0026-895X/09/7502-318–323$20.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 75:318–323, 2009

Vol. 75, No. 2
49486/3428251
Printed in U.S.A.

Thermostable Variants of Cocaine Esterase for Long-Time Protection against Cocaine Toxicity

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Received June 5, 2008; accepted November 4, 2008

ABSTRACT

Enhancing cocaine metabolism by administration of cocaine esterase (CocE) has been recognized as a promising treatment strategy for cocaine overdose and addiction, because CocE is the most efficient native enzyme for metabolizing the naturally occurring cocaine yet identified. A major obstacle to the clinical application of CocE is the thermoinstability of native CocE with a half-life of only a few minutes at physiological temperature (37°C). Here we report thermostable variants of CocE developed through rational design using a novel computational approach followed by in vitro and in vivo studies. This integrated computational-experimental effort has yielded a CocE variant with a ~30-fold increase in plasma half-life both in vitro and in vivo. The novel design strategy can be used to develop thermostable mutants of any protein.

Cocaine strongly reinforces self-administration (Mendelson and Mello, 2000; Singh, 2000; Paula et al., 2004), and the disastrous medical and social consequences of cocaine addiction have made the development of an effective pharmacological treatment a high priority (Gorelick, 1997; Redish, 2004). The classic central nervous system receptor-antagonist approach has failed to yield an anticocaine therapeutic, but we developed a proof of principle for a peripheral blocker to accelerate cocaine metabolism in the circulation (Landry et al., 1993; Mets et al., 1998), producing biologically inactive metabolites via hydrolysis of cocaine benzoyl ester (Gorelick, 1997; Zhan et al., 2003; Meijler et al., 2005; Pan et al., 2005, 2007; Rogers et al., 2005; Zheng et al., 2008). The bacterial cocaine esterase (CocE) (Bresler et al., 2000), the most efficient native cocaine hydrolase yet identified (Larsen et al., 2002), is particularly promising. In rodent models, CocE can both prevent and reverse extreme cocaine toxicity (Cooper et al., 2006; Ko et al., 2007). However, a major obstacle to the clinical application of CocE is the thermoinstability of native CocE with a half-life of only a few minutes at physiological temperature (37°C) (Cooper et al., 2006). It is highly desirable to develop thermostable mutants of CocE for therapeutic treatment of cocaine toxicity.

Protein engineering for thermostability is a particularly exciting and challenging field (Lehmann and Wyss, 2001). In addition to directed evolution, various rational methods have been developed for thermostable mutant design, and a key factor considered in these methods is the interactions of amino acids within a protein's core. Enzyme engineering for thermostability poses additional challenges, because the active site structure of an enzyme and its dynamic behavior during an enzymatic reaction often seem fine-tuned for the optimum catalytic efficiency (Korkegian et al., 2005). To stabilize an enzyme without losing catalytic efficiency, a computational design method must be capable of predicting thermostable mutations within a given fold while minimizing any shift in the backbone that might structurally disrupt the active site structure or quench its flexibility. Computational design has been used successfully to thermostabilize noncatalytic proteins (Malakauskas and Mayo, 1998; Dahiyat, 1999; Luo et al., 2002; Dantas et al., 2003), redesign binding pockets (Benson et al., 2002; Reina et al., 2002; Shifman and Mayo, 2002; Looger et al., 2003), create a protein fold (Kuhlman et al., 2003), and design catalytic activity into a bacterial receptor (Dwyer et al., 2004). Stoddard and associates recently demonstrated that computational design could also

This work was supported by the National Institutes of Health [Grants DA021416, DA025100, DA013930, and GM007767].

D.G. and D.L.N. contributed equally to this work.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.108.049486.

ABBREVIATIONS: CocE, cocaine esterase; MD, molecular dynamics; RMSD, root-mean-square deviation; wt, wild type; PAGE, polyacrylamide gel electrophoresis.

help to thermostabilize an enzyme, because they successfully designed thermostable mutants of a small enzyme (homodimer) (i.e., yeast cytosine deaminase) (Korkegian et al., 2005). The 153 amino acid protein displays irreversible unfolding behavior at high temperature. RosettaDesign (Kuhlman and Baker, 2000) was used to evaluate the fitness of a particular sequence for a given fold, and a Monte Carlo search algorithm was used for sampling sequence space. The program requires a backbone structure as input, uses an empirical energy function, and generates sequences predicted to have the lowest energy for that fold. Excluding the residues located within 4 Å of the active site or involved in the dimer interface, or examined in other published studies, their computational design of the yeast cytosine deaminase mutants only needed to consider possible mutations on 33 amino acid residues.

