Determination of acetylcholinesterase activity by a new chemiluminescence
assay with the natural substrate

Serge BIRMAN
Département de Neurochimie, Laboratoire de Neurobiologie Cellulaire du C.N.R.S., 91190 Gif-sur-Yvette, France

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A chemiluminescence method for detecting acetylcholinesterase activity is described. It is an adaptation of the chemiluminescence assay of acetylcholine described by Israël & Lesbats [(1981) Neurochem. Int. 3, 81–90; (1981) J. Neurochem. 37, 1475–1483]. The acetylcholinesterase activity is measured by monitoring the increase in light emission produced by the accumulation of choline or by determining the amount of choline generated after a short interval. The assay is rapid and sensitive, and uses the natural substrate of the enzyme. Kinetic data obtained with this procedure for acetylcholinesterase from Torpedo and Electrophorus electric organs were comparable with those obtained by using the method of Ellman, Courtney, Andres & Featherstone [(1961) Biochem. Pharmacol. 7, 88–95]. In addition, it was shown that sodium deoxycholate totally inactivated Torpedo acetylcholinesterase but not the Electrophorus enzyme. Competitive inhibitors of acetylcholinesterase protected the enzyme from inactivation.

Assay of acetylcholinesterase (AChE; EC 3.1.1.7) activity may be carried out with analogues of acetylcholine (e.g. acetylthiocholine; Ellman et al., 1961) or radiolabelled acetylcholine (Johnson & Russel, 1975), or by titrating the acid formed by hydrolysis (pH-stat assay; Jacobson et al., 1957). More recently, a chemiluminescence procedure for measuring acetylcholine has been described (Israël & Lesbats, 1981a,b, 1982). The choline formed by hydrolysis of acetylcholine is converted into betaine and H₂O₂ by choline oxidase. The H₂O₂ generated is detected with a luminescence reaction. It was decided to attempt an adaptation of this procedure to the measurement of AChE activity. It is shown in the present paper that AChE activity can indeed be monitored, either continuously or discontinuously, with a simple chemiluminescence assay, suitable for a large range of activity. As an advantage, this method is based on the hydrolysis of the natural substrate in a buffered solution. AChE from Torpedo and from Electrophorus electric organs were compared by using this chemiluminescence procedure. Despite close structural similarity (for a review see Massoulié & Bon, 1982), their kinetic properties were different, and sodium deoxycholate was found to inactivate rapidly Torpedo AChE but not Electrophorus AChE.

Materials and methods

Torpedo marmorata AChE was either extracted by homogenizing the electric organ in 40 mM-MgCl₂/10 mM-Tris/HCl buffer, pH 7.0 (low-salt-soluble form; Bon & Massoulié, 1980), or it was contained in a fraction of isolated nerve endings (synaptosomes) purified from the electric organ as described by Israël et al. (1976) and Morel et al. (1977) (detergent-soluble form; Morel & Dreyfus, 1982). AChE from Electrophorus electricus was purchased from Boehringer and passed through Sephadex G-50 (coarse grade) (5 ml column) before use (Israël & Lesbats, 1981b).

Two methods were used for the determination of AChE activity: the colorimetric procedure of Ellman et al. (1961) and the chemiluminescence method. In the first case, portions of enzyme were added to 1 ml of 0.1 M-sodium phosphate buffer, pH 8.0, containing 0.5 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and 0.5 mM-acetylthiocholine. The increase in absorbance at 412 nm was immediately monitored at 18°C.

For the chemiluminescence assay, the reaction medium contained 1.5 units of choline oxidase (EC
Germany) ml, 10 μg of horseradish peroxidase (EC 1.11.1.7) (type II; Sigma Chemical Co.) ml and 30 μM-luminol (5-amino-1,2,3,4-tetrahydrophthalazin-1,4-dione) (Merck) in 0.1 M-sodium phosphate buffer, pH 8.0. A 0.5 ml portion of this medium was constantly stirred with a small magnet bar in a tube facing a photomultiplier. The instrument for measuring luminescence reaction was identical with the one used by Israël & Lesbats (1981a). Upon addition of acetylcholine (0.5 mM final concentration), the choline it contained led to a small and transient light emission. After the addition of a portion of the AChE sample, a linear increase in light emission was recorded, whose slope was a relative measurement of the AChE activity.

