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A MONOMERIC FORM OF HUMAN ERYTHROCYTE MEMBRANE ACETYLCHOLINESTERASE

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Purified detergent solubilized dimeric human erythrocyte acetylcholinesterase (6.3 S form) was converted to a stable monomeric 3.9 S species when treated with 2-mercaptoethanol and iodoacetic acid. More than 60% of the enzymatic activity were recovered after this treatment. A decreased susceptibility to reduction and alkylation was observed with purified, detergent depleted acetylcholinesterase aggregates. When erythrocyte membranes (ghosts) were subjected to the same treatment, acetylcholinesterase could subsequently be solubilized as monomeric 3.9 S form and and more than 90% of the activity were recovered. Monomeric acetylcholinesterase was less reactive towards antibodies raised against (dimeric) human erythrocyte membrane acetylcholinesterase and towards antibodies against human erythrocyte membranes. The results suggest that acetylcholinesterase is present as dimeric species in human erythrocyte membranes despite the fact that fully active monomers can be obtained.

Introduction

Detergent-depleted human erythrocyte membrane acetylcholinesterase exists in several multiple forms with molecular weights up to 1 237 100 [1]. In presence of non-denaturing detergents, only a homogenous dimeric enzyme species with a molecular weight of approx. 151 000 could be observed [2]. The *s* value obtained, either by analytical ultracentrifugation or by sucrose density gradient centrifugation, was approx. 6.0 S. Cross-linking studies have further confirmed the structure of the enzyme and have established that the dimer is built up of two identical subunits which have molecular weights of approx. 73 000 and which are interlinked by at least one disulfide bond [3,4]. Dimeric enzyme in presence of non-denaturing detergents, as well as aggregated, detergent depleted enzyme, showed a specific activity of 5700 I.U./mg protein [2].

For acetylcholinesterases from several other species *s* values as low as 4 S have been described, which suggested the occurrence of monomeric forms. Some of these enzyme species were obtained either by freezing and thawing [5], limited proteolysis or treatment with SH-reagents [6]. In addition the occurence of native monomeric forms in tissue extracts from various sources has been suggested [7,8].

Based on these results the possibility had to be considered that monomeric acetylcholinesterase might be the enzyme form present in the native erythrocyte membrane. This idea was further corroborated by reports which showed that oxidative impurities were present in detergents used for membrane solubilization [9], suggesting that oxidative cross-linking was responsible for the occurrence of several oligomeric erythrocyte membrane protein species in vitro [10].

The present communication shows that a monomeric form of human erythrocyte membrane acetylcholinesterase can be obtained by treatment of either erythrocyte membranes or purified enzyme with 2-mercaptoethanol and iodoacetic acid.

The process of monomerization takes place without significant loss of enzymatic activity. Immunological evidence, however, suggests that dimeric acetylcholinesterase is the predominant species present in the native erythrocyte membrane.

Materials and Methods

Erythrocyte membranes (ghosts) were prepared from fresh human red blood cells according to Dodge et al. [11] and stored at −20°C. Acetylcholinesterase was prepared by affinity chromatography and enzymatic activity assayed as previously described [12]. One unit of enzyme activity (U) corresponds to 1 μmol substrate (acetylthiocholineiodide) hydrolyzed per min. Rabbit anti-human erythrocyte membrane antibody was obtained from Dako-Immunoglobulins, Copenhagen, Denmark. Anti-albumin activity was removed according to Ref. 13. To prepare antibodies against human erythrocyte membrane acetylcholinesterase, rabbits were injected with 125 μg of enzyme suspended in 0.7 ml of Freund's incomplete adjuvant which contained 0.064% Triton X-100. After the initial injection, the next boosters followed at intervals of 10, 15, 20 and 38 days. Approx. 30 ml of blood were routinely collected 7 days after booster injections from the rabbits ear-vein. Antiserum was purified by ammonium-sulfate precipitation, dialysis and DEAE-cellulose chromatography according to the method of Harboe and Inglid [14]. The anti-acetylcholinesterase antibody titre was 286 μg/ml, determined by rocket immunoelectrophoresis [15]. Catalase was obtained from Boehringer (Mannheim, F.R.G.), Agarose A from Pharmacia Fine Chemicals (Uppsala, Sweden) and Freund's adjuvant (incomplete) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other reagents were standard commercial products obtained either from Fluka (Buchs, Switzerland) or from Merck AG (Darmstadt, F.R.G.).

