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Cocrystallization studies of full-length recombinant butyrylcholinesterase (BChE) with cocaine

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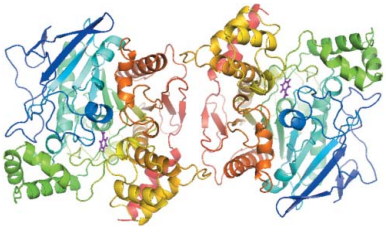

Human butyrylcholinesterase (BChE; EC 3.1.1.8) is a 340 kDa tetrameric glycoprotein that is present in human serum at about 5 mg l^{-1} and has well documented therapeutic effects on cocaine toxicity. BChE holds promise as a therapeutic that reduces and finally eliminates the rewarding effects of cocaine, thus weaning an addict from the drug. There have been extensive computational studies of cocaine hydrolysis by BChE. Since there are no reported structures of BChE with cocaine or any of the hydrolysis products, full-length monomeric recombinant wild-type BChE was cocrystallized with cocaine. The refined 3 Å resolution structure appears to retain the hydrolysis product benzoic acid in sufficient proximity to form a hydrogen bond to the active-site Ser198.

1. Background

The use of cocaine in its various forms stimulates the nervous system by interfering with the reabsorption of dopamine. The build-up of dopamine results in continuous neuron stimulation and the associated euphoria reported by cocaine abusers. Other physical effects of cocaine use include hyperstimulation, restlessness, irritability, constricted blood vessels, increased heart rate, increased blood pressure, reduced fatigue, reduced mental clarity and increased anxiety. Once addicted, cocaine users constantly search for the ever more elusive high or euphoria and need increasing amounts of cocaine to reach that high. Life-threatening symptoms of cocaine toxicity include grand mal seizures, cardiac arrest, stroke and drug-induced psychosis accompanied by elevated body temperature. Cocaine use during pregnancy can have severe detrimental effects including spontaneous abortion, low birth weight, pre-term births and neonates who need to be detoxified of the drug (Minor *et al.*, 1991; Hollander *et al.*, 1995; Zimmerman *et al.*, 1991; Wolfe *et al.*, 1992).

A potential treatment for cocaine toxicity and addiction is the administration of endogenous scavengers such as human BChE (Gorelick, 1997; Hoffman *et al.*, 1996; Lynch *et al.*, 1997). BChE is a tetrameric glycoprotein comprising four identical subunits, each with a molecular weight of 85 kDa from 574 amino acids and nine N-linked glycans (Lockridge *et al.*, 1987). The C-terminal 40 amino acids constitute the tetramerization domain (Blong *et al.*, 1997; Altamirano & Lockridge, 1999*a*,*b*). Human serum BChE has a concentration of up to 5 mg l^{-1} and a half-life of 12 d (Ostergaard *et al.*, 1988). BChE is synthesized by the liver and is present in the highest amounts in serum, intestine, liver and lung (Jbilo *et al.*, 1994). BChE hydrolyzes cocaine to the inactive products benzoic acid and ecgonine methyl ester. In addition, it has been shown that rodents pretreated with human BChE are protected from cocaine toxicity (Carmona *et al.*, 1998; Koetzner & Woods, 2002; Mesulam *et al.*, 2002; Duysen, Bartels *et al.*, 2002; Hoffman *et al.*, 1996). Mice pretreated with either wild-type or mutant human BChE were protected from cocaine-induced hyperactivity and exhibited reduced locomotor activity compared with rodents treated with saline (Duysen, Stribley *et al.*, 2002). It has also been shown that rodents treated with human BChE are protected from cocaine-induced seizures and death (Hoffman *et al.*, 1996; Lynch *et al.*, 1997). Furthermore, treatment with human BChE ameliorates the hypertensive effects of cocaine in rodents (Mattes *et al.*, 1997).

