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## Subunit stabilization and PEGylation of cocaine esterase improves *in vivo* residence time.

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**RUNNING TITLE:** Subunit stabilization and PEGylation of cocaine esterase

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**ABBREVIATIONS:**

ED, emergency department; NSDUH, National Survey on Drug Use and Health; DSMMD, Diagnostic and Statistical Manual of Mental Disorders; BchE, butyrylcholinesterase; CocE, Cocaine esterase; wt, wild-type; CC-CocE, G4C/S10C-CocE; CCRQ-CocE, G4C/S10C/T172R/G173Q-CocE; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, DTT, dithiothreitol, FPLC, performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Km, Michaelis constant; PEG, polyethylene glycol; RMSD, roots mean squared deviation; MAP, mean arterial blood pressure; HR, heart rate; AUC, analytical ultracentrifugation; VEGF, vascular endothelial growth factor; ANS, anilinonaphthalene-8-sulfonic acid; FR, fixed ratio.

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## Abstract

No small molecule therapeutic is available to treat cocaine addiction, but enzyme-based therapy to accelerate cocaine hydrolysis systemically has recently gained momentum. Bacterial cocaine esterase (CocE) is the fastest known native enzyme that hydrolyzes cocaine. However, its lability at 37 °C has limited its therapeutic potential. Crosslinking subunits through disulfide bridging is commonly used to stabilize multimeric enzymes. Herein we utilize structural methods to guide the introduction of two cysteine residues within dimer interface of CocE to facilitate intermolecular disulfide bond formation. The disulphide-crosslinked enzyme displays improved thermostability, particularly when combined with previously described mutations that enhance stability (T172R-G173Q). The newly modified enzyme yielded an extremely stable form of CocE (CCRQ-CocE) that retained greater than 90% of its activity after 41 days at 37°C, representing greater than a 4700-fold improvement over the wild-type enzyme. CCRQ-CocE could also be modified by polyethylene glycol (PEG) polymers, which improved its *in vivo* residence time from 24 to 72 hours, as measured by a cocaine lethality assay, by self-administration in rodents, and by measurement of inhibition of cocaine-induced cardiovascular effects in *Rhesus* monkeys. PEG-CCRQ elicited negligible immune response in rodents. Subunit stabilization and PEGylation has thus produced a potential protein therapeutic with markedly higher stability both *in vitro* and *in vivo*.

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## Introduction

Cocaine use continues to be a serious public health problem. Emergency department (ED) visits due to cocaine abuse lead the charts among all other drugs. In 2006, cocaine was involved in 553,530 in a total of 974,272 ED visits involving an illicit drug (56.8%) (SAMHSA, 2011). Cocaine remains the most co-abused drug along with alcohol again leading all other illicit drugs. In 2008 an astonishingly large number of Americans age 12 and older (5.3 million) were reported to have abused cocaine in any form and 1.1 million had abused crack at least once in the year prior to being surveyed (SAMHSA, 2011). The National Survey on Drug Use and Health (NSDUH) estimates that in 2007 there were 2.1 million current (past-month) cocaine users (SAMHSA, 2008). Moreover, nearly 1.6 million Americans met Diagnostic and Statistical Manual of Mental Disorders (DSMMD) criteria for dependence or abuse of cocaine (in any form) in the past 12 months (SAMHSA, 2011). Despite being a highly addictive and widely abused drug, there is no FDA approved medication for treatment of cocaine abuse or toxicity.

The primary mechanism by which cocaine exerts its behavioral effects is through binding to monoamine transporters and blocking the reuptake of dopamine in the synaptic junctions, thus potentiating the effects of dopamine in the synapse. Chronic and prolonged blockade of dopamine transporters can lead to reinforcement of self-administration, and thereby to various forms of addiction (Benowitz, 1993). At higher concentrations, cocaine also blocks norepinephrine and serotonin reuptake transporters, which contributes to its toxic effects,

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including seizures, tachyarrhythmia and sudden death (Benowitz, 1993; Crumb et al., 1990; Johanson and Fischman, 1989; Uhl et al., 2002).

Discovery of a small molecule antidote for cocaine has been hampered by the pleiotropic actions of cocaine. Approaches for treating cocaine abuse, *ie.* discovering agonists to replace cocaine (Grabowski et al., 2004), antagonists to block cocaine at the site of action (Newman et al., 2005; Rothman et al., 2008) and modulators of cocaine, aimed at altering the effects of cocaine by acting at sites other than monoamine transporters (Dackis and O'Brien, 2003; Mello, 1990; Platt et al., 2002; Roberts and Brebner, 2000) have failed to produce an effective pharmacotherapeutic drug.

An alternative approach using protein biologics to sequester cocaine in the serum (antibodies) or hydrolyze cocaine in the serum before it reaches its site of action (enzymes or catalytic antibodies) has shown some promise. Passive immunization with monoclonal antibodies reduced cocaine self-administration in rats (Fox et al., 1996; Kantak et al., 2000) and blocked cocaine toxicity in overdose models (Carrera et al., 2005). Active immunization with cocaine conjugates resulted in the development of cocaine antibodies capable of sequestering cocaine in the periphery. The antibody titers were sufficient to block reinstatement induced by a single dose of drug, but this protective effect was overcome with either repeated cocaine dosing or increasing the dose (Carrera et al., 2001; Carrera et al., 2000). The most effective vaccines to date have been against BSA- or cholera toxin B-conjugated norcocaine (TA-CD)(Fox et al., 1996) (Kantak et al., 2000) and their success in eliciting good antibody titers has resulted in the initiation of clinical trials in humans (Kinsey et al., 2010; Kosten et al., 2002). A variant of

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passive immunization using catalytic mAbs, which not only can sequester but also hydrolyze cocaine were, unfortunately not therapeutically active for more than 72 hours post administration (Landry et al., 1993; Matsushita et al., 2001; Yang et al., 1996). Other than TA-CD, none of the other immunotherapeutic-based biologics has shown promise.

Extensive work has gone into developing faster acting enzymes that can hydrolyze cocaine more rapidly. Cocaine is hydrolyzed primarily by butyrylcholinesterase (BchE) in humans and considerable effort has been put in to designing highly active mutant form of BchE, which has a 2000-fold improvement in catalytic efficiency over wt-BchE in hydrolyzing cocaine (Zheng et al., 2008).

Cocaine esterase (CocE) isolated from *Rhodococcus* which grow in the rhizosphere soil surrounding coca plants remains the fastest natural enzyme that can hydrolyze cocaine (Bresler et al., 2000; Larsen et al., 2002). Previously we demonstrated that CocE is thermolabile and thermostabilization of CocE is essential for its development into a therapeutic (Cooper et al., 2006). We used *in silico* methods to predict mutations that would stabilize the protein and showed that three mutations T172R, G173Q and L169K either alone in combination stabilized the protein (Brim et al., 2010; Gao et al., 2009; Narasimhan et al., 2010). Double mutants T172R/G173Q and G173Q/L169K have a half-life ( $\tau_{1/2}$ ) of 370 min and 2.9 days at 37°C, respectively, whereas wt-CocE lasts only 12.2 min (Brim et al., 2010; Gao et al., 2009; Narasimhan et al., 2010). More importantly we demonstrated that that these mutations afforded protection against cocaine-induced lethality for greater than four hours in rodent (Narasimhan et al., 2010). We also provided crystallographic and biochemical evidence that

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CocE is a dimer (Narasimhan et al., 2010) and that upon heat inactivation, the protein aggregates. The loss of CocE activity correlates with a transition to a much higher order aggregated state. This observation led to the hypothesis that strengthening the dimer interface with intramolecular disulfide bonds would further stabilize the protein. Disulfide cross-linking of subunits has been utilized to stabilize tetrameric malate dehydrogenase (Bjork et al., 2003),  $\alpha$  and  $\beta$  subunits of human choriogonadotropin (Heikoop et al., 1997), and tetrameric streptavidin (Reznik et al., 1996).

In this report we demonstrate that incorporating disulphide bonds between cysteine residues substituted at Gly4 and Ser10, both located on the  $\beta$ 1 strand of Domain I of CocE, conveys significant improvements to the thermostability and the  $\tau_{1/2}$  at 37°C. Moreover in combination with T172R/G173Q the disulphide-stabilized dimer (CCRQ-CocE) remains greater than 90% active for longer than 40 days at 37°C, representing a >4700-fold improvement over wt-CocE. The enhanced stability serves as a better substrate for modification with polyethylene glycol (PEG) moieties providing the therapeutic with stealth properties. PEGylated CCRQ-CocE retained full *in vitro* enzymatic activity, protected rodents up to 72 hours in a cocaine overdose model, diminished self-administration for 72 hours in rats, reduced cocaine-induced cardiovascular effects and locomotor functions in monkeys for up to 48 hours, and displayed reduced immunogenicity in mice.

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## Materials and Methods

**Materials:** Cocaine was purchased from Mallinckroft Inc. Branched 40 kDa PEG was purchased from JENKEM Technology USA Inc. (Allen, TX). All other reagents are of analytical grade and were obtained from Fisher Scientific or Sigma-Aldridge Corp.

**Site directed mutagenesis:** pET22b (+) CocE plasmid was kindly provided by Dr. Nick Larson (The Scripps Institute, CA) (Turner et al., 2002). Wild-type (wt-CocE) and CocE mutants were expressed as C-terminal hexahistidine-tagged proteins containing the exogenous sequence KLAALAEHHHHHH at the C-terminus. Point mutations were generated using a modified QuikChange™ (Stratagene) mutagenesis protocol. To generate double mutants, cDNAs with single point mutations were used as templates for a second round of mutagenesis. Oligonucleotide primer sequences are available on request. All mutants were confirmed by sequencing of both strands over the entire coding region. CocE preparations were expressed in *E. coli* BL-21 Gold (DE3) cells grown at 37 °C to an OD<sub>600</sub> of 0.8. Protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside and cells were harvested after incubation for 12 hours at 18 °C.