Sucrose density gradient centrifugation was carried out in 10 mM sodium phosphate buffer, pH 7.4, which contained 0.1% Triton X-100, as previously described [16]. In experiments with enzyme aggregates the detergent was omitted. Rocket immunoelectrophoresis of isolated acetylcholinesterase forms was carried out in 1% agarose gels at 2 V/cm for 17 h in a buffer containing 38 mM Tris, 100 mM glycine and 0.5% Triton X-100, pH 8.7 [15]. The gels were stained for esterase activity with α-naphthylacetate and fast red TR according to Bjerrum et al. [17].

For incubation with SH-reagents, purified acetylcholinesterase was dialyzed overnight against a 20 mM Tris-HCl buffer, pH 8.5, which contained 4 mM EDTA and 0.1% Triton X-100. The detergent was omitted in experiments with aggregated acetylcholinesterase. An aliquot of the dialyzed enzyme (80 I.U., corresponding to approx. 14 μg protein) was diluted with buffer to a total volume of 1 ml. 2-Mercaptoethanol was added to a concentration of 14 mmol/l and the mixture incubated for 40 min at room temperature with gentle stirring. A 10-fold excess (= 140 mmol/l) of iodoacetic acid recristallized immediately before use from petroleum ether/ether (5 : 1, v/v) and adjusted to pH 8.5 with 10 M NaOH was then added and the mixture incubated for another 40 min with gentle stirring at room temperature in the dark. Excess iodoacetic acid was removed from the incubation mixture by gel filtration on a Sephadex G-25 column (180 ml bed volume) at a flow rate of 60 ml/h. The elution buffer was 10 mM sodium phosphate, pH 7.4, with 0.1% Triton X-100. Fractions of 2 ml were collected and assayed for acetylcholinesterase activity. The active fractions were pooled and used for density gradient centrifugation. In one set of experiments the enzyme was incubated only with 140 mmol/l iodoacetic acid. Control experiments were carried out without the addition of 2-mercaptoethanol and iodoacetic acid but otherwise under identical conditions.

Basically the same incubation procedure was used for isolated erythrocyte membranes (ghosts). To remove excess iodoacetic acid the suspension was subsequently washed three times by centrifugation at 15000 rpm at 4°C for 20 min and resuspension in 10 mM sodium phosphate buffer, pH 7.4. The pellet obtained in the last centrifugation step was solubilized at 4°C by suspension in ten volumes of 10 mM sodium phosphate buffer, pH 7.4 which contained 1% Triton X-100. Unsolubilized material was removed by centrifugation at 15000 rpm for 30 min at 4°C. The resulting supernatant was used for density gradient centrifugation. One series of experiments was carried out with iodoacetic acid only (140 mmol/l)

and in control experiments 2-mercaptoethanol and iodoacetic acid were both omitted.

Results

Density gradient analysis of purified dimeric 6.3 S acetylcholinesterase (Fig. 1A) treated with mercaptoethanol and iodoacetic acid revealed the conversion of the enzyme to a predominant species with an apparent *s* value of 3.9 S while only minor amounts of dimeric enzyme form remained (Fig. 1B). The recovery of enzymatic activity was better than 60%. When fractions pooled from the 3.9 S peak were subjected to a second density gradient centrifugation experiment the presence of a homogenous 3.9 S enzyme species with no contaminating 6.3 S form was observed (Fig. 1C). Enzyme treated only with iodoacetic acid showed no conversion to the 3.9 S form. Purified detergent depleted aggregated acetylcholinesterase displayed a considerably different behavior. The quantitative distribution of the multiple molecular forms [1] was not significantly changed by reduction and alkylation (Fig. 2A). Only when Triton X-100 (0.5%) was added to the treated aggregates it was possible to detect a fraction of the enzyme which had been converted to the 3.9 S form while more than 50% were still present as dimeric species (Fig. 2B).

