Subunit Stabilization and Polyethylene Glycolation of Cocaine Esterase Improves In Vivo Residence Time⒮

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ABSTRACT

No small-molecule therapeutic is available to treat cocaine addiction, but enzyme-based therapy to accelerate cocaine hydrolysis in serum has 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. Cross-linking subunits through disulfide bridging is commonly used to stabilize multimeric enzymes. Herein we use structural methods to guide the introduction of two cysteine residues within dimer interface of CocE to facilitate intermolecular disulfide bond formation. The disulfide-crosslinked enzyme displays improved thermostability, particularly when combined with previously described mutations that enhance stability (T172R-G173Q). The newly modified enzyme yielded an ex-tremely stable form of CocE (CCRQ-CocE) that retained greater than 90% of its activity after 41 days at 37°C, representing an improvement of more than 4700-fold 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 h, 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.

Introduction

Cocaine use continues to be a serious public health problem. Cocaine abuse, as a cause of visits to the emergency department, leads all other drugs. In 2006, cocaine was involved in 553,530 of a total of 974,272 emergency department visits involving an illicit drug (56.8%) (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2011). Cocaine remains the drug most often coabused 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 (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2011). The National Survey on Drug Use and Health estimates that in 2007 there were 2.1 million current (past-month) cocaine users (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2008). Moreover, nearly 1.6 million Americans met Diagnostic and Statistical Manual of Mental Disorders criteria for dependence or abuse of cocaine (in any form) in the past 12 months (Substance Abuse and Mental Health Services Administration, Office of Applied Studies, 2011). Despite being a highly addictive and widely abused drug, there is no U.S. Food and Drug Administration-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. Long-term and prolonged blockade of dopamine transporters are associated with various forms of addiction (Benowitz, 1993). At higher concentrations, cocaine also

ABBREVIATIONS: BchE, butyrylcholinesterase; wt, wild type; CocE, cocaine esterase; PEG, polyethylene glycol; PAGE, polyacrylamide gel electrophoresis; MALDI, matrix-assisted laser desorption ionization; CC, G4C/S10C; RMSD, root-mean-squared deviation; CCRQ, G4C/S10C/T172R/G173Q; T_m, melting temperature; TOF, time of flight; RQ, T172R/G173Q; KQ, L169K-G173Q; MAP, mean arterial pressure; HR, heart rate.

blocks norepinephrine and serotonin reuptake transporters, which contributes to its toxic effects, including seizures, tachyarrhythmia, and sudden death (Johanson and Fischman, 1989; Crumb et al., 1990; Benowitz, 1993; 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 [i.e., 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 (Mello, 1990; Roberts and Brebner, 2000; Platt et al., 2002; Dackis and O'Brien, 2003)] 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 by increasing the dose (Carrera et al., 2000, 2001). The most effective vaccines to date have been against bovine serum albumin- or cholera toxin B-conjugated norcocaine (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 (Kosten et al., 2002; Kinsey et al., 2010). A variant of passive immunization using catalytic monoclonal antibodies, which can not only sequester but also hydrolyze cocaine, was unfortunately not therapeutically active for more than 72 h after administration (Landry et al., 1993; Yang et al., 1996; Matsushita et al., 2001). Other than the vaccines against bovine serum albumin- or cholera toxin B-conjugated norcocaine, 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 wild-type (wt) BchE in hydrolyzing cocaine (Zheng et al., 2008).

Cocaine esterase (CocE), isolated from *Rhodococcus* spp. that grow in the rhizosphere soil surrounding coca plants, remains the natural enzyme that can hydrolyze cocaine the fastest (Bresler et al., 2000; Larsen et al., 2002). We have demonstrated previously 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 (Gao et al., 2009; Brim et al., 2010; 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 (Gao et al., 2009; Brim et al., 2010; Narasimhan et al., 2010). More importantly, we demonstrated that that these mutations afforded protection against cocaine-induced lethality for greater than 4 h in rodents (Narasimhan et al., 2010). We also provided crystallographic and biochemical evidence that CocE is a dimer (Narasimhan et al., 2010) and that the protein aggregates upon heat inactivation. 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 intermolecular disulfide bonds would further stabilize the protein. Disulfide cross-linking of subunits has been used to stabilize tetrameric malate dehydrogenase (Bjørk et al., 2003), α and β subunits of human choriogonadotropin (Heikoop et al., 1997), and tetrameric streptavidin (Reznik et al., 1996).

In this report, we demonstrate that incorporating disulfide bonds between cysteine residues substituted at Gly4 and Ser10, both located on the β1 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 disulfide-stabilized dimer (CCRQ-CocE) remains more 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 h in a cocaine overdose model, diminished self-administration for 72 h in rats, reduced cocaine-induced cardiovascular effects and locomotor functions in monkeys for up to 48 h, and displayed reduced immunogenicity in mice.

Materials and Methods

Materials. Cocaine was purchased from Mallinckrodt (Hazelwood, MO). Branched 40-kDa PEG was purchased from JenKem Technology USA Inc. (Allen, TX). All other reagents are of analytical grade and were obtained from Thermo Fisher Scientific (Waltham, WA) or Sigma-Aldrich (St. Louis, MO).

Site-Directed Mutagenesis. pET22b (+) CocE plasmid was kindly provided by Dr. Nick Larson (The Scripps Institute, La Jolla, CA) (Turner et al., 2002). Wild-type (wt-CocE) and CocE mutants were expressed as C-terminal hexahistidine-tagged proteins containing the exogenous sequence KLAAALEHHHHHH at the C terminus. Point mutations were generated using a modified QuikChange (Stratagene, La Jolla, CA) 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 *Escherichia coli* BL-21 Gold (DE3) cells grown at 37°C to an OD_{600} of 0.8. Protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside and cells were harvested after incubation for 12 h at 18°C.

Purification of Cocaine Esterase and Mutants. Cells were pelleted, resuspended in 50 mM Tris-HCl, pH 8.0, and 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., Palo Alto, CA), followed by anion exchange chromatography on a Q-Sepharose fast performance liquid chromatography column (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). CocE was eluted from the Q-Sepharose column with a 150 to 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_2 and stored at $-80°C$.