**Purification of cocaine esterase and mutants:** Cells were pelleted, resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl supplemented with protease inhibitors (3 μg/mL each of leupeptin and lima bean or soybean trypsin inhibitor), and lysed using a French press. Wild type or mutant CocE was enriched using Talon™ metal chelate affinity chromatography (Clontech Laboratories, Inc.), followed by anion-exchange chromatography on a Q-Sepharose fast



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performance liquid chromatography (FPLC) column (GE Healthcare, Piscataway, NJ). CocE was eluted from the Q-Sepharose column with a 150-450 mM NaCl linear gradient in buffers containing 50 mM Tris-HCl, pH 8.0. The peak fractions were pooled and concentrated, typically to 5 mg/mL, using Centricon-30 concentrators (Millipore), and then snap frozen in liquid N<sub>2</sub> and stored at -80 °C.

**Oxidation:** Purified proteins were oxidized in 50 mM Tris-HCl, pH 8.0 buffer containing 100 μM CuCl<sub>2</sub> overnight at 4 °C. CuCl<sub>2</sub> was then removed by chelating it by adding 1 mM EDTA to the above mixture. Protein was further purified by passing it through Q Sepharose column and eluting off the column with 150-500 mM NaCl gradient 50 mM Tris-HCl, pH 8.0. Fractions containing purified oxidized protein were identified by SDS-PAGE under non-reducing conditions and then pooled and concentrated to 5 mg/ml and snap frozen in liquid N<sub>2</sub> and stored at -80 °C.

**PEGylation:** Oxidized proteins were conjugated with maleimide-linked branched PEG 40K (GenKem Technology) over night in phosphate buffered saline (pH 8.0) at different PEG to protein ratio. The mixture was then loaded onto a Q sepharose column and eluted off the column over a 100 – 500 mM NaCl gradient in 50 mM Tris-HCl, pH 8.0. PEGylated CCRQ-CocE proteins eluted off the column earlier than unPEGylated CCRQ-CocE. Fractions collected were analyzed by SDS PAGE and fractions of PEGylated CCRQ-CocE were pooled and concentrated. PEGylated and non-PEGylated CocE preparations were analyzed by MALDI mass spectroscopy at the Department of Chemistry Mass Spectrometry Core at the University of Michigan.

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**Michaelis-Menten kinetics of cocaine hydrolysis:** A spectrophotometric real-time assay was used to monitor cocaine hydrolysis (Xie et al., 1999). The initial rates of decay were determined by following the change in the intrinsic absorbance of cocaine at 240 nm ( $6700 \text{ M}^{-1} \text{ cm}^{-1}$ ) (Xie et al., 1999) on a SpectraMax Plus 384 UV plate reader (Molecular Devices) using SOFTmax Pro software (Version 3.1.2). The reaction was initiated by the addition of 100  $\mu\text{L}$  of wt-CocE or mutant CocE (50 ng/mL, 25 ng/mL, 10 ng/mL or 5 ng/mL) in phosphate buffered saline (PBS) pH 7.4 to 100  $\mu\text{L}$  of a 2X cocaine solution made in PBS. The final enzyme concentration was 25 ng/mL, 12.5 ng/mL, 5 ng/mL or 2.5 ng/mL and the final cocaine concentrations were: 100, 50, 25, 12.5, 5, 2.5, 0.5, and 0.25  $\mu\text{M}$ .  $V_{\text{max}}$  and  $K_{\text{m}}$  values were calculated using Prism (GraphPad Software). For stability measurements, CocE variants were diluted to 50 ng/mL (2X) concentration and incubated at 37 °C. Aliquots were removed at varying time points and assayed for activity against cocaine as described above (n of 5). Temperature-dependent decay in esterase activity was measured by pre-incubating wt-CocE and mutant forms of CocE (50 ng/mL) at various temperatures (0, 25, 35, 37, 42, 45, 48, 50 and 55 °C) for 30 min and then the remaining activity was measured as described above in two separate experiments.

**Melting Temperatures ( $T_m$ ) measurements using Thermo Fluor:** Purified CocE variants were buffer exchanged into 50 mM Tris-HCl, pH 8.0. 5  $\mu\text{L}$  of 0.2 mg/mL protein were placed in an ABgene 384-well PCR plate (Thermo-Fisher) in triplicate. Five  $\mu\text{L}$  of 200  $\mu\text{M}$  dye 1-anilinonaphthalene-8-sulfonic acid (Sigma) was subsequently placed on top of the protein solution. The plates were spun on Beckman-Coulter centrifuge at 1000-x g for 2 min to remove air bubbles. 1  $\mu\text{L}$  of mineral oil was overlaid on top of the protein-dye mix to prevent evaporation. Protein unfolding was monitored on a Thermo Fluor 384 reader (Johnson and

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Johnson) using a temperature gradient ranging from 25 to 85 °C. The fluorescence emission data were analyzed using Thermo Fluor Acquire 3.0 software as per manufacturer's guidelines.

***Crystallization and Data Collection:*** Crystals were grown by hanging-drop vapor diffusion in VDX plates. 1  $\mu$ L of CC-CocE at 8 mg/ml was added to 1  $\mu$ L of mother liquor on a siliconized glass cover slip and incubated above 1 mL of mother liquor. Crystals containing one monomer per asymmetric unit were grown and harvested as described previously (Narasimhan et al., 2010). A second crystal form was obtained with wt-CocE over mother liquor consisting of 20% PEG 3,350, 100 mM 2-(N-morpholino)-ethane sulfonic acid, pH 6.0, and 1 M NaCl. Drops were incubated on siliconized cover slips at 4° over 1 mL of mother liquor. The PEG crystal-form was harvested in cryoprotectant containing 70% mother liquor and 30% glycerol. Diffraction data were collected at LS-CAT of the Advanced Photon Source and indexed and scaled using HKL2000 (Otwinowski and Minor, 1997). Atomic models were built using Coot (Emsley and Cowtan, 2004) and refined with REFMAC 5.2.2 (Vagin et al., 2004). Due to the X-ray sensitivity of the disulfide bonds in the CC-CocE crystals, data was limited to the first 25 degrees of data collection.

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## RESULTS

**Design of a disulfide-stabilized CocE dimer.** We previously reported that wt-CocE exists as a homodimer in solution (Narasimhan et al., 2010). However, in prior crystal structures, CocE crystallized with one monomer in the asymmetric unit. In this crystal lattice, a CocE dimer is formed by a crystallographic symmetry axis such that a continuous  $\beta$  sheet is formed by the  $\beta$ 1 strands in Domain I, which likely corresponded to the solution dimer. We have now also determined a crystal structure of CocE in a unique space group with a homodimer in the asymmetric unit (Table II & **FIG 1**). This homodimer superimposes onto the ammonium-sulfate monomer with an RMSD of 1.00 Å over the active-site containing domain I, and 0.93 Å for domain III. The RMSD for superposition of the two homodimers is 1.8 Å for all C $\alpha$  atoms. The higher RMSD value for the entire homodimer corresponds to a slight opening of the active-site cleft by a rigid-body movement of domain III away from domain I. Residue Phe408 is moved 1.1 Å away from the catalytic Ser117, resulting in a slightly more open active site, without disturbing the catalytic triad or oxyanion hole. The high similarity of the homodimers derived from these two unique crystal forms confirms that the biological unit of CocE is generated by homodimerization at the N-terminal  $\beta$ -strand, and that this model could be used to rationally design disulfide bridges. We predicted that Gly4 in one monomer chain and Ser10 in the dimer-related subunit, when substituted to cysteines, would have the right bond angles and distances (Sowdhamini et al., 1989) to form a disulfide bridge (**FIG 1**). Thus, G4C/S10C (CC-CocE) double mutations were incorporated into both wt-CocE and the previously described thermostabilizing mutant, T172R-G173Q (Narasimhan et al., 2010) to create CC-CocE and CCRQ-CocE.

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**Stability assessment.** Crosslinking CC-CocE with  $\text{CuCl}_2$  resulted in a DDT-sensitive  $\sim 140$  kDa species, as assessed by SDS-PAGE (**See Supplemental Data, Fig S1**). The purified proteins were assessed for cocaine hydrolyzing activity and were as active as wt-CocE (Table I) (Narasimhan et al., 2010). Impressively, the CC-CocE protein retained 100% of its enzymatic activity after 7 days incubation at  $37^\circ\text{C}$  (**See supplemental data Fig S2**). In CCRQ-CocE, the four substitutions acted synergistically to create a very stable form of CocE that retained activity for a significantly longer time than observed with any other form of CocE. The activity data were fit to a single exponential decay curve using Prism (GraphPad, San Diego, CA) and reveal an apparent plateau at 93% after 41 days at  $37^\circ\text{C}$  (**FIG 2A**). Because  $>90\%$  of enzymatic activity was retained even after 41 days at  $37^\circ\text{C}$ , it was difficult to ascertain a true half-life for this variant of CocE. Thermofluor analysis was performed on oxidized CC-CocE and CCRQ-CocE to determine melting temperatures ( $T_m$ ). CC-CocE had a  $\sim 3^\circ\text{C}$  elevated  $T_m$  as compared to wt-CocE ( $34.8^\circ\text{C}$  Vs  $38.0^\circ\text{C}$ ) and CCRQ-CocE had an even higher  $T_m$  of  $43.9^\circ\text{C}$ , consistent with the increased half-life of these forms of CocE at  $37^\circ\text{C}$  (**FIG 2B & the table**). We performed thermal inactivation assays as described in before (Narasimhan et al., 2010), in which we pre-incubated of proteins at temperatures above  $37^\circ\text{C}$  prior to measuring their activity. We have shown that wt-CocE is inactivated within 30 min at  $30^\circ\text{C}$ , whereas RQ-CocE and L169K variants were inactivated at higher temperatures ( $40\text{-}45^\circ\text{C}$ ) (Narasimhan et al., 2010). CCRQ-CocE retained 90% of activity even after 15 min incubation at  $47.5^\circ\text{C}$ , indicating its improved thermostability over RQ-CocE (**FIG 2C**). Size-exclusion chromatography analysis revealed that even after 7-day incubation at  $37^\circ\text{C}$ , the majority of CCRQ-CocE protein resolved at approximately 140 kDa, corresponding to a homodimer, with very little aggregate in the void volume (See **Supplemental data, Fig S3**), as

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opposed to wt-CocE which aggregated within an 1 hr incubation at 37 °C (Narasimhan et al., 2010).

