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DNA sequence of butyrylcholinesterase from the rat: expression of the protein and characterization of the properties of rat butyrylcholinesterase☆

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Abstract

The rat is the model animal for toxicity studies. Butyrylcholinesterase (BChE), being sensitive to inhibition by some organophosphorus and carbamate pesticides, is a biomarker of toxic exposure. The goal of this work was to characterize the purified rat BChE enzyme. The cDNA sequence showed eight amino acid differences between the active site gorge of rat and human BChE, six clustered around the acyl binding pocket and two below the active site serine. A prominent difference in rat was the substitution of arginine for leucine at position 286 in the acyl pocket. Wild-type rat BChE, the mutant R286L, wild-type human BChE, and the mutant L286R were expressed in CHO cells and purified. Arg286 was found responsible for the resistance of rat BChE to inhibition by Triton X-100. Replacement of Arg286 with leucine caused the affinity for Triton X-100 to increase 20-fold, making it as sensitive as human BChE to inhibition by Triton X-100. Wild-type rat BChE had an 8- to 9-fold higher K_m for the positively charged substrates butyrylthiocholine, acetylthiocholine, propionylthiocholine, benzoylcholine, and cocaine compared with wild-type human BChE. Wild-type rat BChE catalyzed turnover 2- to 7-fold more rapidly than human BChE, showing the highest turnover with propionylthiocholine (201,000 min^{-1}). Human BChE does not reactivate spontaneously after inhibition by echothiophate, but rat BChE reactivates with a half-life of 4.3 hr. Human serum contains 5 mg/L of BChE and 0.01 mg/L of AChE. Male rat serum contains 0.2 mg/L of BChE and approximately 0.2 mg/L of AChE.

Keywords: Steady-state kinetics; Triton X-100; Cocaine; Phosphorylation reactivation

1. Introduction

The rat is the most commonly used animal for toxicity testing. The results from toxicity tests in rats are then extrapolated to predict toxicity in humans. The enzyme components of the blood have a major influence on the potency of toxic agents. The major detoxifying enzyme in rat blood is carboxylesterase (EC 3.1.1.1, carboxylic-ester hydrolase), but human blood contains no carboxylesterase. In humans, serum BChE (EC 3.1.1.8, acylcholine acylhydrolase) plays a role in the detoxication of cocaine, organophosphorus pesticides, carbamate pesticides, and chemical warfare agents [1,2].

Human BChE has been studied extensively [3,4]; however, relatively little is known about rat BChE. From what is known, it is clear that the reactivity of rat BChE differs from that of human BChE. For example: (a) human BChE shows a preference for the hydrolysis of BTC over PTC, while rat BChE shows the opposite preference [5,6]; (b) with 1 mM BTC as substrate, the reaction of human BChE is inhibited 39% by 0.025% Triton X-100, while rat BChE appears to be unaffected [7]; (c) reactivation of diethylphosphate-inhibited rat BChE proceeds 100-fold faster than diethylphosphate-inhibited human BChE [8]; and (d) rat serum has very little BChE activity compared with that in human serum [7], but whether the low activity was due to less BChE protein or to a BChE with intrinsically lower activity was unknown.

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Abbreviations: BChE, butyrylcholinesterase enzyme; *BCHE*, butyrylcholinesterase gene; TNB, 5-thio-2-nitrobenzoic acid; ONPB, *o*-nitrophenylbutyrate; PCR, polymerase chain reaction; DTNB, 5,5′-dithio-bis(2-nitrobenzoic) acid; BzCh, benzoylcholine; BTC, butyrylthiocholine; ECHO, echothiophate; ATC, acetylthiocholine; PTC, propionylthiocholine; AChE, acetylcholinesterase enzyme.

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These observations prompted us to investigate the kinetic properties of rat BChE in detail. Since BChE is present at low levels in rat blood, we chose to use recombinant BChE for our studies. Therefore, we determined the nucleotide sequence for the rat BCHE cDNA (and consequently the amino acid sequence of the protein), and expressed it in cell culture. We found that the structure of the active site of rat BChE is substantially different from the active site of human BChE. Most of the differences are localized to the acyl-binding pocket. The most prominent of these differences was an arginine at position-286 in rat BChE, where a leucine is found in the human. We tested the importance of this difference on the kinetic properties of rat BChE by making mutants of both rat (R286L) and human (L286R) BChE. We found that the presence of an arginine at position-286 conferred resistance to Triton X-100 inhibition onto rat BChE. However, Arg286 alone does not account for the substrate selectivity of rat BChE, nor is it responsible for the increased rate of reactivation found with diethylphosphate-inhibited rat BChE.

2. Methods and materials

2.1. DNA amplification and sequencing

PCR amplification was performed on rat heart Marathon-Ready cDNA (Sprague–Dawley rat) and on rat genomic DNA (Sprague–Dawley rat) from Clontech, using a panel of oligonucleotide primers directed against human BChE sequences. After obtaining partial rat cDNA sequences, new primers specific for rat BChE were made for further DNA amplification. *Taq* polymerase (Promega), *HotStarTaq* polymerase (Qiagen), or Platinum *Pfx* polymerase (Gibco BRL) were used for PCR. All of the rat BCHE cDNA sequences were amplified and sequenced at least twice, to identify potential errors caused by polymerases.

2.2. Mutagenesis and expression of rat and human BChE

Site-directed mutagenesis to make human L286R and rat R286L was performed with the PCR and *Pfu* DNA polymerase (Stratagene). Expression from CHO-KI cells was performed essentially as previously described [9].

