

COMPARISON OF BIOCHEMICAL AND PHARMACOKINETIC PROPERTIES OF NATIVE AND RECOMBINANT BOVINE ACETYLCHOLINESTERASE

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ABSTRACT

The successful demonstration of plasma-derived cholinesterases (ChEs) as organophosphorus compound (OP) scavengers is attributed to their ability to rapidly sequester a wide variety of OPs and to their long residence time in circulation. The production of large quantities of these enzymes is limited by high cost. However, the utilization of recombinant enzymes with similar biochemical and pharmacokinetic properties to those of the plasma-derived enzymes would provide a therapeutic solution. To further understand the biochemical and pharmacokinetic properties of different molecular forms of acetylcholinesterase (AChE), we cloned the mature T-subunit of bovine (Bo) brain AChE (1851 base pairs) including the bovine signal peptide sequence in exon2. The full-length cDNA clone was truncated at the C-terminus to obtain a 1733 base pair cDNA clone for the monomeric subunit of bovine brain AChE. Functional expression and secretion of the Bo AChE enzyme was achieved by transfecting CHO-K1 cells. The secreted recombinant Bo AChE proteins, tetramer and truncated monomer forms, were purified, characterized and compared with fetal bovine serum (FBS) AChE. The catalytic and inhibitory properties of the two recombinant forms were very similar to those of FBS AChE. However, differences were observed in the reactivation of OP-inhibited recombinant forms by different oximes compared to FBS AChE. As expected, the two recombinant forms displayed different pharmacokinetic profiles, which were different from the plasma-derived enzyme. These results confirm our previous suggestion that the subunit assembly makes a significant contribution to the circulatory stability of ChEs.

INTRODUCTION

The direct decontamination of OP chemical weapons over wide areas is a primary concern of military, antiterrorism units, and governments worldwide. Enzymatic degradation of OP nerve agents has been considered as a potential means of “environmentally friendly” decontamination [1]. However, practical applications of large-scale enzymatic decontamination have always been limited by the cost, the stability of the enzymes, and the mass transport limitation of substrates and products across the cell membrane [2]. Several studies in the last ten years have demonstrated that enzymes such as cholinesterases can successfully be used as scavengers of highly toxic OP nerve agents [3]. These enzymes were found to be effective bioscavengers

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against a variety of OP compounds in rodents as well as non-human primates [4]. Genetic engineering is a suitable method not only for improving bioscavenger performance by site-directed mutagenesis but also for generating large quantities of the bioscavenger. Therefore, the characterization of recombinant enzymes for their use as potential bioscavengers, decontamination, or detoxification agents against OP nerve agents (weapons of mass destruction) is paramount.

Acetylcholinesterase (E.C.3.1.1.7) is an esterase that is responsible for the hydrolysis of the neurotransmitter acetylcholine in the neuromuscular junctions and brain cholinergic synapses [5]. The enzyme is found to exist in many molecular forms in various vertebrate tissues, but is encoded by a single gene [5-8]. Since there are different molecular forms of AChE that may function in different physiological capacities in vivo, it is important to understand their kinetic, biochemical and inhibitory properties. The importance of subunit assembly and glycan structure was also shown in the clearance of AChE from the circulation of animals [13]. To further investigate the role of oligomerization on the catalytic properties as well as its role in the clearance of AChE from the circulation of mice, we cloned and expressed monomeric and tetrameric forms of recombinant (r)Bo AChE.

The cloning of AChEs from different species and their expression in various systems: mammalian, bacterial, yeast, and Baculovirus has been described previously [5,9-12]. For example, Mendelson et al. expressed recombinant Bo AChE in HEK 293 cells [10] using a clone in which the human AChE signal peptide sequence was attached to the Bo AChE mature T-subunit sequence. Here we cloned the Bo AChE signal peptide sequence with the Bo mature T-subunit sequence for the translation, expression and secretion of the rBo AChE in CHO-K1 cells. The tetrameric and monomeric forms of rBo AChE were purified by affinity chromatography on procainamide-Sepharose columns, and their kinetic, inhibitory and circulatory stability were compared with those of native FBS AChE.

EXPERIMENTAL PROCEDURES

1. Purification of Bo AChE

Recombinant Bo AChE (monomeric and tetrameric forms) were purified from serum-free culture medium (Ultraculture) using the procainamide affinity chromatography method of Ralston et al. [14].