**Confirmation of disulfide bond formation.** The 1.53 Å resolution X-ray crystal structure of oxidized CC-CocE was determined, in which good electron density was present at the two engineered cysteine positions. Two conformations of the introduced cysteines were observed. Both conformations have sulfur-sulfur distances of 2.0 angstroms, and a dihedral angle of either 86.5 or -71.9 °, consistent with the presence of disulfide bonds. The Cys4 sulfur in the 86.5 ° conformation is distinguishable in omit-map density contoured up to 5  $\sigma$ , while the other three sulfur conformations are observed at up to 9.5  $\sigma$  omit density (**Fig 3**). The RMSD deviation between the wt-CocE and CC-CocE structures was negligible, indicating that introduction of the two disulfide bonds does not dramatically alter the overall conformation of the proteins. MALDI-TOF mass spectrometry assessment suggests the molecular mass of oxidized and reduced CC-CocE to be 126.79 and 66.43 kDa, respectively (not shown).

***In vivo* protection against cocaine-induced lethality in rodents:** We next examined the ability of CCRQ-CocE to protect rodents from injections of lethal doses of cocaine as described in Narasimhan *et al* (Narasimhan et al., 2010). Mice pretreated with wt-CocE succumbed to a lethal dose of cocaine as shown before (Narasimhan et al., 2010). CC-CocE afforded protection against cocaine-induced lethality to 80% of animals pretreated 4-hr whereas few survived following a 6 hour pretreatment (**data not shown**). To ascertain if the more thermally stable CCRQ-CocE (**Fig 4**) performed better *in vivo* mice were pretreated with CCRQ at 0.1 mg/kg (data not shown), 1 mg/kg (triangles), 10 mg/kg (open squares), or 32 mg/kg (inverted triangles) at

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various times prior to lethal cocaine injection (i.p.). Pretreatment of animals with CCRQ-CocE was not effective for longer than 24 hours even at the highest dose tested (Fig 4, Left top panel) while lower doses of CCRQ-CocE protecting the rodents for shorter pre treatment periods in a dose dependent manner. Similar results were obtained with 32 mg CCRQ-CocE (inverted triangles) in rats (Fig 4, Right top panel).

**Conjugation of PEG polymers to CCRQ-CocE to prolong its *in vivo* life:** The Incorporation of stealth-like properties of 40-kDa branched PEG molecules in to CCRQ-CocE (PEG CCRQ-CocE) using maleimide cross-linking prolonged the *in vivo* lifetime of the protein in agreement with previous PEGylation studies using wt-and RQ-CocE (Park et al., 2010). PEGylation of CC-CocE or CCRQ-CocE with 40-Kda branched PEGS at CocE:PEG ratios 1:1, 1:5, 1:10 or 1:20 revealed little difference in the appearance or yield of PEGylated CocE when CocE:PEG ratio was greater than 1:5 (See **Supplementary Data, Fig S4**). PEG CCRQ-CocE was separated from native-CocE by ion-exchange chromatography and purified to homogeneity. PEG-CCRQ-CocE migrated slower than CCRQ-CocE on a 4-20% gradient SDS-PAGE gel (See **Supplementary Data, Fig S4**) consistent with their higher molecular weight and larger hydrodynamic radius. PEG-CCRQ CocE was analyzed on MALDI-TOF mass spectrometry to discern the stoichiometry of protein to PEG in the final preparation. Two species of PEG-CCRQ-CocE were observed with molecular weights of ~107 kDa and ~210 kDa under reducing and non-reducing conditions, respectively, while the unPEGylated versions have molecular weights of 66.4 and 126.8 kDa, respectively (Data not shown). This is suggestive that monomeric CCRQ-CocE (reduced) contains a single 40k PEG (107-66.4 kDa=40 kDa), whereas the CCRQ-CocE dimer contains 2-40k PEG moieties (210-126.8 = 2x 40 kDa). No discernible non-PEGylated protein was present in the purified species.

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PEG-CCRQ is catalytically as active as wt-CocE in hydrolyzing cocaine (Table I). PEG-CCRQ predictably had higher melting temperature ( $T_m$ ) as compared to wt-CocE (43.86 °C Vs 34.77 °C) when observed by Thermofluor experiments (**Fig 2B and the Table I**).

**PEG-CCRQ-CocE Rodent Lethality studies** To observe protection of rodents against from lethal doses of cocaine, we pretreated mice (**Fig 4B**) and rats (**Fig 4D**) with PEG-CCRQ-CocE (1, 10 or 32 mg/kg per mice and 3.2, 10.0 or 32 mg/kg per rat) intravenously 1-min prior to the initial dose of cocaine (180.0 mg/kg; *i.p.*), with subsequent doses of cocaine (180mg/kg) administered at 24-hr intervals. At this dose of cocaine, rodents die within 3-5 minutes of injection if no CocE was given either prior or subsequent to cocaine injection. Although CCRQ-CocE (*ie.* unPEGylated) provided a dose-dependent protection against the lethal effects of cocaine (**Fig 4A & C**), pretreatment with PEG-CCRQ-CocE provided a dose- and time-dependent protection against an LD<sub>100</sub> dose of cocaine in both rats and mice (**Fig 4B & D**). Unlike the protective effects of CCRQ-CocE (3.2 -32.0 mg/kg), which had fully dissipated 24-hrs after administration, the protective effects of pretreatment with PEG-CCRQ-CocE were much longer lasting. When administered at a dose of 10 mg/kg dose (open squares) PEG-CCRQ-CocE provided a complete protection against a second challenge dose of 180.0 mg/kg; *i.p.* cocaine when administered 24-hr later, with all mice and rats dying by the fourth challenge dose administered 72 hours later. Similar effects were observed with a dose of 32 mg of PEG-CCRQ-CocE (open, inverted triangles), with all mice and surviving the third challenge dose administered 48 hours after pretreatment with PEG-CCRQ-CocE, and 100% lethality observed at the fifth challenge dose 96-hrs later. Doses of 1, 3.2, 10 and 32 mg/kg in rodents translates to approximately to 68 nM, 217 nM, 680 nM and 2.18  $\mu$ M, respectively, based on the blood volume of rodents. A direct



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conversion to humans (75 kg) with 5-liter blood volume would correspond to approximately 0.95 mg/kg, 3.04 mg/kg, 9.52 mg/kg and 30.4 mg/kg human dose, respectively. However, we have recently determined that significantly lower (10-fold) doses of PEG-CCRQ-CocE are required in non-human primates (Collins et al, manuscript under review). We have also determined that lower doses (0.32-3.2 mg/kg) of a double mutant of CocE (RQ-CocE) could ameliorate the cardiovascular effects of cocaine in rhesus monkeys.(Collins et al.) We therefore predict that a dose of PEG-CCRQ-CocE in the 0.32-3.2 mg/kg range would be suitable for humans.

**The effects of PEG-CCRQ-CocE on Cocaine Self-administration.** Rats were trained to respond for increasing doses of cocaine under a fixed ratio (FR) 5 schedule of reinforcement during five sequential components. To evaluate whether PEG-CCRQ-CocE can attenuate responses for longer periods of time (**Fig 5**), rats were pretreated with saline, 3.2 mg/kg, 10 mg/kg or 32 mg/kg PEG-CCRQ at immediately before the start of a session and re-tested at 24-hr increments until responding for cocaine returned to baseline-like levels. During baseline sessions in which rats were pretreated with saline, the dose-response for cocaine-maintained responding showed very low rates of responding maintained by either no drug, or a low unit-dose of cocaine. Peak responding was maintained by 0.1 mg/kg/inj cocaine (Figure 5), and displayed dose-dependent decreases in responding maintained by doses of 0.32, and 1.0 mg/kg/inj cocaine (not shown). Conversely, when saline was substituted for cocaine, low rates of responding were observed in each of the five components, with returning to baseline levels with the reintroduction of cocaine 24-hr or 48-hr later (**Figure 5**). Similar to when saline was substituted for cocaine, low rates of responding were observed when rats were pretreated with 3.2 mg/kg PEG-CCRQ-CocE

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and allowed to respond for 0.1 mg/kg/inj cocaine with a partial recovery of cocaine-maintained responding observed 24-hr later, and baseline-like levels of responding observed 48-hr after pretreatment with PEG-CCRQ-CocE. A similar suppression of responding was observed immediately after pretreatment with 10 mg/kg PEG-CCRQ-CocE however, unlike with 3.2 mg/kg PEG-CCRQ, these low rates of cocaine-maintained responding were also observed 24-hr later, with a partial recovery of responding observed 48-hr later, and baseline-like rates of responding observed 72-hr after PEG-CCRQ-CocE administration. At the highest dose tested (32 mg/kg PEG-CCRQ-CocE) cocaine-maintained responding occurred at saline-like rates for the first 48-hr after administration, with a partial recovery of responding observed at the 72-hr time point, however, baseline-like rates of responding were not fully recovered until five days, or 120-hr after the administration of PEG-CCRQ-CocE.

**PEG-CCRQ-CocE decreases cardiovascular effects of cocaine in rhesus monkey.** In comparison to saline (filled circles), cocaine (3.2 mg/kg i.v, open circles) produces a long-lasting increase in mean arterial pressure (MAP, **Fig 6A**) and heart rate (HR, **Fig 6B**). As shown in Figure 6, these increases in MAP and HR were rapidly decreased following the administration of 3.2 mg/kg PEG-CCRQ-CocE, 10-min after cocaine (red, inverted triangles). In addition to this immediate amelioration of the cardiovascular effects of cocaine, the protective effects of PEG-CCRQ-CocE against the increases in MAP and HR induced by additional challenge doses of 3.2 mg/kg cocaine were still apparent at the 24-hr (yellow, inverted triangles) and 48-hr (green, inverted triangles) time points, however, these effects had diminished by the 72-hr time point (blue inverted triangles), and the cardiovascular effects of cocaine were no different than during the baseline condition.