CocE represents a considerably more challenging enzyme to computationally derive a thermostable enzyme because of its relatively large size (574 amino acids). First, the computational design and subsequent experimental tests need to take into account many more amino acid residues to identify the best possible thermostable mutations. In addition, it is unnecessary for the enzyme to unfold before it becomes inactive. Enzyme inactivation could be merely associated with minor structural changes on the least stable region of the protein, without completely unfolding the enzyme. In this case, a mutation lowering the total folding energy of an enzyme does not necessarily lead to a longer half-life of the active enzyme structure. Hence, it is crucial to uncover the inactivation mechanism while modeling the thermostabilization of an enzyme.

In the present study, molecular modeling and simulations have led to important mechanistic insights into the inactivation process of CocE. Herein we report thermostable variants of CocE developed through structure- and mechanism-based design followed by in vitro and in vivo studies. This integrated computational-experimental effort has yielded CocE variants with melting temperatures up to 7° higher than the native enzyme and a ~30-fold increase in plasma half-life both in vitro and in vivo. This robust method can be used to develop thermostable mutants of any protein.

Materials and Methods

Molecular Modeling and Simulation. The mechanism of enzyme inactivation is very difficult to unveil through computational simulation. For example, CocE has a half-life ($\tau_{1/2}$) of a few minutes (see below), which is too short for use as a drug, but too long for performing a molecular dynamics (MD) simulation to simulate the inactivation process. This is because a practical, fully relaxed MD simulation (with a required time step of 1 or 2 fs) on a protein system like CocE can be performed for as long as nanoseconds using currently available supercomputers. Instead, we based our computational design strategy on an analysis of the kinetic relationship between the inactivation rate constant (k_{ina}) and the temperature (T) for a given inactivation free-energy barrier (ΔG_{ina}) of the inactivation process:

$$k_{ina} = (k_B T/h)\exp(-\Delta G_{ina}/RT), \quad (1)$$

which is analogous to the rate constant equation used in the well known variational transition state theory (Corchado et al., 1998). In the equation, k_B is Boltzmann's constant, h is Planck's constant, and R is the gas constant. According to the equation, the rate constant k_{ina} of the enzyme inactivation is dependent on the temperature for a given inactivation free-energy barrier. The higher the temperature, the larger the rate constant, and therefore, the half-life of the active enzyme is shorter. This kinetic understanding enables us to reveal the inactivation pathway of CocE through performing MD simulation at an appropriately high temperature. The high temperature used in the MD simulations is physiologically irrelevant but can considerably shorten the time of the protein inactivation process and, thus, allows us to observe the protein inactivation process within nanoseconds.

The initial structures constructed from the X-ray crystal structure (Larsen et al., 2002) were neutralized by adding sodium counterions and were solvated in a rectangular box of TIP3P water molecules with a minimum solute-wall distance of 10 Å. All of the MD simulations were performed by using the Sander module of Amber 8 package (http://ambermd.org/). The solvated systems were carefully equilibrated and fully energy-minimized. These systems were gradually heated from T = 10 K to T = 298 K in 30 ps before production MD simulations at 298 K (in the constant temperature and constant pressure ensemble with P = 1 atm) and then at 575 K (in the constant temperature and constant volume ensemble). The time step used for the MD simulations was 2 fs. Periodic boundary conditions with Berendsen temperature coupling (Berendsen et al., 1984) with isotropic molecule-based scaling (Berendsen et al., 1984) were applied. The SHAKE algorithm (Ryckaert et al., 1977) was used to fix all covalent bonds containing hydrogen atoms. The nonbonded pair list was updated every 10 steps. The particle mesh Ewald method (Darden et al., 1993) was used to treat long-range electrostatic interactions. A residue-based cutoff of 10 Å was used to the noncovalent interactions. Furthermore, the protein structures simulated at 298 K were subjected to the energy minimization before the interaction energy calculations in the gas phase. The solvent shifts of the interaction energies were calculated by using the Delphi program (Gilson et al., 1988). In addition, the empirical scoring function implemented in RosettaDesign program (Kuhlman and Baker, 2000) was also used to estimate the interaction energies.