2. Assay for AChE Activity

The activity of rBo AChE and FBS AChE was assayed by the method of Ellman et al. using 0.5 mM acetylthiocholine as the substrate [15]. The concentrations of the enzyme subunits were determined using active-site titration with DEPQ. Interactions of rBo AChE with specific inhibitors and reactivators (oximes) were conducted as previously described [17-18].

3. Sucrose gradient centrifugation

Analytical sucrose-gradient centrifugation was conducted using 5-20% sucrose gradients containing 50 mM sodium phosphate buffer, pH 8.0. Centrifugation was carried out using a SW41 Ti rotor (Beckman) at 30,000 rpm for 18 h at 4°C. Fractions of 0.2 ml were collected and assayed for AChE activity. Catalase (11S) was used as an internal sedimentation marker in all gradients.

4. SDS-PAGE analysis was conducted according to the system of Laemmli [16].

5. Pharmacokinetics

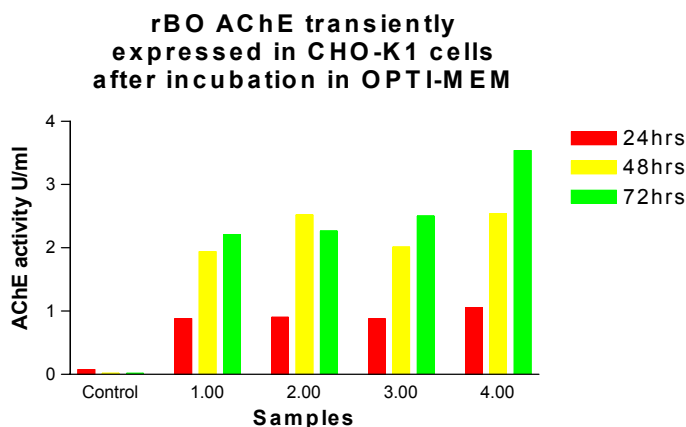
Balb/c mice were obtained from Charles River. Eighteen mice (~ 8 weeks old, 18-22 g) equal numbers of males and females were divided into 3 groups, n=6. Each group received one of the following three enzymes: (1) FBS-AChE; (2) rBo AChE tetramer; and (3) rBo AChE truncated monomer. 100 U of enzyme in a volume of 0.3 ml saline was administered to each mouse by intraperitoneal (i.p.) injection. Blood was drawn at specific time points and assayed for AChE activity.

RESULTS AND DISCUSSION

1. Expression of tetrameric and monomeric forms of rBo AChE subunit in CHO-K1 cells.

Figure 1 shows the activity levels for the transient expression of rBo AChE in CHO-K1 cells. Each sample (1 to 4) contained a different concentration of Bo AChE cDNA (from 0.9 μ g, 1.8 μ g, 2.7 μ g, 3.6 μ g, respectively). At 24h post transfection, all samples were expressing 1 U/ml of AChE activity. After 72h post transfection, sample 4 which contained 3.6 μ g of DNA yielded the highest level of AChE activity at 3.5U/ml. Stable cell lines were established from each transiently expressing CHO-K1 sample. After co-transfecting with PRAD (proline rich attachment domain) DNA (which helps with the association of the monomeric subunits into tetramers), each cell line was again selected using G-418 and stable CHO-K1 cell line isolates were established. These cells had AChE activity levels of 5 to 10 U/ml each (data not shown). Stable cell lines were also established for the expression of monomeric rBo AChE using the truncated rBo AChE cDNA. The level of Bo AChE activity in these cells reached 10-15 U/ml (data not shown).

FIGURE 1.