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**Measurement of immune response to PEG-CCRQ-CocE.** PEGylation of proteins or peptides have been shown to reduce immunogenicity of foreign proteins (Caliceti and Veronese, 2003). To determine if PEG-CCRQ-CocE elicited an immune response in mice and thereby lost its effectiveness after repeated exposure (**Fig 7**), CCRQ (open bars) or PEG-CCRQ (dark bars) was administered at a 10 mg/kg dose four times, once every two weeks, and at every time point anti-enzyme antibody and any diminution of the effectiveness of the enzyme were measured. Effectiveness of the enzyme in protecting mice against lethal doses of cocaine was measured using the standard lethality assay. As illustrated in **Fig 7B** only after fourth exposure with CCRQ-CocE were anti-CocE antibodies increased (~1000-fold) whereas at this time point very little increase in antibody titer with PEG-CCRQ-CocE administration were observed. As shown in **Fig 7A**, CCRQ-CocE loses its effectiveness after the third and the fourth injections, where it fails to protect 40 % (3<sup>rd</sup> exposure) and approximately 50% (4<sup>th</sup> exposure) of the animals from lethal dose of cocaine (180 mg/kg). In contrast only after the fourth exposure did PEG-CCRQ-CocE is fail to protect (25%) even though anti-CocE antibody titer at this stage was negligible.

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## Discussion

Cocaine esterase has great potential to be developed as a protein therapeutic to combat cocaine abuse. We previously determined that thermally stabilized CocE (Brim et al., 2010; Gao et al., 2009; Narasimhan et al., 2010), to make it suitable for therapy and showed that RQ-CocE not only resists the toxic effects of cocaine but also inhibited the reinforcing properties of cocaine in a rodent self-administration model (Brim et al., 2010; Collins et al., 2009; Cooper et al., 2006; Ko et al., 2007). Moreover biochemical and biophysical analyses of these thermostable forms revealed that the mutants and wt-CocE exists as dimers (Narasimhan et al., 2010).

Analysis of the crystal-packing in a new crystal form reveals a dimer interface encompassing  $1172 \text{ \AA}^2$  of accessible surface area per monomer. This large interface includes residues from all three domains of CocE. The dimer interface interactions are mainly van der Waals or hydrophobic in nature. Domain I constitutes the majority of the dimerization interface, accounting for 87% of the buried surface area. For domain II, only helix 3 contributes to dimerization. The jelly-roll  $\beta$ -barrel fold of domain III contributes only residues 410, 411, and 412 to dimerization. The most striking feature of this interface is the sharing of a  $\beta$ -sheet between the two monomers. Residues 6-8 of each monomer form anti-parallel  $\beta$ -strands to create a continuous  $\beta$ -sheet composed of two  $\beta$ -strands (**FIG 1, Top view**). Analytical ultracentrifugation (AUC) analysis also confirmed dimerization and that the molecular weight of wt-CocE is approximately 127,000 Da (data not shown).

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Incubation of CocE variants at 37 °C induces protein aggregation and subsequent loss of activity (Narasimhan et al., 2010). We demonstrated that stabilizing mutations delayed the aggregation, and that time to aggregation correlated well with the in vitro half-lives of the mutants (Narasimhan et al., 2010). Furthermore the two-phase inactivation kinetics seen with the mutants at 37 °C suggests that thermal denaturation is due to two events; local unfolding and dimer disruption. We hypothesized that any transient disruption of the dimer interface leads to global protein misfolding and aggregation.

For many multimeric enzymes the first step in denaturation seems to be subunit dissociation e.g.: alkaline phosphatase and  $\beta$ -galactosidases (Fernandez-Lafuente, 2009; Pilipenko et al., 2007; Poltorak et al., 1999; Wong and Wong, 1992). Thermophilic proteins have evolved to employ multimerization and strong interactions between multimers as strategy to survive extreme temperatures (Ogasahara et al., 2003; Tanaka et al., 2001). Some thermophilic proteins utilize disulfide bridges across subunits to strengthen subunit interaction as compared to homologous mesophilic proteins (Kabashima et al., 2001; Nakka et al., 2006). Oligomer stabilization by disulfide crosslinking across the interface is a widely used rational approach to engineer stability in industrial biocatalysts (Mateo et al., 2008) (Bjork et al., 2003; Heikoop et al., 1997; Reznik et al., 1996).

The  $\beta$ -sheet formed by  $\beta$ 1-strands from both subunits is the most conspicuous facet of the dimer interface of CocE and residues 4 and 10 in those strands are positioned ideally to substitute into cysteines in order to create a disulfide bridge. Residue 295 (Alanine) in the loop in the interface also can be mutated to cysteine to create a disulfide bridge (See Supplemental

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Data, **Fig S1**). G4CS10C- CocE (CC-CocE) retained almost all activity after 7 days of 37 °C treatment, a significant improvement over T172R-G173Q ( $\tau_{1/2}$  = 370 min) and L169K-G173Q ( $\tau_{1/2}$  = 2.9 days) variants of CocE whose mutations were predicted by molecular modeling analysis (Brim et al., 2010; Gao et al., 2009; Narasimhan et al., 2010). Combining these mutations that stabilize intramolecular interactions with dimer stabilizing mutations, led to a meta-stable form, CCRQ-CocE, which retained >90% of activity after 41 days at 37°C and seems more resistant to aggregation. However, when this variant was tested *in vivo* for its durability in protecting rodents from toxic doses of cocaine, it failed to protect for longer than 24 hours (**Fig 4, top panels**). These data indicate that thermostability is no longer the half-life determining step *in vivo*. Clearly recombinant CocE is cleared from the rodents through either immunosurveillance and/or proteolysis (data not shown).

Modification such as PEGylation is necessary to overcome these shortfalls and to make CocE a viable therapeutic alternative. PEGylation has been shown to prolong *in vivo* life of protein biologics, reduce immunogenicity and protect proteins and peptides from proteolytic digestion (Caliceti and Veronese, 2003). Many PEGylated molecules have successfully been introduced in the market including, PEG-interferon  $\alpha$ 2a (Pegasys<sup>®</sup>, Roche, Nutley NJ) and branched PEG-anti-VEGF aptamer (Pegaptanib, Macugen<sup>™</sup>, OSI Pharmaceuticals, Melville, NY). Our group has previously reported that PEGylation of wt-CocE and the T172R-G173Q mutant CocE with 40 kDa branched PEG with maleimide coupling was feasible and PEG-CocE retained its *in vitro* and *in vivo* function (Park et al., 2010). Due to the inherent lack of stability of wt-CocE and T172R-G173Q mutant CocE as compared to CCRQ-CocE, we observed that conjugation with PEG-40K was detrimental to the enzyme activity (Park et al., 2010). In contrast, PEG-CCRQ

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retained 100% of activity as compared to unPEGylated CCRQ-CocE (Table I) and that we believe is because of higher overall stability of the CCRQ-CocE.

Compared to the effects of T172R-G173QCocE (RQ-CocE) and L169K-G173QCocE (KQ-CocE) (Brim et al., 2010; Narasimhan et al., 2010), PEG-CCRQ-CocE provided a much longer lasting protection against the lethal effects of cocaine with 100% of rats and mice surviving not only the immediate 180.0 mg/kg; i.p. dose of cocaine, but also at least two additional challenges with this LD<sub>100</sub> dose of cocaine over the next 48-hrs. A similarly prolonged duration of action was observed in the rodent self-administration studies as PEG-CCRQ completely inhibited responding that was maintained by doses of cocaine as large as 1.0 mg/kg/inj for at least 48 hr, with a significant antagonism of the reinforcing effects of cocaine apparent for at least 96 hours post treatment. Not only are these effects the longest lasting of any of the CocE variants (Brim et al., 2010; Collins et al., 2009), but when taken together, the results of the lethality and self-administration studies suggest that PEG-CCRQ retains its ability to rapidly metabolize large amounts of cocaine for at least 48-hrs *in vivo*. Repeated wt-CocE exposures in rats increased antibody titers after the fourth exposure by 100- to 1000- fold and after fourth exposure the effectiveness of wt-CocE in preventing cocaine induced lethality was diminished (Ko et al., 2007). PEGylated CCRQ-CocE showed negligible increase in anti-CocE titer even after fourth exposure but after the fourth exposure its effectiveness in protecting rodents was diminished (**Fig 7**). Other factors like damage to myocardium due to repeated injections of toxic doses of cocaine could result in lethality and could have diminished the effectiveness of PEG-CCRQ-CocE at the fourth exposure. Importantly, a similarly prolonged duration of action was also observed in a third, larger species, the rhesus monkey. When administered 10 minutes

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after cocaine, 3.2 mg/kg PEG-CCRQ produced a rapid amelioration of the MAP and HR increasing effects of 3.2 mg/kg; i.v. cocaine to a degree that was similar to that which was previously reported with RQ-CocE (Collins et al., 2011). However, unlike with RQ-CocE, PEG-CCRQ-CocE retained its capacity to reduce the cardiovascular effects of cocaine for at least 48-hrs. Interestingly, that this duration of action was similar to those observed with 32.0 mg/kg PEG-CCRQ-CocE in rodents, despite the 10-fold lower dose, suggests that PEGylation may be more effective at increasing the *in vivo* residence times in the rhesus monkey, a much larger species that is more closely related to the human.

Maleimide coupling PEG-40K could potentially yield products with three PEG molecules per monomer because a CocE monomer has three solvent exposed cysteines. MALDI-TOF analysis (not shown) and site-directed mutagenesis (Narasimhan and Sunahara, personal communication) indicate that cysteine at position 551 is conjugated to PEG-40K resulting in two PEG-40K molecules per CCRQ-CocE dimer. These conjugated PEG molecules mask enough charged residues in native CCRQ-CocE to enable us to purify the protein effectively on an anion exchange chromatography column to 100% homogeneity. The lower than expected *in vivo* residence time and effectiveness of PEG-CCRQ may be explained by insufficient shielding of the CocE dimer from immunosurveillance and proteases by PEGylation. Efforts are currently underway to engineer new sites on the CocE dimer, which are diametrically opposite to residue 551 in an effort to provide a more complete mask around the dimer, and provide more protection from immune response and proteases.



