Biochimica et Biophysica Acta, 775 (1984) 71–76
Elsevier

BBA 72204

AMPHIPHILE DEPENDENCY OF THE MONOMERIC AND DIMERIC FORMS OF ACETYLCHOLINESTERASE FROM HUMAN ERYTHROCYTE MEMBRANE

PETER OTT and URS BRODBECK

Medizinisch-chemisches Institut der Universität, P.O. Box, CH-3000 Berne 9 (Switzerland)

(Received March 26th, 1984)

Key words: Acetylcholinesterase; Monomeric structure; Detergent; Lipid vesicle; Amphiphile dependence; Density gradient centrifugation; (Erythrocyte membrane)

Human erythrocyte membrane-bound acetylcholinesterase was converted to a monomeric species by treatment of ghosts with 2-mercaptoethanol and iodoacetic acid. After solubilization with Triton X-100, the reduced and alkylated enzyme was partially purified by affinity chromatography and separated from residual dimeric enzyme by sucrose density gradient centrifugation in a zonal rotor. Monomeric and dimeric acetylcholinesterase showed full enzymatic activity in presence of Triton X-100 whereas in the absence of detergent, activity was decreased to approx. 20% and 15%, respectively. Preformed egg phosphatidylcholine vesicles fully sustained activity of the monomeric species whereas the dimer was only 80% active. The results suggest that a dimeric structure is not required for manifestation of amphiphile dependency of membrane-bound acetylcholinesterase from human erythrocytes. Furthermore, monomeric enzyme appears to be more easily inserted into phospholipid bilayers than the dimeric species.

Introduction

Human erythrocyte membrane acetylcholinesterase interacts strongly with detergents. The enzyme is found as homogeneous dimeric species in detergent solution and aggregates via hydrophobic interactions to multiple molecular forms when the detergent is removed [1,2]. Furthermore, the enzyme was shown to be influenced in its catalytic activity by an amphiphilic environment. When acetylcholinesterase, solubilized in a buffer solution containing detergents slightly above their critical micellar concentration, was diluted at least 1000-fold into an assay medium without amphiphile, it rapidly lost its activity due to dissociation of the detergent from the enzyme molecule. Amphiphiles included in the assay medium were able to preserve enzymatic activity to an extent which depended on the nature and the amount of amphiphile added to the medium [3]. The stabilizing environment could be provided in vitro either by detergents or lipids or even amphiphilic proteins [3,4]. Above an enzyme concentration of 2.5 μg/ml, activity was stabilized by self aggregation when the detergent was removed [3]. Moreover, acetylcholinesterase in intact rat erythrocytes was modulated by its lipid surrounding [5–7]. Similar observations were made for purified human erythrocyte acetylcholinesterase incorporated into lipid vesicles [8]. In this study, Arrhenius plots showed a breakpoint which coincided with the gel to liquid-cristalline phase transition of the lipid [8]. The breakpoint was no longer observed, when cholesterol/phospholipid mixtures at a molar ratio which is known to abolish the phase transition [9] were used in the reconstitution process.

Although dimeric acetylcholinesterase appears to be the predominant form present in the membrane of human erythrocytes, a monomeric en-

zyme species could recently be obtained by treatment of erythrocyte membranes with 2-mercaptoethanol and iodoacetic acid [10]. This monomer showed full catalytic activity when solubilized from the erythrocyte membranes with detergent but no information was available with respect to amphiphile requirements of this enzyme form. On the other hand, limited proteolysis of acetylcholinesterases from human erythrocytes and from *Torpedo marmorata* was shown to abolish interactions with amphiphiles although catalytic activity of these modified enzymes was partially preserved [11,12].

In order to further characterize the structural prerequisites for the expression of amphiphile dependency, monomeric and dimeric acetylcholinesterase solubilized from human erythrocyte membranes were partially purified and their behavior towards detergent and lipid vesicles was compared. The results demonstrate that a dimeric structure is not necessary for the expression of amphiphile dependency of the catalytic activity in human erythrocyte membrane acetylcholinesterase.