2.3. Purification of BChE

Recombinant proteins of wild-type rat BChE, wild-type human BChE, and mutants R286L (rat) and L286R (human) were purified from serum-free culture medium using the affinity chromatography method of Lockridge [4], followed by ion exchange chromatography on DE52 essentially as described [9]. The concentration of the recombinant BChE proteins was determined by titration with chlorpyrifos-oxon [diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphate, from ChemService Inc.] as proposed by Amitai *et al.* [10].

2.4. Steady-state kinetics

Substrate turnover was followed spectrophotometrically in a temperature-controlled, single-beam Gilford spectrophotometer, which was interfaced via a MacLab data recorder (ADInstruments) to a Macintosh computer. Initial rates for all reactions were measured in 0.1 M potassium phosphate buffer, pH 7.0, at 25°. All rates were corrected for spontaneous degradation of substrates and reagents. Unless otherwise indicated, all chemicals were obtained from the Sigma Chemical Co.

The hydrolysis of thioester substrates, ATC, PTC, or BTC, was measured by the method of Ellman *et al.* [11] as previously described [9]. Hydrolysis of the other substrates was followed by established methods: BzCh [9]; ONPB [12]; (+)-cocaine (National Institute on Drug Abuse Research Resources Drug Supply System) [13]; and (−)-cocaine [14].

Inhibition of BChE turnover by Triton X-100, Tween 20 (Fisher), and Brij 96 V (Fluka) was determined in the presence of BTC.

2.5. Analysis of steady-state turnover data

Steady-state data (velocity versus substrate concentration) for ATC, PTC, BTC, and BzCh were fit to an equation for excess substrate activation/inhibition [15], Eq. (1):

$$k_{app} = \frac{k_{cat} + \dfrac{bk_{cat}[S]}{K_{ss}}}{1 + \left(\dfrac{K_m}{[S]}\right)1 + \left(\dfrac{[S]}{K_{ss}}\right)} \quad (1)$$

In this equation, k_{app} is the apparent rate, in terms of moles of product per mole of BChE per minute; [S] is the concentration of substrate; k_{cat} is the turnover number (min^{-1}) when $[S] \ll K_{ss}$; K_m is the Michaelis constant; bk_{cat} is the turnover number (min^{-1}) when $[S] \gg K_{ss}$; and K_{ss} is the dissociation constant for excess substrate. The parameter b reflects the efficiency of product formation from the ternary complex (SES). When $b > 1$, there is substrate activation. When $b < 1$, there is substrate inhibition. When $b = 1$, the enzyme follows Michaelis–Menten kinetics. The k_{cat}, K_m, K_{ss}, and b values were obtained by non-linear, least-squares fitting of the apparent rate versus substrate concentration data to Eq. (1), using SigmaPlot v4.16 (Jandel Scientific). The value for bk_{cat} was obtained by multiplying k_{cat} by b.

Turnover data (velocity versus substrate concentration) for (+)-cocaine, (−)-cocaine, and ONPB were fit to the Michaelis–Menten equation [16], using a non-linear, least-squares algorithm with SigmaPlot v4.16, in order to extract K_m and k_{cat}.

2.6. Phosphorylation of BChE

Inhibition of BChE by ECHO (from Wyeth Ayerst) was performed in the absence of substrate as described by

Aldridge and Reiner [17]. BChE (0.3 to 3.0 nM) was incubated in 1,980 μL of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM DTNB and variable amounts of ECHO (0.05 to 1.0 mM for L286R and 0.0125 to 0.1 μM for wild-type rat, wild-type human, and R286L), at 25°, in a series of quartz spectrophotometer cuvettes. At intervals, 20 μL of 100 mM ATC was added to a cuvette, and the BChE activity remaining at that time was determined by the rate of product formation (ΔA_{412}/min). ATC rather than BTC was used because ATC is a better substrate for rat BChE. The time interval for incubation of BChE with ECHO ranged from 10 sec to 10 min. The apparent rate of inhibition (k_{phos}) was determined by plotting log(ΔA_{412}/min) versus incubation time. This inhibition protocol relies on the substrate (ATC) to displace non-covalently bound ECHO from the BChE. The evidence that this was accomplished successfully is that extrapolation of the semilog plots to time zero yielded the uninhibited ΔA_{412}/min in every case (see Ref. [17]). A secondary plot of k_{app} versus the ECHO concentration was linear, yielding an apparent second order rate constant for phosphorylation.

2.7. Reactivation of diethylphosphorylated BChE

BChE (60–80 nM) was inhibited by reacting with 95 nM ECHO in 20 mM potassium phosphate buffer plus 1 mM EDTA, pH 7.0, at 25° for 30 min, after which time the activity was inhibited by greater than 95%. Then, 10-μL aliquots of the inhibited BChE were diluted into 1,920 μL of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1% bovine serum albumin, in a series of quartz spectrophotometer cuvettes. The cuvettes were sealed with Parafilm and incubated at 25°. At intervals, 20 μL of 100 mM ATC and 50 μL of 20 mM DTNB were added to a cuvette, and the activity of the reactivated BChE (ΔA_{412}/min) was determined. The bovine serum albumin stabilized the BChE against spontaneous loss of activity during the extended incubation required to follow reactivation. A parallel incubation of uninhibited BChE was also made to control for spontaneous loss of activity. Reactivation rate constants were extracted by fitting the activity versus incubation time data to a single exponential expression with SigmaPlot v4.16, using a non-linear, least-squares algorithm. We are employing the term reactivation to describe the process we are following in this section, because no effort was made to separate dephosphorylation from aging.