Oxidation. Purified proteins were oxidized in 50 mM Tris-HCl, pH 8.0 buffer containing 100 μM $CuCl_2$ overnight at 4°C. $CuCl_2$ 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 to 500 mM NaCl gradient 50 mM Tris-HCl, pH 8.0. Fractions containing purified oxidized protein were identified by SDS-PAGE under nonreducing conditions and then pooled and concentrated to 5 mg/ml and snap-frozen in liquid N_2 and stored at $-80°C$.

PEGylation. Oxidized proteins were conjugated with maleimide-linked branched PEG 40-kDa (JenKem 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 to 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.

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 M^{-1} cm^{-1}) (Xie et al., 1999) on a SpectraMax Plus 384 UV plate reader (Molecular Devices, Sunnyvale, CA) using SOFTmax Pro software (version 3.1.2). The reaction was initiated by the addition of 100 μl of wt-CocE or mutant CocE (50, 25, 10, or 5 ng/ml) in phosphate-buffered saline, pH 7.4 to 100 μl of a 2X cocaine solution made in phosphate-buffered saline. The final enzyme concentration was 25, 12.5, 5,l or 2.5 ng/ml and the final cocaine concentrations were: 100, 50, 25, 12.5, 5, 2.5, 0.5, and 0.25 μM. V_{max} and K_m values were calculated using Prism (GraphPad Software, San Diego, CA). For stability measurements, CocE variants were diluted to 50 ng/ml (2×) concentration and incubated at 37°C. Aliquots were removed at various time points and assayed for activity against cocaine as described above ($n = 5$). Temperature-dependent decay in esterase activity was measured by preincubating 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 Temperature Measurements Using Thermo Fluor. Purified CocE variants were buffer exchanged into 50 mM Tris-HCl, pH 8.0. Five microliters of 0.2 mg/ml protein were placed in an ABgene 384-well polymerase chain reaction plate (Thermo Fisher Scientific) in triplicate. Five microliters of 200 μM dye 1-anilinonaphthalene-8-sulfonic acid (Sigma) was subsequently placed on top of the protein solution. The plates were spun on a centrifuge (Beckman Coulter, Fullerton, CA) at 1000g for 2 min to remove air bubbles. One microliter 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 & Johnson, New Brunswick, NJ) 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. One microliter of G4CS10C-CocE (CC-CocE) at 8 mg/ml was added to 1 μl of mother liquor on a siliconized glass coverslip 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 3350, 100 mM 2-(N-morpho-

lino)-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). Because of the X-ray sensitivity of the disulfide bonds in the CC-CocE crystals, data were limited to the first 25 degrees of data collection.

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 β sheet is formed by the β1 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 (Fig. 1; Supplemental Table). 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 Cα 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
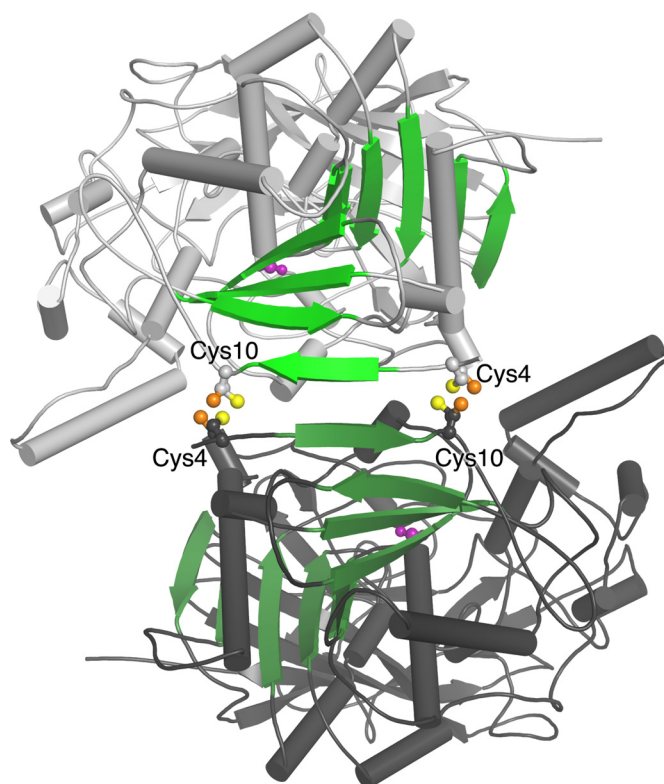

Fig. 1. The dimer interface of wt-CocE and position of β1-strands that forms a continuous β-sheet. Introduction of cysteine at positions 4 and 10 creates two covalent bonds to stabilize the α/β hydrolase domain. Oxidation of these two cysteines (conformation A, orange; conformation B, yellow) cements a continuous β-sheet that includes the active-site serine 117 (purple) of both monomers (light gray, dark gray). The homodimer β-sheet consists of 20 β-strands (light green, dark green).

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 β-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.

Stability Assessment. Cross-linking CC-CocE with $CuCl_2$ resulted in a dithiothreitol-sensitive ~140-kDa species, as assessed by SDS-PAGE (Supplemental Fig. S1). The purified proteins were assessed for cocaine hydrolyzing activity and were as active as wt-CocE (Table 1) (Narasimhan et al., 2010). Impressively, the CC-CocE protein retained 100% of its enzymatic activity after 7 days incubation at 37°C (Supplemental 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 software and reveal an apparent plateau at 93% after 41 days at 37°C (Fig. 2A). Because >90% of enzymatic activity was retained even after 41 days at 37°C, it was difficult to ascertain a true half-life for this variant of CocE. Thermo Fluor analysis was performed on oxidized CC-CocE and CCRQ-CocE to determine melting temperatures (T_m). CC-CocE had a ~3°C elevated T_m compared with wt-CocE (34.8 versus 38.0°C) and CCRQ-CocE had an even higher T_m of 43.9°C, consistent with the increased half-life of these forms of CocE at 37°C (Fig. 2B). We performed thermal inactivation assays as described previously (Narasimhan et al., 2010), in which we preincubated of proteins at temperatures above 37°C before measuring their activity. We have shown that wt-CocE is inactivated within 30 min at 30°C, whereas RQ-CocE and L169K variants were inactivated at higher temperatures (40–45°C) (Narasimhan et al., 2010). CCRQ-CocE retained 90% of activity even after 15 min incubation at 47.5°C, indicating its improved thermostability over RQ-CocE (Fig. 2C). Size-exclusion chromatography analysis revealed that even after 10-day incubation at 37°C, the majority of CCRQ-CocE protein resolved at approximately 140 kDa, corresponding to a homodimer, with very little aggregate in the void volume (Supplemental Fig. S3), as opposed to wt-CocE, which aggre-