Site-Directed Mutagenesis. Point mutations were generated using QuikChange (Stratagene, La Jolla, CA) and CocE cDNA cloned in the bacterial expression vector, pET-22b (+). Double mutants were generated using single-point mutations as templates. Oligonucleotide sequences are available on request. All mutants were sequenced in both directions over the entire coding region. All enzymes were expressed as 6xHis-tagged proteins in *Escherichia coli* BL-21 (DE3) cells grown at 37°C. Protein expression was induced with 1 mM isopropyl-β-thiogalactopyranoside (Thermo Fisher Scientific, Waltham, MA) for 12 h at 18°C.

Purification of Cocaine Esterase and Mutants. Cells were pelleted, resuspended in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM dithiothreitol, and a protease inhibitor cocktail (34 μg/ml each of L-tosylamido-2-phenylethyl chloromethyl ketone, 1-chloro-3-tosylamido-7-amino-2-heptanone, and phenylmethylsulfonyl fluoride, and 3 μg/ml each of leupeptin and lima bean trypsin inhibitor) and lysed using a French press (Thermo Fisher Scientific). 6xHis-tagged enzymes were enriched using Talon metal affinity chromatography (Clontech, Mountain View, CA) and purified using anion exchange (Q-Sepharose; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) chromatography. CocE was eluted from the Q-Sepharose column with 150 to 450 mM NaCl linear gradient buffers containing 20 mM HEPES, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol. The peak fractions were pooled and concentrated by Centricon-30 (Millipore, Billerica, MA), snap-frozen in liquid nitrogen, and stored at −80°C.

Michaelis-Menten Kinetics of Cocaine Hydrolysis. Cocaine hydrolysis was monitored as described previously (Turner et al., 2002). The initial rates of decay were estimated by following the change in the intrinsic absorbance of cocaine at 240 nm with time using a SpectraMax Plus 384 UV plate reader (Molecular Devices, Sunnyvale, CA) and SOFTmax Pro software (version 3.1.2). The initial rates were estimated from the linear portion of the progress curves and spanned no longer than 5 min. The reaction was initiated

by adding 100 μl of a 2× enzyme solution (100 mM phosphate buffer, pH 7.4, and 300 mM NaCl) to 100 μl of a 2× cocaine solution (50 ng/ml enzyme, 100 mM phosphate buffer, pH 7.4, and 300 mM NaCl). Final cocaine concentrations were as follows: 125, 62.5, 31.25, 15.63, 7.81, 3.91, 1.95, and 0.977 μM. V_{max} and K_m values were calculated using Prism (GraphPad Software Inc., San Diego, CA). Stability measurements were performed using the native and mutant enzymes diluted to 2× concentration and incubated at 37°C for various times. At the end of each time point, an aliquot was removed, and kinetic behavior was observed as mentioned above. The percentage of remaining activity was plotted against time to assess the half-life ($\tau_{1/2}$) of each enzyme.

Circular Dichroism Measurement and Thermal Denaturation Experiments. CD data were collected on a J-810 spectropolarimeter (JASCO, Easton, MD). Near UV wavelength scans were run from 250 to 320 nm with a 0.2-nm pitch, varying protein concentrations (5–30 μM), temperatures increasing from 5 to 70°C in 2.5°C increments, and a 1-cm pathlength water-jacketed cylindrical quartz cell. Four elliptical curves were generated per temperature measurement, and denaturation was recorded as a change in average ellipticity over temperature. Apparent T_m values were determined by deconvolution to component spectra using the convex constraint algorithm (Perczel et al., 1992) into 2 curves using 30 iterations, and subsequent nonlinear regression curve-fitting using Prism 5 (GraphPad Software).

In vivo Protection against Cocaine Lethality. Male NIH-Swiss mice (25–32 g; Harlan Inc., Indianapolis, IN) were housed in groups of 6 mice per cage, allowed ad libitum access to food and water, and were maintained on a 12-h light/dark cycle with lights on at 6:30 AM in a room kept at a temperature of 21 to 22°C. Mouse body temperatures are assumed to be in the well established range of 3.65 to 38°C (Harkness and Wagner, 1989). All protocols and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health and were approved by the University Committee on the Use and Care of Animals at the University of Michigan.