3. Results

3.1. BCHE sequence

Sequence information from both rat cDNA and rat genomic DNA was combined to produce the nucleotide sequence of the cDNA for the rat *BCHE* gene (GenBank Accession Number AF244349). The 1,791 nucleotides, which corresponded to the mature protein-coding sequence and the signal peptide, showed 81% identity with human *BCHE* at the nucleotide level [18] and 80% identity at the amino acid sequence level [19]. Fifty-seven percent of the nucleotides in this region were AT.

Translation of the nucleotide sequence yielded a mature protein-coding sequence of 574 amino acids, and a 23 amino acid signal peptide. The rat signal peptide is five amino acids shorter than the human signal peptide [18]. At 574 residues, rat BChE was exactly the same length as human BChE, which makes numbering for the two enzymes identical.

Human BChE carries nine asparagine-linked carbohydrates, which are located at positions 17(19), 57(59), 106(108), 241(243), 256(258), 341(343), 455(457), 481(483), and 486(488) [19]. By convention, the numbering of BChE from all species is referenced to the numbering of AChE (EC 3.1.1.7, acetylcholine hydrolase) from *Torpedo californica* [20]. The *T. californica* number is given in parentheses, following the number for the enzyme of interest. Rat BChE showed the Asn-X-Thr/Ser consensus sequence for an asparagine-linked, carbohydrate attachment site at seven of these locations. Positions 17 and 256 were missing. The rat sequence showed one additional site at position 87, for a total of eight potential carbohydrate attachment sites.

The six cysteines that form interchain disulfide bridges in human BChE, i.e. 65(67), 92(94), 252(254), 263(265), 400(402), and 519(521) [21], were all conserved in rat BChE. This strongly suggests that the same structurally important disulfide bridges are present in rat BChE. In addition, a cysteine at position 571(573), which is responsible for a disulfide bridge between monomers in human BChE [21], was conserved in rat BChE. This suggests that rat BChE retains the same dimer-of-dimers quaternary structure as human BChE [22]. An additional cysteine at position 210(212) was found in the rat sequence.

By analogy with the structure of *T. californica*, there are approximately 55 residues in the active site gorge of human BChE. The essential catalytic triad, consisting of S198(200), E325(327), and H438(440), was conserved in rat BChE. Out of the remaining 52 residues, eight differences were found between rat and human BChE. A schematic representation of those changes is shown in Fig. 1. Two of the changes were located below the catalytic triad, away from the substrate binding locus. One, F398(400)I, was a relatively conservative replacement. The other, Q223(225)E, was not. This latter change introduced a potential negative charge into the vicinity of the catalytic glutamate. The remaining six differences were clustered in the acyl binding pocket and along the gorge directly above the acyl binding pocket: V288(290)I, L286(288)R, P285(287)I, T284(286)S, V280(282)L, and A277(279)K. Three of these were conservative replacements, but L286R, P285I, and A277K were not.

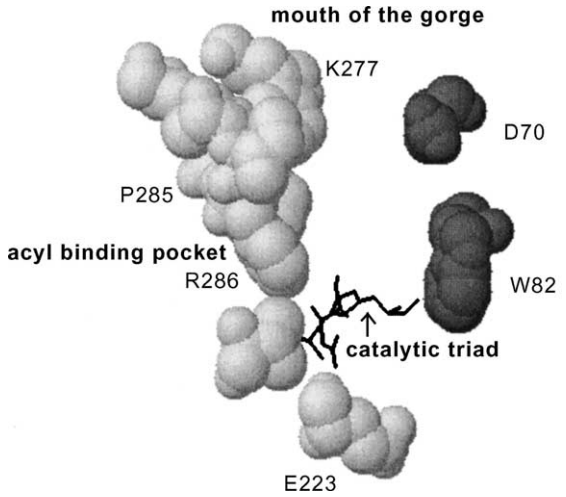

Fig. 1. Schematic representation of the amino acid differences between rat and human BChE in the active site and active site gorge. The catalytic triad is indicated by the stick structures. It is located at the bottom of a 10 Å deep gorge, the mouth of which is indicated by the residues at the top of the figure. The dark residues on the right side of the gorge are aspartate 70 (at the top) and tryptophan 82 (at the bottom). These residues are conserved in both rat and human BChE. Tryptophan 82 is generally considered to associate with the positively charged portion of cationic substrates. The acyl binding pocket, on the left side of the gorge, is generally considered to associate with the acyl end of the substrate. The light colored residues represent those amino acids that differ between rat and human BChE.

The 277 position is located at the mouth of the gorge. As such, the positive charge from the lysine in rat BChE might be expected to interact with the bulk solvent. If this is so, then this change in the rat structure might be relatively innocuous. Consistent with this interpretation is the fact that replacing A277 in human BChE with histidine had no significant effect on K_m or K_{ss} for the turnover of BTC [23].

Position 285 is in the midst of the acyl binding pocket. Replacing proline 285 with isoleucine undoubtedly will affect the structure of the acyl binding pocket, but the exact nature of this effect is unclear. In fact, with so many changes clustered in the vicinity of the acyl binding pocket, it would be difficult to accurately predict their overall effect on the structure of that region.

However, the L286R change is particularly intriguing. The special significance of this residue, in part, is extrapolated from studies on mouse and human AChE, where the size of the residue in the position equivalent to 286 defines the substrate specificity [24,25]. In addition, we have found that substituting a histidine into position-286 of human BChE increased the rate of reactivation for diethylphosphate-inhibited human BChE. Thus, we considered it possible that the presence of the arginine in position-286 could account for: (a) the substrate specificity of rat BChE; (b) the increased rate for reactivation from organophosphate inhibition by rat BChE; and (c) the lack of sensitivity of rat BChE toward inhibition by Triton X-100.