gated within a 1-h 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
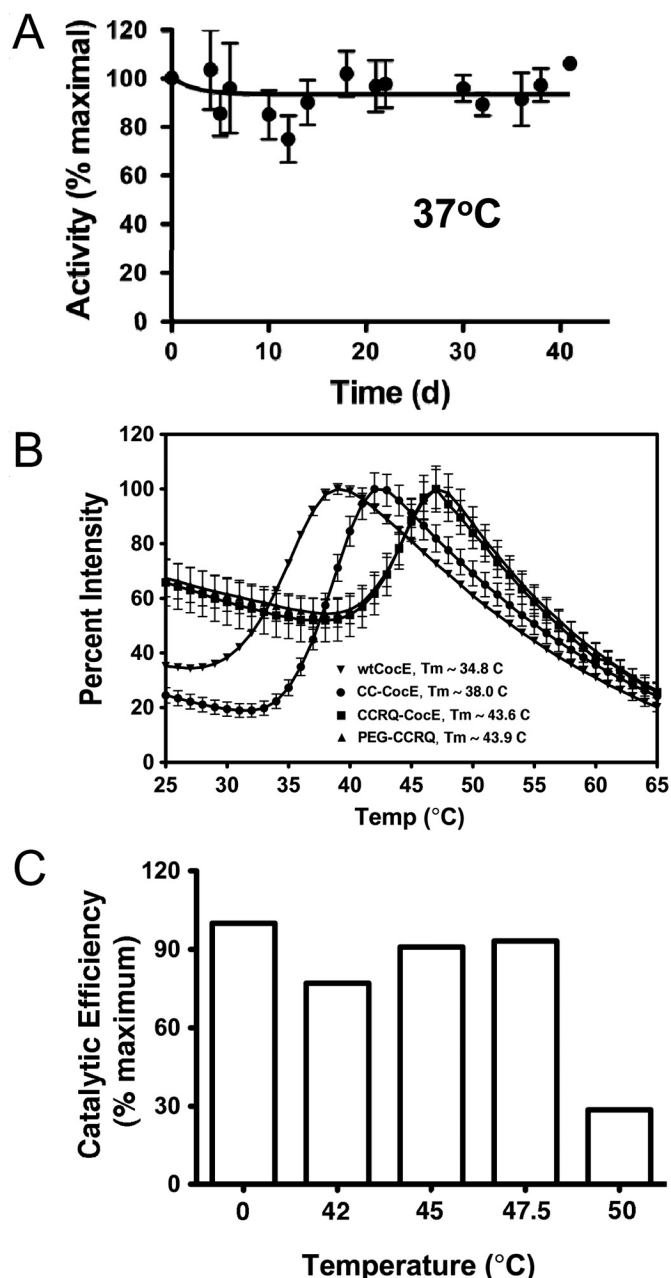

Fig. 2. Enzymatic stability of CCRQ-CocE. A, decay in the capacity to convert cocaine to egonine methyl ester and benzoic acid was measured at 37°C. CCRQ-CocE (50 ng/ml) was incubated at 37°C, and the activity was calculated over time ($n = 3$). Percentage remaining activity was plotted against days of incubation at 37°C. B, melting temperatures of wild-type and mutants forms of CocE. Protein unfolding was monitored on a Thermo Fluor 384 reader using 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) (100 μM final) at a protein concentration of 100 mg/ml. Melting curves of wt-CocE (▼), CC-CocE (●), CCRQ-CocE (■), and PEG-CCRQ-CocE (▲) 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 preincubated for 15 min at temperatures indicated (°C), and activity was measured as described previously. The activities of each mutant remaining (as a percentage of the catalytic efficiency, without preincubation) after preincubation are shown.

TABLE 1
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 under *Materials and Methods.*

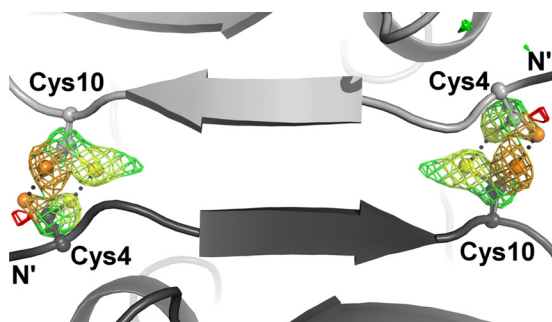| CocE Variant	K_{cat}	K_m	K_{cat}/K_m
	s^{-1}	mM	$s^{-1}\ M^{-1}$
wt-CocE	51.4 ± 16.8	0.0057 ± 0.0019	8.99×10^6
CC-CocE	49.9 ± 4.66	0.021 ± 0.0042	2.38×10^6
CCRQ-CocE	56.6 ± 4.90	0.026 ± 0.0018	2.12×10^6
PEG-CCRQ-CocE	40.1 ± 3.24	0.019 ± 0.0020	2.11×10^6

Fig. 3. X-ray crystallography reveals two conformations of the engineered intermolecular disulfide bridge. When two CocE monomers (light gray, dark gray) dimerize, they form an antiparallel β-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°. Conformation B, yellow, has a dihedral angle of −71.9°. FoFc density, generated by omitting the β carbon and γ sulfur of both cysteine 4 and 10, is shown contoured to 5 σ (green) and −5 σ (red). Both conformations have a sulfur-sulfur distance of 2.0 Å.

was determined, and 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 Å, 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 σ, whereas the other three sulfur conformations are observed at up to 9.5 σ 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 (data 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. (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 for 4 h, whereas few survived after a 6-h pretreat-

ment (data not shown). To ascertain whether 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 (△), 10 mg/kg (□), or 32 mg/kg (▽) at various times before lethal cocaine injection (intraperitoneal). Pretreatment of animals with CCRQ-CocE was not effective for longer than 24 h even at the highest dose tested (Fig. 4, left top). Similar results were obtained with 32 mg of CCRQ-CocE (▽) in rats (Fig. 4, right top).

