Target size of neurotoxic esterase and acetylcholinesterase as determined by radiation inactivation

Clark D. CARRINGTON,* Donald J. FLUKE† and Mohamed B. ABOU-DONIA*‡

*Department of Pharmacology, Duke University Medical Center, Durham, NC 27710, and †Department of Zoology, Duke University, Durham, NC 27706, U.S.A.

The target size of neurotoxic esterase (NTE), the putative target site for the initiation of organophosphorus-compound-induced delayed neurotoxicity, and acetylcholinesterase (AChE) from hen brain were examined by determining the rate at which the activities of the esterases were destroyed by ionizing irradiation. Samples of hen brain were prepared by slowly drying a microsomal preparation under vacuum. The dried samples were then irradiated with electrons from a 1 MeV Van de Graaff generator. The doses ranged from 0 to 28 Mrad. The radiation doses were calibrated by the rate of inactivation of T₁-bacteriophage plaque induction. Following the irradiation procedure, the samples were resuspended in buffer and enzymic activity was measured. The target size of NTE from hen brain was determined to be about 105 kDa, whereas hen brain AChE was found to have a target size of about 53 kDa. The target size of NTE was found to be similar in experiments with rat brain and cat brain. In addition, commercial preparations of electric-eel electric-organ AChE and horse serum butyrylcholinesterase were found to have target sizes that were identical with each other, and also were very similar to that of AChE from hen brain.

INTRODUCTION

Although most organophosphorus pesticides are direct or indirect inhibitors of acetylcholinesterase (AChE), a few of these compounds also produce delayed neurotoxicity in certain animal species (Smith *et al.*, 1930; Abou-Donia, 1981). Neurotoxic esterase (NTE) is a protein that is postulated to be the target site for the initiation of organophosphorus-compound-induced delayed neurotoxicity (Johnson, 1982). A protein with a molecular mass of between 155 and 178 kDa has been isolated, by using either SDS/polyacrylamide-gel electrophoresis (Williams & Johnson, 1981; Carrington & Abou-Donia, 1985a) or gel column electrophoresis (Williams, 1983), that binds ³H-labelled di-isopropyl phosphorofluoridate. This protein is similar to NTE in its sensitivity to inhibitors, regional distribution in brain, aging characteristics, rate of phosphorylation by the neurotoxic compound mipatopox, and its rate of recovery *in vivo* after inhibition by di-isopropyl phosphorofluoridate. We have also detected small amounts of a 115 kDa protein with organophosphorus-compound-binding characteristics similar to those of NTE (Carrington & Abou-Donia, 1985a).

In the present study we have determined the target size of the NTE activity in the brain of the hen and of two mammalian species by measuring the sensitivity of inactivation by ionizing radiation. This method has been found to be useful for determining the molecular mass of proteins in non-purified samples (for review see Kempner & Schlegel, 1979). In addition, we determined the target size of AChE from hen brain and of commercial preparations of electric-eel AChE and horse serum butyrylcholinesterase (BuChE).

METHODS

Animals

White Leghorn hens (1.5–2 kg) were obtained from Featherdown Farms, Raleigh, NC, U.S.A. Female Long Evans rats (300–400 g) were obtained from Charles River Laboratories (Wilmington, MA, U.S.A.). Fischer 344 rats (250–350 g) were obtained from Harlan Sprague-Dawley Laboratories (Indianapolis, IN, U.S.A.). The cats were female and weighed about 2 kg.

Materials

The paraoxon and mipafox used in the NTE assay were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and the Midwest Research Institute (Kansas City, MO, U.S.A.) respectively. Electric-eel AChE (type VI-S) and horse serum BuChE (type IV-S) were obtained from Sigma Chemical Co.

Protein preparation

The protein was prepared in either 50 mM-Tris/HCl buffer, pH 8.0 (four of the eight NTE hen brain experiments, and the experiments with rat and cat brain) or 50 mM-sodium phosphate buffer, pH 7.0 (the remaining NTE experiments and the experiments with AChE and BuChE). Brain tissue was homogenized in buffer (10%, w/v) with a Polytron (Brinkmann Instruments) and centrifuged at 1000 *g* for 10 min. The supernatant was removed and spun at 100000 *g* for 10 min. The pellet was then resuspended in buffer (approx. 20%, w/v). This procedure increased the specific activity (NTE per mg of protein) 3–5-fold, and also increased the mipafox-sensitive portion of the paraoxon-insensitive phenyl valerate-

Abbreviations used: NTE, neurotoxic esterase; AChE, acetylcholinesterase; BuChE, butyrylcholinesterase; SDS, sodium dodecyl sulphate.

