SOME DIFFERENCES BETWEEN SOLUBLE AND MEMBRANE-BOUND ACETYLCHOLINESTERASE FROM *ELECTROPHORUS ELECTRICUS*

B. ROBAIRE and G. KATO

Department of Pharmacology and Therapeutics, and the Department of Research in Anaesthesia, McIntyre Medical Building, McGill University 3655 Drummond Street, Montreal 109, Quebec, Canada

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1. Introduction

In the electric eel, *Electrophorus electricus*, acetylcholinesterase (Acetylcholine acetyl-hydrolase EC 3.1.1.7) activity is associated with the membrane structure of the electroplaque [1, 2]. Acetylcholinesterase (AChE) has most commonly been solubilized from membranes of *Electrophorus electricus* by treating the electric organ with toluene for periods of time ranging from six weeks [3] to two years [4] or with detergent [4]; the physicho-chemical and kinetic properties of different soluble forms of the enzyme have recently been thoroughly investigated [4, 5]. However, there have been very few studies on the properties of the membrane-bound enzyme or comparative studies of membrane-bound and solubilized AChE obtained from the same source.

We have investigated this problem and in this letter we report that a crude membrane preparation obtained from the electric organ of *Electrophorus electricus* releases about 50% of its total AChE activity into solution when stored for two days at 4°C in a salt solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, pH 7.20. This solubilized enzyme has a two-fold greater apparent affinity (K_M) for the substrate acetylcholine iodide (AChI) and exhibits greater inhibition by excess substrate than does the membrane-bound enzyme.

2. Materials and methods

2.1. *Preparation of membranes from the electric organ of Electrophorus electricus*

Selections of 2–5 cm were cut from the rostral end of the eel [6]. The electric organ was isolated and cleaned of connective tissue, washed in a medium containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$, pH 7.20, blotted and weighed.

The tissue was homogenized for 15 sec in the salt medium (4 ml/g tissue) in an Omni Mixer type OM (Ivan Sorvall Incorporated). The brei was then homogenized at 5700 rev/min in a Potter-Elvehjem homogenizer by 10 up and down strokes of the pestle. The homogenate was filtered through a Chrom-a-cord mesh (Travenol Laboratories) and then through a stainless steel sieve with openings of 96 microns (Newark Wire Company). The filtrate was then re-homogenized in a Potter-Elvehjem homogenizer as above and then by three strokes of the Ten Broeck tissue grinder; this was then centrifuged (20 000 *g* for 30 min) in a Sorvall RC-2B refrigerated centrifuge. The pellet was resuspended in the salt medium (4 ml/g of original tissue) by applying three strokes of the Ten Broeck tissue grinder. This procedure was repeated three times. The final pellet was resuspended by gently homogenizing it with three strokes of a Ten Broeck tissue grinder in a volume of salt solution equal to that of the original weight of the tissue.

2.2. *Acetylcholinesterase assay*

Acetylcholinesterase was assayed with a pH stat assembly (Radiometer corporation). The substrate used was acetylcholine iodide (AChI) (Sigma Chemical Company). The assays were done at 30°C under a nitrogen atmosphere in a solution containing 180 mM NaCl, 5 mM KCl, 6 mM $CaCl_2$, 1.5 mM $MgCl_2$ and 2.0 mM phosphate buffer, pH 7.20. The acetic acid formed was neutralized by 0.010 N NaOH.

Table 1
Distribution of AChE activity in fractions prepared from the electric organ of *Electrophorus electricus.*

Fraction	Rate of hydrolysis of AChI*
Low-speed supernatant of homogenate-S_1 (20 000 *g* 30 min)	5.16** ± 0.04†
High-speed supernatant of S_1-S_2 (100 000 *g* 2 hr)	1.77 ± 0.05
Low-speed pellet of homogenate-P_1 (20 000 *g* 30 min)	13.10 ± 0.19
First wash of P_1 with salt medium (20 000 *g* 30 min)	1.51 ± 0.05
Second wash of P_1 with salt medium (20 000 *g* 30 min)	0.12 ± 0.01
Third wash of P_1 with salt medium (20 000 *g* 30 min)	0.07 ± 0.01

*The rate is expressed as μmoles of acid formed/min/g wet weight tissue.
**All rates were measured at 2 mM AChI in the salt medium described in the method section.
†Standard Error.