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Cleary subunit stabilization and subsequent PEGylation of the stable dimer CocE has resulted in a protein therapeutic that has shown significant advancements in improving the *in vivo* duration of action compared to earlier versions of CocE. Further improvements with site-specific PEGylation, encapsulation in nanoparticles or both could result in extremely stable biotherapeutic. These improvements and strategies are promising first steps in developing a safe, effective and long lasting protein biologic that combats cocaine addiction and abuse in humans.

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## FIGURE LEGENDS

**Figure 1. The dimer interface of wt-CocE and position of  $\beta$ 1-strands that forms a continuous  $\beta$ -sheet.** A) Introduction of cysteine at positions 4 and 10 creates two covalent bonds to stabilize the  $\alpha/\beta$  hydrolase domain. Oxidation of these two cysteines (conformation A, orange; conformation B, yellow) cements a continuous  $\beta$ -sheet that includes the active site serine 117 (purple) of both monomers (light gray, dark gray). The homodimer  $\beta$ -sheet consists of 20  $\beta$ -strands (light green, dark green).

**Figure 2. Enzymatic stability of CCRQ-CocE.** A) Decay in the capacity to convert cocaine to ecgonine methylester and benzoic acid was measured at 37°C. CCRQ-CocE (50 ng/mL) was incubated at 37°C and the activity calculated over time (n = 3). Percent remaining activity was plotted against days of incubation at 37 °C. B) Melting temperatures of wild-type and mutant forms of CocE. Protein unfolding was monitored on a ThermoFluor 384 reader using 1-anilinonaphthalene-8-sulfonic acid (1, 8-ANS) (100  $\mu$ M final) at a protein concentration of 100 mg/mL. Melting curves of wt-CocE (inverted triangles), CC-CocE (closed circles), CCRQ-CocE (closed squares), and PEG-CCRQ-CocE (triangles) are shown. The melting temperatures observed from the melting curves are shown in the table. C) Temperature-dependent decay in esterase activity. CCRQ-CocE (50 ng/mL ) was pre-incubated for 15 min at temperatures indicated (°C) and activity measured as described previously. The activities of each mutant

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remaining (as a percentage of the catalytic efficiency, without pre-incubation) following pre-incubation are shown).

**Figure 3. X-ray crystallography reveals two conformations of the engineered intramolecular disulfide bridge.** When two CocE monomers (light gray, dark gray) dimerize, they form an antiparallel beta-sheet at their extreme N-termini. The flexibility at the extreme N-terminus allows two conformations of this bridge. Conformation A, orange, has a dihedral angle of 86.5 degrees. Conformation B, yellow, has a dihedral angle of -71.9 degrees. FoFc density, generated by omitting the beta carbon and gamma sulfur of both cysteine 4 and 10, is shown contoured to 5 sigma (green) and -5 sigma (red). Both conformations have a sulfur-sulfur distance of 2.0 angstroms.

**Figure 4. Efficacy of CCRQ-CocE or PEG-CCRQ CocE in vivo.** 1 mg/kg (triangles), 10 mg/kg (squares) or 32 mg/kg (inverted triangles) of CCRQ-CocE (A & C) or 1 mg/kg (triangles), 3.2 mg/kg (open circles), 10 mg/kg (squares) or 32 mg/kg (inverted triangles) of PEG-CCRQ-CocE (B & D) were injected into mice (A & B) or rats (C & D) at different times prior to 180 mg/kg i.p. cocaine injection at time -1 minute. Efficacy of CCRQ-CocE and PEG-CCRQ-CocE in protecting the rodents was assessed and percent survival was plotted against the pretreatment times.

**Figure 5. PEG-CCRQ-CocE mediated suppression of cocaine self-administration in rats.** Rats were trained to respond for increasing doses of cocaine under a fixed ratio (FR) 5 schedule of



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reinforcement during five sequential components. Rats ( $n = 6$  for each condition) were intravenously pretreated with saline (**open bars**), 3.2 mg/kg (**shaded bars**), 10 mg/kg (**hatched bars**), or 32 mg/kg (**filled bars**) and tested immediately, 1 day, 2 days, 3 days, 4 days or 5 days with 0.1 mg/kg/inj cocaine. Baseline testing and saline injections are shown in top right panel.

**Figure 6. Effect of PEG-CCRQ-CocE on the cardiovascular effects of cocaine in monkeys.**

Monkeys were either given saline (closed circles) or 3.2 mg/kg cocaine (open circles) intravenously and mean arterial pressure (A, **MAP**) and heart rate (B, **HR**) were observed. To observe the effect of PEG-CCRQ-CocE, we injected 3.2 mg/kg of the enzyme 10 minutes after the cocaine injection (**red inverted triangles**). Challenge doses of cocaine (3.2 mg/kg) were given 24-hr (**magenta inverted triangles**), 48-hr (**green inverted triangles**) or 72-hr (**blue inverted triangles**) after PEG-CCRQ-CocE injection and mean arterial pressure and heart rate were observed. Changes in mean arterial pressure ( $\Delta$ MAP, left panel) and heart rate ( $\Delta$ HR, right panel) were plotted against time.

**Figure 7. Effectiveness of CCRQ-CocE and PEG-CCRQ-CocE in preventing cocaine-induced toxicity in mice following repeated administration. A)** The protective effects of *i.v.* enzyme (10 mg/kg, 1 min pretreatment) against *i.p.* cocaine 180 mg/kg-induced lethality were tested in the same mice ( $n=11$ ) every two weeks. **B)** The anti-enzyme antibodies were determined from serum samples collected 24 hr before the toxicity test. Each value represents the percentage of

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mice affected (A) or the mean  $\pm$  SEM (B). The asterisk represents a significant difference from the value determined in the first trial (\*,  $p < 0.05$ ).

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**Table I. Kinetic behavior of wt-CocE and mutant CocE.** The metabolism of cocaine by purified preparations of wt-CocE, CC-CocE, CCRQ-CocE, or PEG-CCRQ-CocE was measured as described in Materials and Methods Section.

CocE variant	$k_{cat}$ ( $s^{-1}$ )	$K_m$ (mM)	$k_{cat}/K_m$ ( $s^{-1} M^{-1}$ )
wt-CocE	$51.4 \pm 16.8$	$0.0057 \pm 0.0019$	$8.99 \times 10^6$
CC-CocE	$49.9 \pm 4.66$	$0.021 \pm 0.0042$	$2.38 \times 10^6$
CCRQ-CocE	$56.6 \pm 4.90$	$0.026 \pm 0.0018$	$2.12 \times 10^6$
PEG-CCRQ-CocE	$40.1 \pm 3.24$	$0.019 \pm 0.0020$	$2.11 \times 10^6$

