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MODULATION OF ERYTHROCYTE ACETYLCHOLINESTERASE BY CARDIOLIPIN: EFFECT ON SUBUNIT COUPLING REVEALED BY IRRADIATION INACTIVATION.

Guy Beauregard, Michel Potier and Basil D. Roufogalis*
Section de Génétique Médicale, Centre de Recherche Pédiatrique, Hôpital Sainte-Justine, Université de Montréal, Montréal, Québec, Canada H3T 1C5

*Laboratory of Molecular Pharmacology, Faculty of Pharamceutical Sciences, University of British Columbia, Vancouver, B.C., Canada V6T 1W5

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SUMMARY: The functional molecular weights of two kinetically distinct forms of bovine erythrocyte acetylcholinesterase were determined by irradiation inactivation. Whereas both forms have similar molecular weights by hydrodynamic measurements and contain 33 molecules of cardiolipin, the functional molecular weight of form α (140,000) was found to be twice that of form β (73,000). As form β is derived from form α by treatment with high salt concentration in alkaline Ca^{2+}-chelating conditions, a procedure which is considered to disrupt the functional association of a Ca^{2+}-cardiolipin complex with the enzyme, it is suggested that cardiolipin mediates the energy transfer between enzyme subunits, thereby modulating the kinetic properties of the lipoprotein.

INTRODUCTION

We reported previously that solubilized bovine erythrocyte acetylcholinesterase (AChE), (acetylcholine hydrolase, EC. 3.1.1.7) is a lipoprotein (1) whose molecular weight of 190,000 computed from hydrodynamic properties is made up of two subunits of about 72,000 each (2) and 33 molecules of cardiolipin of about 46,000 (3). In solution bovine erythrocyte AChE exists in two states (4) with different kinetic properties (5). The properties of the enzyme in dilute aqueous salt solutions (form α) are modulated by the presence of cardiolipin, charge-linked to the protein via a divalent metal ion bridge (4). A second form (form β) exists at high ionic strength and in the presence of a calcium chelator at alkaline pH. In contrast to form α, form β shows no break in the Arrhenius plot at 20°C (1,4,5), shows no change in its substrate and inhibitor binding properties above and below 20°C (5) and may be depleted of cardiolipin by organic solvent extraction (3). The alkaline high salt concentration treatment does not remove cardiolipin from the enzyme, as both forms of AChE have

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similar molecular weights when determined from their hydrodynamic properties, R_e, $S°_{20,w}$ and $\bar{v}_2$ (3). Irradiation inactivation analysis, also called target size anlaysis, is sensitive to the functional rather than the structural size of a molecule. In this study we have used the technique to obtain information on the size of the functional AChE molecule in form α and form β. We report that cardiolipin regulates the properties of erythrocyte AChE by modulating subunit coupling.

MATERIALS AND METHODS

Materials: The irradiation inactivation was performed with a Gammacell 220 (Atomic Energy of Canada, Ottawa, Canada) equipped with a ^{60}Co source. The samples were irradiated in 1.5 ml Eppendorf plastic capped tubes. Bovine erythrocyte AChE and bovine serum albumin (Cohn fraction V) were from Sigma Chemical Co.(St. Louis, MO.). Beef liver catalase was from Pharmacia (Montreal, Canada) and beef liver β-glucuronidase was from Worthington (Freehold, N.J.). Sodium (4-methylumbelliferyl-α-D-N-acetylneuraminate) was synthesized in one of our laboratories (6). Neuraminidase was obtained by published methods (7) from male Sprague Dawley rat liver cytosolic fraction.

