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Recent progress in protein drug design and discovery with a focus on novel approaches to the development of anticocaine medications

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Abstract

Cocaine is highly addictive and no anticocaine medication is currently available. Accelerating cocaine metabolism, producing biologically inactive metabolites, is recognized as an ideal anticocaine medication strategy, especially for the treatment of acute cocaine toxicity. However, currently known wild-type enzymes have either too low a catalytic efficiency against the abused cocaine, in other words (–)-cocaine, or the *in vivo* half-life is too short. Novel computational strategies and design approaches have been developed recently to design and discover thermostable or high-activity mutants of enzymes based on detailed structures and catalytic/inactivation mechanisms. The structure- and mechanism-based computational design efforts have led to the discovery of high-activity mutants of butyrylcholinesterase and thermostable mutants of cocaine esterase as promising anticocaine therapeutics. The structure- and mechanism-based computational strategies and design approaches may be used to design high-activity and/or thermostable mutants of many other proteins that have clear therapeutic potentials and to design completely new therapeutic enzymes.

Cocaine abuse is a major public health problem. This widely abused drug is highly addictive and may be the most reinforcing of all drugs of abuse [1]. Despite huge advances in the neuroscience of drug abuse and dependence in the past decades, there is still no approved pharmacological treatment for cocaine abuse. Cocaine reinforces self-administration in relation to the peak serum concentration of the drug, the rate of rise to the peak and the degree of change of the serum level [2]. Potent CNS stimulation is followed by depression [3]. Respiratory depression, cardiac arrhythmia and acute hypertension are common effects of drug overdose. The disastrous medical and social consequences of cocaine abuse have made the development of an effective pharmacological treatment a high priority [4,5]. Pharmacological treatment for cocaine overdose and addiction can be either pharmacodynamic or pharmacokinetic. Most currently employed anti-addiction strategies use the classical pharmacodynamic approach, that is, developing small molecules that interact with one or more neuronal binding sites, with the goal of blocking or counteracting a drug's neuropharmacological actions without blocking normal physiological processes. It is well known that cocaine binds to the dopamine transporter (DAT) and other transporters/receptors. In the main target, DAT, the cocaine-binding site is considered to overlap with the dopamine-binding site [6,7]. Thus, it would be extremely difficult to develop a DAT antagonist/inhibitor that can potently block DAT–cocaine binding without affecting the DAT–dopamine interaction and downstream signal transduction. Hence, despite decades of effort, existing pharmacodynamic approaches to treat cocaine abuse have

not yet proven successful [4,8]. Novel pharmacological approaches to the treatment of cocaine overdose and addiction are highly desirable.

The inherent difficulties in antagonizing a blocker such as cocaine in the CNS have led to the development of the pharmacokinetic approach, which aims to act directly on the drug itself to alter its distribution or accelerate its clearance [4]. Pharmacokinetic antagonism of cocaine could be implemented by administration of a molecule, such as a cocaine antibody, which binds tightly to cocaine so as to prevent the drug from crossing the blood–brain barrier [4]. The antibody approach, with either active prophylaxis (vaccine) or passive prophylaxis (monoclonal antibody produced in another host), is expected to work well in low-dose cocaine use. However, in the case of cocaine overdose, one cannot expect to have a sufficient amount of cocaine antibody molecules available to bind with all cocaine molecules. Nevertheless, the blocking action could also be implemented by administration of an enzyme or a catalytic antibody (regarded as an artificial enzyme) that not only binds to cocaine but also accelerates cocaine metabolism, thereby freeing itself for further binding [9]. In the case of cocaine overdose, after an enzyme molecule metabolizes a cocaine molecule and the metabolites leave the active site of the enzyme, the enzyme molecule can bind with and metabolize another cocaine molecule. Thus, an enzyme molecule can be used repeatedly until all cocaine molecules are metabolized. Hence, **enzyme therapy** with a sufficiently efficient enzyme would be an ideal approach for therapeutic treatment of acute cocaine toxicity in the case of cocaine overdose. Usually, a pharmacokinetic agent would not be expected to cross the blood–brain barrier and thus would itself have no direct pharmacodynamic action, such as abuse liability [4,8].