Materials and Methods

Phosphatidylcholine from egg yolk was obtained from Koch Light Laboratories, Colnbrook, U.K. Catalase was obtained from Boehringer (Mannheim, F.R.G.). All other reagents were standard commercial products obtained either from Fluka (Buchs, Switzerland) or from Merck AG (Darmstadt, F.R.G.) and were of the highest purity available.

Protein determinations were carried out according to Wang and Smith [13] and acetylcholinesterase activity was determined following the procedure of Ellman [14], unless stated otherwise. One unit of enzyme activity corresponds to 1 μmol substrate (acetylthiocholineiodide) hydrolyzed per min. Erythrocyte membranes (ghosts) were prepared from fresh human red blood cells according to Dodge et al. [15] and stored at $-20\,^{\circ}C$.

For monomerization of acetylcholinesterase, erythrocyte ghosts (1000 ml) were washed twice with a 20 mM Tris-HCl buffer (pH 8.5), containing 4 mM EDTA. The washing steps were carried out by centrifugation at 9000 rpm at 4 °C with a GS-3 rotor in a Sorval RC-5B centrifuge, and resuspension in buffer. The pellet obtained after the washing procedure was then suspended in 1000 ml buffer. Mercaptoethanol was added to a concentration of 14 mM and the mixture incubated for 40 min at room temperature with gentle stirring. Iodoacetic acid, recrystallized immediately before use from petroleum ether/ether (5 : 1; v/v) and adjusted to pH 8.5 with 10 M NaOH, was then added to a final concentration of 140 mM and the mixture incubated for another 40 min with gentle stirring at room temperature in the dark. To remove excess iodoacetic acid the suspension was subsequently washed three times by centrifugation at 9000 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 with 4000 ml of 10 mM sodium phosphate buffer (pH 7.4) which contained 1% (w/v) Triton X-100. Unsolubilized material was removed by centrifugation as described above.

Prior to enzyme purification 1 M NaCl (final concentration) was added to the resulting supernatant which was then readjusted to pH 7.4. Sepharose CL-4B to which the affinity ligand had been coupled as previously described [16] was then added (50 ml) and the suspension was stirred for 60 h. The Sepharose was removed from the suspension by filtration, degassed and packed into a column with an inner diameter of 2 cm. Acetylcholinesterase was eluted with a buffer made of 10 mM sodium phosphate, 1 M NaCl, 1% (w/v) Triton X-100, 0.05% (w/v) sodium azide, and 20 mM decamethoniumbromide (pH 7.4). The flow rate was 5 ml/h and fractions of 3 ml were collected. The peak fractions were pooled and extensively dialyzed against 10 mM sodium phosphate buffer (pH 7.4), containing 0.1 M NaCl, 1% (w/v) Triton X-100 and 0.05% sodium azide.

To separate the monomeric and dimeric forms of partially purified acetylcholinesterase, a total amount of 2000 IU obtained from two individual preparations were concentrated to a volume of 15 ml and subjected to density gradient centrifugation in a zonal rotor. The use of this rotor allowed separation of the entire sample in one single centrifugation step. A B-XIV titanium zonal rotor with a total volume of approx. 670 ml was used in

a MSE superspeed 65 centrifuge. Rotor filling operations were carried out at 2000 rpm. The sucrose density gradient from 5% to 30% (w/v) sucrose in a buffer of 10 mM sodium phosphate, 0.1% (w/v) Triton X-100 and 0.05% sodium azide, pH 7.4, was linear with the radius of the rotor and was filled into the rotor at a flow rate of 20 ml/min. A LKB Ultrograd 11 300 gradient mixer was used to form the gradients. The enzyme preparation was layered on top of the gradient and buffer without sucrose was used as an overlay to displace the enzyme sample from the rotor core. Separation was carried out at 45 000 rpm for 44 h at 4°C. At the end of the run the rotor speed was reduced to 2000 rpm and the gradient was displaced from the rotor with a 50% (w/v) sucrose solution at a flow rate of 12 ml/min. Fractions of 6 ml were collected. The peak fractions of the monomer and the dimer peak were pooled individually and concentrated to 5 ml each, with an Amicon ultrafiltration cell using a Diaflo PM-10 membrane. Homogeneity of monomer and dimer was checked by conventional sucrose density gradient centrifugation in a swinging bucket rotor as previously described [1].