Molecular weight determinations: Aliquots (0.1 ml) of control enzymes were lyophilyzed in the following solutions: catalase, 0.1 mg/ml of water in the presence of 1 mg/ml bovine serum albumin; rat liver cytosolic fraction, 10% w/v in 0.25 M sucrose with or without dialysis against deionized water; β-glucuronidase, 4 mg/ml with dialysis against deionized water. The tubes were quickly capped after the contents were flushed gently with nitrogen. During the course of irradiation, the tubes were immersed upside down in a sand bath, leaving the residue above the sand surface. The sand bath temperature remained constant at 26° ± 2°C without any temperature control in the apparatus, or it was maintained at 7°C ± 5°C by immersing the sand bath in crushed ice. Appropriate controls were performed in the same conditions, except for the variation in irradiation exposure. The Gammacell 220 was calibrated in our conditions with standard enzymes. The relationship between molecular weight and dose (empirical equation) is: $M = 6.4 \times 10^5/D_{37}$, where D_{37} is the dose (in Megarad) at which 37% of the original activity remains. The molecular weight found by this method corresponds to that of the functional molecule, usually the minimal functional subunit complex (8). To eliminate secondary inactivation phenomena by the milieu, this method should be used on dry samples in the absence of oxygen (8).

Preparation of AChE samples for irradiation: Commercial AChE contains endogenous sodium phosphate, pH 7.6. To obtain form α, AChE was dissolved in deionized water (2.74 mg/ml) and dialyzed against deionized water at 4°C overnight prior to lyophilization. Form β was obtained either by dissolving AChE in a concentrated form (100 mg/ml of water) or by treating a 27.4 mg/ml water solution by dialysis against 1 M $NaHCO_3$, as previously described (4). In all three cases 0.274 mg original weight aliquots were lyophilyzed (form α: 0.1 ml; form β: 2.74 µl and 10 µl, respectively).

Enzyme assays: Irradiated or control enzyme powders were taken up in 0.1 ml of water. Catalase was measured according to (9) using a Pye Unicam

Spectrophotometer SP1800; β-glucuronidase according to (10) and neuraminidase as described in (6), except that a 0.1 M cacodylate buffer, pH 6.0 was used. AChE was measured with 5 µl enzyme aliquots by a colorimetric method (11), as modified in (12).

RESULTS

We determined that the Gammacell 220 (at 5,150 Ci of ^{60}Co) had a dose rate of 0.64 Mrad/h. This was determined by comparing the time required to decrease the activity of control enzymes to 37% of their original value (D_{37}) to previously reported values, as follows: catalase (D_{37} = 2.78 Mrad (13)), 0.65 Mrad/h; β-glucuronidase (D_{37} = 7.9 Mrad (14)), 0.63 Mrad/h; rat liver cytosolic neuraminidase (M = 55,000, D_{37} = 11.67 Mrad (M. Potier, unpublished result), 0.64 Mrad/h). The dose rate did not change by more than 3.6% during the time we performed the experiments and was corrected when necessary.

The irradiation inactivation of AChE is shown in Fig. 1, where the log remaining activity (%) is plotted against irradiation dose. Irradiation performed above (26°C) and below (7°C) the temperature of the break in the Arrhenius plot were not significantly different and the data was pooled to calculate D_{37} values. Each experimental point represents at least triplicate experiments, and in each experiment each tube was assayed at least in triplicate and usually in quadruplicate.

Form β displays curvature at the highest doses (longest times of exposure to ^{60}Co). The molecular weight calculated from the dose range between 0 and 5 Mrad was 73,000 (D_{37} = 8.8 Mrad). This compares very well with the value of 72,000 already obtained by Levinson and Ellory (15) in similar experimental conditions, except for the dose rate. Form α of AChE has a molecular weight of 140,000, almost twice the value obtained for form β.

DISCUSSION

The molecular weight of an enzyme determined by irradiation inactivation reflects the functional rather than the structural entity of the molecule (8).