This encouraged us to investigate the role of the R286 in the activity of BChE. Consequently, we prepared the rat mutant R286L and the complementary human mutant L286R. We compared the steady-state kinetics of these mutants to those of wild-type rat and wild-type human BChE. In addition, we examined the kinetics for reactivation from diethylphosphate inhibition, and the kinetics of Triton X-100 inhibition with each enzyme.

3.2. Steady-state turnover kinetics (ATC, PTC, BTC, BzCh, and ONPB)

Qualitatively, rat BChE behaved much the same as human BChE (Table 1). Both showed marked substrate activation with positively charged, acyl-choline substrates, ATC, BTC, and PTC, as reflected in b values ranging from 1.3 to 4.0. Both had normal Michaelis–Menten behavior with the neutral ONPB, as shown by b values equal to 1. Wild-type rat BChE showed a higher K_m for all substrates than did wild-type human. On the other hand, both k_{cat} and bk_{cat} generally were higher for wild-type rat BChE than they were for the wild-type human enzyme, suggesting that the catalytic machinery of the rat enzyme is more highly optimized. Our results are in reasonable agreement with reports from the literature (see Table 1).

When k_{cat}/K_m is used as a measure of catalytic efficiency, wild-type human BChE is more efficient with BTC, BzCh, ONPB, (+)-cocaine, and (−)-cocaine, whereas the wild-type rat enzyme is more efficient with ATC. Comparing efficiency within the acyl-choline series of substrates (ATC, PTC, and BTC), wild-type human BChE becomes increasingly efficient as the size of the acyl portion of the substrate increases (ATC < PTC < BTC). Wild-type rat BChE is least efficient with the larger BTC and equally efficient with the two smaller substrates. These findings suggest that the acyl-binding pocket in the active site of rat BChE is smaller than that of human BChE.

We had suggested that the arginine in position-286 of rat BChE might be responsible for the turnover differences between rat and human BChE. Accordingly, we examined the steady-state properties of the rat mutant, R286L, where the arginine in position-286 was replaced with the residue normally found in human BChE. The K_m values for R286L were reduced by about 2-fold, with all substrates (Table 1). The k_{cat} (bk_{cat}) values were also reduced (Table 1). The k_{cat}/K_m values indicated that the overall catalytic efficiency was improved only slightly by replacing Arg286 with leucine, and that the relative efficiency within the acyl-choline series of substrates was unchanged. Thus, the unique steady-state properties of rat BChE cannot be attributed simply to Arg286.

The converse mutation, L286R, in human BChE resulted in an enzyme with K_m values for positively charged substrates ATC, PTC, BTC, and BzCh, which were 20- to 50-fold higher for L286R than for wild-type human BChE (Table 1). The K_m value for the neutral ONPB was actually 2-fold lower for L286R. Although K_m is not a reliable measure of substrate affinity, these findings suggest that the

Table 1
Steady-state kinetic constants (determined in 0.1 M potassium phosphate at pH 7.0 and 25°)

Substrate	Species	Variant	K_m (μM)	K_{ss} (μM)	b	k_{cat} (min^{-1})	bk_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1} min^{-1})
ATC	Human	WT	57 ± 6.4	2,890 ± 495	2.47 ± 0.1	20,200 ± 910	49,900 ±3,000	354
ATC[a]	Human	WT	33	620	2.7	NR[b]	NR	NR
ATC	Human	L286R	2,340 ± 68	None[c]	1	11,800 ± 98	11,800 ± 98	5
ATC	Rat	WT	61 ± 6.7	2,580 ± 330	2.66 ± 0.11	66,200 ± 3,500	176,000 ± 11,800	1,090
ATC[d]	Rat	WT	80	NR	NR	NR	NR	NR
ATC	Rat	R286L	37 ± 6.8	1,630 ± 253	2.93 ± 0.23	47,000 ± 4,100	138,000 ± 16,200	1,270
PTC	Human	WT	25 ± 2.2	2,630 ± 130	4.09 ± 0.12	26,600 ± 860	109,000 ± 4,800	1,060
PTC[a]	Human	WT	24	410	2.2	NR	NR	NR
PTC	Human	L286R	510 ± 81	5,820 ± 3,600	1.59 ± 0.12	5,350 ± 610	8,510 ± 1,200	10
PTC	Rat	WT	189 ± 18	6,180 ± 1,200	1.99 ± 0.07	201,000 ± 9,300	400,000 ± 23,000	1,060
PTC	Rat	R286L	112 ± 6.8	4,800 ± 517	2.12 ± 0.05	144,000 ± 4,000	305,000 ± 11,000	1,290
BTC	Human	WT	14 ± 1.8	1,320 ± 78	3.60 ± 0.14	29,500 ± 1,100	106,000 ± 5,700	2,110
BTC[e]	Human	WT	23	1,400	2.5	33,900	84,900	1,470
BTC[f]	Human	WT	20	300	2.4	24,000	58,000	1,200
BTC	Human	L286R	738 ± 38	None	1	14,300 ± 410	14,300 ± 410	19
BTC	Rat	WT	134 ± 12	3,600 ± 2,500	1.32 ± 0.04	53,000 ± 26,500	70,000 ± 35,000	395
BTC[d]	Rat	WT	100	NR	NR	NR	NR	NR
BTC	Rat	R286L	94 ± 7.2	7,390 ± 2,900	1.63 ± 0.07	59,500 ± 1,900	97,000 ± 5,200	633
BzCh	Human	WT	5.6 ± 0.23	?[g]	<1	15,400 ± 140	?	2,750
BzCh[f]	Human	WT	8	?	<1	14,500	?	1,810
BzCh	Human	L286R	240 ± 54	None	1	23,300 ± 5,200	23,300 ± 5,200	97
BzCh	Rat	WT	43 ± 1.6	?	<1	23,300 ± 800	?	542
BzCh	Rat	R286L	21 ± 0.7	?	<1	14,600 ± 340	?	695
ONPB	Human	WT	106 ± 4.8	None	1	33,100 ± 420	33,100 ± 420	312
ONPB[h]	Human	WT	130	NR	NR	36,000	NR	277
ONPB	Human	L286R	42 ± 2	None	1	54,100 ± 830	54,100 ± 830	1,288
ONPB	Rat	WT	695 ± 36	None	1	76,900 ± 3,700	76,900 ± 3,700	111
ONPB	Rat	R286L	342 ± 14	None	1	66,500 ± 240		194
(+)-Coc[i,j]	Human	WT	13 ± 0.7	?	<1	8,900 ± 230	?	680
(+)-Coc[e,j]	Human	WT	10	None	1	7,500	7,500	750
(+)-Coc	Rat	WT	100 ± 15	None	1	7,800 ± 1,100	7,800 ± 1,100	78
(−)-Coc[i,j]	Human	WT	6.0 ± 1.4	None	1	1.8 ± 0.15	1.8 ± 0.15	0.30
(+)-Coc[e,j]	Human	WT	14	?	<1	3.9	?	0.27
(+)-Coc	Rat	WT	8.4 ± 3.0	None	1	0.43 ± 0.07	0.43 ± 0.07	0.05