Cocaine-induced toxicity was characterized by the occurrence of lethality, as defined by the cessation of observed movement and respiration. Lethality was recorded after intraperitoneal cocaine administration (LD_{100}, 180 mg/kg) on mice subjected to pretreatment of purified native CocE or mutants (intravenous injection of 0.2 ml) for various times (1, 5, 10, or 30 min; 1, 2, 3, 4, and 5 h after enzyme administration). Each treatment used eight mice to assess the percentage of lethality (i.e., protection) in mice pretreated with a single dose of an esterase at a single time point.

Blood Collection and Western Blotting. Blood was collected 1 and 2 h after treatment from the submandibular area. This area was punctured using a mouse bleeding lancet (GoldenRod 4.0-mm animal lancet; MEDIpoint Inc., Mineola, NY). Blood was collected into BD Microtainer collection tubes (BD Diagnostics Serums Seperator Tubes; BD Biosciences, San Jose, CA) and placed on ice. Samples were spun at 4000 rpm for 5 min to separate the plasma. Plasma proteins (10 μg) were loaded onto a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were then probed with anti-CocE rabbit antibody followed by horseradish peroxidase-linked anti-rabbit secondary antibody. As a loading control, blots were stripped and then reprobed with mouse anti-apolipoprotein-A1 antibody.

Results

We used Amber 8 (http://ambermd.org/) to carry out MD simulations on native CocE in water. The simulations, starting from the X-ray crystal structure (Larsen et al., 2002) of CocE, were performed first at room temperature (298 K) for 250 ps followed by an increase in temperature to 575 K for 1050 ps (i.e., 250 to 1300 ps in Fig. 1A). Depicted in Fig. 1A is the time-dependence of the root-mean-square deviation (RMSD) of the atomic positions in the simulated CocE structure from the corresponding positions in the X-ray crystal structure. The RMSD value reflects the magnitude of the overall change of the protein structure during the MD simulation. A stable protein structure is usually expected to have a reasonable RMSD value of ~2 Å for the overall protein structure because of the dynamics of protein in water. A significantly larger RMSD value may be associated with some major structural change that could cause the protein to lose its normal function. Figure 1A shows that the CocE structure was quickly stabilized during the MD simulation at 298 K. The RMSD value became increasingly larger during the further MD simulation at 575 K and reached a value for the overall protein structure of 4 Å at ~1000 ps. To identify the pathway for the major structural change of CocE during the MD simulation at 575 K, we further visualized the detailed structural changes during the MD simulation and found that amino acid residues Gly165 to Ile195 in domain II (consisting of amino acid residues Asp145 to Leu240) of CocE first started to have the major structural changes. Figure 1B depicts the three domains (I to III) of CocE. As seen in Fig. 1B, part of the substrate-binding pocket belongs to domain II. Based on the MD simulation, the region from residues Gly165 to Ile195 in domain II is the least stable region of the CocE structure. As