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 PEGyation studies using wt-and RQ-CocE (Park et al., 2010). PEGylation of CC-CocE or CCRQ-CocE with 40-kDa branched PEG 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 (Supplemental 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 to 20% gradient SDS-PAGE gel (Supplemental Fig. S4) consistent with their higher molecular mass 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 masses of ~107 and ~210 kDa under reducing and nonreducing conditions, respectively, whereas the non-PEGylated versions have molecular masses of 66.4 and 126.8 kDa, respectively (data not shown). This is suggestive that monomeric CCRQ-CocE (reduced) contains a single 40-kDa PEG (107–66.4 kDa = 40 kDa), whereas the CCRQ-CocE dimer contains two 40-kDa PEG moieties (210–126.8 = 2× 40 kDa). No discernible non-PEGylated protein was present in the purified species.

PEG-CCRQ is catalytically as active as wt-CocE in hydrolyzing cocaine (Table 1). PEG-CCRQ predictably had higher melting temperature (T_m) than wt-CocE (43.86 versus 34.77°C) when observed by Thermo Fluor experiments (Fig. 2B).
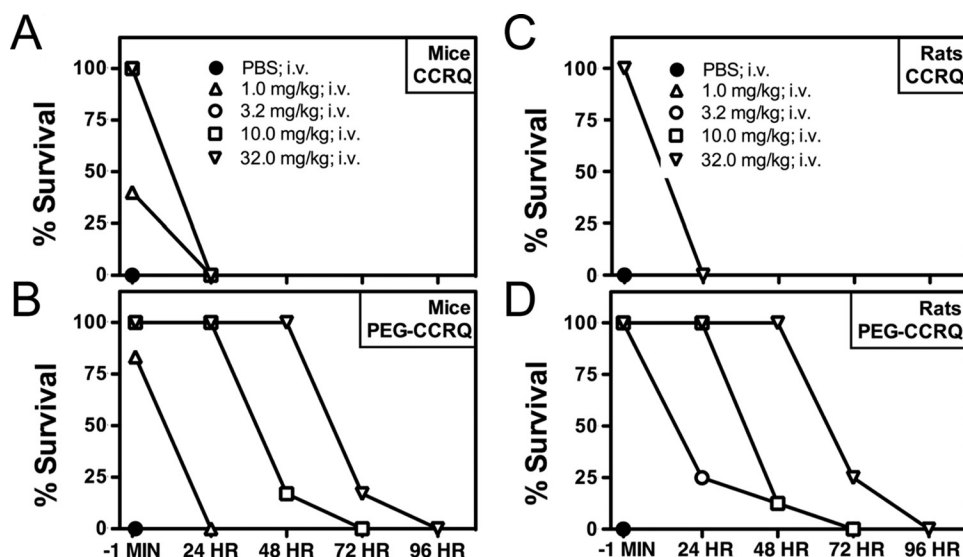

Fig. 4. Efficacy of CCRQ-CocE or PEG-CCRQ CocE in vivo. CCRQ-CocE at 1 (△), 10 (□), or 32 mg/kg (▽) (A and C) or PEG-CCRQ-CocE at 1 (△), 3.2 (○), 10 (□), or 32 mg/kg (▽) (B and D) were injected into mice (A and B) or rats (C and D) at different times before 180 mg/kg i.p. cocaine injection at time −1 min. Efficacy of CCRQ-CocE and PEG-CCRQ-CocE in protecting the rodents was assessed and percentage survival was plotted against the pretreatment times.

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 mouse and 3.2, 10.0, or 32 mg/kg per rat) intravenously 1 min before the initial dose of cocaine (180.0 mg/kg i.p.), with subsequent doses of cocaine (180 mg/kg) administered at 24-h intervals. At this dose of cocaine, rodents die within 3 to 5 min of injection if no CocE was given either prior or subsequent to cocaine injection. Although CCRQ-CocE (i.e., non-PEGylated) provided a dose-dependent protection against the lethal effects of cocaine (Fig. 4, A and C), pretreatment with PEG-CCRQ-CocE provided a dose- and time-dependent protection against an LD_{100} dose of cocaine in both rats and mice (Fig. 4, B and D). Unlike the protective effects of CCRQ-CocE (3.2–32.0 mg/kg), which had fully dissipated 24 h 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 (□) PEG-CCRQ-CocE provided a complete protection against a second challenge dose of 180.0 mg/kg i.p. cocaine when administered 24 h later, with all mice and rats dying by the fourth challenge dose administered 72 h later. Similar effects were observed with a dose of 32 mg of PEG-CCRQ-CocE (▽), all mice surviving the third challenge dose administered 48 h after pretreatment with PEG-CCRQ-CocE, and 100% lethality was observed at the fifth challenge dose 96 h later. Doses of 1, 3.2, 10, and 32 mg/kg in rodents translates to approximately to 68, 217, 680, and 2.18 μM, respectively, based on the blood volume of rodents. A direct conversion to humans (75 kg) with 5-l blood volume would correspond to approximately 0.95, 3.04, 9.52, 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 nonhuman primates (Collins et al., 2011). 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., 2011) We therefore predict that a dose of PEG-CCRQ-CocE in the 0.32 to 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 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, 10, or 32 mg/kg PEG-CCRQ at immediately before the start of a session and retested at 24-h 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/injection cocaine (Fig. 5) and displayed dose-dependent decreases in responding maintained by doses of 0.32, and 1.0 mg/kg/injection 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 or 48 h later (data not shown). Similar to when saline was substituted for cocaine, low rates of responding were observed when rats were pretreated with 3.2 mg/kg PEG-
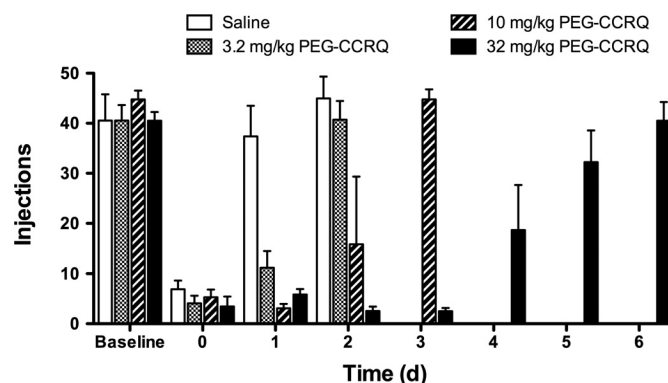