Vesicles from egg phosphatidylcholine were prepared by sonication of a lipid dispersion (5 mg/ml) in 100 mM sodium phosphate buffer (pH 7.4), for a total time of 15 min (15 s sonication followed by an interruption of 15 s). A MSE Ultrasonic Desintegrator (Mk 2) was used at an amplitude setting of 12 μm. The resulting suspension was centrifuged for 90 min at 100 000 $\times g$ at 10°C with an 10 $\times$ 10 ml titanium angle rotor in a MSE Superspeed 65 centrifuge and the supernatant was used as vesicle stock solution. Fresh vesicles were prepared for each experiment. Alternatively, vesicles were prepared according to the procedure of Brunner et al. [17]. The lipid concentration in vesicle solutions was determined following the method of Rouser et al. [18] after lipid extraction according to Renkonen et al. [19].

To compare the amphiphile dependency of dimeric and monomeric acetylcholinesterase, both enzyme solutions were adjusted to 0.06% (w/v) Triton X-100 and to activities between 2.5 and 10 IU/ml. Enzyme (1 μl) was then added to 3 ml of 100 mM sodium phosphate buffer (pH 7.4), which contained variable amounts of amphiphile, either Triton X-100 or lipid, and equilibrated for 30 min. The enzymatic reaction was started by addition of acetylthiocholine iodide as substrate and 5,5′-dithiobis(2-nitrobenzoic acid) (3,3′-6) (DTNB; Ellman's reagent) to a final concentration of 1 mM and 125 μM, respectively. These reagents were added as a 10-fold concentrated solution in buffer. Each experiment was performed with two individual enzyme specimens and each measurement was carried out in triplicate.

Results

Treatment of human erythrocyte ghosts with 2-mercaptoethanol and iodoacetic acid, followed by incubation with Triton X-100-containing buffers resulted in a release of predominantly monomeric acetylcholinesterase from the membranes [10]. In the present study, 2000 IU of partially purified enzyme were prepared from a total of 2000 ml human erythrocyte ghosts. This corresponds to an overall yield of approx. 14% of the starting activity (14 400 IU), which is relatively low compared to earlier published observations [11,20]. Since predominantly monomeric acetylcholinesterase and hardly any dimeric species was found in the filtrate obtained after the affinity adsorption step, the low yield must be due to a

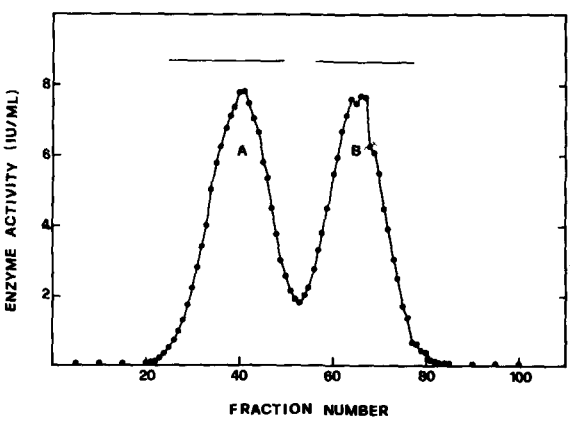

Fig. 1. Density gradient centrifugation in a zonal rotor of partially purified acetylcholinesterase. Enzyme was monomerized, purified and subjected to centrifugation as described in Materials and Methods. The top of the gradient is on the left hand side in this figure. Peak A represents the monomer and peak B the dimer of acetylcholinesterase. The pooled fractions are indicated by horizontal bars.