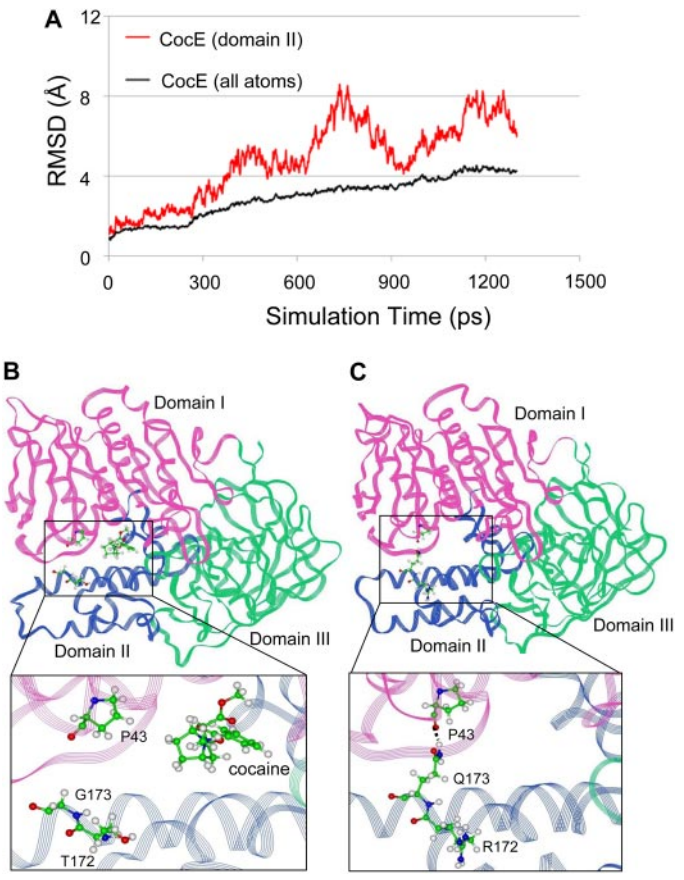

Fig. 1. A, time-dependence of the root-mean-square deviation (in angstroms) of the MD-simulated atomic positions of CocE from those in the initial structure built from the X-ray crystal structure. The temperature used in the MD simulation was 298 K for first 250 ps and 575 K for the remaining 1050 ps (including temperature increase for 50 ps). Domain II refers to the plot for the atoms of residues Gly165 to Ile195 in domain II. B, CocE-(−)-cocaine binding structure modeled by molecular docking and MD simulation at 298 K. C, optimized structure of T172R/G173Q mutant of CocE. The hydrogen bond between Gln173 domain II and Pro43 of domain I is indicated by a broken line.

seen in Fig. 1A, the RMSD value for residues Gly165 to Ile195 in domain II is much larger than the corresponding RMSD value for the overall CocE structure. Elevation to 575 K imposes distortions in domain II that would probably lead to the disruption of the substrate-binding pocket and loss of catalytic activity because of their relative close proximity. Eventually, the distortion may lead to global alterations in protein structure. The enzyme is expected to be inactive after disruption of domain II structure, regardless of how stable domains I and III are. Without altering the side chains on residues Gly165 to Ile195 in domain II, decreasing the total folding energy by stabilizing domain I or III should not increase the half-life of the active enzyme structure.

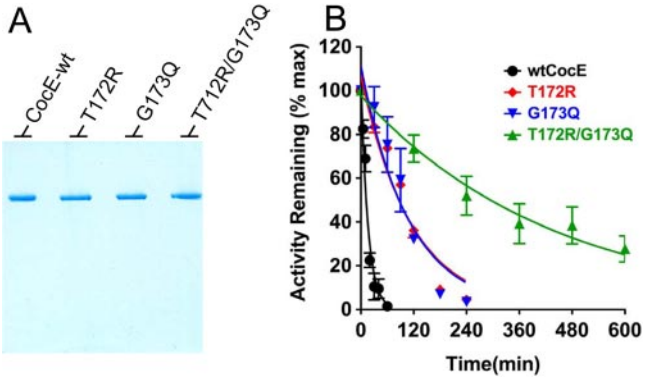

Fig. 2. A, SDS-PGE of purified CocE-wt, T172R, G173Q, and T172R/G173Q. B, decay in activity at 37°C; 50 ng/ml wt-CocE and the mutants were incubated at 37°C, and enzymatic activity was measured over time. The half-lives for each mutant were determined by analyzing the catalytic efficiency at different preincubation times. Data were fitted using a signal phase exponential decay (Prism 5; GraphPad Software). Native CocE (black), T172R(red), G173Q (blue), and T172R/G173Q (green) showed 11.2 ± 0.9, 78 ± 6.5, 75 ± 9.9, and 305 ± 38 min half-lives, respectively.

These mechanistic insights obtained from the MD simulations suggest that rational design of possible thermostable mutants of CocE should therefore focus on domain II, particularly residues Gly165 to Ile195. This may be accomplished by increasing either hydrogen bonding between domains I and II or by increasing some favorable interactions between amino acid residues within domain II. Hence, our current computational design only targets residues of domain II that are at least 5 Å away from (−)-cocaine atoms according to our modeled CocE-(−)-cocaine complex structure. The energetics of possible mutations on these amino acid residues were estimated by using two different approaches. The first approach calculates the shift in the interaction energy between the mutated residue and the remaining part of the enzyme through a combined application of Amber and Delphi (Gilson et al., 1988) programs. The structures of both native CocE and mutants were optimized (through energy minimization) before the interaction energy calculations.