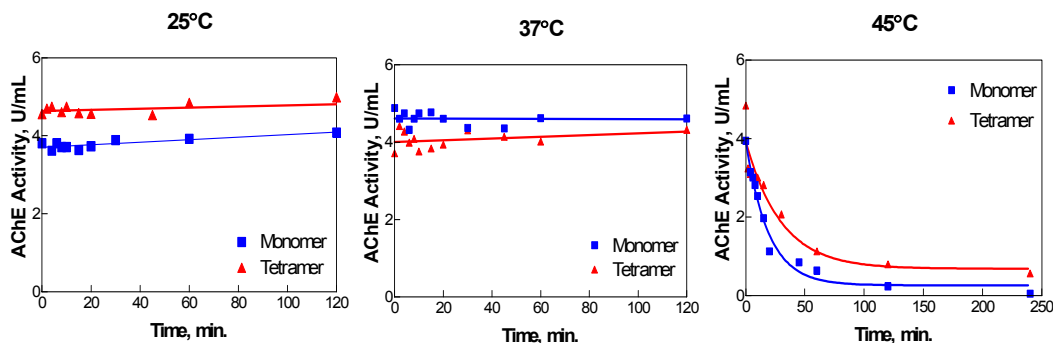
2. Characterization of recombinant tetrameric and monomeric forms of rBo AChE

The kinetic constants determined for the rBo AChE tetrameric and truncated monomeric forms are shown in Table 1. The K_m , K_{ss} , and k_{cat} values of the rBo AChE enzymes are similar to those of the native FBS AChE. Therefore, the truncated monomeric and tetrameric forms of rBo AChE have similar catalytic properties to those of the native FBS AChE.

TABLE 1. CATALYTIC PROPERTIES

	FBS AChE	rBo Tetra AChE	rBo Mono AChE
K_m (mM)	0.121	0.122	0.118
K_{ss} (mM)	4.917	5.033	4.614
k_{cat} (min ⁻¹)	1,000,000	998,000	999,000

FIGURE 2. THERMAL STABILITY STUDIES



The data shows that the activities of both forms of rBo AChE are not affected at 25°C or 37°C. However at 45°C there is a drastic decrease in activity for both recombinant forms. The recombinant truncated monomeric form denatures faster than the recombinant tetrameric form at 45°C. These studies show that the tetrameric form of the rBo AChE is more stable at higher temperatures than the truncated form.

TABLE 2. TITRATION RESULTS WITH DEPQ

	Titration Result	
FBS AChE	rBo Tetramer AChE	rBo Monomer AChE
380 U/nmole	395 U/nmole	378 U/nmole

The data shows that approximately 400 Units of each enzyme neutralizes 1nmole of DEPQ.

TABLE 3. INHIBITION STUDIES

Inhibitor	Native Tetramer K_I (μ M)	Rec. Tetramer K_I (μ M)	Rec. Monomer K_I (μ M)
Edrophonium	0.46 ± 0.03	0.5200 ± 0.04093	0.2641 ± 0.01281
Tacrine	0.023 ± 0.001	0.02036 ± 0.004260	0.01412 ± 0.002370
(-)-Huperzine A	0.00027 ± 0.00003	0.00045 ± 0.00008	0.00052 ± 0.00022
E2020	0.0029 ± 0.0002	0.001418 ± 0.0001424	0.001343 ± 0.0002395
Propidium	0.36 ± 0.18	0.3878 ± 0.1045	0.5862 ± 0.1720

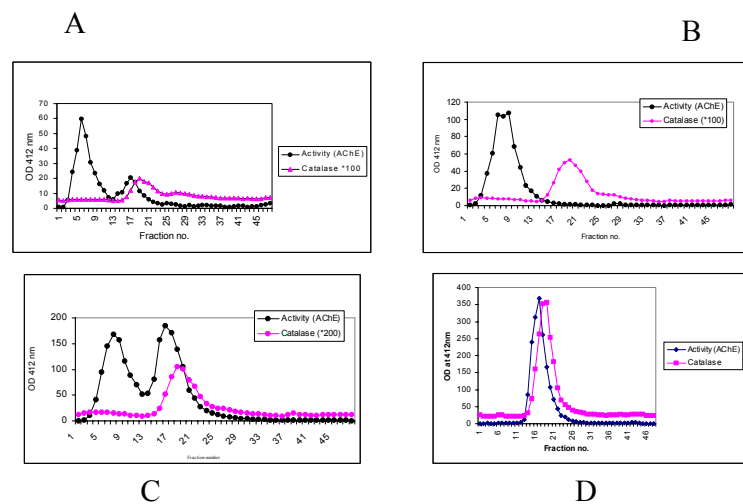
The results of inhibition studies conducted with edrophonium, tacrine, (-) huperzine A, E2020, and propidium are shown in Table 3. With the classical peripheral anionic-site inhibitor propidium the recombinant forms gave similar results as compared to the native FBS AChE. The inhibition constants for the active-site inhibitors (edrophonium, tacrine, huperzine A, and E2020) showed no significant differences between the recombinant forms and the native FBS AChE. From this data we can conclude that the recombinant tetramer is as catalytically efficient as native FBS AChE tetramer.