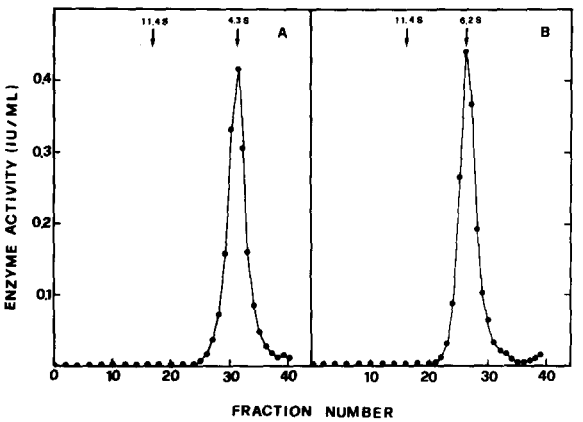

Fig. 2. Density gradient centrifugation in a swinging bucket rotor of separated acetylcholinesterase monomer and dimer. Centrifugation was carried out as described in Materials and Methods. The arrow at 11.4 S indicates the position of catalase which was used as marker protein. (A) Monomeric enzyme; (B) dimeric enzyme.

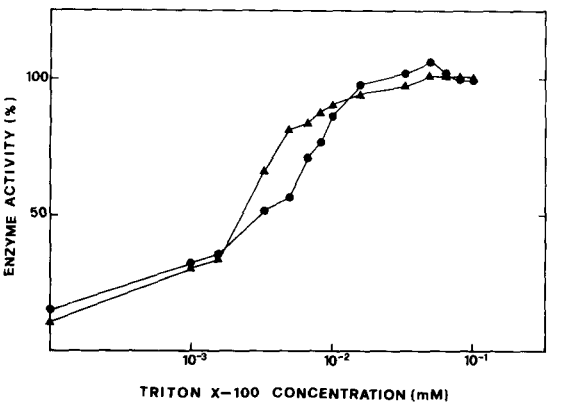

Fig. 3. Influence of Triton X-100 on the activity of human erythrocyte acetylcholinesterase. The characterization of amphiphile dependency was carried out for monomeric (●———●) and dimeric (▲———▲) enzyme as described in Materials and Methods.

rather weak binding of monomerized enzyme to the affinity resin. Since monomerization was not complete and dimeric enzyme bound more readily to the affinity resin, the preparation contained considerable amount of dimers. Therefore, monomers and dimers had to be separated prior to the comparison of their respective amphiphilic behavior. This was achieved by sucrose density gradient centrifugation in a zonal rotor, a method which had previously been used to separate on a preparative scale the various multiple molecular forms of detergent depleted acetylcholinesterase [2]. Almost complete separation of monomer and dimer could be obtained (Fig. 1). The pooled peak fractions, which are indicated in Fig. 1, were submitted to conventional sucrose density gradient centrifugation, to establish the homogeneity of the enzyme species in the two pools (Fig. 2). The monomeric and dimeric acetylcholinesterase obtained by these purification and separation procedures had specific activities of, respectively, 1580 IU/mg protein and 2012 IU/mg protein.

The two pools obtained after zonal centrifugation were compared with respect to their amphiphile dependency. With Triton X-100 as stabilizing agent, almost identical curves were obtained for monomeric and dimeric acetylcholinesterase (Fig. 3). With increasing detergent concentration in the assay medium the enzymes were increasingly stabilized. With both preparations full activity was obtained at a detergent concentration of approx. 55 μM. The activity measured in a control experiment for each individual enzyme pool, in an assay medium containing 0.78 mM (= 0.05%; w/v) Triton X-100 was taken as the 100% value.

On the other hand, differences were observed when egg phosphatidylcholine vesicles were used as stabilizing amphiphile. Monomeric acetylcholinesterase interacted more readily with the lipid vesicles and reached full activity, which corresponds to 100% of the control, at a lipid con-

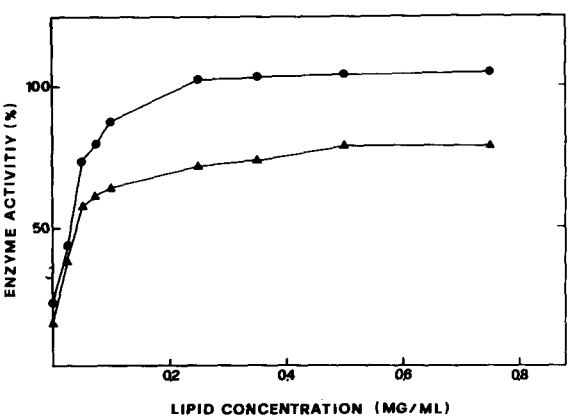

Fig. 4. Influence of egg phosphatidylcholine on the activity of human erythrocyte acetylcholinesterase. Lipid dependency of monomeric (●———●) and dimeric (▲———▲) enzyme was characterized as described in Materials and Methods.