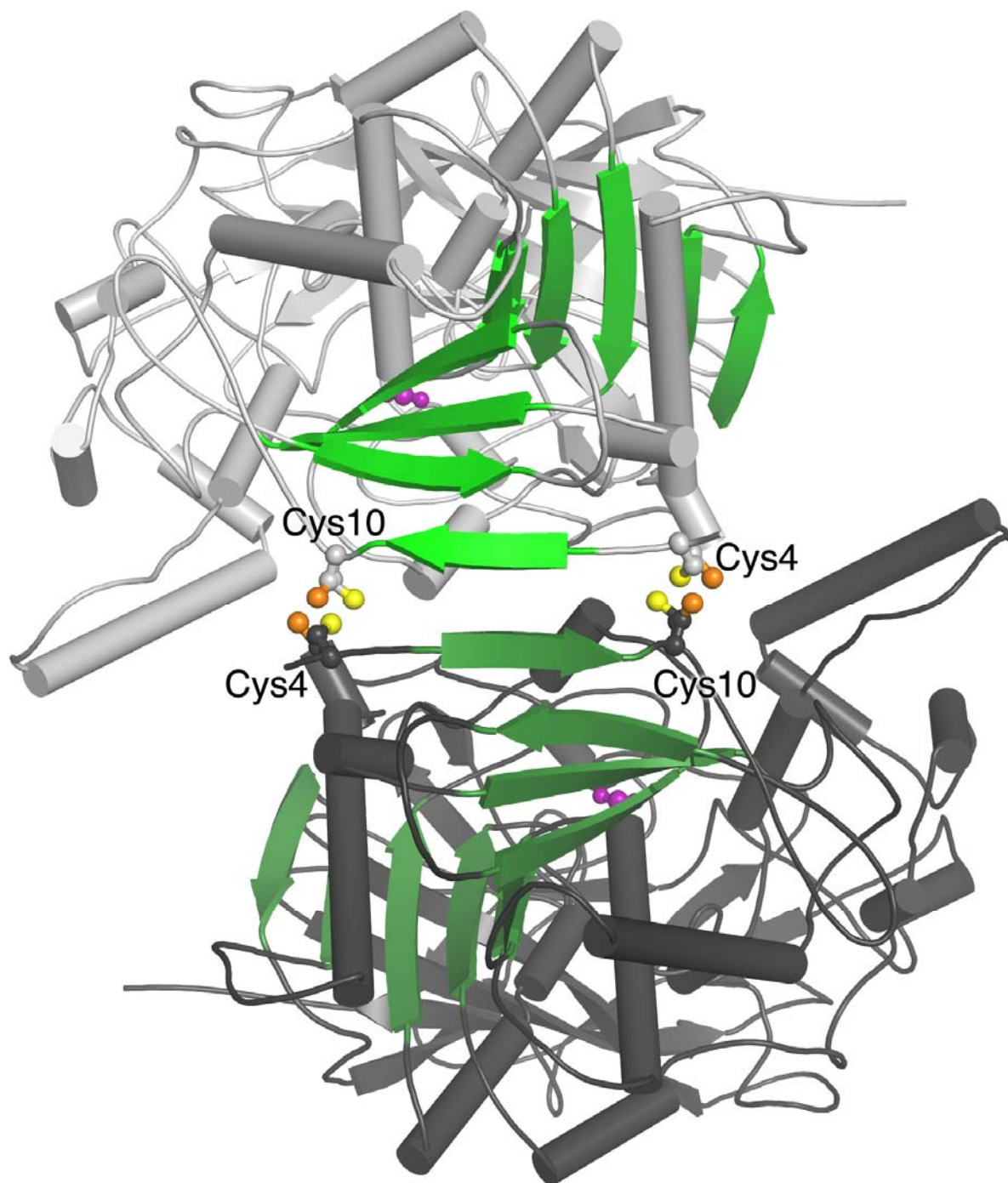


Figure 1

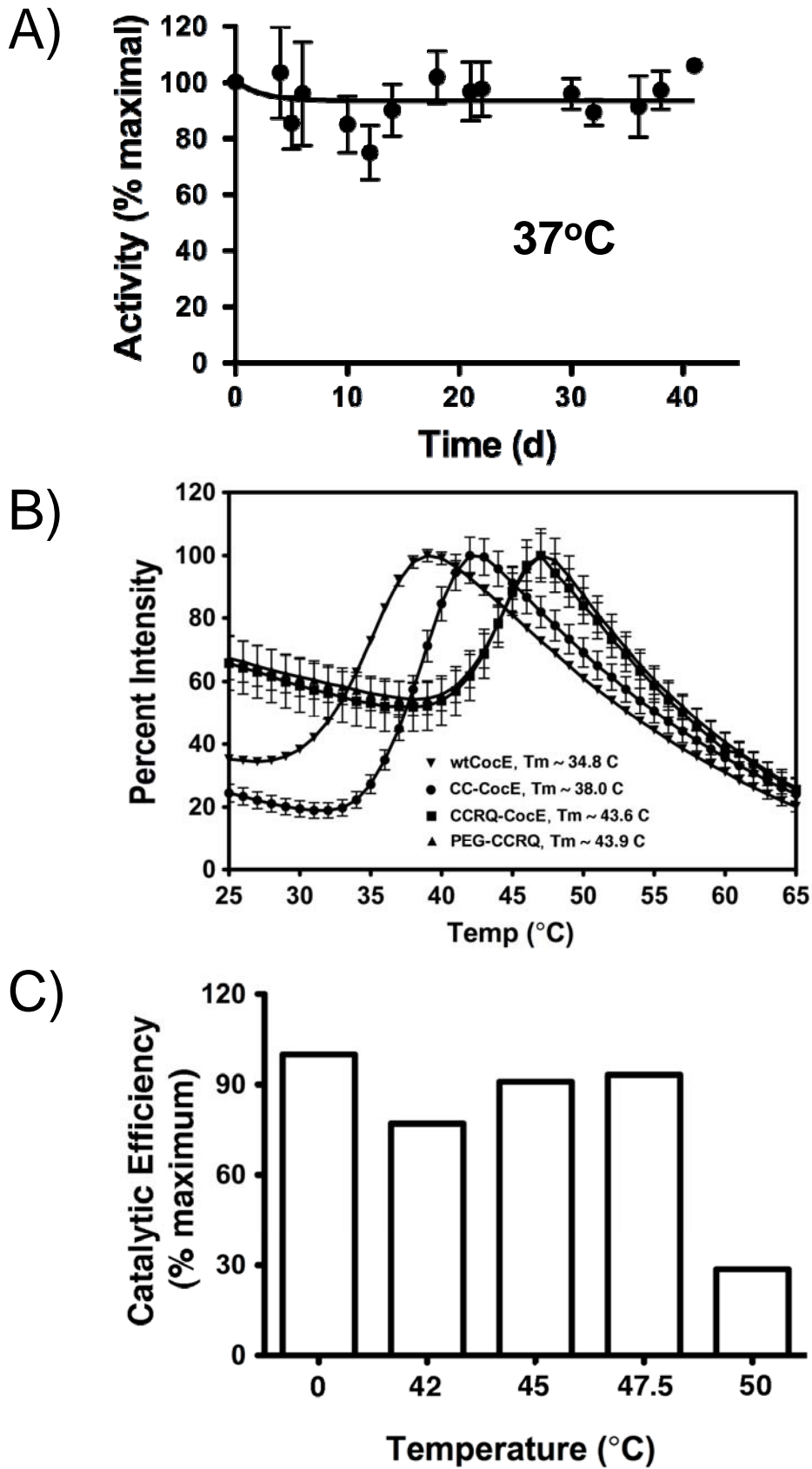


Figure 2

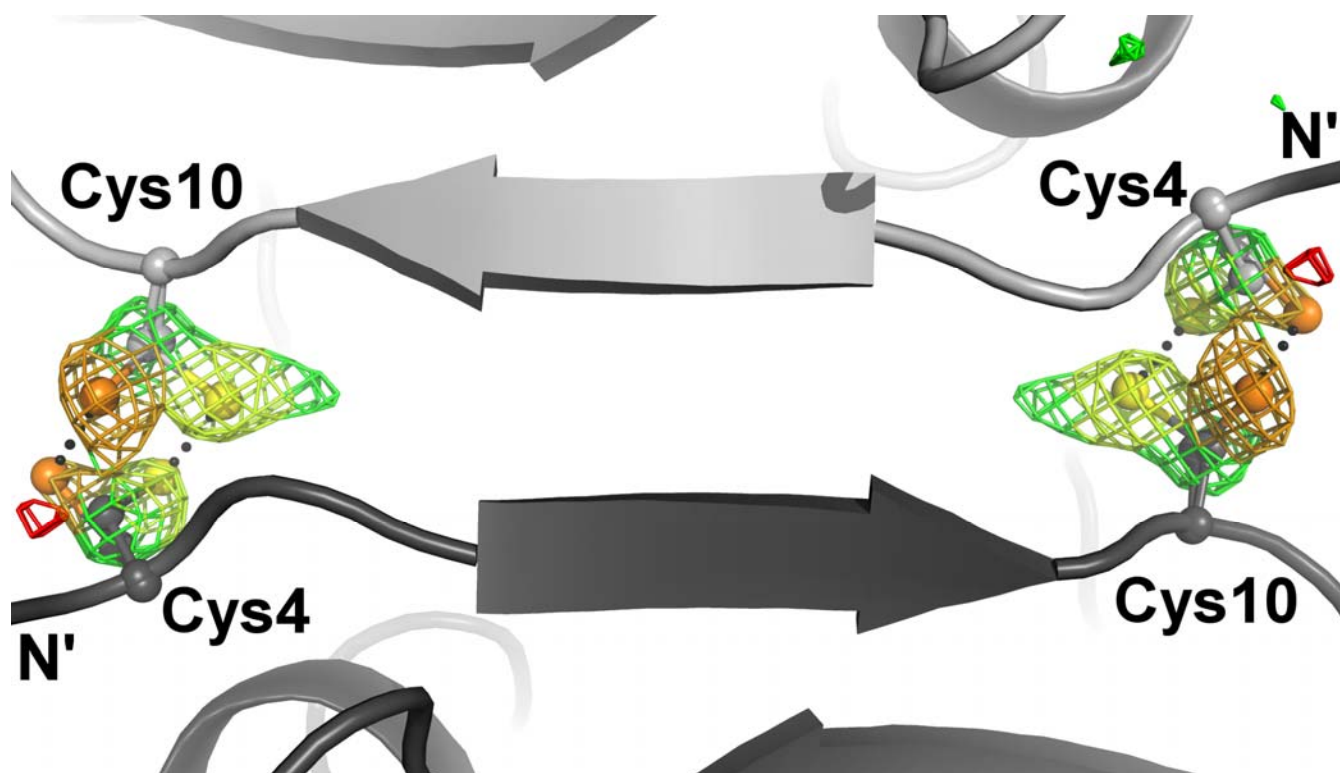


Figure 3

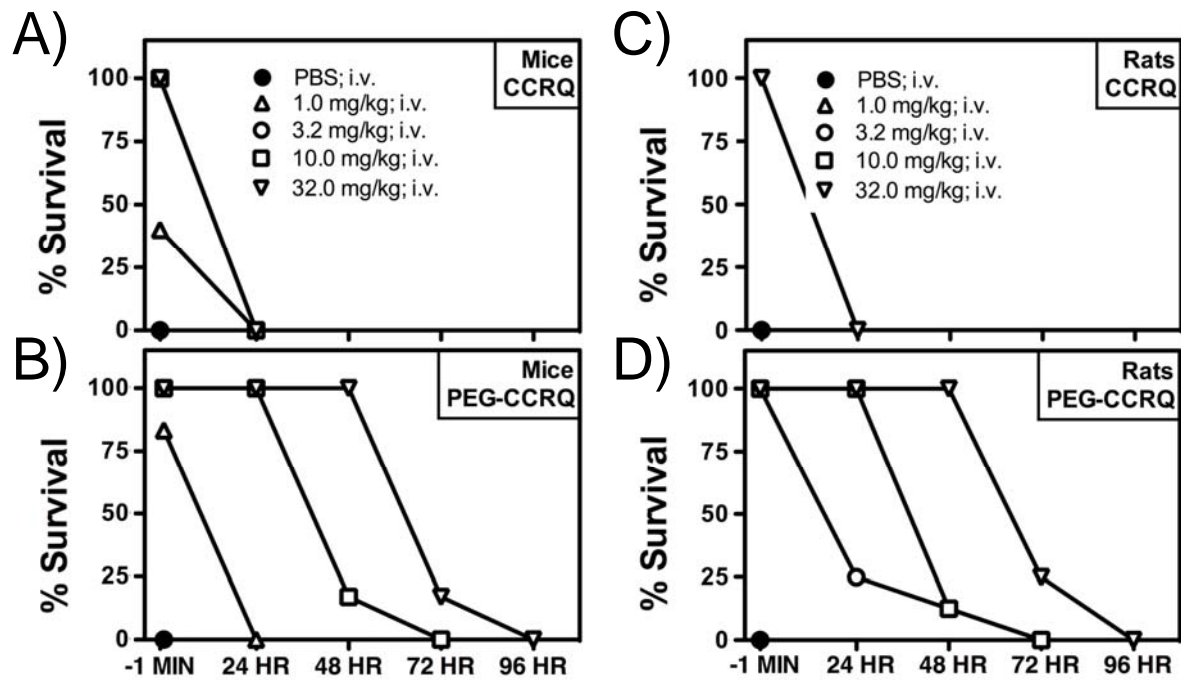


Figure 4

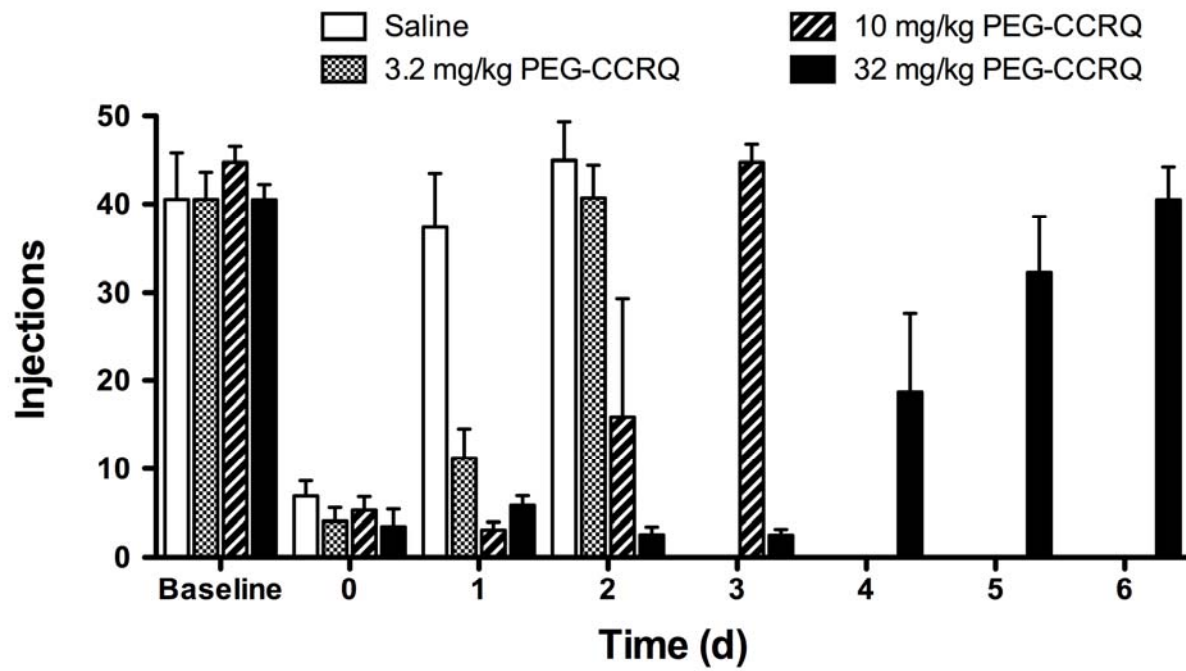


Figure 5



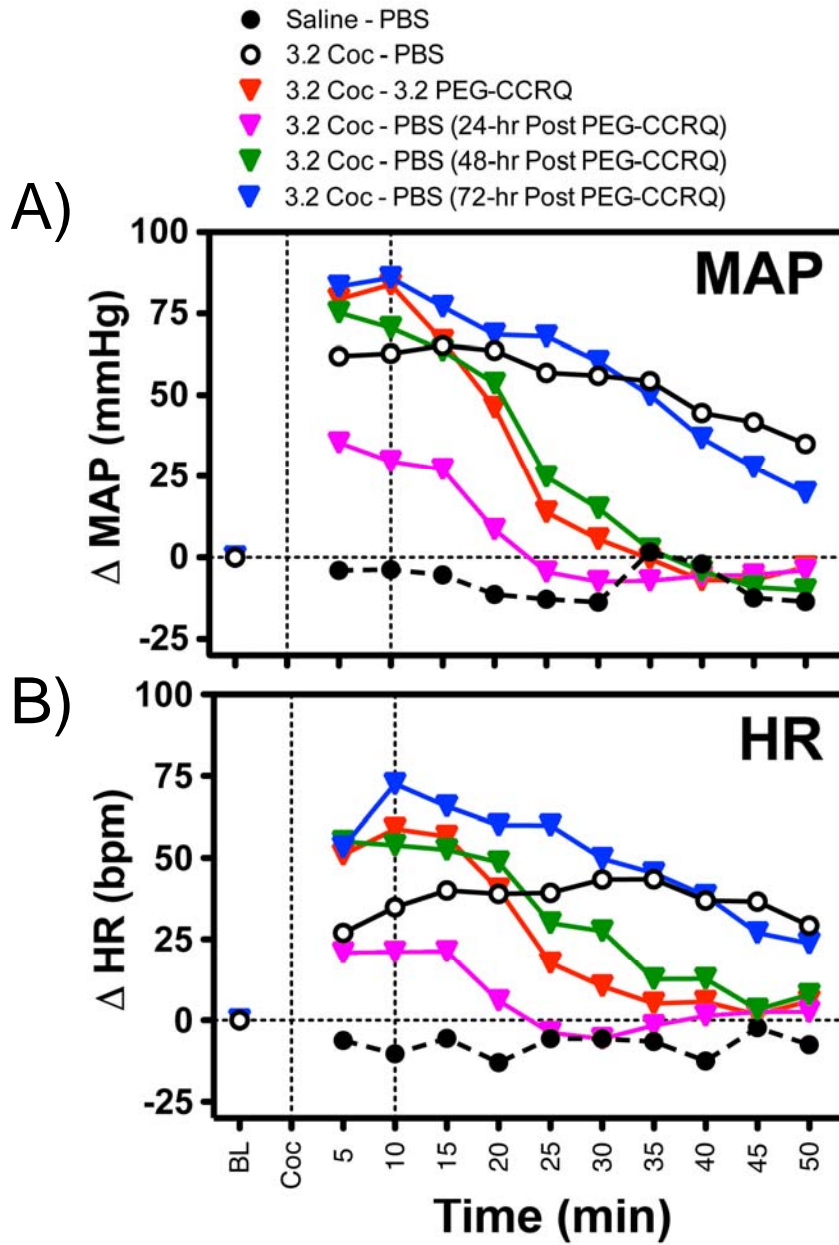


Figure 6

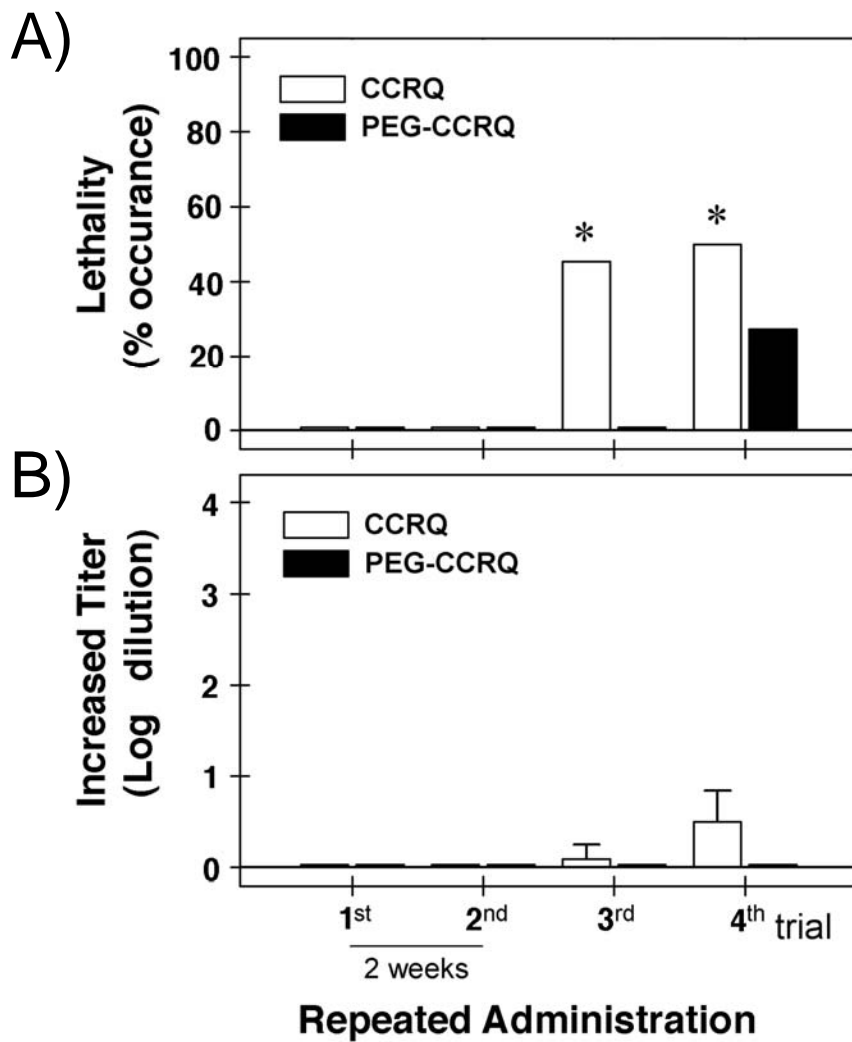


Figure 7

## Supplemental Data

### **Subunit stabilization and PEGylation of cocaine esterase improves *in vivo* residence time.**

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## Molecular Pharmacology

**Table S1**

Crystallographic data and refinement statistics

	<b>wt CocE (PEG)</b> <i>PDB ID: 3PUH</i>	<b>CC-CocE (AMS)</b> <i>PDB ID: 3PUI</i>
X-ray source	APS 21-ID-D	APS 21-ID-F
Wavelength (Å)	1.033	0.979
$D_{\min}$ (Å)	2.30	1.53
Space group	P 2 <sub>1</sub> 2 <sub>1</sub> 2 <sub>1</sub>	P 6 <sub>5</sub> 2 2
