, leading to the suggestion that the tail structure may somehow be involved in the immobilization of the enzyme on some sort of membrane structure (11,12). The type of membrane structure or structures with which acetylcholinesterase is associated is not known with certainty. Based on the solubilization of acetylcholinesterase

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from Torpedo californica by collagenase it has been suggested that the enzyme in this tissue is anchored via its tail to the collagen-laced basement membrane surrounding the cells (12). On the other hand erythrocyte acetylcholinesterase has lipid binding properties which suggest that its primary association may be with the plasma membrane (13,14).

At present little is known about the interaction of Electrophorus electricus acetylcholinesterase and lipids. Phospholipase C has been shown to affect the sedimentation behavior of purified acetylcholinesterase (16) implying the presence of phospholipids, but the interpretation of this phenomenon is not clear. We have recently isolated a membrane bound acetylcholinesterase fraction in which the enzyme is tightly bound to a lipid-containing membrane (15). Solubilization of the enzyme from the membrane occurs only in the presence of detergent, suggesting a possible enzyme:lipid interaction. In this communication we report on the interaction of 11.8S, 18S and a newly reported 21.4S species of acetylcholinesterase with sphingomyelin and phosphatidylcholine. The results are interpreted in terms of an interaction between the tail structure of the enzyme and sphingomyelin.

METHODS

Purification of "Native" Acetylcholinesterase. Acetylcholinesterase was purified from fresh electric tissue of Electrophorus electricus by affinity chromatography on 9-[N^{γ}-(ε-Sepharose 4B-aminocaproyl)-γ-aminopropylamino] acridinium according to Rosenberry and Richardson (6).

Collagenase Treatment of Purified Enzyme. Affinity purified acetylcholinesterase (0.7 mg) was incubated in a total volume of 0.6 ml for 1 hour at 37°C with 0.025 mg of Clostridium histolyticum collagenase (EC 3.4.24.3), grade I from Boehringer Mannhein in 0.01 M sodium phosphate buffer, pH 7.0 containing 1.0 M sodium chloride, 0.5 mM calcium chloride, and 0.3 mM magnesium chloride.

Preparative Sucrose Gradient Centrifugation. The 18S, 14S, and 8.5S enzyme species present in the affinity chromatography purified acetylcholinesterase or the 21.4S, 17.1S and 11.8S species in the collagenase treated samples were separated by centrifugation of a 0.6 ml sample on a sucrose gradient consisting of a 32 ml linear gradient from 0.15 to 0.63 M sucrose layered over 5.0 ml of a 2.0 M sucrose solution. All solutions contained 0.01 M sodium phosphate buffer, pH 7.0 with 1.0 M sodium chloride. Centrifugation was carried out for 21 hours at 27,000 rpm in a Beckman SW27 rotor. Fractions were collected from the bottom of the cellulose nitrate tubes and assayed using acetylthiocholine as substrate (17). One unit of enzyme hydrolyzes one mmole of substrate per hour.

Binding Experiments. Sphingomyelin from bovine brain and phosphatidylcholine from egg yolk (Type III-E) were obtained from Sigma Chemical Co. Ten mg of sphingomyelin or phosphatidylcholine were suspended in 1.0 ml of the appropriate buffer at 0°C and containing 1.2 M sucrose. The lipid was dispersed with a small glass homogenizer until the suspension was uniform, 2-3 minutes. Enzyme samples, 25 or 50 µl in volume, were added to the suspension and allowed to stand for 30 minutes at 0°C before centrifugation. Sucrose gradients consisted of 0.5 ml of 2.0 M sucrose, 0.7 ml of enzyme:lipid sample in 1.2 M sucrose, 3.1 ml of 1.0 M sucrose, and 0.5 ml of buffer. All sucrose solutions contained the same buffer as that of the sample layer. Centrifugation was carried out for