Table 1
Crystallographic data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

PDB code	3o9m
Space group	$P42_12$
Unit-cell parameters (Å)	$a = b = 150.39$, $c = 142.45$
Resolution limits (Å)	31.7–2.98 (3.09–2.98)
$\langle I/\sigma(I)\rangle$	32.7 (2.5)
No. of unique reflections	34051 (3549)
Multiplicity	7.0 (6.8)
R_{merge}† (%)	8.3 (53.5)
Completeness (%)	99.6 (98.2)
R_{cryst}‡ (%)	22.3 (22.7)
R_{free}§ (%)	25.4 (30.3)
Correlation coefficient	
$F_o - F_c$	0.912
$F_o - F_c$ (free)	0.881
R.m.s. deviations	
Bond lengths (Å)	0.017
Bond angles (°)	1.736
No. of atoms	
Protein atoms	8451
Benzoic acid	18
Solvent	2
Overall mean B factor (Å^2)	
Protein atoms	54.3
Benzoic acid	58.7
Solvent	32.0
Ramachandran (%)	
Favored	93.4
Allowed	5.7
Outliers	0.9

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl)\rangle$ are the intensity of measurement i and the mean intensity of the reflection with indices hkl, respectively. ‡ $R_{cryst} = \sum_{hkl} \big||F_{obs}| - |F_{calc}|\big| / \sum_{hkl} |F_{obs}|$, where F_o are observed and F_c are calculated structure-factor amplitudes. § R_{free} uses a randomly chosen 5% set of reflections.

BChE is an effective detoxifier of cocaine because it has a binding affinity for cocaine in the physiological range: $K_m = 14$ µM (Xie *et al.*, 1999). While wild-type human BChE hydrolyzes cocaine slowly, with a k_{cat} of 4 min^{-1} (Xie *et al.*, 1999), mutants that are more efficient at cocaine hydrolysis have been developed, including A328W, which has a 15-fold faster hydrolysis rate (Duysen, Bartels *et al.*, 2002), and a double mutant, A328W/Y332A, that hydrolyzes cocaine 40-fold faster than the wild type (Sun *et al.*, 2002). Both mutant forms of BChE provide greater protection from cocaine-induced locomotor activity than wild-type BChE (Duysen, Stribley *et al.*, 2002). This protective effect of BChE against cocaine toxicity stimulated interest in large-scale BChE production for clinical use and recently a quintuple mutant A199S/F227A/S287G/A328W/Y332G that hydrolyzes cocaine 2000-fold faster than wild-type BChE was designed (Zheng *et al.*, 2008). The quintuple-mutant BChE selectively blocks reinstatement of drug seeking in rats (Brimijoin *et al.*, 2008). There are 11 reported structures of BChE and their PDB codes are 1xlu, 1xlv, 1xlw, 3djy, 3ddk, 2wsl, 1p0q, 1p0m, 1p0i, 1p0p and 2pm8 (Carletti *et al.*, 2008, 2009; Nachon *et al.*, 2005; Nicolet *et al.*, 2003; Sanson *et al.*, 2009; Ngamelue *et al.*, 2007). These structures are either complexes with organophosphate inhibitors or analogues. There are no structures available of BChE with cocaine or hydrolysis products; thus, we carried out structural studies of monomeric full-length recombinant wild-type BChE with cocaine.

2. Materials and methods

2.1. Crystallization of BChE

The expression, purification and crystallization of full-length recombinant human BChE has been described elsewhere (Ngamelue *et al.*, 2007). All crystals were grown at 293 K by vapor diffusion in sitting drops. Soaking was accomplished by adding 1 mM cocaine hydrochloride in precipitant solution to preformed crystals. Soaking damaged the preformed crystals and resulted in a reduction of the diffraction resolution to below 4.8 Å. Consequently, all structural analyses were performed on cocrystals.