For developing a really useful enzyme therapy for anticocaine medication, it is highly desirable to identify a cocaine hydrolase (CocH) that has a sufficiently high catalytic efficiency for hydrolysis of the abused cocaine, namely, (–)-cocaine [10]. We will first consider natural enzymes. The primary reaction pathway for cocaine metabolism in primates is hydrolysis at the benzoyl ester or methyl ester group [4]. Hydrolysis of the benzoyl ester generates ecgonine methyl ester, whereas hydrolysis of the methyl ester yields benzoylecgonine (BE). The major cocaine-metabolizing enzymes in humans are butyrylcholinesterase (BChE), which catalyzes benzoyl ester hydrolysis (Figure 1), and two liver carboxylesterases (denoted by hCE-1 and hCE-2) that catalyze hydrolysis at the methyl ester and the benzoyl ester, respectively. Among the three, BChE is the principal cocaine hydrolase in human serum. Hydrolysis accounts for approximately 95% of cocaine metabolism in humans. The remaining 5% are deactivated through oxidation by the liver microsomal cytochrome P450 system, producing norcocaine [4]. Ecgonine methyl ester appears to be the least pharmacologically active of the cocaine metabolites and may even cause vasodilation, whereas both BE and norcocaine appear to cause vasoconstriction and lower the seizure threshold, similar to cocaine itself. Norcocaine is hepatotoxic and a local anesthetic [11]. Thus, hydrolysis of cocaine at the benzoyl ester by an enzyme is the pathway most suitable for amplification. Ignoring hCE-2, so far, two types of known native enzymes may be used to catalyze hydrolysis of cocaine at the benzoyl ester: BChE and cocaine esterase (CocE).

Butyrylcholinesterase is synthesized in the liver and widely distributed in the body, including in plasma, brain and lung [4]. Studies in animals and humans have demonstrated that enhancement of BChE activity by administration of exogenous enzyme substantially decreases cocaine's half-life [4]. Clinical studies suggest that BChE has unique advantages. First, human BChE has a long history of clinical use [4], and no adverse effects have been noted with increased BChE plasma activity. Second, more than 20 different naturally occurring mutants of human BChE have been identified, and there is no evidence that these mutants are antigenic [4]. BChE also has potential advantages over active immunization since its administration would immediately enhance cocaine metabolism and would not require an immune response

to be effective. For these reasons, enhancement of cocaine metabolism by administration of BChE is considered a promising pharmacokinetic approach for the treatment of cocaine abuse and dependence [4,8]. However, the catalytic activity of this plasma enzyme is three orders of magnitude lower against naturally occurring (–)-cocaine than against the biologically inactive (+)-cocaine enantiomer [4]. (+)-cocaine can be cleared from plasma in seconds and prior to partitioning into the CNS, whereas (–)-cocaine has a plasma half-life of approximately 47 min or longer (for an intravenous dose of cocaine 0.2 mg/kg), long enough for the manifestation of the CNS effects, which peak in minutes [9]. Thus, PET mapping of the binding of (–)-cocaine and (+)-cocaine in baboon CNS showed marked uptake corresponding to (–)-cocaine at the striatum along with other areas of low uptake, whereas no CNS uptake corresponding to (+)-cocaine was observed [4]. (+)-cocaine was hydrolyzed by BChE so rapidly that it never reached the CNS for PET visualization. Furthermore, the actual half-life of (–)-cocaine in plasma is dependent on the dose of cocaine received. This is because the enzyme BChE should be eventually saturated when (–)-cocaine distribution reaches the equilibrium and when the equilibrium concentration of (–)-cocaine in plasma is much larger than the Michaelis–Menten constant ($K_M = 4.1 \mu\text{M}$) of BChE against (–)-cocaine. When the enzyme is saturated, the (–)-cocaine hydrolysis speed has already reached its maximum and will no longer change with an increasing dose of (–)-cocaine. Thus, the higher the dose, the longer the actual half-life of (–)-cocaine in plasma in the case of a large overdose.

Cocaine esterase, found in *Rhodococcus spp.* strain MB1, a bacterium isolated from the rhizosphere soil of coca plants, hydrolyzes the benzoyl ester of cocaine (Figure 1) [12]. This bacterial enzyme hydrolyzes (–)-cocaine with a k_{cat} value of 468 min^{-1} (7.8 s^{-1}) and a K_M value of 640 nM. Therefore, the catalytic efficiency (k_{cat}/K_M) of native CocE is approximately 800-fold higher than that of native BChE against (–)-cocaine. The potential of CocE as an enzyme-based therapy for cocaine abuse was demonstrated originally in rodent models. In a rat model, 1 mg of CocE intravenously, when administered 1 min prior to cocaine injection (180 mg/kg intraperitoneally), protected 100% of rats as opposed to 13 mg of BChE administered intravenously, which offered no protection from the lethal dose of cocaine [13]. In a mice model, pretreatment of CocE at 0.32 and 1-mg doses intravenously, resulted in ten- and 18-fold shifts in the dose–response curve for cocaine-induced convulsions, and eight- and 14-fold shifts in the dose–response curve for cocaine-induced lethality, respectively [14]. CocE has a relatively short half-life *in vivo*, and the main reason for this short half-life is thought to be its low thermostability. CocE evolved to function at a much lower temperature than a physiological temperature of 37°C . In rat plasma, CocE has a half-life of approximately 10 min, and the effectiveness of CocE decreases with time *in vivo*. In mice, CocE administered 10 min before cocaine only protected 50% of animals [14]. In addition, being a bacterial protein, CocE could elicit a robust immune response.