TABLE 4. REACTIVATION ANALYSIS

OXIMES	FBS AChE $k_r \text{ mM}^{-1} \cdot \text{Min}^{-1}$	rBo Tetra AChE $k_r \text{ mM}^{-1} \cdot \text{Min}^{-1}$	rBo Mono AChE $k_r \text{ mM}^{-1} \cdot \text{Min}^{-1}$
2-PAM	3.214	1.182	0.367
TMB ₄	21.895	9.763	3.106
Tox	14.099	9.314	2.727

The three oximes used reactivate DEPQ-inhibited FBS AChE three times faster than either DEPQ-inhibited rBo AChE form (3-fold differences were observed in the reactivation of DEPQ inhibited recombinant forms by different oximes). A 2-fold difference in the reactivation rate constant was observed between the native FBS AChE and the tetrameric form of rBo AChE.

FIGURE 3. SUCROSE GRADIENTS



The data shows that without co-transfection with PRAD DNA, the 10 % of rBo AChE is expressed in tetrameric form and 90% in monomeric form (panel A). As expected, PRAD has no effect on the expression of rBo AChE monomer which is 100% monomeric (panel B). Co-transfection of the PRAD DNA with the rBo AChE cDNA yields 50 to 60% of the enzyme in tetrameric form (panel C). Incubation of the rBo AChE co-transfected protein with synthesized PRAD peptide shown in panel A yields 100% tetramers (panel D).

Non-denaturing PAGE shows that cells co-transfected with the full-length Bo AChE cDNA and PRAD express a mixture of tetramers and monomers (lane 2). If additional synthetic PRAD peptide is added to this mixture of purified recombinant enzyme the yield of tetramers becomes 100% (lane 3). High molecular weight aggregates of the rBo AChE are also seen above the position of the tetramer in both lanes 2 and 3. The native FBS AChE appears to be 100% tetramers as seen in lane 1.

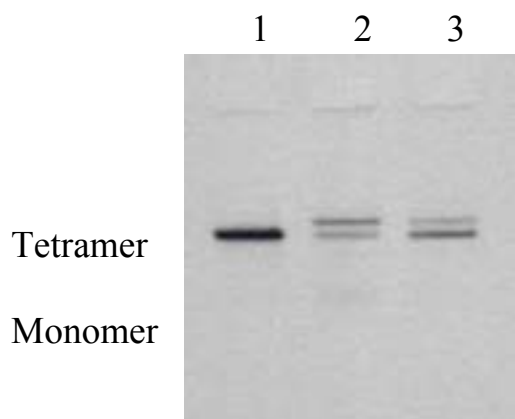


FIGURE 4. 10% Non-denaturing PAGE gel stained with Karnovsky and root stain.

3. Pharmacokinetics

To study the pharmacokinetic behavior of FBS AChE and rBo AChEs, the purified enzymes (100 U of each enzyme) were administered into mice by ip injections and the enzyme levels were determined at different time intervals. The time course profiles clearly show that native FBS AChE that is tetrameric and fully glycosylated remains the longest in the bloodstream. Of the two rBo AChEs, monomeric rBo AChE enzyme peaks higher initially than the tetrameric form, but the rBo AChE tetrameric form remains seven times longer in the bloodstream than the monomeric form.

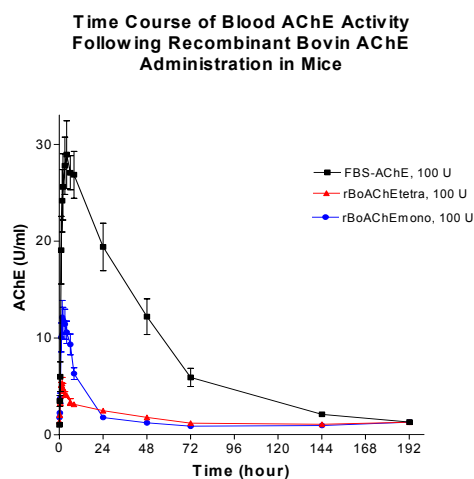


FIGURE 5. Shows the time course for the enzymes in the mouse circulatory system. The mean residence time for the native FBS AChE enzyme was 43 h, for the rBo AChE tetramer it was 20 h, and for the rBo AChE truncated monomer it was 3 h.