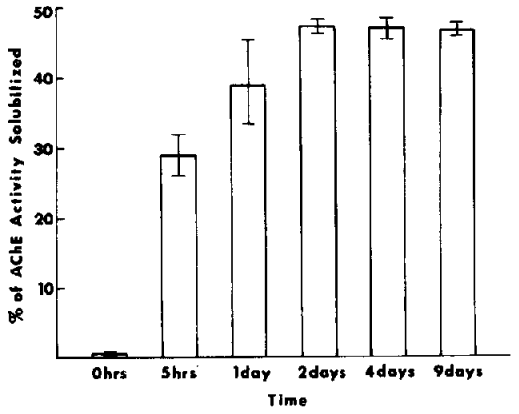

Fig. 1. Time course of solubilization of AChE from membranes of the electric organ of *Electrophorus electricus.* Each bar represents the mean ± one S.E. Rates were measured as in table 1. The time represents the time from preparation of the membranes. All rates were measured at 2 mM AChI.

3. Results

3.1. *Solubilization of AChE*

When the electric tissue is fractionated as described above, over 70% of the AChE activity is associated with the 20 000 *g*-30 min pellet; only about one third (28%) of the activity in the 10 000 *g* 30 min supernatant does not sediment at 100 000 *g* 2 hr. After washing the 20 000 *g* pellet three times with salt medium, less than 1% of the total activity of the pellet remains in the supernatant of the last wash (table 1).

Membranes were prepared as described above and stored in the salt medium at 4°C. Fig. 1 shows the percent enzyme activity solubilized from these membranes with time. AChE was assayed at 2 mM AChI concentration unless otherwise specified. One ml aliquots of a membrane preparation that had been stored in the cold (4°C) were removed at the time intervals indicated in fig. 1. Membrane-bound enzyme was recovered by centrifuging the aliquot at 20 000 *g* for 30 min and resuspending the pellet in 1 ml of the salt solution. Solubilized enzyme was recovered by centrifuging the 20 000 *g* for 30 min supernatant at 100 000 *g* for 2 hr and collecting that supernatant. The percent activity solubilized expressed as a percentage of the total enzyme activity, membrane plus solubilized enzyme, increases to 50% in 2 days and remains constant for at least another 7 days. Resuspended membranes do not release any more activity if they are stored under similar conditions, for an extended period of time (up to 7 days). The total enzyme activity diminishes by 25.0 ± 2.1% over a 10-day period.

3.2. *Comparison of kinetic parameters of membrane-bound and solubilized enzyme*

An enzyme that does not exhibit sigmoidal kinetics can be described by determining its apparent Henri-Michaelis constant (K_M) and its maximum velocity (V_{max}) [7]. AChE is such an enzyme, however, it is inhibited by excess substrate and since there is no adequate parameter to quantitate this aspect of its kinetics, we have defined the term percent substrate inhibition (%SI) as the rate of hydrolysis at 2 mM AChI, where the maximum rate is measured, minus the rate of hydrolysis at 20 mM AChI divided by the rate of hydrolysis at 2 mM AChI, $\%\,SI = (R_2 - R_{20})/R_2$, where, R is the rate of hydrolysis of AChI in μmoles of acid formed/min/g wet weight tissue and the subscripts 2 and 20 are the substrate concentrations in mM. K_M and V_{max} are determined by the method of fitting, by computer analysis, the data to a rectangular hyperbola, ignoring the points above the maximum velocity as Cleland [8] has described.

Table 2 shows the K_M's and the % SI of the solu-

Table 2
K_M and % substrate inhibition (SI) in solubilized and membrane-bound AChE

	K_M (μM AChI)	% SI
Membrane-bound AChE	449 ± 77*	16 ± 4
Solubilized AChE	230 ± 53	36 ± 5

*Standard errors.

bilized and membrane-bound enzymes. The K_M is about twice and the % SI about half as great in the membrane-bound enzyme, as in the solubilized form of AChE.