Sitting-drop cocrystallization experiments in VDX plates were prepared by mixing 8 µl protein solution (8 mg ml^{-1} in MES pH 6.5) with 1.5 µl reservoir solution (2.0 M ammonium sulfate in 0.1 M Tris–HCl pH 8.5) and 0.5 µl 25 mM cocaine hydrochloride in water. This ratio of protein to precipitant is higher than that required for a truncated BChE construct (Nicolet *et al.*, 2003), but is consistent with the ratio required for crystallization of the full-length protein (Ngamelue *et al.*, 2007). The largest plate-like crystals (0.6 × 0.2 × 0.07 mm) were obtained in about two weeks. The cocrystals had the same physical morphology, thickness and space group as and similar lattice parameters to the crystals grown in the absence of cocaine and reported previously (Ngamelue *et al.*, 2007). The crystals were cryoprotected with 5 min soaks in solutions containing 15% glycerol and equivalent amounts of precipitants as mother liquor. The crystals were then flash-cooled directly in a stream of N_2 prior to data collection at 100 K.

2.2. Data collection and structure determination

X-ray diffraction data extending to 2.98 Å resolution were collected from a single crystal on synchrotron beamline 14-BM-C at the Advanced Photon Source, Argonne National Laboratory, Chicago, USA at a wavelength of 0.9 Å. Data were collected using an ADSC Quantum-315 CCD detector operating at 13.77 keV. The crystal-to-detector distance used was 450 mm, with an exposure time of 3 s per 1° oscillation. Data were processed using *MOSFLM* (Leslie, 1999, 2006) and merged with *SCALA* (Leslie, 1999, 2006). Statistics are listed in Table 1. The structure was solved by molecular replacement with *Phaser* (McCoy *et al.*, 2005; Storoni *et al.*, 2004; Read, 2001) using a monomer of the uncomplexed structure of full-length monomeric BChE (PDB entry 2pm8; Ngamelue *et al.*, 2007) stripped of ligands and waters as the search model. The molecular-replacement solution had a dimer in the asymmetric unit and 62% solvent content, corresponding to a Matthews coefficient V_M of 3.24 Å^3 Da^{-1} (Matthews, 1968). Despite the low resolution, the main chain and many of the side-chain residues were clearly visible in the $2F_o - F_c$ electron-density map calculated from molecular-replacement phases. The greatest disorder was furthest away from the catalytic Ser and in the longer amino acids (Lys and Arg). The final

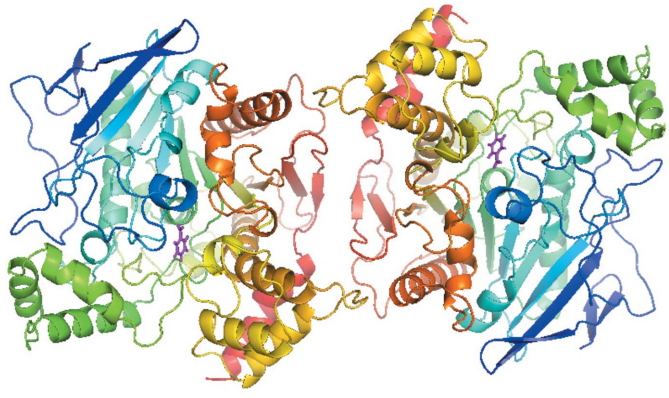

Figure 1
Ribbon representation of a dimer of BChE. Each monomer is rainbow colored from blue at the C-terminus to red at the N-terminus. The benzoic acid moieties are shown in magenta.

model was obtained through iterative cycles of manual model building in *Coot* (Emsley & Cowtan, 2004) and structure refinement using a maximum-likelihood refinement procedure and NCS phase restraints in the program *REFMAC*5 (Pannu *et al.*, 1998; Murshudov *et al.*, 1997, 1999). The resulting structure was validated using *PROCHECK* (Laskowski *et al.*, 1993). Data and model statistics are shown in Table 1. The presence of outliers was confirmed by visual inspection and analysis of the electron-density maps. There is visible density for several carbohydrates which were difficult to model and so were not included in the refinement. The atomic coordinates and structure factors have been deposited in the Protein Data Bank with accession code 3o9m. Figs. 1 and 3 were generated with *PyMOL* (DeLano, 2002), while Fig. 2 was generated with *Coot*. All alignments were performed with the program *LSQKAB* (Kabsch, 1976).

