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

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A chemiluminescence method for determining 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, 1981*a,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, 1981*b*).

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

Abbreviation used: AChE, acetylcholinesterase.

1.1.3.17) (Wako Chemical Industries, Düsseldorf, Germany)/ml, $10\mu\text{g}$ of horseradish peroxidase (EC 1.11.1.7) (type II; Sigma Chemical Co.)/ml and $30\mu\text{M}$ -luminol (5-amino-1,2,3,4-tetrahydrophthalazine-1,4-dione) (Merck) in 0.1M-sodium phosphate buffer, pH 8.0. A 0.5ml 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 (0.5mM 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 (echothiophate iodide) ($100\mu\text{M}$), 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 $1\mu\text{mol}$ 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 H_2O_2 , luminol and peroxidase, which catalysed the luminescent detection of the H_2O_2 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 ($50\mu\text{M}$) 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

Fig. 1. Chemiluminescence estimation of the synaptosomal AChE activity

(a) When acetylcholine (final concn. 0.5mM) is added to 0.5ml of the reaction mixture containing choline oxidase, peroxidase and luminol in 0.1M-sodium phosphate buffer, pH 8.0, the choline formed by its spontaneous hydrolysis induces a small light emission, which decays rapidly. The AChE activity introduced by a few microlitres of the synaptosomal fraction leads to a rapid and linear increase in light-intensity, whose slope is a relative measure of AChE activity. (b) No linear increase in light emission is recorded when the reaction mixture contains the esterase inhibitor Phospholine ($50\mu\text{M}$) because of the rapid inactivation of the synaptosomal AChE.

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.

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 μ mol 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 μ M) 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,

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)

The Michaelis constants were graphically determined from Lineweaver-Burk plots (mean $\pm$ range of two determinations), and they are expressed in μ M. The maximal activity of an enzyme sample was determined first by the chemiluminescence method and then by the colorimetric assay. Results are means ($\pm$ S.D.) for three determinations and they are expressed in nmol of substrate hydrolysed/h. The *Torpedo* AChE sample was obtained from hypototically shocked synaptosomes passed through Sephadex G-50.

Substrate ...	Acetylcholine		Acetylthiocholine	
	K_m (μ M)	v (nmol/h)	K_m (μ M)	v (nmol/h)
<i>Electrophorus</i> AChE	90 $\pm$ 5	69.9 $\pm$ 0.3	80 $\pm$ 7	62.8 $\pm$ 3.5
<i>Torpedo</i> AChE	130 $\pm$ 10	9.2 $\pm$ 0.6	77 $\pm$ 3	14.7 $\pm$ 0.6

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

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 (○ and ●) and *Electrophorus* AChE (△ and ▲) were incubated at 18°C in water with (○ 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 10mM-Tris/HCl buffer, pH 7.7, containing 0.5% sodium deoxycholate and various concentrations of choline chloride: 0mM (●), 1mM (○), 5mM (▲) and 10mM (△). Choline chloride was compensated by NaCl to a total of 100mM. 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).

& 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 90min of incubation in the presence of 0.5% deoxycholate and 1mM 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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