Absolute activities could be easily determined in the same conditions by using a discontinuous method. Portions of the enzyme were added to the medium in the absence of choline oxidase. After 1 min, the reaction was terminated by injection of the cholinesterase inhibitor Phospholine (echthiophate iodide)(100 μM), and choline oxidase was added. The choline generated led then to a light emission, which was immediately calibrated by injecting known amounts of choline in the medium. A blank without enzyme allowed an assessment of the amount of spontaneous hydrolysis, which was subtracted. For the determination of very diluted AChE activities, the reaction was performed at pH 7.0 for much longer periods. One enzyme unit hydrolyses 1 μmol of substrate/min at 18°C.

Results and discussion

Chemiluminescence determination of AChE activity

The reaction medium contained choline oxidase, which oxidized any generated choline to betaine and H₂O₂, luminol and peroxidase, which catalysed the luminescent detection of the H₂O₂ formed. Upon addition of AChE, a rapid and linear increase in light emission was recorded. Fig. 1(a) shows a typical light-response obtained by AChE inherently present in a synaptosomal fraction from Torpedo electric organ. When Phospholine (50 μM) was added, the response was abolished because of the rapid inactivation of the AChE activity (Fig. 1b).

With this continuous method, the increase in light-intensity that is recorded reflects the accumulation of choline in the medium. This accumulation depends on the relative rates of choline production by AChE and of choline consumption during the chemiluminescent process. Thus too much choline oxidase in the reaction mixture clearly decreased the sensitivity of the assay (results not shown). As shown in Fig. 2(a), the slope of the recorded signal was dose-dependent with respect to AChE for a large range of activity. Therefore this method is very convenient for the determination of relative amounts of AChE activity. When required, absolute measurements of activity can be readily obtained by using a simple discontinuous procedure described in the Materials and methods section.

The sensitivity of the continuous method was good and comparable with that of the colorimetric assay method of Ellman et al. (1961) (Fig. 2a). Higher sensitivity could be obtained with the discontinuous procedure, as shown in a dose-response curve obtained after 3 h of incubation (Fig. 2b). Consequently, the chemiluminescence AChE assay is simple and can be very sensitive. Moreover, it can be particularly useful in some cases when AChE activity has to be determined with the natural substrate. In addition, this method can be adapted to the continuous determination of choline acetylase activity by making use of the reversibility of this enzyme (M. Israël, personal communication). CoA in the presence of acetylcholine starts the enzymic reaction, and the choline formed is detected by the luminescence process.
Chemiluminescence assay for acetylcholinesterase

![Graph](image)

Fig. 2. Dose–response curves for AChE measured by the chemiluminescence procedures (logarithmic scale)

(a) The slope of the recorded increase in light intensity was measured and plotted for the different AChE activities. The power supply was set at 800 V. AChE prepared from Electrophorus electricus was diluted in water (1000 units/ml) and passed through Sephadex G-50 before use. It was serially diluted in 0.1 m-sodium phosphate buffer, pH 8.0. One enzyme unit hydrolyses 1 μmol of substrate/min. (b) Further dilutions of AChE activity were assayed discontinuously. AChE was incubated with acetylcholine (final concn. 0.5 mM) for 3 h at pH 7.0. Then the enzymic reaction was stopped with Phospholine (200 μM) and the choline produced was measured by the chemiluminescence method (see the Materials and methods section). Each point was determined in duplicate. A control without enzyme allowed assessment of the background due to the spontaneous hydrolysis of acetylcholine, which was subtracted. This method gives absolute amounts of activity.

**Kinetic properties of Torpedo AChE and Electrophorus AChE**

Table 1 compares kinetic data obtained with the chemiluminescence method and the colorimetric assay method of Ellman et al. (1961). Values are in good agreement; however, they are not identical, because the substrate used is not the same for the two methods. Torpedo AChE showed similar \( K_m \) values for acetylcholine (0.10–0.12 mM) when determined by the pH-stat assay (Lee et al., 1982). Electrophorus AChE hydrolyses acetylcholine more rapidly than it does acetylthiocholine, but, interestingly, the opposite is true for Torpedo AChE.