3. Results and discussion

Our cocrystallization studies of full-length recombinant BChE with cocaine yielded a 2.98 Å resolution structure with two monomers in the asymmetric unit (Fig. 1). As was observed in the uncomplexed structure of full-length BChE, the dimer is oriented such that the active-site openings of each monomer are on opposite sides and the main chains of both structures overlay well, with an r.m.s. deviation of 0.312 Å. The resulting structure appears to be the complex with the hydrolysis product benzoic acid. This is evident from unbiased OMIT electron-density maps for both monomers, which have sufficient electron density to model benzoate but not the substrate cocaine or the other hydrolysis product ecgonine methyl ester (Fig. 2). This electron density is substantially larger than what we had previously observed in proximity to Ser198 in the structure of uncomplexed full-length BChE (PDB entry 2pm8), which was modeled as a water molecule. The electron density in the cavity of the uncomplexed structure is not large enough to model benzoate or any of the substrates or products of cocaine hydrolysis. Additionally, in the structure of the cocrystal with cocaine there is a small amount of electron density in proximity to the benzoate that lies in the location in which the butyrate had been previously observed in the structure of native truncated BChE (PDB code 2p0p; Nicolet *et al.*, 2003). This density is present in both monomers of BChE in the asymmetric unit and is too small to accommodate a glycerol molecule or butyrate; we therefore modeled it as a water molecule per monomer (Fig. 2). The two water molecules have a lower *B* factor than the average for the entire molecule. However, the *B* factors are consistent with those of residues in the binding cavity such as Ser198.