Values are means ± SD, obtained using SigmaPlot.
[a] Data taken in 67 mM potassium phosphate, pH 7.0, at 25°, from [26].
[b] NR: not reported.
[c] "None" indicates that no indication of an excess substrate effect was detected at the highest concentration of substrate used.
[d] Data taken in 10 mM potassium phosphate, 150 mM sodium chloride, pH 7.4, at 25°, from [27].
[e] Data taken in 100 mM potassium phosphate, pH 7.0, at 25°, from [9].
[f] Data taken in 100 mM potassium phosphate, pH 7.4, at 25°, from [28].
[g] The "?" indicates that evidence for substrate inhibition was detected, but that insufficient data were collected to accurately define K_{ss} or b.
[h] Data taken in 100 mM potassium phosphate, pH 7.0, containing 5.5% methanol at from [12].
[i] (+)-Coc stands for (+)-cocaine, and (−)-Coc stands for (−)-cocaine.
[j] Substrate inhibition with cocaine becomes detectable at concentrations greater than 100 μM; refer to the present work with (+)-cocaine and to Xie *et al.* with (−)-cocaine [9]. We did not detect substrate inhibition with (−)-cocaine because we did not exceed 25 μM cocaine in our radioactive assay. Xie *et al.* did not report substrate inhibition with (+)-cocaine because they did not exceed 100 μM.

increased size of the arginine in position-286 did not obstruct substrate binding in L286R. Rather, the new positive charge in the active site of human BChE may selectively repel the positively charged substrates. Thus, the active site of human BChE tolerates the presence of a new positive charge poorly.

A similar charge-repulsion in rat BChE does not seem to occur. This is indicated by the fact that replacing Arg286 with leucine in rat BChE did not result in a large decrease in K_m for positively charged substrates. It follows that the positive charge on the arginine at position-286 in the rat enzyme is probably neutralized, most likely by the negatively charged Glu223.

Overall, the turnover results with the mutant BChE enzymes indicate that the structure of the active site of rat BChE is significantly different from the structure of the active site of human BChE. The observed differences in kinetic properties cannot be explained by a simple, single amino acid substitution. Furthermore, the active site of rat BChE is adapted to accommodate the presence of the

positively charged Arg286 in ways that cannot be easily mimicked by human BChE.

3.3. BChE levels in rat serum

It is well known that the measured activity of BChE in rat serum is low relative to that of human serum [5–7,29]. Having determined the specific activity of purified rat BChE to be 146 BzCh-units/mg, we were in a position to quantitate the amount of BChE protein in rat serum. Rat serum (adult male Sprague–Dawley, from RJO Biologicals Inc.), when measured with 50 μM BzCh in 0.1 M potassium phosphate, pH 7.0, at 25°, contained 0.027 units/mL of BChE activity. This is equivalent to 0.185 mg of BChE/L of rat serum. In contrast, there are 5 mg of BChE/L of human serum.

3.4. Inhibition of BChE by Triton X-100

Li *et al*. [7] showed that the monomeric form of Triton X-100 could inhibit activity of human BChE, but that rat serum BChE was resistant to Triton X-100 inhibition. It was proposed that the arginine in position-286 of rat BChE might be responsible for the rat's resistance. In those experiments, Triton X-100 appeared to behave as a competitive inhibitor of human BChE turnover. The investigation into Triton X-100 inhibition of wild-type BChE has been extended with a traditional steady-state inhibition study, using multiple concentrations of both substrate (BTC) and Triton X-100. BChE mutants at the 286-position of both rat and human BChE have been included in the study to test the proposal that resistance to Triton X-100 inhibition in rat BChE is due to the presence of arginine in position-286.

Both wild-type rat and human BChE showed classical competitive inhibition with Triton X-100. Plots of 1/[substrate] versus 1/velocity at different concentrations of Triton X-100 were linear and converged on the y-axis. Re-plots of the slopes as a function of Triton X-100 concentration were linear, yielding competitive inhibition constants of 13.1 ± 2.9 and 194 ± 19 μM for Triton X-100 binding to human and rat BChE, respectively. Thus, either Triton X-100 or BTC can bind to the enzyme, but not both. It was not clear whether this mutually exclusive binding results from direct competition between Triton X-100 and BTC for the same binding site, or whether it occurs allosterically with Triton X-100 and BTC binding to separate loci.