The susceptibility of membrane-bound acetylcholinesterase to reduction and alkylation was investigated using ghost preparations. In this case, Triton X-100 was not included in the buffers used for the treatment with mercaptoethanol and iodoacetic acid but only in the buffer used for enzyme solubilization. The predominant enzyme species observed after incubation and solubilization was again the 3.9 S form (Fig. 3B) and 96% of the initial enzymatic activity were recovered. Interestingly, even the controls which had been incubated in absence of reducing and alkylating agents contained some of this enzyme species (Fig. 3A). After incubation of ghosts with iodoacetic acid only, a slightly increased fraction of the low molecular weight form could be detected besides

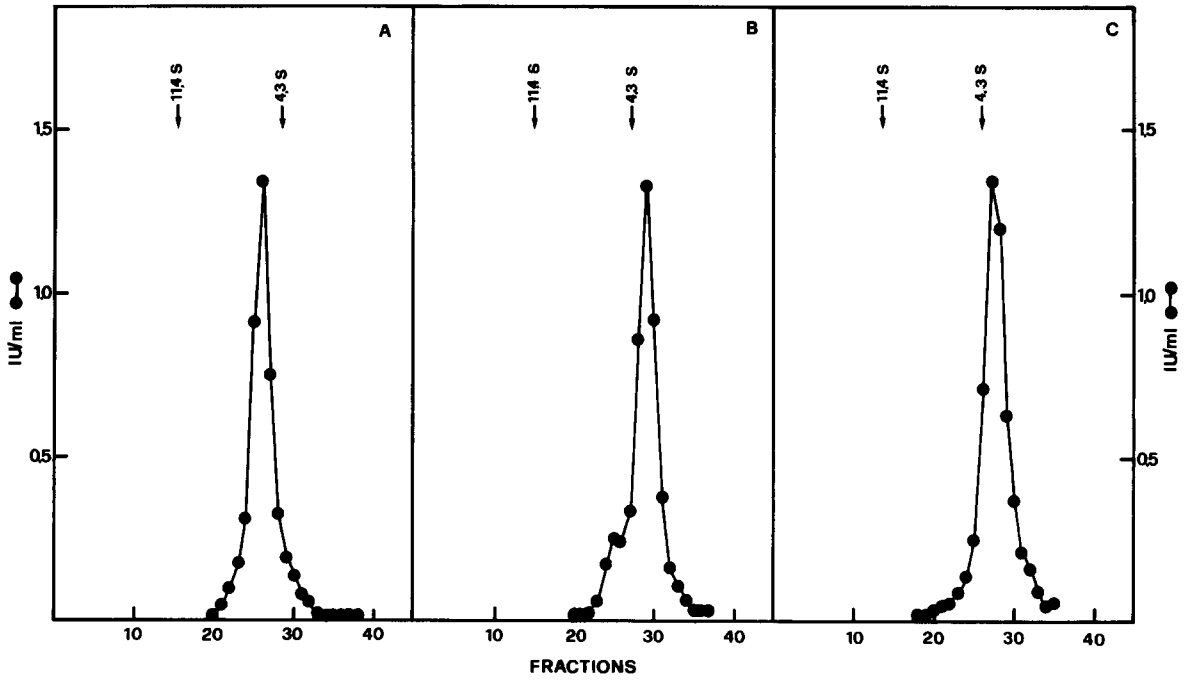

Fig. 1. Density gradient centrifugation of purified human erythrocyte membrane acetylcholinesterase in presence of Triton X-100. A: Untreated control. B: Enzyme incubated with mercaptoethanol and iodoacetic acid in presence of detergent as described in Materials and Methods. C: The peak fractions of the 3.9 S peak were pooled from six density gradients, dialyzed extensively and subjected to a second density gradient centrifugation. The arrows indicate the positions of the marker proteins catalase and hemoglobin.