Cell constants (Å)	a=73.3, b=105.4, c=224.5; $\alpha=\beta=\gamma=90.0$	a=b= 105.7, c= 222.4; $\alpha=\beta= 90.0, \gamma=120.0$
Unique reflections	78,582	110,683
Redundancy	7.9 (6.4) <sup>a</sup>	3.2 (3.0)
$R_{\text{sym}}$ (%)	10.8 (63.0)	6.6 (59.9)
Completeness (%)	100 (100)	94.6 (90.9)
$I/\sigma_1$	33.8 (3.5)	17 (1.4)
Refinement statistics		
Resolution (Å)	25-2.30	25-1.53
Total reflections	74,428 (5255) <sup>b</sup>	99,518 (7070)
Protein atoms	8,819	4,717
Non-protein atoms	816	697
RMSD bond length (Å)	0.014	0.007
RMSD bond angles (°)	1.384	1.157
Est. coordinate error (Å) <sup>c</sup>	0.146	0.040
Ramachandran plot		
Most favored (%)	96.2	96.7
Disallowed (%)	0.2	0.2
$R_{\text{work}}^{\text{d}}$	20.8 (25.0)	15.5 (25.6)
$R_{\text{free}}^{\text{e}}$	24.9 (31.7)	17.7 (29.0)
Mean B values for all protein atoms	23.0	13.28

• <sup>a</sup>Numbers in parentheses correspond to the highest resolution shell of data; wt-CocE: 2.38-2.30 Å ; cc-CocE: 1.56-1.53.