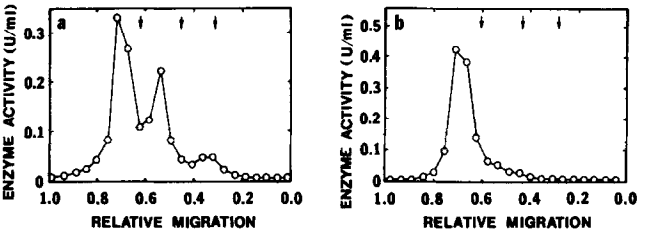


Figure 1 Sedimentation profiles of "native" acetylcholinesterase in 1.0 M sodium chloride in 0.01 M sodium phosphate, pH 7.0. Centrifugation was carried out in a 5 - 20% linear sucrose gradient for 7 hours at 50,000 rpm in a Beckman SW50 rotor. Arrows indicate migration of marker enzymes. a) Affinity purified enzyme, b) the 18S species.

three hours at 50,000 rpm ($204,000 x g_{av}$) in a Beckman SW50 swinging bucket rotor. Fractions were assayed immediately after collection.

Sedimentation Analysis. Sedimentation coefficients of the various enzyme forms were determined according to Martin and Ames (18). Centrifugation was carried out for 7 hours at 50,000 rpm in a Beckman SW50 rotor. Internal marker enzymes consisting of β-galactosidase (EC 3.2.1.23), 16S; yeast alcohol dehydrogenase (EC 1.1.1.1), 7.4S; and beef liver catalase (EC 1.11.1.6), 11.3S were included in each determination. Relative migration was defined as the fractional migration of the sample in question from the center of the original sample layer to the center of the peak at one half maximum peak height.

RESULTS

Preparation of the Various Enzyme Species. The acetylcholinesterase which was purified by affinity chromatography had a specific activity of 245 units $ml^{-1}A_{280}^{-1}$, which compares favorably to 240 units $ml^{-1}A_{280}^{-1}$, the specific activity of the enzyme purified by Rosenberry and Richardson (6) expressed in the units used in this laboratory. The sedimentation profile of our enzyme preparation is shown in Figure 1, a. The experimentally determined sedimentation coefficients of 17.9S, 13.6S, and 8.6S agree well with those reported for the "native" enzyme forms, 18.4S, 14.2S, and 8.5S (9). Since the 18S species is the most prevalent and can be converted into any of the other species by the action of proteolytic enzymes, we chose to study its lipid binding properties in preference to the other species. The 18S species was separated from the 14S and 8.5S by preparative sucrose gradient centrifugation. The enzyme peak corresponding to the 18S species was collected and used in subsequent experiments. As shown in Figure 1, b, the preparation consisted almost entirely of a single enzyme species with a sedimentation coefficient of 18.1S.

In order to prepare tailless forms of acetylcholinesterase, the purified enzyme was treated with collagenase. Collagenase has been reported to convert the "native" species of Torpedo californica acetylcholinesterase into the

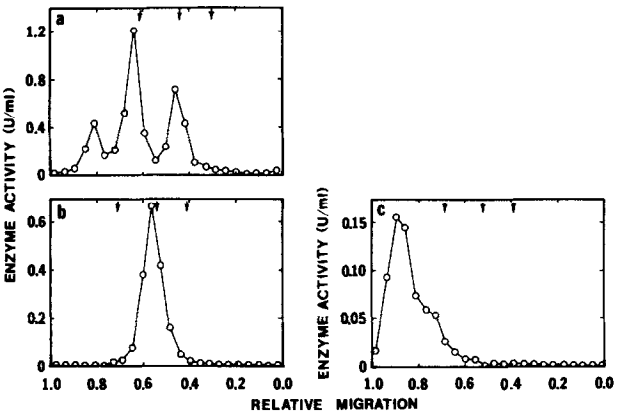
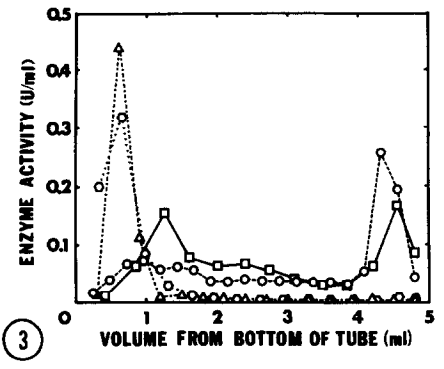


Figure 2 Sedimentation profiles of the collagenase treated acetylcholinesterase. The centrifugation conditions were the same as in Figure 1. a) Collagenase treated enzyme, b) and c) the lightest and heaviest products of collagenase treatment separated from the reaction mixture by preparative sucrose gradient centrifugation.