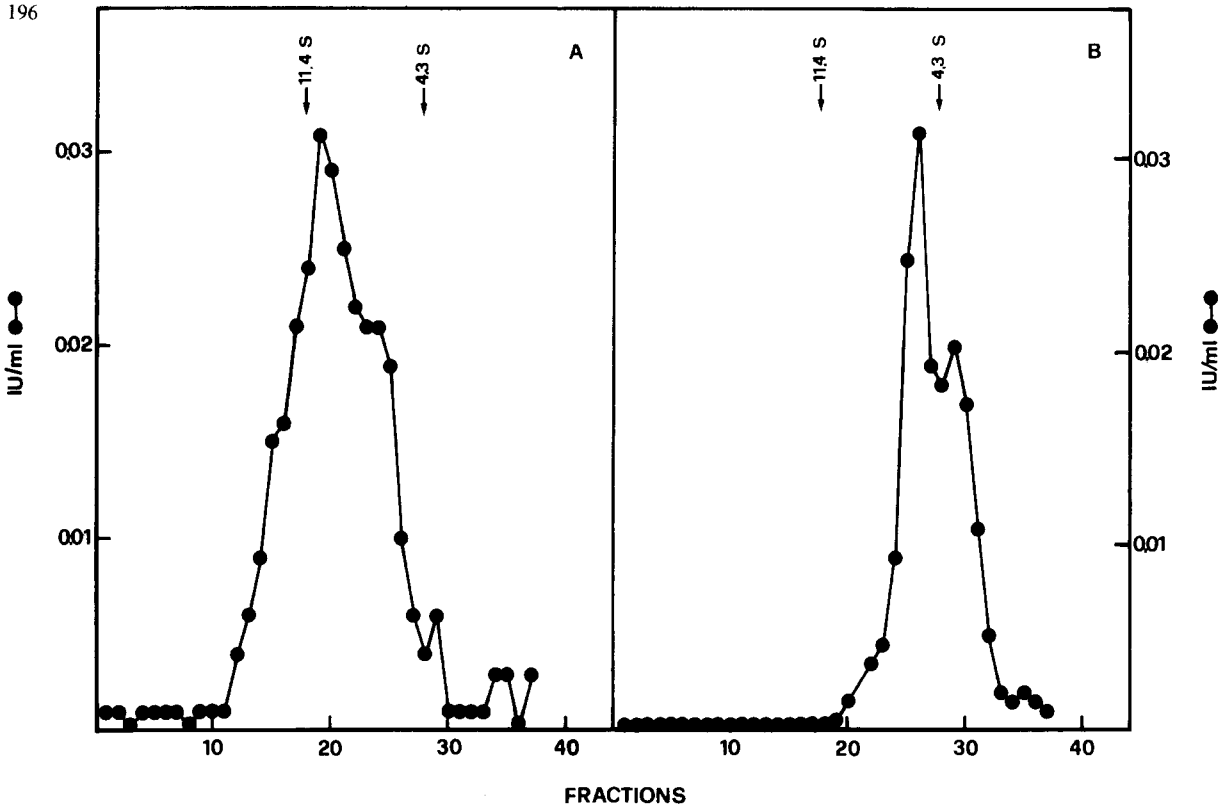

Fig. 2. Density gradient centrifugation of reduced and alkylated acetylcholinesterase. The enzyme was incubated with mercaptoethanol and iodoacetic acid in absence of Triton X-100. A: Centrifugation was carried out in absence of detergent. B: Centrifugation was carried out in presence of 0.5% Triton X-100. The arrows indicate the position of the marker proteins catalase and hemoglobin.