Fig. 5. PEG-CCRQ-CocE-mediated suppression of cocaine self-administration in rats. Rats were trained to respond for increasing doses of cocaine under a fixed ratio 5 schedule of reinforcement during five sequential components. Rats ($n = 6$ for each condition) were intravenously pretreated with saline (open bars), 3.2 (shaded bars), 10 (hatched bars), or 32 mg/kg (filled bars) and tested immediately, 1, 2, 3, 4, or 5 days with 0.1 mg/kg/injection cocaine.

CCRQ-CocE and allowed to respond for 0.1 mg/kg/injection cocaine with a partial recovery of cocaine-maintained responding observed 24 h later, and baseline-like levels of responding observed 48 h 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 h later, with a partial recovery of responding observed 48 h later, and baseline-like rates of responding observed 72 h 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 h after administration, with a partial recovery of responding observed at the 72-h time point; however, baseline-like rates of responding were not fully recovered until 5 days or 120 h after the administration of PEG-CCRQ-CocE.

PEG-CCRQ-CocE Decreases Cardiovascular Effects of Cocaine in Rhesus Monkey. In comparison to saline (●), cocaine (3.2 mg/kg i.v., ○) produces a long-lasting increase in mean arterial pressure (MAP; Fig. 6A) and heart rate (HR; Fig. 6B). As shown in Fig. 6, these increases in MAP and HR were rapidly decreased after 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 h (magenta inverted triangles) and 48 h (green inverted triangles) time points; however, these effects had diminished by the 72-h time point (blue inverted triangles), and the cardiovascular effects of cocaine were no different from during the baseline condition.

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 whether 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 2 weeks, and at every time point anti-enzyme antibody and any diminution of the effec-
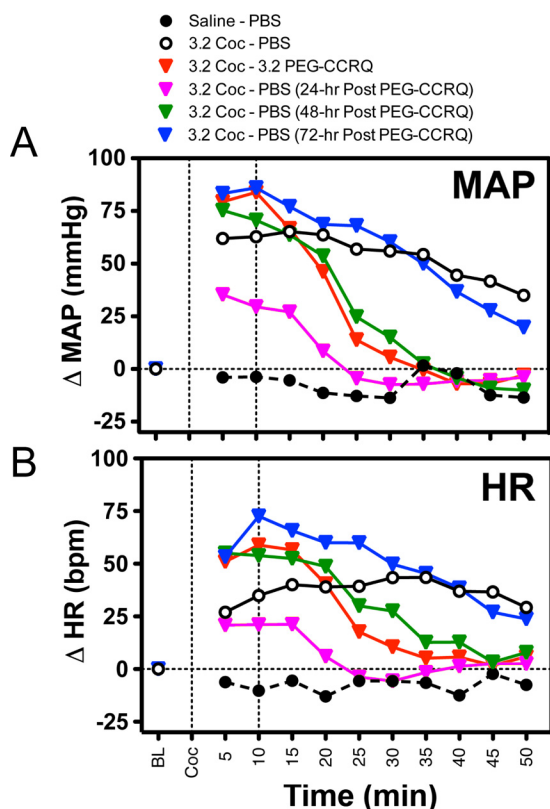
Fig. 6. Effect of PEG-CCRQ-CocE on the cardiovascular effects of cocaine in monkeys. Monkeys were either given saline (●) or 3.2 mg/kg i.v. cocaine (○) and HR (B) were observed. To observe the effect of PEG-CCRQ-CocE, we injected 3.2 mg/kg enzyme 10 min after the cocaine injection (red inverted triangles). Challenge doses of cocaine (3.2 mg/kg) were given 24 h (magenta inverted triangles), 48 h (green inverted triangles), or 72 h (blue inverted triangles) after PEG-CCRQ-CocE injection and mean arterial pressure and heart rate were observed. Changes in mean arterial pressure (ΔMAP, left) and heart rate (ΔHR, right) were plotted against time.

Fig. 7. Effectiveness of CCRQ-CocE and PEG-CCRQ-CocE in preventing cocaine-induced toxicity in mice after repeated administration. A, the protective effects of enzyme (10 mg/kg i.v., 1-min pretreatment) against 180 mg/kg i.p. cocaine-induced lethality were tested in the same mice ($n = 11$) every 2 weeks. B, the anti-enzyme antibodies were determined from serum samples collected 24 h before the toxicity test. Each value represents the percentage of mice affected (A) or the mean ± S.E.M. (B). The asterisk represents a significant difference from the value determined in the first trial (∗, $p < 0.05$).

tiveness 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% (3rd exposure) and approximately 50% (4th exposure) of the animals from lethal dose of cocaine (180 mg/kg). In contrast, only after the fourth exposure did PEG-CCRQ-CocE fail to protect (25%), even though anti-CocE antibody titer at this stage was negligible.