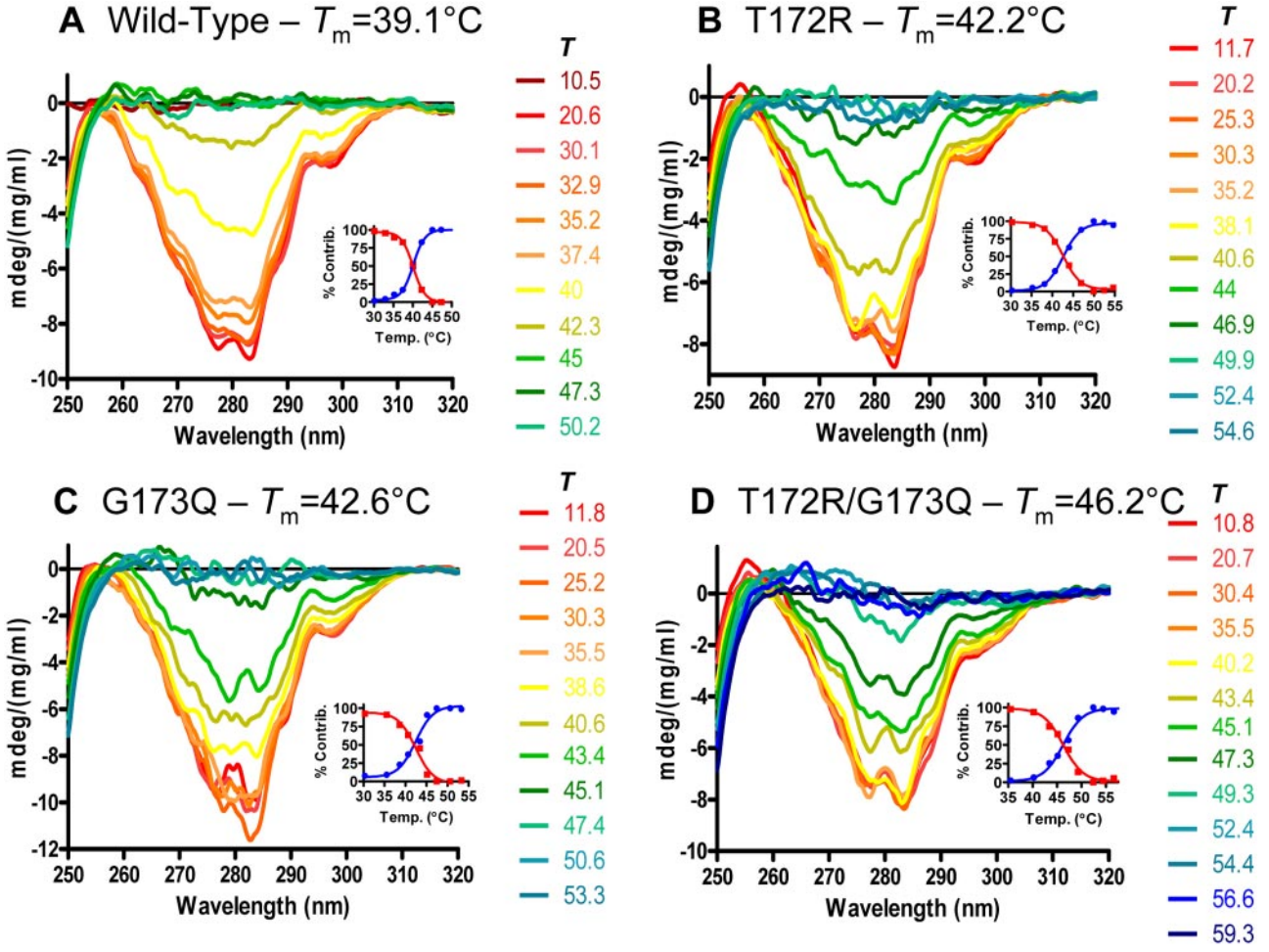

Fig. 3. Data from circular dichroism measurements. A, native CocE. B, T172R mutant. C, G173Q mutant. D, T172R/G173Q mutant. The temperatures of each scan for each mutant are listed in color-coded form.

The other approach estimates the change in folding energy of the mutant by using the RosettaDesign program for folding energy calculations on the optimized structures of native CocE and its mutants. These two computational approaches can complement each other. Whereas the former uses a more sophisticated theoretical energetic approach, the latter uses the empirical scoring function developed specifically for folding energy calculations. We can reasonably expect that when both approaches consistently predict that a mutation on a residue in domain II can stabilize domain II, the prediction should be more reliable. Indeed, both computational approaches consistently predicted that several mutations, including T172R and G173Q, should stabilize domain II. Mutation G173Q was predicted to build a bridge between domains I and II through a hydrogen bond between residues Pro43 of domain I and Gln173 of domain II (Fig. 1C), whereas mutation T172R was predicted to have an improved interaction between residue 172 and the other residues in domain II (with a lower interaction/folding energy). The CocE-wt crystal structure (Larsen et al., 2002) reveals that the alkyl chain of Thr172 on helix five points toward helix 6 within domain II but away from domain I. Substitution of arginine at this location introduces a longer alkyl moiety and a charge that we rationalize may act in concert to contact and stabilize helix 6. The stabilization of domain II itself may influence the overall stability of the protein. Although the computational evaluations suggest that the effects of mutations T172R and G173Q on the energetics should be additive, improving the integrity of domain II itself may help G173Q to facilitate interactions with domain I in a more cooperative manner.