Replacing the arginine at position-286 in rat BChE with leucine (R286L) caused the inhibition to become mixed, with both the slopes and the y-axis intercepts of the double-reciprocal plot showing a dependence on Triton X-100 concentration. Analysis indicated classical, non-competitive, mixed-type inhibition with the lines on the double-reciprocal plot converging on the x-axis. The inhibition constant obtained from the slopes was 9.3 ± 0.79 μM, and that from the intercepts was 9.6 ± 1.1 μM. Thus, Triton X-100 bound to R286L 20-fold more tightly than it did to wild-type rat BChE. This strongly suggests that the arginine in position-286 is responsible for the weak binding of Triton X-100 to rat BChE.

Replacing the leucine at position-286 in human BChE with arginine (L286R) resulted in an enzyme that retained competitive Triton X-100 inhibition, but with an inhibition constant, which was increased 3-fold, to 40.1 ± 3.6 μM. The decrease in affinity for Triton X-100 upon introduction of arginine into position-286 of human BChE is consistent with the results from rat BChE. However, the difference in affinity for human BChE was much smaller than for rat BChE. This result supports our suggestion, taken from the steady-state turnover findings, that the structures of the active sites of human and rat BChE are significantly different.

It is noteworthy that 250 μM is the critical micellar concentration for Triton X-100 [30]. The fact that all of the Triton X-100 inhibition constants were below the critical micellar concentration supports the proposal by Li *et al*. [7] that the monomeric form of Triton X-100 is responsible for the inhibition of BChE.

3.5. Triton X-100 and tissue extracts

It is common for investigators to extract tissues with 0.5% Triton X-100 when assaying for BChE activity. Tissue extracts containing 0.5% Triton X-100 are typically diluted directly into the assay mixture in order to measure BChE activity. For tissues with low activity, such as those from the rat, 50 μL of extract may be diluted into 1 mL of reaction medium. This results in a 40-fold dilution, or 0.025% Triton X-100 remaining in the assay [7]. We have determined the effect of Triton X-100 on the activity of wild-type rat and human BChE with 1 mM BTC (Fig. 2). Rat BChE was inhibited 24% at Triton X-100 concentrations greater than 250 μM (0.016%). Human BChE was inhibited 40% under the same conditions. Thus, in the

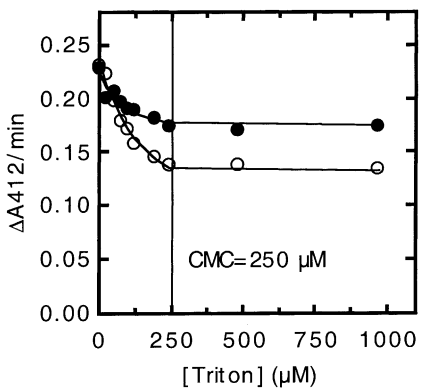

Fig. 2. Inhibition of wild-type BChE from rats and humans by Triton X-100. BChE was reacted with 1 mM BTC (0.1 M potassium phosphate buffer, pH 7.0, at 25°) in the presence of various concentrations of Triton X-100 (0–1 mM). The effect of Triton X-100 on BChE activity (ΔA_{412}/min) is shown: closed circles for rat BChE, open circles for human BChE. CMC denotes the critical micellar concentration for Triton X-100. The points are the average of triplicate assays.

presence of 0.025% Triton X-100, even rat BChE would be inhibited markedly by Triton X-100.

Li *et al*. [7] reported that BChE in rat serum was resistant to Triton X-100 inhibition, under conditions comparable to those described here. The data (see Table 4 from Ref. [7]) actually show a 23% inhibition of rat BChE activity in serum, in the presence of 0.025% Triton X-100. However, the low levels of activity with which Li *et al*. were working made this difference unreliable. In the present experiments, sufficiently high levels of the recombinant rat BChE were used that the observed 24% inhibition is reliable.

3.6. Other detergents

In addition to studying Triton X-100, the inhibition of BChE by Tween 20 was examined. It was found that with 1 mM BTC and 0.125% Tween 20 (1 mM) neither wild-type rat nor wild-type human BChE was inhibited. This is equivalent to the findings of Li *et al*. [7]. Inhibition by Brij 96 V was also examined. It was found that with 1 mM BTC and 0.07% Brij (1 mM) neither wild-type rat nor wild-type human BChE was inhibited.

3.7. Phosphorylation of BChE by ECHO

In the present work (in 0.1 M potassium phosphate buffer, pH 7.0, at 25°), the apparent rate constant for phosphorylation (k_{phos}) of wild-type human BChE, up to a value of 1.6 min^{-1}, was linearly dependent upon the ECHO concentration. Concentrations of ECHO ranged from 0.1 to 0.6 μM. The apparent second order rate constant was $2.5 \pm 0.2 \times 10^6$ M^{-1} min^{-1}. Masson *et al*. [31] have reported that phosphorylation of human BChE by ECHO is not linearly dependent upon ECHO in this concentration range. Rather, it showed saturating behavior, reaching a limiting rate at 0.48 min^{-1} (in 0.1 M potassium phosphate buffer, pH 7.0, plus 0.5% ethanol, at 25°). The reaction of wild-type human BChE with ECHO was repeated in the presence of 1% ethanol. The k_{phos} values were still linearly dependent upon the ECHO concentration, up to 1.5 min^{-1}, though the apparent second order rate constant was reduced to $1.23 \pm 0.04 \times 10^6$ M^{-1} min^{-1}. The reason for the discrepancy between these results and those of Masson *et al*. [31] is unclear.