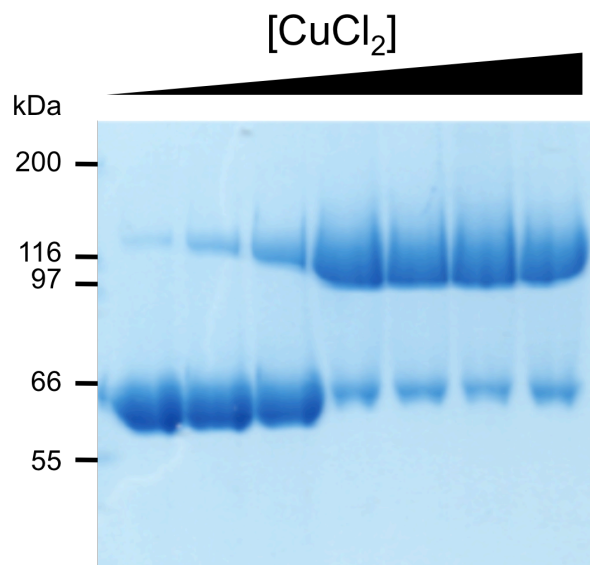
• <sup>b</sup>Numbers in parentheses correspond to the highest resolution shell of data; wt-CocE (PEG): 2.36-2.30 ; CC-CocE (AMS): 1.57-1.53 Å.

• <sup>c</sup>Error determined using maximum likelihood in Refmac 5.5.01.2.

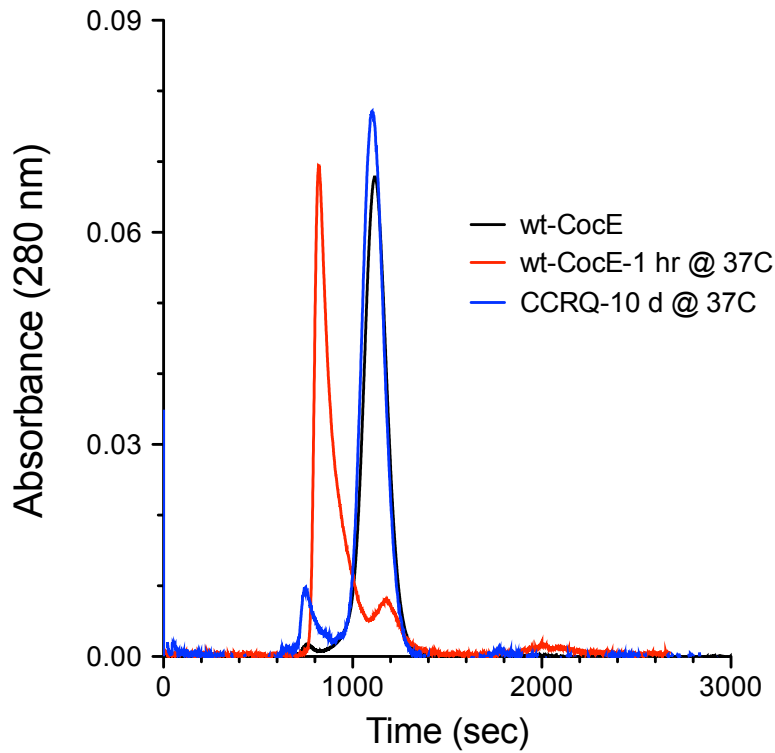
• <sup>d</sup> $R_{\text{work}} = \sum_{\text{hkl}} \|F_{\text{obs}}(\text{hkl}) - |F_{\text{calc}}(\text{hkl})|\| / \sum_{\text{hkl}} |F_{\text{obs}}(\text{hkl})|$ ; no  $I/\sigma$  cutoff was used during refinement. •

• <sup>e</sup>5% of the truncated data set was excluded from refinement to calculate  $R_{\text{free}}$ .

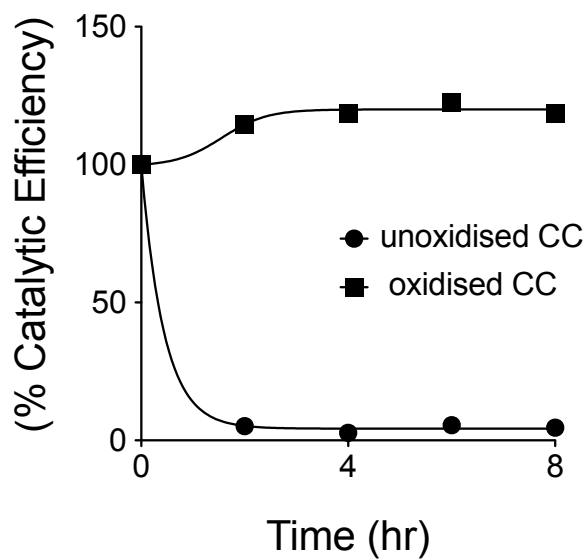
## Supplemental Data



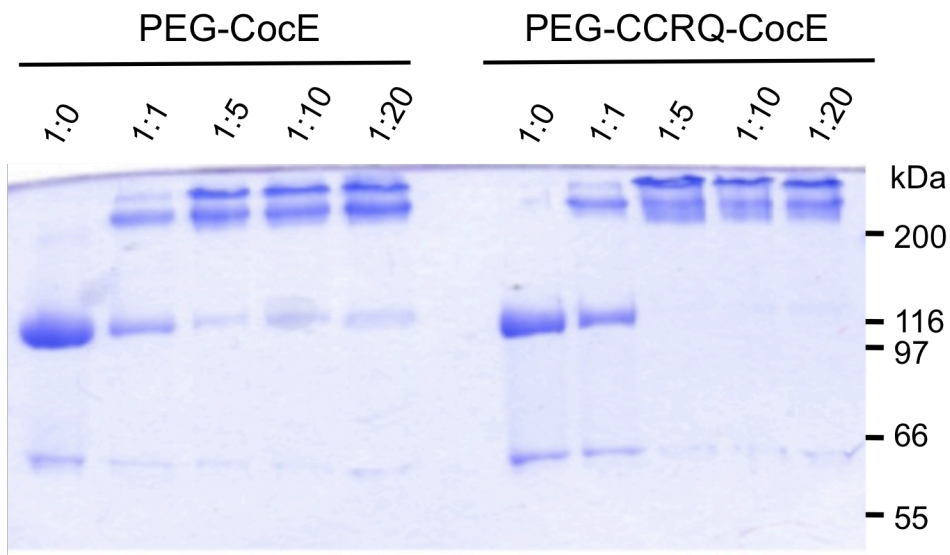
**Supplemental Figure 1:** Oxidation-induced disulfide bridge cross-linking of CC-CocE. Purified CC-CocE was incubated overnight with increasing concentrations of copper chloride ( $\text{CuCl}_2$ ) at  $4^\circ\text{C}$ . Cross-linking of CC-CocE monomers were analyzed by running the protein on an 8% SDS-PAGE gel without the reducing agent DTT. Molecular weight markers were run on lane M and the approximate sizes are indicated on the left. Lanes 1 to 7 show samples of mixtures from reactions containing 1, 5, 10, 25, 50, 75 and 100  $\mu\text{M}$  copper chloride.



**Supplemental Figure 2:** Size exclusion chromatography of wt-CocE treat for 1 hr at 4C (black) or 37C (red) compared to CCRQ-CocE incubated for 10 days at 37°C (blue). to assess the dimerization status of the protein. Absorbance (280 nm) of the eluate was plotted against volume (ml). Aggregate peak is seen at 820 seconds (~8.3 ml) whereas the dimer peak is seen at 1100 seconds (~13 ml).



**Supplemental Figure 3:** Oxidation of CC-CocE retains activity. CC-CocE was oxidized with 100  $\mu\text{M}$   $\text{CuCl}_2$  overnight at 4°C to cross-link the monomers. Oxidized CC-CocE (dark squares) and unoxidized CC-CocE (dark circles) were incubated for several lengths of time at 37°C and aliquots taken from different time points were assessed for CocE hydrolyzing activity. Catalytic activity (% max) was plotted against time (hours). Oxidized CC-CocE lost no activity over a period of 8-hour incubation while unoxidized CC-CocE lost all activity in less than 2 hours.



**Supplemental Figure 4:** SDS-PAGE gel depicting the mobility and the approximate size of PEGylated CC-CocE (lanes 2, 3, 4, and 5) and PEGylated CCRQ-CocE (lanes 7, 8, 9 and 10). Lane 1 and lane 6 contains non PEGylated native CC-CocE and CCRQ-CocE, respectively. CC-CocE and CCRQ-CocE were subjected to PEGylation reaction with 1:1 (lanes 2 and 7), 1:5 (lanes 3 and 8), 1:10 (lanes 4 and 9) or 1:20 (lanes 5 and 10) ratio of protein to 40 KDa branched PEG and purified over ion-exchange chromatography. Lane labeled M, depicts the mobility of molecular weight markers with the approximate sizes of the bands displayed on the right.