‡ To whom requests for reprints should be addressed.

hydrolysing activity to about 70%. The commercial AChE and BuChE preparations were dissolved in buffer containing 5 mg of egg albumin/ml. The latter was added in order to facilitate the assessment of recovery after the drying and resuspension procedure. Portions (50–100 μ l) of the homogenate were then pipetted on to a 2.5 cm $\times$ 2.5 cm square of 5 mm Mylar and dried as described previously (Fluke, 1966b).

Sample irradiation

The samples were irradiated with electrons from a 1 MeV Van de Graaff generator (model J5; High Voltage Engineering Corp., Burlington, MA, U.S.A.) as described previously (Fluke, 1966b). Groups of eight to 14 samples were irradiated with doses that ranged from 0 to 25 Mrad.

Dosimetry

A nominal radiation exposure can be calculated by measuring the surface area of the target transected by the beam of electrons and the number of electrons in the beam over the exposure period. However, a value so derived is dependent on the assumption that the collection of electrons is absolute, and is also dependent on accurate calibration of the equipment used to monitor the electrons. Consequently, we calibrated the electronic measurements by measuring the sensitivity of inactivation of T₁-bacteriophage plaque-forming ability in *Escherichia coli* B. The D₃₇ for the loss of T₁-phage activity has been determined to be 570 $\pm$ 15 krad with γ -rays or several other types of ionizing radiation (Fluke, 1966a). Since the activity of a phage sample can be accurately measured over several orders of magnitude, T₁-phage inactivation is a particularly accurate method of calibrating a radiation source.

Enzyme assays

After irradiation, the samples were resuspended in 1 ml of buffer. NTE activity was assayed by using the method of Johnson (1977). Cholinesterase activity was measured by using the method of Ellman *et al.* (1961) with either 1 mM-acetylthiocholine or 0.2 mM-butyrylthiocholine as substrate. Activities were corrected for recovery from the Mylar by expressing the velocity of the reaction as ΔA /mg of protein. Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. T₁-phage activity was assayed as described previously (Fluke, 1966a).

Calculations

The data were fitted by a non-linear repression program (SAS, 1982; NLIN procedure) to the model $A = A_0 e^{kD}$, where A is the measured activity, A_0 is the activity at the zero dose, D is the dose in rads, and k is a constant that is characteristic of the activity. The constant is equal to $1/D_{37}$, where D_{37} is the dose that inactivates 63% of the activity. It has been empirically determined that k , in rad^{-1} , is approximately equal to the relative molecular mass of the target divided by 6.4×10^{11} (Kepner & Macey, 1968). This value is close to the theoretical value calculated by Hutchinson & Pollard (1961). Standard errors and 95% confidence intervals were used to compare the estimates and were derived from the SAS analysis.

Fig. 1. Inactivation of T₁-bacteriophage-induced plaque formation as a function of irradiation with 1 MeV electrons

The electronic measurements were calibrated for the experiments with the esterases by assuming the D₃₇ of the phage to be 570 krad.

Fig. 2. Activities of NTE (●) and AChE (○) from hen brain samples as a function of irradiation with 1 MeV electrons

The D₃₇ values for NTE and AChE were calculated to be 6.1 and 12.1 Mrad respectively.

RESULTS

The data used to calibrate the 1 MeV Van de Graaff generator for the experiments reported in this paper are shown in Fig. 1. The radiation exposure as calculated from the physical calibration differed from the phage calibration by 26%.

The D₃₇ values for NTE and AChE were found to be 6.1 ± 0.3 and 12.1 ± 0.9 Mrad respectively (Fig. 2). The indicated target sizes for the two enzymes are 105 kDa and 53 kDa (Table 1). The target sizes for NTE from cat and rat brain were found similar and statistically indistinguishable from those of the chicken (Table 1). The rates of inactivation at a constant radiation dose of the commercial preparations of electric-eel AChE and horse serum BuChE were essentially identical and were very

Fig. 3. Activities of horse serum BuChE (●) and electric-eel AChE (○) as a function of irradiation with 1 MeV electrons

The difference in the lines that best fit the data is beyond the resolution of the graph. The D_{37} values for BuChE and AChE were calculated to be 10.8 and 10.9 Mrad respectively.

close to the rate of inactivation of AChE from hen brain (Fig. 3 and Table 1).