Regarding the use of these enzymes in anti-cocaine therapeutics, both BChE and CocE have their own advantages and disadvantages. Concerning the advantages of using human BChE, first, BChE from a human source can be tolerated easily in the human body. In addition, BChE is very stable under physiological conditions and, thus, has a relatively longer half-life in human body. The disadvantages of using BChE are associated with the low catalytic efficiency of native BChE against naturally occurring (–)-cocaine. Compared with native BChE, the catalytic efficiency of native CocE against (–)-cocaine is approximately 800-fold higher. A disadvantage of the use of native CocE is its bacterial origin and low thermostability. Hence, the two potential treatment agents, BChE and CocE, could complement each other. Both BChE and CocE could be engineered to become valuable anticocaine therapeutic agents. The use of CocE as an anticocaine therapeutic requires a decrease in the immunogenicity and an increase in the thermostability of the enzyme, whereas the use of an engineered BChE as an anticocaine therapeutic requires an improved catalytic efficiency against (–)-cocaine.

The recent developments of state-of-the-art computational techniques of molecular modeling and simulation related to rational design of protein mutants have provided a great opportunity to more accurately design protein mutants with significantly improved function and/or thermostability. The most recently reported state-of-the-art computational designs of protein mutants are based not only on the protein structures, but also on detailed mechanisms. As discussed later, the development of novel computational design approaches has led to the discovery of the desirable new mutants of CocE and BChE.

Two published articles have reviewed the rational design of anticocaine catalytic antibodies and BChE mutants reported earlier [11,15]. Thermostable mutants of CocE and new mutants of human BChE with higher catalytic activity against (–)-cocaine have been reported since their publication. The new mutants of BChE and CocE were designed and discovered using novel computational design approaches. This review focuses on the most recent progress in the area. Below, we discuss the most recently developed novel computational design approaches and their applications to the development of thermostable mutants of CocE and high-activity mutants of human BChE against (–)-cocaine. Based on the known successes and emerging advances in protein drug discovery and design, we will further provide a perspective of future developments in the field of rational design and discovery of novel protein drugs.

Design & discovery of thermostable mutants of CocE

Protein engineering for thermostability is crucial for broadening the industrial use of recombinant proteins [16]. 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 appear fine-tuned for optimum catalytic efficiency [17]. 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 [18], redesign binding pockets [19] and create a protein fold [20]. Stoddard and associates recently demonstrated that computational design could also help to thermostabilize an enzyme, as they successfully designed thermostable mutants of a small enzyme (homodimer), yeast cytosine deaminase [17]. The 153 amino acid protein displays irreversible unfolding behavior at high temperatures. RosettaDesign 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 [17]. 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.

Cocaine esterase, depicted in Figure 2, represents a considerably more challenging enzyme from which to derive a thermostable enzyme computationally, due to its relatively large size (consisting of 574 amino acids). First, the computational design and subsequent experimental tests need to take into account many more amino acid residues in order to identify the best possible thermostable mutations. In addition, it is unnecessary for an 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 thermostabilization of an enzyme. However, the mechanism of enzyme inactivation is very difficult to unveil through computational simulation. For example, CocE has a half-life 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 model the inactivation process. This is because a practical, fully relaxed MD simulation (with a required time step of 1 or 2 fs) of a protein system such as CocE can be performed for as long as nanoseconds using currently available supercomputers.

A recently developed computational design strategy is based on an analysis of the proposed (hypothesized) 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 [22], that is,

$$k_{\text{ina}} = (k_{\text{B}}T/h) \exp(-\Delta G_{\text{ina}}/RT) \quad (\text{Equation 1})$$

In Equation 1, k_{B} is Boltzmann's constant and h is Planck's constant. Equation 1 was proposed through an extended use of the rate constant equation [22] used in the well-known variational transition state theory [23], which originally applies to chemical kinetics. According to Equation 1, 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 is and, therefore, the shorter the half-life of the active enzyme. 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 observation of the protein inactivation process within nanoseconds [22].