non-aggregating tetrameric species presumably by its collagenolytic action on its collagen-like tail structure (12). The sedimentation profile of the collagenase treated acetylcholinesterase is shown in Figure 2,a. Three peaks of enzyme activity, with sedimentation coefficients of 21.6S, 16.8S, and 11.8S are seen. These three species were separated by preparative sucrose gradient centrifugation. The homogeneity of the separated species was examined by sucrose gradient centrifugation. Figure 2,b shows the sedimentation profile of the material from the uppermost portion of the preparative gradient. It consists of a single species with a sedimentation coefficient of 11.8S, the same as that reported by Massoulie and Rieger (3) for the globular tetrameric species produced by trypsin treatment of the "native" forms. As shown in Figure 2,c the enzyme recovered from the lowermost portion of the preparative gradient consists mostly of enzyme with a sedimentation coefficient of 21.4S and a small amount of enzyme having a somewhat smaller sedimentation coefficient. Although not shown in Figure 2, the material from the center part of the preparative gradient appeared to be homogeneous and had a sedimentation coefficient of 17.1S. At low ionic strength none of these forms exhibited the aggregation phenomenon observed with the 18S and 14S species. The possible structures of the products of collagenase treatment will be addressed in the Discussion section.

The Binding of Acetylcholinesterase to Lipids The binding of the various enzyme species to phosphatidylcholine and sphingomyelin was investigated using a flotation type binding assay. The advantage of this type of assay over



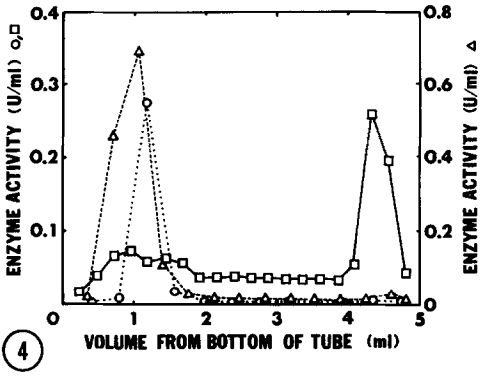


Figure 3 The binding of 18S acetylcholinesterase to sphingomyelin and phosphatidylcholine. ○ , enzyme alone in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride (μ=0.1); △ , enzyme plus 1% phosphatidylcholine in same buffer; ○ , enzyme plus 1% sphingomyelin in same buffer; □ , enzyme plus 1% sphingomyelin in same buffer with 1.0 M sodium chloride (μ=1.0).

Figure 4 The binding of various acetylcholinesterase species to 1% sphingomyelin in 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 M sodium chloride (μ=0.1) See Figure 3 for conditions. □ , 18S enzyme; △ , 11.8S enzyme; ○ , 21.4S enzyme.

a sedimentation type of assay is that ambiguity caused by aggregated enzyme masquerading as membrane-bound is avoided. Only aggregates with a density less than 1.13 g/cm^3 will "float" to the upper part of the gradient. More dense material will remain at or below the sample layer.

Results from several binding experiments with the 18S enzyme are shown in Figure 3. At low ionic strength in the absence of any added lipid, the enzyme remains at the bottom of the tube. Addition of 1% (w/v) phosphatidylcholine leads to no change except the finding of considerable turbidity associated with the lipid dispersion near the top of the tube. However, when 1% sphingomyelin is added, much of the enzyme activity and the turbidity associated with the sphingomyelin dispersion are found near the top of the gradient, indicative of an enzyme:sphingomyelin interaction. At high ionic strength this binding interaction still takes place, although at somewhat diminished level. Recovery of total enzyme units when based on assays of non-centrifuged controls was generally greater than 90%[1].

[1]It should be noted that the 18S and 21.4S non-centrifuged controls lost 30-60% of their activity over a period of 3-5 hours subsequent to transfer and dilution of the enzyme from stock solutions. The phenomenon has been reported by Rosenberry and Richardson (6). We have observed that the loss of activity can be partially prevented by 1% (w/v) sodium cholate.

The results of binding experiments with the 11.8S, 18S, and 21.4S species and sphingomyelin at low ionic strength are shown in Figure 4. Neither the 11.8S nor the 21.4S enzyme species shows any evidence of binding to this lipid.