A similar, linear relationship between the concentration of ECHO and the rate of phosphorylation was found for wild-type rat BChE, and mutants R286L (rat) and L286R (human). The apparent second order rate constant for the phosphorylation of wild-type rat BChE by ECHO ($1.86 \pm 0.04 \times 10^7$ M^{-1} min^{-1}) was 10-fold faster than that for wild-type human BChE. Replacement of Arg286 with leucine (R286L) did not have much effect on that value ($1.73 \pm 0.07 \times 10^7$ M^{-1} min^{-1}), despite the fact that ECHO is positively charged. This finding supports our interpretations of the steady-state turnover data from rat BChE, which concluded that: (a) the arginine does not create a major steric hindrance in the active site; and (b) the charge on the arginine is neutralized. The increased rate for phosphorylation of rat BChE is consistent with the increased rates observed for substrate turnover by rat BChE.

In contrast, introducing an arginine into the active site of human BChE at position-286 (L286R) reduced the apparent second order rate constant for phosphorylation by ECHO by 3 orders of magnitude (to $3.5 \pm 0.4 \times 10^4$ M^{-1} min^{-1}). Since ECHO is positively charged, this result is consistent with significant charge repulsion for positively charged compounds in the active site of human L286R.

3.8. Reactivation of diethylphosphorylated BChE

Davison [8] has shown that the reactivation rate for diethylphosphate-inhibited wild-type rat BChE (2.0×10^{-3} min^{-1} at pH 7.8 and 37°) is 100-fold faster than that for wild-type human BChE (1.6×10^{-5} min^{-1}). In the present study, reactivation of wild-type rat BChE after inhibition by ECHO (a diethylphosphate containing organophosphate) occurred at $2.7 \pm 0.08 \times 10^{-3}$ min^{-1} (half-life of 4.3 hr), which is in good agreement with the Davison report. Seventy-three percent of the starting activity was recovered after reactivation was complete, suggesting that aging was not a major contributor to the reactivation rate.

Replacing the arginine at position-286 in rat BChE with leucine (R286L) slowed the reactivation rate by only 30% (to $1.9 \pm 0.40 \times 10^{-3}$ min^{-1}), while yielding a 60% recovery of the starting activity. Thus, Arg286 is not the factor responsible for the fast reactivation of rat BChE. Consistent with this conclusion, the human L286R mutant showed no measurable reactivation after 6.5 hr, indicating that the arginine at this position did not promote reactivation in human BChE.

3.9. Hydrolysis of cocaine

The non-pharmacologically active (+)-cocaine has been reported to react well with wild-type human BChE, with a K_m of 6–10 μM and a k_{cat} of 7,500–8,900 min^{-1} [9,13]. Similar values for wild-type human BChE (Table 1) were found in this study. With wild-type rat BChE, the K_m for (+)-cocaine was 10-fold weaker (K_m = 100 μM), although the k_{cat} value was essentially the same as that for human BChE (Table 1). The higher K_m value for rat BChE reacting with (+)-cocaine is consistent with the increase in K_m found for rat BChE reacting with other substrates. When k_{cat}/K_m was used as a measure of relative catalytic efficiency, rat BChE was 10-fold less efficient than human BChE at hydrolyzing (+)-cocaine.

Xie *et al*. [9] have reported that wild-type human BChE hydrolyzes the pharmacologically active (−)-cocaine 2,000-fold more slowly than (+)-cocaine, although the (−)-cocaine bound with the same affinity as (+)-cocaine (Table 1). Similar results were obtained in the current work (Table 1). In addition, it was found that with wild-type rat

BChE the K_m for (−)-cocaine was much the same ($K_m = 8.4 \pm 3.0$ μM) as that for wild-type human BChE (6.0 ± 1.4 μM), and that the turnover rate with rat BChE (0.43 ± 0.07 min^{-1}) was nearly 4-fold slower than with human BChE (1.8 ± 0.15 min^{-1}). The net effect is that rat BChE is about 5-fold less efficient than human BChE at hydrolyzing (−)-cocaine.

Since the K_m values for both (+)- and (−)-cocaine were the same for human BChE, it was unexpected to find a 10-fold difference in the K_m for (+)-cocaine (100 μM) and (−)-cocaine (8.4 μM) for rat BChE. To test the reliability of the numbers, the affinity of (−)-cocaine for rat BChE was determined by using this slow substrate as an inhibitor for the hydrolysis of the faster substrate, ATC. Plots of 1/velocity versus 1/[ATC] at different (−)-cocaine concentrations were linear. Both the slopes and the y-axis intercepts were dependent upon the (−)-cocaine concentration, indicating mixed-type inhibition. The inhibition constant calculated from the slopes was 14.5 ± 1.2 μM, while the inhibition constant calculated from the intercepts was 24.8 ± 8.1 μM. These values are in reasonable agreement with the K_m of 8.4 μM for the reaction of (−)-cocaine with rat BChE.

3.10. AChE in rat serum

In addition to BChE, rat serum contains AChE. The presence of AChE is demonstrated in Fig. 3, where non-denaturing polyacrylamide gels have been stained for activity. The band labeled AChE was identified as AChE by its disappearance when the gel was treated with the AChE-selective inhibitor BW [1,5-bis(4-allyldimethylammoniumphenyl)-pentan-3-one]. Fetal bovine serum, known to contain only AChE, served as a control. The BChE bands were identified by treating the gel with the BChE-selective inhibitor iso-OMPA [tetraisopropylpyrophosphoramide]. Rat serum, but not human serum or fetal bovine serum, contains carboxylesterase. The carboxylesterase migrates behind albumin.