Discussion

Cocaine esterase has great potential to be developed as a protein therapeutic to combat cocaine abuse. We previously stabilized CocE (Gao et al., 2009; Brim et al., 2010; 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 (Cooper et al., 2006; Ko et al., 2007; Collins et al., 2009; Brim et al., 2010). Moreover, biochemical and biophysical analyses of these
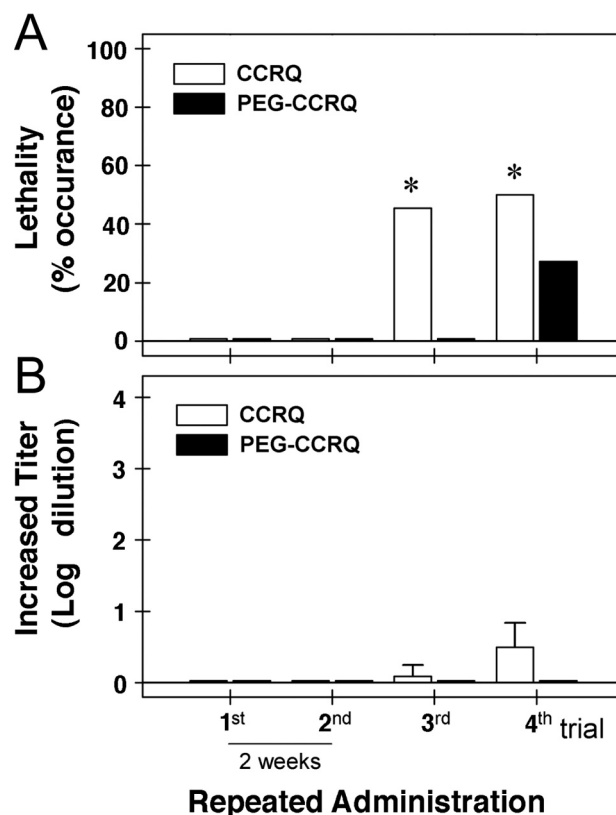
thermostable forms revealed that the mutants and wt-CocE exists as dimers (Narasimhan et al., 2010).

Analysis of the crystal-packing in a new crystal form reveals a dimer interface encompassing 1172 Å² 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 β-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 β-sheet between the two monomers. Residues 6 to 8 of each monomer form anti-parallel β-strands to create a continuous β-sheet composed of two β-strands (Fig. 1). Analytical ultracentrifugation analysis also confirmed dimerization and that the molecular mass of wt-CocE is approximately 127 kDa (data not shown).

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 hypothe-

sized 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 β-galactosidases] (Wong and Wong, 1992; Poltorak et al., 1999; Pilipenko et al., 2007; Fernandez-Lafuente, 2009). Thermophilic proteins have evolved to employ multimerization and strong interactions between multimers as strategy to survive extreme temperatures (Tanaka et al., 2001; Ogasahara et al., 2003). Some thermophilic proteins use disulfide bridges across subunits to strengthen subunit interaction compared with homologous mesophilic proteins (Kabashima et al., 2001; Nakka et al., 2006). Oligomer stabilization by disulfide cross-linking across the interface is a widely used rational approach to engineer stability in industrial biocatalysts (Reznik et al., 1996; Heikoop et al., 1997; Bjørk et al., 2003; Mateo et al., 2008).

The β-sheet formed by β1-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 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. 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 (Gao et al., 2009; Brim et al., 2010; 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 h (Fig. 4, top). 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.

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 α2a (Pegasys; Roche, Nutley NJ) and branched PEG-anti-VEGF aptamer (pegaptanib; Macugen, 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). Because of the inherent lack of stability of wt-CocE and T172R-G173Q mutant CocE compared with CCRQ-CocE, we observed that conjugation with 40-kDa PEG was detrimental to the enzyme activity (Park et al., 2010). In contrast, PEG-CCRQ retained 100% of activity compared with non-PEGylated CCRQ-CocE (Table 1) and that, we believe, is because of higher overall stability of the CCRQ-CocE.

Compared with the effects of T172R-G173Q-CocE (RQ-CocE) and L169K-G173Q-CocE (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; 100% of rats and mice survived not only the immediate 180 mg/kg i.p. dose of cocaine but also at least two additional challenges with this LD_{100} dose of cocaine over the next 48 h. 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/injection for at least 48 h, with a significant antagonism of the reinforcing effects of cocaine apparent for at least 96 h after treatment. Not only are these effects the longest lasting of any of the CocE variants (Collins et al., 2009; Brim et al., 2010), 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 h in vivo. Repeated wt-CocE exposures in rats increased antibody titers after the fourth exposure by 100- to 1000-fold, and after the 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 such as damage to myocardium as a result of 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. It is noteworthy that a similarly prolonged duration of action was also observed in a third, larger species, the rhesus monkey. When administered 10 min 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 similar to that 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 h. It is noteworthy that 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; this 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 40-kDa PEG 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 (D. Narasimhan and R. K. Sunahara, personal communication) indicate that cysteine at position 551 is conjugated to 40-kDa PEG resulting in two 40-kDa PEG 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 under way 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.

Clearly 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 with earlier versions of

CocE. Further improvements with site-specific PEGylation, encapsulation in nanoparticles or both could result in an 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.

Authorship Contributions

Participated in research design: Narasimhan, Collins, Ko, Woods, Tesmer, and Sunahara.

Conducted experiments: Narasimhan, Collins, Nance, Nichols, Edwald, and Chan.

Performed data analysis: Narasimhan, Collins, and Nance.

Purified and prepared protein: Narasimhan and Nichols.

Wrote or contributed to the writing of the manuscript: Narasimhan, Collins, Woods, Tesmer, and Sunahara.

References

Benowitz NL (1993) Clinical pharmacology and toxicology of cocaine. *Pharmacol Toxicol* **72:**3–12.

Bjørk A, Dalhus B, Mantzilas D, Eijsink VG, and Sirevåg R (2003) Stabilization of a tetrameric malate dehydrogenase by introduction of a disulfide bridge at the dimer-dimer interface. *J Mol Biol* **334:**811–821.