centration of approx. 0.3 mg/ml. The activity of the dimer reached the plateau at a lipid concentration of approx. 0.5 mg/ml but never exceeded 80% of the control value obtained in Triton X-100 (Fig. 4). The maximum activities observed varied when different batches of lipid were used but always ranged between 60% to 80% for dimeric enzyme and between 80% to 100% for the monomeric form.

Discussion

Monomeric and dimeric acetylcholinesterase could not be separated by gel filtration on a Sepharose CL-4B column in buffers which contained Triton X-100, as both enzyme species were eluted with comparable elution volumes (Ott, P., unpublished observation). This was probably due to enzyme-detergent mixed micelles with similar Stokes radii. Therefore, separation by density gradient centrifugation in a zonal rotor was attempted. This technique has mainly been used for the separation of subcellular fractions of cell homogenates but only rarely for isolation of individual components of a protein mixture. In an earlier study the method had been successfully applied to the separation of the four most prominent multiple molecular forms of aggregated acetylcholinesterase in detergent-free medium [2]. An even better separation was obtained in the present study (Fig. 1) with only two components. Due to the relatively low sedimentation coefficients of the enzyme forms (6.3 S and 3.9 S; Ref. 10), optimal separation could be achieved only after a rather long centrifugation time of 44 h. The homogeneity of these enzyme forms and the quality of the separation could be confirmed by recentrifugation (Fig. 2). The separation was clearly better than with a swinging bucket rotor [10] and a sample volume of 15 ml could be applied instead of 0.1 ml.

Monomeric and dimeric acetylcholinesterase isolated from the same batch of erythrocyte ghosts showed a very similar amphiphile dependency of catalytic activity. It is interesting to note that stabilization by Triton X-100 appears to be easily comparable with most acetylcholinesterase species so far investigated. With monomeric and dimeric human erythrocyte enzyme (Fig. 3), as well as the tetrameric form isolated from human brain [21], detergent concentrations needed to obtain full activity were between 0.01 and 0.1 mM. Somewhat higher concentrations were required for the enzyme from *Torpedo marmorata* [12]. With egg phosphatidylcholine as stabilizing amphiphile corresponding results were obtained for erythrocyte acetylcholinesterase monomer (Fig. 4) and brain acetylcholinesterase tetramer [21]. The observation that no complete stabilization of activity of the dimeric enzyme from erythrocytes could be obtained is in contrast to previous results [4] and may be ascribed to differences in the experimental approach. In the present study enzyme was incubated for 30 min in buffer which contained amphiphile, to allow equilibration prior to the addition of substrate. Since interaction of enzyme with lipid vesicles is not expected to occur immediately and because irreversible inactivation of amphiphile-depleted enzyme is known to occur rapidly [3] one has to assume that a certain amount of enzyme is inactivated during the incubation period. On the other hand, in earlier experiments enzyme was diluted into a solution which already contained substrate. In this case enzyme-substrate interaction may have resulted in a stabilization of the enzyme prior to enzyme-lipid interaction. This hypothesis is supported by a recent study of Rosenberry et al. [11] who have observed stabilization of acetylcholinesterase activity by an active-site directed inhibitor during treatment with disulfide reducing agents or proteases.