To validate our computational predictions we assessed the effect of T172R, G173Q, and T172R/G173Q compared with native CocE (CocE-wt) using in vitro and in vivo experimentation. Using purified enzyme preparations we directly compared their catalytic activities and half-lives at a physiological temperature (37°C). The purified preparations are greater than 95% pure by SDS-PAGE and Coomassie blue staining (Fig. 2A) and display appropriate Michaelis-Menten kinetics with the following catalytic constants: CocE-wt (K_m ~21 μM, and k_{cat}~2323 min^{-1}), T172R (K_m ~24 μM and k_{cat} ~2502 min^{-1}), G173Q (K_m ~15 μM and k_{cat} ~2384 min^{-1}), and T172R/G173Q (K_m ~24 μM and k_{cat} ~2247 min^{-1}). The effects of temperature on thermostability are illustrated in Fig. 2B and summarized in Fig. 4.

Mutant enzymes were generated by site-directed mutagenesis and expressed and purified as described previously (Cooper et al., 2006). The in vitro enzyme activity depicted in Fig. 2B reveals that preincubation of native CocE at 37°C decreased the enzymatic activity exponentially with time ($\tau_{1/2}$ = ~11 ± 0.9 min). In contrast, the CocE mutants T172R, G173Q, and T172R/G173Q displayed significantly longer half-lives without changing the enzymatic activity before preincubation at 37°C: $\tau_{1/2}$(T172R) = ~78 ± 6.5 min (~7-fold increase), $\tau_{1/2}$(G173Q) = ~75 ± 9.9 min (~7-fold increase), and $\tau_{1/2}$ (T172R/G173Q) = ~305 ± 38 min (~30-fold increase). Careful analysis of the activity of T172R/G173Q after incubation at 37°C suggests a more complicated mechanism of inactivation at 37°C compared with CocE-wt or the single mutants. Although CocE-wt, T172R, and G173Q fit very well to a single-phase exponential decay, T172R/G173Q displays multiphase inactivation kinetics with $\tau_{1/2short}$ ~180 min and $\tau_{1/2long}$ ~4900 min. Although we have few data to pinpoint a precise mechanism for the biphasic appearance, the data do reflect the cooperative, rather than additive, effects of combining T172R and G173Q mutations. These data correlated well with subsequent circular dichroism measurements (Fig. 3) of thermal denaturation, revealing a 3.1 to 3.5°C increase in apparent denaturation temperature (T_m) for single mutants and a 7.1°C increase for the double mutant T172R/G1734Q. The data suggest an overall loss in helicity of the enzyme preparations with respect to temperature and indicates the thermal denaturation of the enzyme structure. CocE is approximately 28% helical in nature; however, domain II accounts for approximately one fifth of the overall helicity. One may therefore rationalize that mutants that stabilize domain II or the interaction with domain I and domain II should preserve the observed helicity at higher temperatures compared with the native enzyme.

Assessment of the thermostability using in vivo models based on protection of native CocE (CocE-wt) and its T172R and T172R/G173Q mutants against cocaine-induced lethality correlated well with the in vitro data. Depicted in Fig. 4A are the determined in vivo half-lives of the enzymes compared with the corresponding in vitro half-lives at 37°C. As shown in Fig. 4A, the in vivo half-life of CocE-wt is ~11 ± 1.4 min, whereas both the T172R and T172R/G173Q mutants have