Bresler MM, Rosser SJ, Basran A, and Bruce NC (2000) Gene cloning and nucleotide sequencing and properties of a cocaine esterase from Rhodococcus sp. strain MB1. *Appl Environ Microbiol* **66:**904–908.

Brim RL, Nance MR, Youngstrom DW, Narasimhan D, Zhan CG, Tesmer JJ, Sunahara RK, and Woods JH (2010) A thermally stable form of bacterial cocaine esterase: a potential therapeutic agent for treatment of cocaine abuse. *Mol Pharmacol* **77:**593–600.

Caliceti P and Veronese FM (2003) Pharmacokinetic and biodistribution properties of poly(ethylene glycol)-protein conjugates. *Adv Drug Deliv Rev* **55:**1261–1277.

Carrera MR, Ashley JA, Wirsching P, Koob GF, and Janda KD (2001) A second-generation vaccine protects against the psychoactive effects of cocaine. *Proc Natl Acad Sci USA* **98:**1988–1992.

Carrera MR, Ashley JA, Zhou B, Wirsching P, Koob GF, and Janda KD (2000) Cocaine vaccines: antibody protection against relapse in a rat model. *Proc Natl Acad Sci USA* **97:**6202–6206.

Carrera MR, Trigo JM, Wirsching P, Roberts AJ, and Janda KD (2005) Evaluation of the anticocaine monoclonal antibody GNC92H2 as an immunotherapy for cocaine overdose. *Pharmacol Biochem Behav* **81:**709–714.

Collins GT, Brim RL, Narasimhan D, Ko MC, Sunahara RK, Zhan CG, and Woods JH (2009) Cocaine esterase prevents cocaine-induced toxicity and the ongoing intravenous self-administration of cocaine in rats. *J Pharmacol Exp Ther* **331:**445–455.

Collins GT, Carey KA, Narasimhan D, Nichols J, Berlin AA, Lukacs NW, Sunahara RK, Woods JH, and Ko MC (2011) Amelioration of the cardiovascular effects of cocaine in rhesus monkeys by a long-acting mutant form of cocaine esterase. *Neuropsychopharmacology* **36:**1047–1059.

Collins GT, Narasimhan D, Cunningham AR, Zaks ME, Nichols J, Ko MC, Sunahara RK, and Woods JH (2011) Long-lasting effects of a PEGylated mutant cocaine esterase (CocE) on the reinforcing and discriminative stimulus effects of cocaine in rats. *Neuropsychopharmacology* doi:10.1038/npp.2011.226.

Cooper ZD, Narasimhan D, Sunahara RK, Mierzejewski P, Jutkiewicz EM, Larsen NA, Wilson IA, Landry DW, and Woods JH (2006) Rapid and robust protection against cocaine-induced lethality in rats by the bacterial cocaine esterase. *Mol Pharmacol* **70:**1885–1891.

Crumb WJ Jr, Kadowitz PJ, Xu YQ, and Clarkson CW (1990) Electrocardiographic evidence for cocaine cardiotoxicity in cat. *Can J Physiol Pharmacol* **68:**622–625.

Dackis C and O'Brien C (2003) Glutamatergic agents for cocaine dependence. *Ann N Y Acad Sci* **1003:**328–345.

Emsley P and Cowtan K (2004) Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **60:**2126–2132.

Fernandez-Lafuente R (2009) Stabilization of multimeric enzymes: strategies to prevent subunit dissociation. *Enzyme Microb Technol* **45:**405–418.

Fox BS, Kantak KM, Edwards MA, Black KM, Bollinger BK, Botka AJ, French TL, Thompson TL, Schad VC, Greenstein JL, et al. (1996) Efficacy of a therapeutic cocaine vaccine in rodent models. *Nat Med* **2:**1129–1132.

Gao D, Narasimhan DL, Macdonald J, Brim R, Ko MC, Landry DW, Woods JH, Sunahara RK, and Zhan CG (2009) Thermostable variants of cocaine esterase for long-time protection against cocaine toxicity. *Mol Pharmacol* **75:**318–323.

Grabowski J, Shearer J, Merrill J, and Negus SS (2004) Agonist-like, replacement pharmacotherapy for stimulant abuse and dependence. *Addict Behav* **29:**1439–1464.

Heikoop JC, van den Boogaart P, Mulders JW, and Grootenhuis PD (1997) Structure-based design and protein engineering of intersubunit disulfide bonds in gonadotropins. *Nat Biotechnol* **15:**658–662.

Johanson CE and Fischman MW (1989) The pharmacology of cocaine related to its abuse. *Pharmacol Rev* **41:**3–52.

Kabashima T, Li Y, Kanada N, Ito K, and Yoshimoto T (2001) Enhancement of the thermal stability of pyroglutamyl peptidase I by introduction of an intersubunit disulfide bond. *Biochim Biophys Acta* **1547:**214–220.

Kantak KM, Collins SL, Lipman EG, Bond J, Giovanoni K, and Fox BS (2000) Evaluation of anti-cocaine antibodies and a cocaine vaccine in a rat self-administration model. *Psychopharmacology* (Berl) **148:**251–262.

Kinsey BM, Kosten TR, and Orson FM (2010) Anti-cocaine vaccine development. *Expert Rev Vaccines* **9:**1109–1114.

Ko MC, Bowen LD, Narasimhan D, Berlin AA, Lukacs NW, Sunahara RK, Cooper ZD, and Woods JH (2007) Cocaine esterase: interactions with cocaine and immune responses in mice. *J Pharmacol Exp Ther* **320:**926–933.

Kosten TR, Rosen M, Bond J, Settles M, Roberts JS, Shields J, Jack L, and Fox B (2002) Human therapeutic cocaine vaccine: safety and immunogenicity. *Vaccine* **20:**1196–1204.

Landry DW, Zhao K, Yang GX, Glickman M, and Georgiadis TM (1993) Antibody-catalyzed degradation of cocaine. *Science* **259:**1899–1901.

Larsen NA, Turner JM, Stevens J, Rosser SJ, Basran A, Lerner RA, Bruce NC, and Wilson IA (2002) Crystal structure of a bacterial cocaine esterase. *Nat Struct Biol* **9:**17–21.