DISCUSSION

The simplest interpretation of the binding experiments is that 18S acetylcholinesterase interacts with sphingomyelin but not with phosphatidylcholine. However, such a broad conclusion must be viewed cautiously. The nature of the binding assay is such that interaction of acetylcholinesterase with relatively few phosphatidylcholine molecules, insufficient to decrease the density of the complex to less than the 1.13 g/cm^3 required for flotation, would not appear as a binding interaction. In the case of the interaction between the 18S enzyme species and sphingomyelin however, it is clear either that acetylcholinesterase interacts with preformed sphingomyelin vesicles or that a sufficiently large number of sphingomyelin molecules bind to the enzyme to result in flotation of the complex. Although we do not presently know why acetylcholinesterase interacts differently with sphingomyelin than with phosphatidylcholine, vesicles composed of these lipids exhibit several surface differences which may be related to the observed differences in binding. Both lipids have zwitterionic phosphocholine head groups exposed to solvent, but they may be in different orientations due to inter- and intramolecular hydrogen bonding (19,20). Furthermore, the sphingomyelin vesicles appear to be much more rigid than the phosphatidylcholine vesicles, for the same reasons.

On the other hand, some information on the role of the enzyme's collagen-like tail is available from the binding experiments involving the collagenase-treated enzyme. The 11.8S species, one of those produced upon collagenase treatment of the affinity purified enzyme has been reported by others as a tailless tetrameric form of acetylcholinesterase (3). However, we also observed a 21.4S and 17.1S species. The possible origin of these other species is as follows. Lytic events occuring at points where tetramers are attached to the tail (designated A in Figure 5) would produce one 11.8S species per attack and leave the corresponding 14S or 8.5S tailed species, which could also be attacked until only 11.8S enzyme remains. Such a series of proteolytic steps has been proposed to occur when the enzyme is exposed to trypsin (3). We would suggest, however, that collagenase preferentially attacks in such a way as to remove the tail structure without separating the tetramers from each other, i.e., in the vicinity of B in Figure 5. An attack on the 18S species would give the 21.4S species, which would sediment more rapidly due to its reduced asymmetry. Similarly, the 14S species would give rise to the 17.1S species, and the 8.5S to the 11.8S. The specificity of

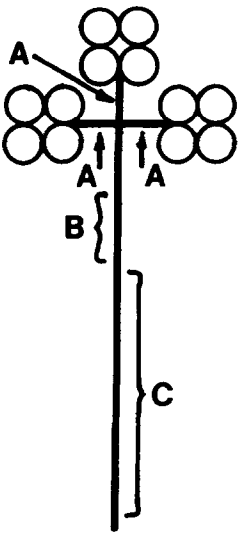


Figure 5 Schematic representation of the 18S acetylcholinesterase species showing two possible modes of proteolytic cleavage, A and B. Region C represents a possible sphingomyelin binding region.

collagenase for attack at B does not appear to be absolute, since the relative amounts of the 21.4S, 17.1S and 11.8S species suggests some lysis at A also occurred. With this interpretation in mind, the failure of either the 11.8S or 21.4S species to bind to sphingomyelin indicates that the collagen-like tail is required for the binding interaction.

The possible physiological significance of the binding of acetylcholinesterase to sphingomyelin is difficult to assess. Schmidt, et al. (20) have recently suggested that the relatively strong intermolecular interactions between sphingomyelin molecules may lead to the formation of "microdomains" in biological membranes. If such microdomains exist in electroplax membranes, which have approximately 5% of their total lipids as sphingomyelin (21), they may provide a means by which acetylcholinesterase may specifically interact with the plasma membrane. We have recently used a flotation technique to isolate a membrane-bound acetylcholinesterase fraction (15) in which the enzyme appears to be associated with the lipid portion of the membrane. In contrast to most of the enzyme in electric tissue homogenates, the membrane-bound acetylcholinesterase is not solubilized in high ionic strength buffer (μ=1.0) but requires detergent in addition. Based on the experimentally determined sedimentation coefficients of 18S, 14S and 8.5S (24), the detergent solubilized enzyme consists of the "native" or tailed species. Although the

properties of the membrane-bound acetylcholinesterase may suggest an enzyme: sphingomyelin complex, other similar complexes cannot be ruled out.

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