DISCUSSION

The indicated target size for NTE of 105 kDa is closer to the minor (115 kDa) SDS/polyacrylamide-gel electrophoresis band with NTE-like specificity than the major 160 kDa band. However, many proteins have a target size that is smaller than the whole molecule (Kempner & Schlegel, 1979). This is often attributed to the fact that a subunit of the enzyme may be wholly responsible for the catalytic activity measured, and may be unaffected by damage to the rest of the molecule. Similarly, if a portion of the NTE molecule is not necessary for the enzymic activity, the 160 kDa SDS/polyacrylamide-gel electrophoresis band could still correspond to NTE. One possibility is that NTE, like many other membrane-bound proteins, is glycosylated. This notion is supported by the fact that many proteins that are fast transported are glycoproteins (Rambourg & Droz, 1980; Grafstein & Forman, 1980), and that NTE is fast transported

(Carrington & Abou-Donia, 1985b). It is also noteworthy that glycoproteins are known to run more slowly than unglycosylated proteins in SDS/polyacrylamide-gel electrophoresis (Segrest *et al.*, 1971). Consequently, glycosylation of NTE could increase the molecular mass of NTE without increasing the target size, and cause overestimation of the molecular mass by SDS/polyacrylamide-gel electrophoresis. It is possible that the 115 kDa band represents the unglycosylated 160 kDa SDS/polyacrylamide-gel electrophoresis band. In any case, it does not appear that the di-isopropyl phosphorofluoridate-binding subunit is part of a larger protein that is responsible for the enzymic activity.

The rates of inactivation of AChE from hen brain and the Sigma electric-eel preparation are similar. Although chicken AChE is considered to have a higher molecular mass (90–110 kDa) than the electric-eel form of the enzyme (70–75 kDa; Bon *et al.*, 1979; Rotundo, 1984), it has been found that digestion of chicken AChE with low concentrations of trypsin yields an active enzyme with properties similar to those of the electric-eel enzyme (Allemand *et al.*, 1981). Our data also suggest a fundamental similarity between chicken and non-avian AChE enzymes.

Our estimate of the D_{37} (10.8 Mrad) for the Sigma type VI electric-eel AChE is significantly greater ($P < 0.05$) than the value of 8.2 Mrad reported by Levinson & Ellory (1974). Unless the preparations are assumed to differ, the discrepancy must be in the dosimetry. Had we relied on our physical calculation, we would have obtained a value of 8.0 Mrad. Although the dosimetry is clearly very important, there is unfortunately no information about the method used for quantifying dosages in the report by Levinson & Ellory (1974). Although the indicated target size (71 ± 4 kDa) reported by Levinson & Ellory (1974) is close to the apparent molecular mass of the catalytic subunit as determined by SDS/polyacrylamide-gel electrophoresis (75 kDa; Rosenberry & Richardson, 1977), our data suggest that the target size is somewhat smaller.

It is interesting that the rates of inactivation of AChE and BuChE are so similar when the molecular mass of BuChE is found to be higher in most species (Massoulié & Bon, 1982): the molecular mass of the catalytic subunit of BuChE has been estimated to be between 90 and 110 kDa (Lee & Harpst, 1973). Our data

Table 1. D_{37} values for NTE, AChE and BuChE from various sources

D_{37} values are given as means $\pm$ S.E.M. The target sizes are given assuming the molecular mass is equal to $6.4 \times 10^{11}/D_{37}$.

Enzyme	Source	D_{37}	Target size (kDa)	No. of observations (no. of experiments)
NTE	Hen brain	6.1 ± 0.3	105	96 (8)
	Cat brain	6.6 ± 0.7	97	28 (2)
	Rat brain			
	Long Evans	5.0 ± 0.4	129	14 (1)
AChE	Fischer 344	6.4 ± 1.1	100	14 (1)
	Hen brain	12.1 ± 0.9	53	52 (4)
	Electric eel (Sigma)	10.9 ± 0.7	59	14 (1)
BuChE	Horse serum (Sigma)	10.8 ± 0.5	59	14 (1)

suggest that there is a central component to each of the two enzymes and that these are similar to each other.

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