**Interaction with anionic bile salts**

Anionic bile salts do not usually denature proteins (Helenius & Simons, 1975). However, the inactivation by deoxycholate of an isoenzyme of lactate dehydrogenase, an hydrophilic enzyme, has already been reported (Lehnert & Berlet, 1979). Sodium deoxycholate (0.5%, w/v) was found to inactivate rapidly AChE activity from Torpedo electric organ (Fig. 3a). In contrast, Electrophorus AChE was not inactivated by deoxycholate, but rather slightly stimulated (Fig. 3a), suggesting that the inactivation was peculiar to Torpedo AChE and might be linked to its hydrophobic properties. However, the water-soluble form of this enzyme obtained after a treatment with Pronase, a proteolytic agent, was also inactivated by deoxycholate (results not shown), whereas it was reported not to interact with any detergent with sucrose-gradient experiments (Bon & Massoulié, 1980; Li & Bon, 1983). This observation indicates that deoxycholate binds to the catalytic and hydrophilic domain of the molecule.

Cholate also inactivated Torpedo AChE, but much more slowly than deoxycholate did: this may be explained by the lower pK_a of cholate (Helenius

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<th>Substrate</th>
<th>Acetylcholine</th>
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<tr>
<td>( K_m ) (( \mu M ))</td>
<td>( v ) (nmol/h)</td>
<td>( K_m ) (( \mu M ))</td>
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<tr>
<td>Electrophi rous AChE</td>
<td>90 ± 5</td>
<td>69.9 ± 0.3</td>
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<tr>
<td>Torpedo AChE</td>
<td>130 ± 10</td>
<td>9.2 ± 0.6</td>
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Table 1. Comparison of kinetic data of Torpedo AChE and Electrophorus AChE measured by using the chemiluminescence method and the colorimetric assay method of Ellman et al. (1961)
& Simons, 1975). Indeed, inactivation by deoxycholate was 4 times slower when the pH increased from 7.3 to 8.0. Therefore it is probably not the anionic, but the protonated, form of deoxycholate or cholate that is responsible for the inactivation.

Strictly competitive inhibitors of AChE such as compound BW 284 C51 [1,5-bis-(4-allyldimethylammoniumphenyl)pentane-3-one dibromide] or choline were found to protect Torpedo AChE from the inactivation by deoxycholate (Fig. 3b). Compound BW 284 C51, which has a smaller $K_i$ than choline, was more efficient. Thus, in the conditions described in Fig. 3(a), 33% of the initial AChE activity was recovered after 90 min of incubation in the presence of 0.5% deoxycholate and 1 mM of compound BW 284 C51. The interaction between deoxycholate and AChE inhibitors is probably not strictly competitive, because of the dissimilarity of their molecular structure. It may be proposed that the occupation of the anionic binding site of Torpedo AChE by a quaternary ammonium stabilizes an active state of the enzyme.

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Fig. 3. Time course of inactivation of Torpedo AChE by sodium deoxycholate: comparison with Electrophorus AChE and protecting effect of choline

(a) Low-salt-soluble Torpedo AChE (O and ●) and Electrophorus AChE (▲ and ▼) were incubated at 18°C in water with (O and ▲) or without (● and ▼) 0.5% sodium deoxycholate. AChE activity was determined at various times by the chemiluminescence method. (b) Protecting effect of choline. Hypo-osmotically shocked synaptosomes (passed through Sephadex G-50) were incubated at 0°C in 10 mM-Tris/HCl buffer, pH 7.7, containing 0.5% sodium deoxycholate and various concentrations of choline chloride: 0 mM (●), 1 mM (O), 5 mM (▲), and 10 mM (▼). Choline chloride was compensated by NaCl to a total of 100 mM. The maximal activity was determined for each condition just before deoxycholate was added. AChE activity was measured by the method of Ellman et al. (1961).

References


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