4. Discussion

4.1. Rat BChE enzyme

We found that wild-type rat BChE generally binds substrates more weakly than wild-type human BChE, and that this effect is more prominent with larger substrates. This selectivity for smaller substrates probably reflects a decrease in the size of the acyl-binding pocket of rat BChE, which is the locus for most of the active site amino acid differences between rat and human BChE. This preference for smaller substrates, which is shared by mouse BChE [6], led Augustinsson to consider the rat enzyme to be a propionylcholinesterase rather than a butyrylcholinesterase [5]. We also found that Arg286, in the acyl-binding pocket of rat BChE, was intimately involved in the resistance of rat BChE to inhibition by Triton X-100, but that it had little-or-no role in determining substrate specificity or in promoting reactivation of the diethylphosphate-inhibited enzyme.

4.2. BChE and AChE in rat serum

Our measured value for male rat serum (0.185 BzCh-units/mg) was 25-fold lower than that of BChE in human serum. However, BChE is not the only cholinesterase in rat

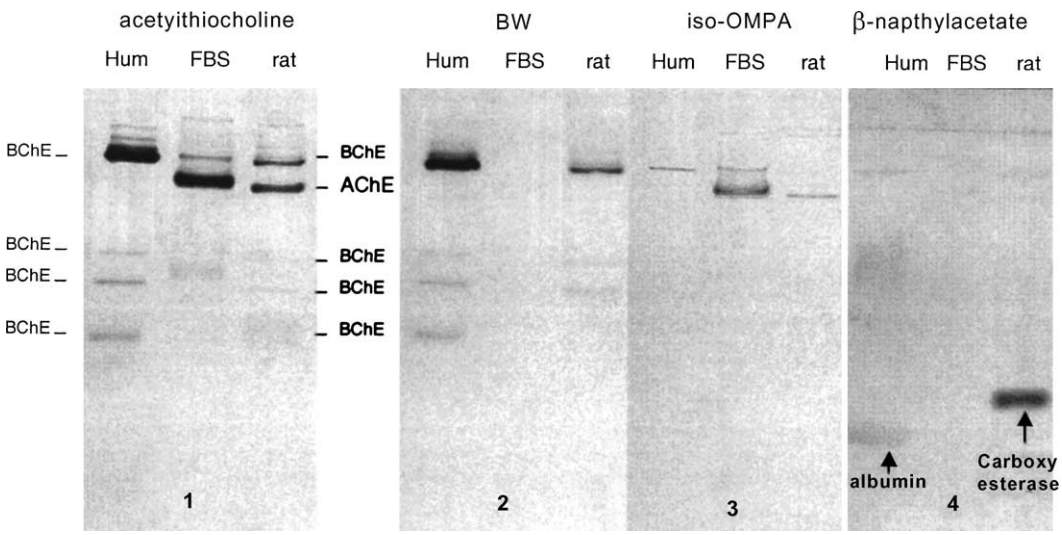

Fig. 3. Visualization of AChE, BChE, and carboxylesterase in rat serum. Lanes of non-denaturing gradient gels, 4 to 30% polyacrylamide, contained 3 μL of human serum, fetal bovine serum (FBS), or rat serum. Gel 1 was stained to reveal both AChE and BChE activity by the method of Karnovsky and Roots [32] using 1.7 mM ATC as substrate. Gel 2 was incubated in 30 μM BW for 30 min to inhibit AChE before ATC was added. Gel 3 was incubated in 0.1 mM iso-OMPA for 30 min to inhibit BChE before ATC was added. Gel 4 was stained for carboxylesterase with β-naphthylacetate and Fast Blue RR. The rat AChE band migrates at the same position as the AChE tetramer in fetal bovine serum. Rat serum has four BChE bands, similar to the four bands in human serum. The most intense BChE band is a tetramer. Carboxylesterase is present in rat serum but not in human serum or fetal bovine serum.

serum. In fact, the AChE activity in rat serum is 2.5-fold higher than the BChE activity with 1 mM ATC [7,29]. Considering the k_{cat} levels of rat AChE and BChE, it can be estimated that rat serum contains approximately the same amounts of AChE and BChE protein. This is an important point that is commonly overlooked when cholinesterase measurements on rat serum are made.

4.3. Triton X-100 inhibition

Triton X-100 is another important factor to consider when working with BChE. Li *et al*. [7] found that Triton X-100 could inhibit BChE from a variety of species. However, by reporting that rat BChE was resistant to Triton X-100, they implied that rat tissues extracted with Triton X-100 could be assayed without the concern of inhibiting BChE activity. We have found that although rat BChE is less sensitive than human BChE to inhibition by Triton X-100, it is still inhibited (see Fig. 2). Under conditions commonly used for the assay of rat BChE in tissue extracts, there is enough residual Triton X-100 to inhibit BChE activity by 24%. Li *et al*. report on BChE activities measured from various tissues, extracted with either Tween 20 or Triton X-100 (see Table 5 from Ref. [7]). They consistently report greater activity in the presence of Triton X-100. We suggest that this higher activity reflects more efficient solubilization of BChE by Triton X-100, and that the correct activities in the Triton X-100 samples are actually 24% higher than reported.

In conclusion, we can say that rat BChE and human BChE are remarkably similar. They are 80% identical in their amino acid sequences, and hydrolyze the same set of substrates, including cocaine, with similar kinetic constants. They are both inhibited by Triton X-100, in a competitive manner, and they are both readily inhibited by ECHO. Rat BChE is distinct from human BChE in that it favors the hydrolysis of PTC over BTC; it reactivates from diethylphosphate inhibition 100-fold more rapidly; and it is 15-fold less sensitive to Triton X-100 inhibition.

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