Fig. 2 shows that the % SI, measured at various time intervals from the time the membranes are first prepared stays the same in the membrane-bound enzyme (about 15%) as well as in the solubilized enzyme (about 36%).

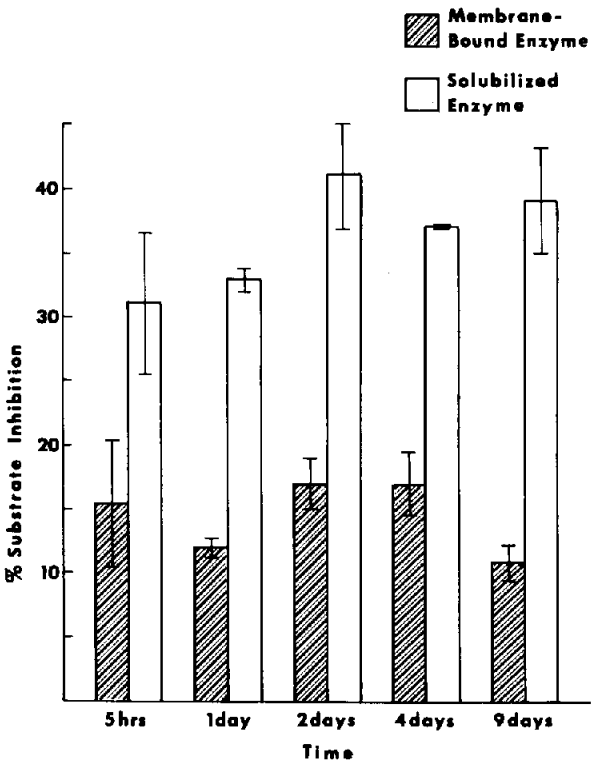

Fig. 2. Percent substrate inhibition as a function of time. The term percent substrate inhibition is defined in the text. Time refers to the time of storage in cold (4°C) from the time of preparation of the membranes.

4. Discussion

Nearly 50% of the enzyme activity that is associated with a membrane preparation from *Electrophorus electricus* (20 000 *g* 30 min pellet) at the time of its preparation is found in a soluble fraction (100 000 *g* 2 hr supernatant) after standing at 4°C for 2 days while most of the remaining activity is in the membrane fraction. This can be explained by either of the following two hypotheses: 1) one portion of the molecules having AChE activity is an integral part of the membrane structure while the other is a peripheral component, or 2) all of the AChE molecules are part of a peripheral protein pool [9] but the ionic strength, pH and temperature conditions in our experiments are such that only half of the enzyme activity is solubilized. Silman and Karlin [10] have shown that pre-incubation of the membranes with 1 M NaCl solubilizes over 90% of the AChE activity. Their data would tend to support the hypothesis of a single pool of peripheral AChE molecules.

The decrease in K_M and increase in the % SI of the solubilized enzyme compared to its membrane-bound state can be explained by assuming that some change in the shape of the catalytic site has occurred upon release of the enzyme from a membrane matrix.

Bar-Eli et al. [11], Pennington et al. [12], and Silman et al. [1], and others [13] have shown that enzymes immobilized by some matrix have different properties from enzymes free in solution. Bar-Eli and Katchalski [11] have shown that when trypsin is bound to an insoluble polyelectrolyte, the enzyme's K_M is much greater than when it is free in solution. The data of Pennington et al. [12] indicate that AChE bound to an artificial matrix is more resistant to longer exposure at 60°C than the free enzyme. Silman and Karlin [10] have established that unlike soluble-AchE, membrane-AChE requires the presence of a low concentration of buffer (e.g., 2 mM phosphate) to exhibit a bell-shaped response to changes in pH. In light of these observations, the different kinetic behavior of the two states of the enzyme is most easily explained by assuming that solubilization induces a change in

the tertiary structure of the active site of the enzyme.

We intend to further study the nature of the solubilization phenomenon and to determine whether inorganic cations, anticholinesterases and cholinergic drugs affect the kinetics of the free and the membrane-bound enzyme in similar or different fashion.

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