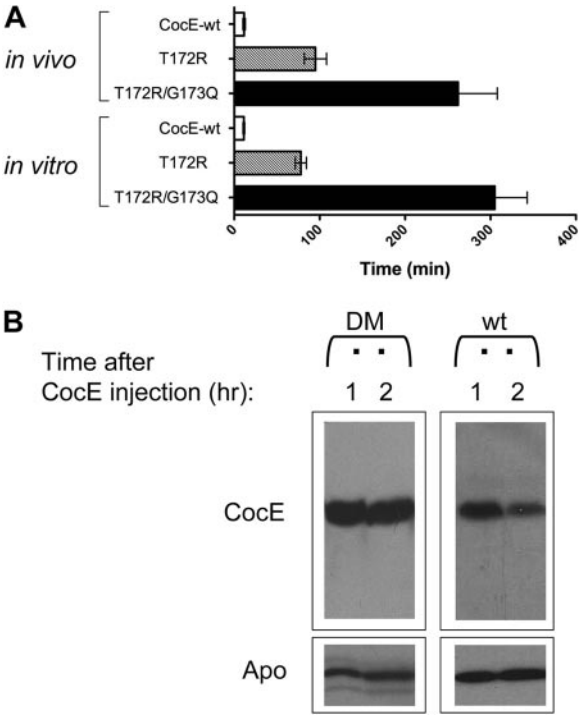

Fig. 4. A, prolonged protection against cocaine toxicity. The time after CocE administration (1 mg i.v.) pretreatment at which the enzyme protects 50% of the animals from a lethal cocaine administration (180 mg/kg i.p.; n = 8 for each dosing condition). The in vivo half-lives for CocE-wt (□), T172R (▨) and T172R/G173Q (■) are compared with the $\tau_{1/2}$ values determined by the in vitro measurements of cocaine hydrolysis at 37°C from Fig. 2. $\tau_{1/2}$ for the in vivo data were determined by analyzing the lethality time course data. Data were fitted using a signal-phase exponential decay (Prism 5; GraphPad Software), and the times that led to 50% lethality were interpolated. B, Western blot analysis of mice plasma collected 1 and 2 h after treatment with and CocE-wt and the T172R/G173Q mutant (1 mg i.v,). Plasma proteins (10 μg) were loaded onto a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Membranes were then probed with anti-CocE rabbit antibody followed by HRP-linked anti-rabbit secondary antibody. As a loading control, blots were stripped and then reprobed with mouse anti-apolipoprotein-A1 antibody.

significantly longer half-lives of ~95 ± 13 min (~9-fold increase) and ~262 ± 46 min (~24-fold increase), respectively, which are consistent with the in vitro data. Western blotting analysis of CocE-wt and the T172R/G173Q mutant in serum after intravenous injection in mice suggests that the intact, immunoreactive species is present for at least 2 h (Fig. 4B) with no detectable proteolysis. Careful analysis of the data in Fig. 4B suggests that CocE-wt seems to disappear faster than the T172R/G173Q mutant (after 1 h) but at a slower rate than suggested by the protection from lethality data (~11 min). In contrast, serum levels of the T172R/G173Q mutant seem to be unchanged 2 h after injection. These data support the notion that the improved thermostability of the T172R/G173Q mutant may account for its prolonged in vivo protective effect on the cocaine-induced lethality. The identification of thermostable mutants of CocE that display significantly longer circulation time in the body provide promising therapeutic potential for the treatment of cocaine overdose. The T172R/G173Q mutant displays an extended half-life beyond the plasma half-life of cocaine in humans (~45–90 min) (Landry et al., 1993). More extensive in vivo studies on these thermostable mutants of CocE will be described elsewhere.

Discussion

Biotechnology-based therapeutics has recently garnered considerable attention in the pharmaceutical industry. The relatively lower toxic and metabolic potential of protein-based therapeutics are their main attraction. One of the most challenging problems complicating the use of proteins as drugs is the issue of thermostability. The success in generating more thermostable forms of CocE described here suggests that our structure- and mechanism-based design approach is a promising and efficient general computational approach for protein redesign. In particular, to design thermostable mutants of a protein, one can first perform the MD simulation on the protein at an appropriately chosen high temperature to understand the protein inactivation pathway. Based on the detailed understanding of the protein structure and inactivation mechanism, one can carry out a virtual screening of various possible mutants through the interaction energy calculations to predict the most likely thermostable mutants for wet experimental tests in vitro and in vivo. This computational approach will probably represent a valuable strategy for the thermostabilization of other proteins and have dramatic implications on their therapeutic potential.

Acknowledgments

We acknowledge the Center for Computational Sciences at University of Kentucky for supercomputing time on an IBM X-series Cluster with 1360 processors.

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