The MD simulation of CocE in water at 298 K showed that the CocE structure was stabilized quickly [22]. However, during the further MD simulation at 575 K, the root mean square deviation (RMSD) of the atomic positions from the corresponding initial positions of the protein became increasingly larger during the simulation and reached a value for the overall protein structure of 4 Å at approximately 1000 ps. The major structural changes of the protein were associated with the atoms in domain II (consisting of amino acid residues D145 to L240). The RMSD value for domain II reached approximately 9 Å at approximately 750 ps, while the RMSD value for the whole protein (including domain II) was only approximately 3.5 Å [22]. The MD simulation at 575 K imposed distortions in domain II that would probably lead to disruption of the substrate binding pocket and loss of catalytic activity due to their relative close proximity (Figure 2). Eventually, the distortion may lead to global alterations in protein structure. The enzyme is expected to be inactive following disruption of the domain II structure, regardless of how stable domains I and III are. Without altering the side chains on residues G165 to I195 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.

The mechanistic insights obtained from the MD simulation at 575 K suggest that the rational design of possible thermostable mutants of CocE should therefore focus on domain II, particularly residues G165 to I195. 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, the reported computational design only targeted residues of domain II that are at least 5 Å away from (–)-cocaine atoms, according to our modeled CocE–(–)-cocaine complex structure [22]. The energetics of possible mutations on these amino acid residues were estimated using two different approaches [22]. The first approach calculates

the shift in the interaction energy between the mutated residue and the remaining part of the enzyme. The structures of both native CocE and mutants were optimized (through energy minimization) prior to the interaction energy calculations. The other approach estimates the change in folding energy of the mutant using RosettaDesign 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 later uses the empirical scoring function developed specifically for folding energy calculations.

One 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 P43 of domain I and Q173 of domain II, 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 computational evaluations also suggested that the effects of mutations of T172R and G173Q on the energetics are additive.

The computational predictions were followed by *in vitro* and *in vivo* experimental tests on the T172R, G173Q and T172R/G173Q mutants of CocE in comparison with the native enzyme. Their catalytic activities and half-lives were compared at a physiological temperature (37°C) [22]. *In vitro* experimental data revealed that preincubation of native CocE at 37°C decreased the enzymatic activity exponentially with time, showing that the half-life of native CocE at 37°C was only approximately 11 ± 0.9 min. The CocE mutants T172R, G173Q and T172R/G173Q displayed significantly longer half-lives without changing the enzymatic activity (baseline activity) prior to preincubation at 37°C [22]:

- Half-life (T172R): approximately 78 ± 6.5 min (approximately sevenfold increase);
- Half-life (G173Q): approximately 75 ± 9.9 min (approximately sevenfold increase);
- Half-life (T172R/G173Q): approximately 305 ± 38 min (an approximately 30-fold increase).

Assessment of the thermostability using *in vivo* models with mice correlated well with the *in vitro* data. The *in vivo* half-life of the active enzyme was determined to be approximately 11 ± 1.4 min for native CocE, approximately 95 ± 13 min (an approximately ninefold increase) for the T172R mutant, and approximately 262 ± 46 min (an approximately 24-fold increase) for the T172R/G173Q mutant [22].

It should be pointed out that the *in vitro* half-life of a (purified) enzyme is determined by its thermostability, whereas the *in vivo* half-life of a protein drug is determined by many factors (see later). The observed good agreement between the *in vitro* and *in vivo* half-lives of CocE and its mutants indicates that the *in vivo* half-lives of these enzymes are also determined by the thermostability. However, it should be noted that the correlation between the thermostability and *in vivo* half-lives observed for CocE and its mutants does not necessarily exist in other protein drugs.

Design & discovery of high-activity mutants of human BChE

Design of a high-activity enzyme mutant is extremely challenging, particularly when the chemical reaction process becomes rate determining for the enzymatic reaction [24–26]. Generally speaking, for rational design of a mutant enzyme with an improved catalytic activity for a given substrate, one needs to design possible mutations that can accelerate the rate-