Mateo R, Luna E, Rincón V, and Mateu MG (2008) Engineering viable foot-and-mouth disease viruses with increased thermostability as a step in the development of improved vaccines. *J Virol* **82:**12232–12240.

Matsushita M, Hoffman TZ, Ashley JA, Zhou B, Wirsching P, and Janda KD (2001) Cocaine catalytic antibodies: the primary importance of linker effects. *Bioorg Med Chem Lett* **11:**87–90.

Mello NK (1990) Preclinical evaluation of the effects of buprenorphine, naltrexone and desipramine on cocaine self-administration. *NIDA Res Monogr* **105:**189–195.

Nakka M, Iyer RB, and Bachas LG (2006) Intersubunit disulfide interactions play a critical role in maintaining the thermostability of glucose-6-phosphate dehydrogenase from the hyperthermophilic bacterium Aquifex aeolicus. *Protein J* **25:**17–21.

Narasimhan D, Nance MR, Gao D, Ko MC, Macdonald J, Tamburi P, Yoon D, Landry DM, Woods JH, Zhan CG, et al. (2010) Structural analysis of thermostabilizing mutations of cocaine esterase. *Protein Eng Des Sel* **23:**537–547.

Newman AH, Grundt P, and Nader MA (2005) Dopamine D3 receptor partial agonists and antagonists as potential drug abuse therapeutic agents. *J Med Chem* **48:**3663–3679.

Ogasahara K, Ishida M, and Yutani K (2003) Stimulated interaction between α and β subunits of tryptophan synthase from hyperthermophile enhances its thermal stability. *J Biol Chem* **278:**8922–8928.

Otwinowski Z and Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* **276:**307–326.

Park JB, Kwon YM, Lee TY, Brim R, Ko MC, Sunahara RK, Woods JH, and Yang VC (2010) PEGylation of bacterial cocaine esterase for protection against protease digestion and immunogenicity. *J Control Release* **142:**174–179.

Pilipenko O, Atyaksheva L, Poltorak O, and Chukhrai E (2007) Dissociation and catalytic activity of oligomer forms of β-galactosidases. *Russ J Phys Chem A* **81:**990–994.

Platt DM, Rowlett JK, and Spealman RD (2002) Behavioral effects of cocaine and dopaminergic strategies for preclinical medication development. *Psychopharmacology* (Berl) **163:**265–282.

Poltorak OM, Chukhrai ES, Kozlenkov AA, Chaplin MF, and Trevan MD (1999) The putative common mechanism for inactivation of alkaline phosphatase isoenzymes. *J Mol Catal B Enzym* **7:**157–163.

Reznik GO, Vajda S, Smith CL, Cantor CR, and Sano T (1996) Streptavidins with intersubunit crosslinks have enhanced stability. *Nat Biotechnol* **14:**1007–1011.

Roberts DC and Brebner K (2000) GABA modulation of cocaine self-administration. *Ann NY Acad Sci* **909:**145–158.

Rothman RB, Baumann MH, Prisinzano TE, and Newman AH (2008) Dopamine transport inhibitors based on GBR12909 and benztropine as potential medications to treat cocaine addiction. *Biochem Pharmacol* **75:**2–16.

Substance Abuse and Mental Health Services Administration, Office of Applied Studies (2008) *Results from the 2007 National Survey on Drug Use and Health: National Findings* (NSDUH Series H-34, DHHS Publication no. SMA 08-4343). Substance Abuse and Mental Health Services Administration, Office of Applied Studies, Rockville, MD.

Substance Abuse and Mental Health Services Administration, Office of Applied Studies (2008) Drug Abuse Warning Network, 2006: National Estimates of Drug-Related Emergency Department Visits. DAWN Series D-30, DHHS Publication no. (SMA) 08-4339. Substance Abuse and Mental Health Services Administration, Office of Applied Studies, Rockville, MD.

Sowdhamini R, Srinivasan N, Shoichet B, Santi DV, Ramakrishnan C, and Balaram P (1989) Stereochemical modeling of disulfide bridges. Criteria for introduction into proteins by site-directed mutagenesis. *Protein Eng* **3:**95–103.

Tanaka H, Chinami M, Mizushima T, Ogasahara K, Ota M, Tsukihara T, and Yutani K (2001) X-ray crystalline structures of pyrrolidone carboxyl peptidase from a hyperthermophile, Pyrococcus furiosus, and its cys-free mutant. *J Biochem* **130:**107–118.

Turner JM, Larsen NA, Basran A, Barbas CF 3rd, Bruce NC, Wilson IA, and Lerner RA (2002) Biochemical characterization and structural analysis of a highly proficient cocaine esterase. *Biochemistry* **41:**12297–12307.

Uhl GR, Hall FS, and Sora I (2002) Cocaine, reward, movement and monoamine transporters. *Mol Psychiatry* **7:**21–26.

Vagin AA, Steiner RA, Lebedev AA, Potterton L, McNicholas S, Long F, and Murshudov GN (2004) REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. *Acta Crystallogr D Biol Crystallogr* **60:**2184–2195.

Wong SS and Wong LJ (1992) Chemical crosslinking and the stabilization of proteins and enzymes. *Enzyme and Microbial Technology* **14:**866–874.

Xie W, Altamirano CV, Bartels CF, Speirs RJ, Cashman JR, and Lockridge O (1999) An improved cocaine hydrolase: the A328Y mutant of human butyrylcholinesterase is 4-fold more efficient. *Mol Pharmacol* **55:**83–91.

Yang G, Chun J, Arakawa-Uramoto H, Wang X, Gawinowicz MA, Zhao K, and Landry DW (1996) Anti-cocaine catalytic antibodies: a synthetic approach to improved antibody diversity. *J Am Chem Soc* **118:**5881–5890.

Zheng F, Yang W, Ko MC, Liu J, Cho H, Gao D, Tong M, Tai HH, Woods JH, and Zhan CG (2008) Most efficient cocaine hydrolase designed by virtual screening of transition states. *J Am Chem Soc* **130:**12148–12155.

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