The structural prerequisites for amphiphilic behaviour of acetylcholinesterases are not yet fully understood. Several investigators have shown that purified acetylcholinesterases from human erythrocytes and from *Torpedo marmorata* could be converted by limited proteolysis to a hydrophilic enzyme species with only a small decrease in molecular weight of approx. 3000 [11,12]. The modified forms did no longer interact with amphiphiles such as detergents or lipids [11,12]. This suggested that a hydrophobic peptide sequence, responsible for anchoring the native enzymes in the lipid bilayer of membrane structures and for amphiphile dependency of the catalytic activity, had been cleaved off. On the other hand, monomerization of the enzyme by disulfide reduction [10] did not affect amphiphile dependency

(Fig. 3 and Fig. 4). Obviously, different regions of human erythrocyte membrane acetylcholinesterase are affected by proteolytic treatment and disulfide reduction, respectively.

Acetylcholinesterase is present as dimer in the human red cell membrane [10]. The observation that the monomeric species retained amphiphile dependency is consistent with the idea that each of the subunits of the native dimer contains a hydrophobic peptide. This supports recent detergent binding studies which suggested that Triton X-100 may bind to both subunits in equal amounts [11]. At present however it is not yet clear, by what mechanism the presumed hydrophobic 'tail' is able to govern the amphiphile dependency of the catalytic activity of detergent soluble acetylcholinesterases.

Acknowledgments

This work was supported by the Swiss National Foundation, grant No. 3.300-0.82 to U.B. and P.O. The skillful technical assistance of Mrs. Y. Binggeli is gratefully acknowledged.

References

1 Ott, P., Jenny, B. and Brodbeck, U. (1975) Eur. J. Biochem. 57, 469–480
2 Ott, P. and Brodbeck, U. (1978) Eur. J. Biochem. 88, 119–125
3 Wiedmer, Th., Di Francesco, C. and Brodbeck, U. (1979) Eur. J. Biochem. 102, 59–64
4 Di Francesco, C. and Brodbeck, U. (1981) Biochim. Biophys. Acta 640, 359–364
5 Morero, R.B., Bloj, B., Farias, R.N. and Trucco, R.E. (1972) Biochim. Biophys. Acta 282, 157–165
6 Bloj, B., Morero, R.B., Farias, R.N. and Trucco, R.E. (1973) Biochim. Biophys. Acta 311, 67–79
7 Farias, R.N., Bloj, B., Morero, R.B., Sineriz, F. and Trucco, R.E. (1975) Biochim. Biophys. Acta 415, 231–251
8 Frenkel, E.J., Roelofsen, B., Brodbeck, U., Van Deenen, L.L.M. and Ott, P. (1980) Eur. J. Biochem. 109, 377–382
9 Demel, R.A. and De Kruijff, B. (1976) Biochim. Biophys. Acta 457, 109–132
10 Ott, P., Ariano, B.H., Binggeli, Y. and Brodbeck, U. (1983) Biochim. Biophys. Acta 729, 193–199
11 Rosenberry, T.L., Scoggin, D.M., Dutta-Choudhury, T.A. and Haas, R. (1984) in Cholinesterases: Fundamental and Applied Aspects (Brzin, M., Kiauta, T. and Barnard, E.A., eds.), W. de Gruyter, Berlin, in the press
12 Stieger, S. and Brodbeck, U. (1984) J. Neurochem. in the press
13 Wang, C.-S. and Smith, R.L. (1975) Anal. Biochem. 63, 414–417
14 Ellman, G.L., Courtney, D.K., Andres, V. and Featherstone, R.M. (1961) Biochem. Pharmacol. 7, 88–95
15 Dodge, J.T., Mitchell, C.B. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130
16 Brodbeck, U., Gentinetta, R. and Ott, P. (1981) in Membrane Proteins (Azzi, A., Brodbeck, U. and Zahler, P., eds.), pp. 85–96, Springer-Verlag, Berlin, New York
17 Brunner, J., Skrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322–331
18 Rouser, G., Fleischer, S. and Yamamoto, A. (1970) Lipids 5, 494–496
19 Renkonen, O., Kosunen, T.U. and Renkonen, O.V. (1963) Ann. Med. Exp. Biol. Fenn. 41, 375–381
20 Ott, P., Lustig, A., Brodbeck, U. and Rosenbusch, J.P. (1982) FEBS Lett. 138, 187–189
21 Sörensen, K., Gentinetta, R. and Brodbeck, U. (1982) J. Neurochem. 39, 1050–1060