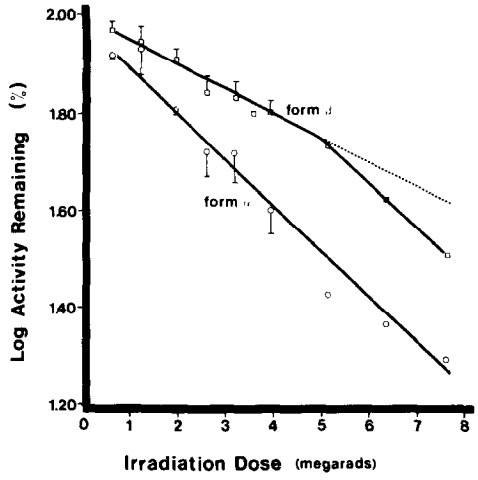

Fig. 1. Dependence of AChE activity on the dose of irradiation exposure. Form β data (□) represents four experiments on a 100 mg/ml protein solution and one experiment after treatment of form α (27.4 mg/ml) with 1 M $NaHCO_3$ (see Methods). Three runs were made at about 7°C and two at about 26°C. Form α data (O) represent a 2.74 mg/ml protein solution dialyzed against water prior to irradiation (see Materials and Methods). Two experiments were made at about 7°C and one at about 26°C; bars when present represent standard error of the mean of usually five or sometimes three results.

We found in the present irradiation inactivation study that two kinetically distinct forms of bovine erythrocyte AChE differ in the size of their functional molecular entities. The functional molecular weight of form α is twice that of form β, despite the fact that both forms contain 33 molecules of cardiolipin (3,4) and have similar molecular weights from hydrodynamic measurements. The long irradiation period necessitated by the low dose rate delivered by the Gammacell 220 used for this study might be responsible for the curvature observed for inactivation of form β of AChE (Fig. 1), although a very slow conversion of form β to form α has been observed in solution (4).

Erythrocyte AChE is known to be a structural dimer of subunits of molecular weight around 72,000 (2,17,18). Whereas both forms α and β of bovine erythrocyte AChE are dimers, they differ in the nature of their association

with cardiolipin; it was considered that in form β a salt bridge between the enzyme and the Ca^{2+}-cardiolipin complex was disrupted by the high salt treatment in alkaline Ca^{2+}-chelating conditions, while a hydrophobic interaction between cardiolipin and the enzyme remained (and was possibly strengthened) after this treatment (4). The Ca^{2+}-cardiolipin bridge appears to allow energy transfer (from ^{60}Co radiation) between the two subunits in form α (8); thus the functional protein molecular weight of form α represents the dimer (140,000). In form β of AChE the two subunits are functionally uncoupled from each other, probably separated by burried cardiolipin in the Na^+ form (14), with a functional molecular weight of 73,000.

The nature of the 'looser' association of protein subunits in form β, a functional monomer, remains unclear (Fig. 2), but the subunits may be held together by disulfide linkages (17) or hydrophobic interactions. As the active sites of forms α and β exhibit different thermodynamics of binding of substrate and inhibitors (5), it appears that coupling between subunits influences the conformation of the active site. The present results also show that the changes in activation energy of V_{max}/K_m with form α at temperatures above and below 20°C (1,3,4,5) are not caused by changes in subunit coupling, as irradiation inactivation proceeds similarly at 26°C and 7°C. Irradiation

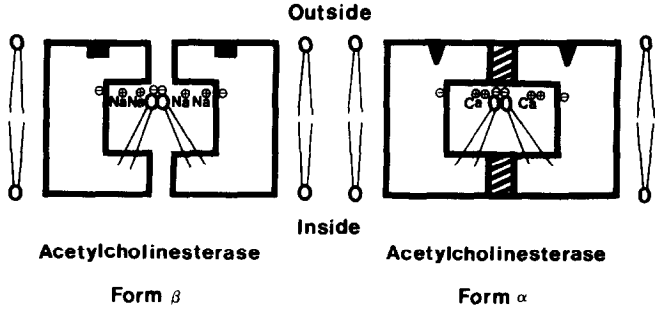

Fig. 2. Models of AChE in the erythrocyte membrane, showing the active sites (dark areas) directed outwards (18,19). 'Hair pins' represent phospholipids and double 'hair pins' represent cardiolipin burried between the two subunits (1,4). The striped areas represent subunit couplings.

inactivation analysis has shown that cardiolipin regulates the subunit coupling of bovine erythrocyte AChE, which in turn affects its kinetic properties.

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