CONCLUSIONS

The expression and secretion of functional rBo AChE tetrameric and monomeric forms were achieved. The catalytic properties of the two forms of rBo AChE were very similar to those of native FBS AChE. The rBo AChE tetramer reactivates faster than the rBo AChE monomeric form with each oxime, showing the importance of oligomerization in reactivation. The two forms

of rBo AChE displayed different pharmacokinetic profiles that were also different from the plasma-derived enzyme. The mean residence time for the native tetramer of FBS AChE was 2 times greater than that of the rBo AChE tetrameric form. This data shows the importance of oligomerization, as well as, the need for a fully glycosylated protein. The rBo AChE enzyme shows similar kinetic and biochemical properties to those of the native FBS AChE. Therefore, enhanced modifications of the recombinant enzyme are needed before it could be used to substitute for the native form of the enzyme.

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REFERENCES

- [1] Mulchandani A, Kaneva I, and Chen W. *Biotechnology & Bioengineering* 1999; 63:216-23.
- [2] Cheng TC, Rastogi VK, DeFrank JJ, and Sawiris GP. *Annals of the New York Academy of Sciences* 1998; 864:253-8
- [3] Doctor BP, Blick DW, Caranto G, Castro CA, Gentry MK, Larrison R, Maxwell DM, Murphy MR, Schutz M, Waibel K, et al. *Chem Biol Interact* 1993; 87:285-93.
- [4] Doctor BP, et al., *Chemical Warfare Agents: Toxicity at Low Levels*. New York: CRC Press, 2001. In: Somani SM and Romano JA, Jr. Editors, pp. 191-214.
- [5] Massoulie J, Pezzementi L, Bon S, Krejci E, and Vallette F-M. *Prog Neurobiol* 1993; 41:31-91.
- [6] Li Y, Camp S, Rachinsky TL, Getman D, and Taylor P. *J Biol Chem* 1991; 266:23083-23090.
- [7] Taylor P. *J Biol Chem* 1991; 266:4025-4028.
- [8] Taylor P, Li Y, Camp S, Rachinsky TL, Ekstrom T, Getman D, Fuentes ME, Vellom DC, and Radic Z. *Chem Biol Interact* 1993; 87:199-207.
- [9] Rachinsky TL, Camp S, Li Y, Ekstrom TJ, Newton M, and Taylor P. *Neuron* 1990; 5:317-27.
- [10] Mendelson I, Kronman C, Ariel N, Shafferman A, and Velan B. *Biochem J* 1998; 334:251-259.
- [11] Heim J, Schmidt-Dannert C, Atomi H, and Schmid RD. *Biochim Biophys Acta* 1998; 1396:306-319.
- [12] Soreq H, Ben-Aziz R, Prody CA, Seidman S, Gnatt A, Neville L, Lieman-Hurwitz J, Lev-Lehman E, Ginzberg D, Lapidot-Lifson Y, and Zakut H. *Proc Natl Acad Sci USA* 1990; 87:9688-9692.
- [13] Kronman C, Velan A, Marcus D, Ordentlich, Reuveny S, and Shafferman A. *Biochem. J.* 1995; 311:959-967.
- [14] Ralston JS, Rush RS, Doctor BP, and Wolfe AD. *J Biol Chem* 1985; 260:4312-4318.
- [15] Ellman GL, Courtney KD, Andres, Jr.V, and Featherstone RM. *Biochem Pharmacol* 1961; 7:88-95.
- [16] Laemmli UK. *Nature* 1970; 227:680-5.
- [17] Luo C, Saxena A, Ashani Y, Leader H, Radic' Z, Taylor P, and Doctor BP. *Chem Biol Interact* 1999; 119-120:129-135.
- [18] Maxwell DM, Saxena A, Gordon RK, and Doctor BP. *Chem Biol Interact* 1999; 119-120:419-428.