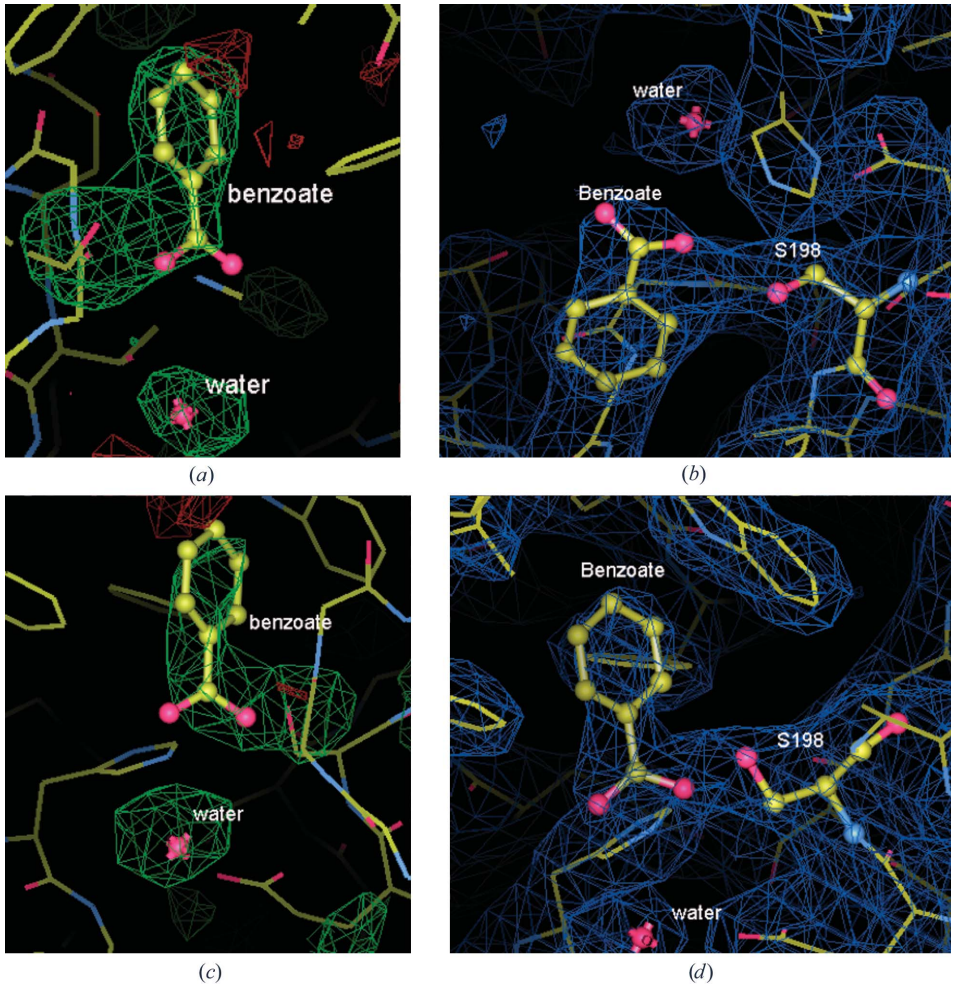

Figure 2
Refined model of 3o9m placed into $F_o - F_c$ electron-density maps calculated from molecular-replacement phases for each monomer at 2.5σ contour levels (green) for both monomers reveals clear density for the hydrolysis product benzoic acid. Additional density is observed adjacent to the proposed benzoate-binding site, into which a water molecule is modeled. The fit of the refined model in the final *REFMAC*5-generated $2F_o - F_c$ maps (in blue) is shown for both monomers. (*a*) and (*b*) correspond to one monomer, while (*c*) and (*d*) correspond to the other monomer in the asymmetric unit.

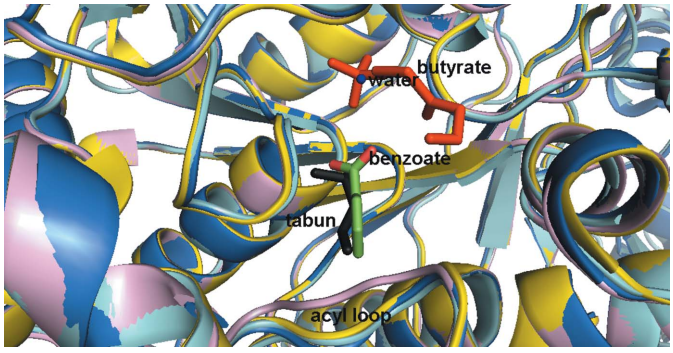

Figure 3
The binding cavity of BChE reveals that the benzoate (green) occupies a position close to that of the irreversible tabun inhibitor (gray) rather than butyrate (red). The aligned structures are BChE cocrystal studies with cocaine (PDB entry 3o9m; aquamarine), uncomplexed full-length BChE (PDB entry 2pm8; Ngamelue *et al.*, 2007; blue), truncated BChE in complex with butyrate (PDB entry 1p0p; Nicolet *et al.*, 2003; salmon) and truncated BChE in complex with tabun (PDB entry 2wsl; Carletti *et al.*, 2009; yellow). Comparing the main chains reveals differences in the acyl loop after cocaine hydrolysis but no significant shifts in the rest of the binding cavity and loop 1.

Two potential binding positions for ligands in the catalytic site have been identified in previous BChE structures. The first is illustrated by the putative butyrate modeled into the cavity of the original truncated BChE structure (Nicolet *et al.*, 2003), while the second is that of the irreversible inhibitors typified by the tabun analogue-inhibited BChE (PDB entry 2wsl; Carletti *et al.*, 2009). The benzoate in our structure occupies a similar position to the latter tabun analogue rather than butyrate (Fig. 3). Additionally, most of the main-chain residues overlay well in all of the structures except for the butyrate-containing truncated structure of BChE, which has a less open cavity owing to a shift of the acyl loop (Fig. 3).

4. Concluding remarks

Our studies show that the full-length recombinant protein is catalytically active and is capable of hydrolyzing cocaine. Our structure reveals that the orientation of the binding cavity remains more similar to both the apo structure of full-length recombinant BChE (2pm8) and structures with inhibitors such as the tabun analogue (PDB entry 2wsl) than to the truncated BChE structure bound to butyrate (PDB entry 2p0p). Cocrystallization studies with cocaine analogues that cannot be hydrolyzed are required in order to clarify the exact mode of cocaine binding by BChE and to explain why the quintuple mutant A199S/F227A/S287G/A328W/Y332G has a 2000-fold increase in cocaine-hydrolysis rate.

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