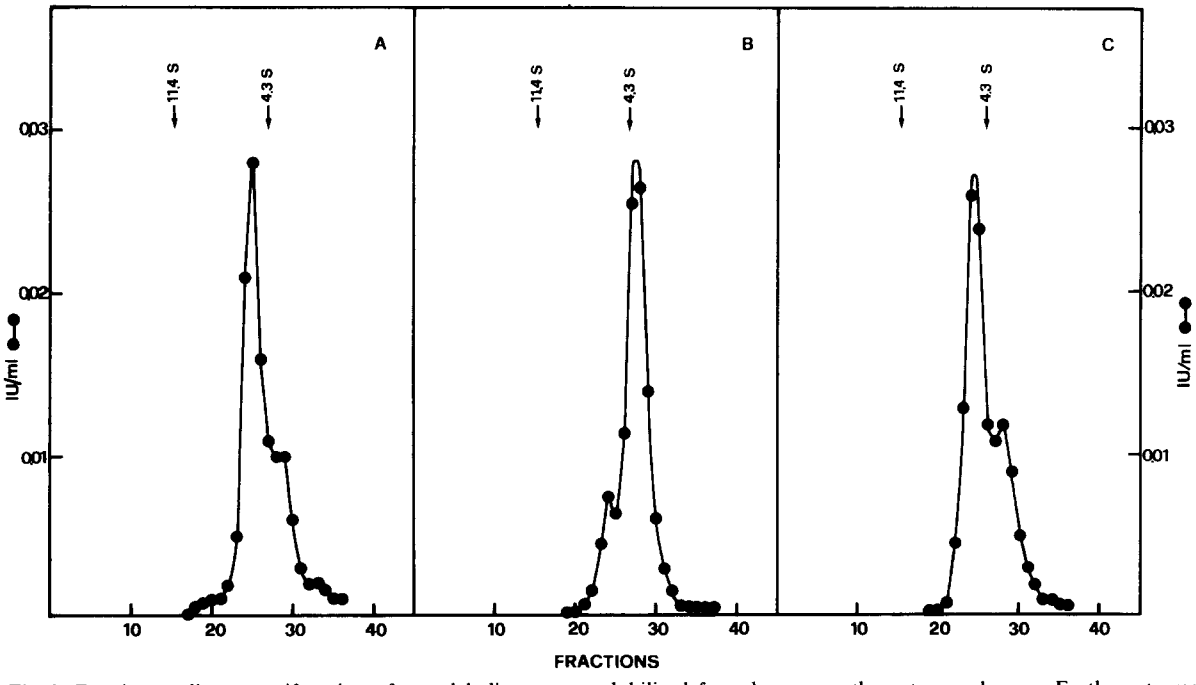

Fig. 3. Density gradient centrifugation of acetylcholinesterase solubilized from human erythrocyte membranes. Erythrocyte membranes (ghosts) were incubated with mercaptoethanol and iodoacetic acid and the enzyme solubilized as described in Materials and Methods. Centrifugation was carried out in presence of Triton X-100. A: Untreated control. B: Enzyme solubilized from ghosts incubated with mercaptoethanol and iodoacetic acid. C: Enzyme solubilized from ghosts incubated with only iodoacetic acid. The arrows indicate the position of the marker proteins catalase and hemoglobin.

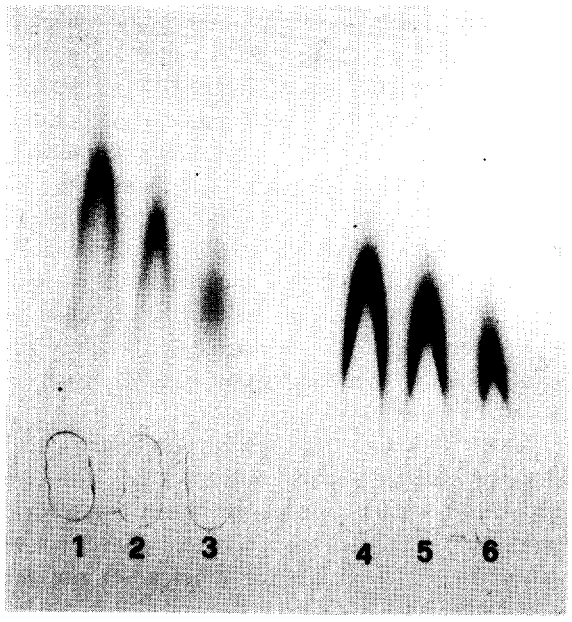

Fig. 4. Rocket immunoelectrophoresis of human erythrocyte acetylcholinesterase. Enzyme forms were isolated by density gradient centrifugation. The 3.9 S form was applied to well Nos. 1–3 (45, 30 and 15 mU, respectively) and the 6.3 S form to well Nos. 4–6 (45, 30 and 15 mU, respectively). The agarose gel contained 0.5 μl of anti-acetylcholinesterase antibody per cm^2. Electrophoresis was carried out as described in Materials and Methods.

the prevailing 6.3 S dimer (Fig. 3C). Similar patterns were observed with ghosts obtained from erythrocytes which had been pretreated with Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid)) to block free SH groups in the native erythrocyte membrane.

Rocket immunoelectrophoresis revealed clear differences between the two enzyme species. With identical amounts of enzyme applied to the gel a considerably increased peak heigh was observed for the 3.9 S form (Fig. 4). A ratio of 1.6 (3.9 S form: 6.3 S form) was calculated when anti-erythrocyte membrane antibody was used and a ratio of 1.5 was obtained with anti-acetylcholinesterase antibody. Furthermore, the staining intensity (activity stain) was greatly decreased with the 3.9 S species.

Discussion

It has been previously shown that acetylcholinesterase exists as homogeneous dimeric species with full catalytic activity in presence of detergents [18]. The dimeric enzyme has a sedimentation coefficient of 5.7 S when determined by analytical ultracentrifugation [2] and of 6.3 S when determined by density gradient centrifugation [16]. The present study shows that this dimeric species can be split into smaller fragments by treatment with reducing and alkylating agents. The 3.9 S form apparently represents monomeric acetylcholinesterase which is produced as a consequence of the reduction of disulfide bonds between the two subunits of the dimer. The results are consistent with studies which have shown by sodium dodecyl sulfate polyacrylamide gel electrophoresis that the enzyme is built up of two identical monomers interlinked by at least one disulfide bond [3]. No information, however, was available on the activity of monomeric acetylcholinesterase since in these earlier experiments monomerization was accompanied by denaturation of the enzyme protein.

The present study shows that more than 60% of the initial activity could be recovered after reduction of purified enzyme and more than 95% when ghosts were treated with mercaptoethanol. The observation that the activity of purified acetylcholinesterase is more susceptible to mercaptoethanol treatment than the membrane bound enzyme indicates that the hydrophobic surrounding of the membrane stabilizes the enzyme structure and protects it from an extensive reduction and concomitant loss of activity. Solubilization of acetylcholinesterase from reduced and alkylated erythrocyte membranes does not diminish it's activity and most of the enzyme is recovered as monomeric species which clearly indicates that a dimeric structure is not required for full catalytic activity.

The structure of human erythrocyte acetylcholinesterase appears to be quite different from the structure of the electric eel (*Electrophorus electricus*) acetylcholinesterase which can be obtained as monomer only after proteolytic degradation combined with reduction or after freezing and thawing [5,7]. On the other hand, a dimeric enzyme species which could also be monomerized by reduction and alkylation without loss of enzymatic activity was obtained from bovine brain nucleus caudatus [6].

Besides these dimers, however, a considerable amount of a native monomeric enzyme species was detected in nucleus caudatus extract [8]. The presence of minor amounts of the 3.9 S form of human erythrocyte acetylcholinesterase after treatment of ghosts with iodoacetic acid suggests that a small fraction of membrane-bound enzyme may be present in the native membrane as monomers or as non-covalently associated dimers (Fig. 3). Such dimers would most probably appear as monomeric species in density gradient centrifugation in presence of Triton X-100 because the hydrophobic interactions responsible for dimer formation would be disrupted by the detergent. It must be emphasized, however, that the amount of native monomer is much smaller in erythrocytes than in bovine brain.

At present if is not possible to decide what interactions promote the formation of a disulfide-linked acetylcholinesterase dimer in erythrocyte membranes. Non-covalently associated dimers may become covalently linked with advancing maturation of the red cell. Monomer-dimer associations have also been reported for the band 3 protein of the erythrocyte membrane. Oxidation with Cu^{2+}-phenanthroline was shown to promote the covalent dimerization via S-S bonds while in the native membrane or in detergent solution non-covalent associations were postulated [10,19]. The situation for the band 3 protein thus is quite different from the one observed with acetylcholinesterase. A possible influence of oxidative impurities in the detergents used for solubilization appears unlikely, as no increased amount of 3.9 S form was observed when intact membranes were pretreated with Ellman's reagent to block free SH groups before solubilization with Triton. Identical results were obtained with Triton freed of oxidative contaminations.

The results obtained with rocket immunoelectrophoresis show clear differences between monomeric and dimeric human erythrocyte acetylcholinesterase. The peak area is considerably increased with the monomer and, at the same time, the staining intensity of the activity stain is significantly decreased despite the fact that with both enzyme preparations (dimer and monomer) identical amounts of enzyme were applied to the gels (Fig. 4). Obviously antibodies raised against intact human erythrocyte membranes react more strongly with the dimeric enzyme species which shows that the antibody recognizes preferentially the dimeric structure. This further documents the preponderance of dimeric enzyme in the native membrane. Acetylcholinesterase oligomers are much less susceptible to reduction and alkylation than either the membrane-bound enzyme or the purified dimers (Fig. 2). This suggests that the structural components of the enzyme are differently arranged in native membranes and in aggregates. It thus appears unlikely, that aggregated enzyme exists in the native membrane.

Taken together the results presented here strongly support the notion that dimeric acetylcholinesterase prevails in native erythrocyte membranes, despite the fact that fully active monomers can be prepared. Whether or not the amphiphilic behavior of the enzyme [18] is dependent on an intact dimeric structure remains to be elucidated.

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