determining step of the entire catalytic reaction process [27–29], while the other steps are not slowed down by the mutations. The detailed catalytic reaction pathways for BChE-catalyzed hydrolysis of (–)-cocaine and (+)-cocaine were uncovered by extensive MD simulations [27, 29] and reaction coordinate calculations [27,28] using the quantum mechanics (QM) and hybrid QM/molecular mechanics (QM/MM). It has been shown that the formation of the prereactive BChE–(–)-cocaine complex (ES) is the rate-determining step of (–)-cocaine hydrolysis catalyzed by wild-type BChE [27,29,30], whereas the rate-determining step of the unnatural, biologically inactive (+)-cocaine hydrolysis catalyzed by the same enzyme is the chemical reaction process consisting of four individual reaction steps [27]. Based on this mechanistic understanding, previous efforts for rational design of BChE mutants were focused on how to improve the ES formation process, and several BChE mutants were found to have an approximately nine- to 34-fold improved catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against (–)-cocaine [29,31,32]. Subsequently reported computational models also suggest that the formation of the prereactive BChE–(–)-cocaine complex (ES) is hindered mainly by the bulky side chain of Y332 residue in wild-type BChE, but the hindering can be removed by a Y332A mutation and a Y332G mutation can produce a more significant improvement [29]. Combined computational and experimental data have revealed that the rate-determining step of (–)-cocaine hydrolysis catalyzed by the A328W/Y332A and A328W/Y332G mutants is the first step of the chemical reaction process [24,29,30,33]. Therefore, starting from the A328W/Y332A or A328W/Y332G mutant, further improving the catalytic efficiency of BChE against (–)-cocaine should aim to decrease the energy barrier for the first reaction step without significantly affecting ES formation and other chemical reaction steps [24–26,28]. It is necessary to simulate the detailed transition state structures associated with various candidate mutants for further computational design of new BChE mutants with a significantly improved catalytic activity against (–)-cocaine.

In theory, the transition state structures can be simulated by performing QM or QM/MM reaction coordinate calculations, or MD simulations using a QM- or QM/MM-based force field. Unfortunately, it would be too computationally demanding to carry out such types of calculations/simulations on a large number of mutants of the enzyme for meaningful virtual screening. Hence, it would be very interesting to simulate the transition state structures by performing MD simulations using a classical force field (molecular mechanics).

However, in principle, MD simulation using a classical force field can only simulate a stable structure corresponding to a local minimum on the potential energy surface, whereas a transition state during a reaction process is always associated with a first-order saddle point on the potential energy surface. Hence, MD simulation using a classical force field cannot directly simulate a transition state without any restraint on the geometry of the transition state. Recently, a convenient computational strategy has been developed to simulate and virtually screen the transition state structures by carrying out MD simulations using a classical force field [33]. The computational strategy is based on the theoretical consideration that if one can technically remove the freedom of imaginary vibration in the transition state structure, then the number of degrees of vibrational freedom (normal vibration modes) for a nonlinear molecule will decrease from $3N$ to 6. The transition state structure is associated with a local minimum on the potential energy surface within a subspace of the reduced degrees of vibrational freedom, although it is associated with a first-order saddle point on the potential energy surface with all of the $3N$ to 6 degrees of vibrational freedom. Theoretically, the degree of vibrational freedom associated with the imaginary vibrational frequency in the transition state structure can be removed by appropriately freezing the reaction coordinate. The reaction coordinate corresponding to the imaginary vibration of the transition state is generally characterized by a combination of some key geometric parameters. These key geometric parameters are bond lengths of the transition bonds, that is, the forming and breaking covalent bonds during this reaction step of BChE-catalyzed hydrolysis of cocaine. Thus, the lengths of the transition bonds need to be maintained

during the MD simulation of a transition state. Technically, one can maintain the lengths of the transition bonds simply by fixing all atoms within the reaction center, by using some constraints on the transition bonds or redefining the transition bonds. Based on the computational strategy of **transition state simulations**, novel computational design strategies/approaches have been developed and used to design high-activity mutants based on detailed structural and mechanistic understanding and virtual screening of the transition states of an enzymatic reaction [33–37].

The initial virtual screening based on the transition state simulations was focused on whether the hydrogen bonding between the carbonyl oxygen of (–)-cocaine benzoyl ester and the oxyanion hole (residues #116, #117 and #199) of the enzyme in the rate-determining transition state (TS1; i.e., the transition state for the first step of the chemical reaction process; Figure 3) can be enhanced by mutation [24,33]. The enhanced hydrogen bonding may potentially stabilize the TS1 structure, thus possibly lowering the energy barrier for the first reaction step of (–)-cocaine hydrolysis. Hydrogen bonding energy-based virtual screening led to the discovery of some high-activity mutants of BChE; the most active being A199S/S287G/A328W/Y332G BChE [33]. This high-activity mutant designed by Zhan and associates [33] has been confirmed and recognized by independent researchers (Brimijoin *et al.*) as “a true CocH with a catalytic efficiency that is 1000-fold greater than wild-type BChE” [38]. The A199S/S287G/A328W/Y332G mutant [33] can selectively block cocaine toxicity and reinstatement of drug seeking in rats [38].

Most recently, a more generalized and systematic computational design approach has been proposed and tested for virtual screening of the transition states for the rate-determining step of BChE-catalyzed hydrolysis of (–)-cocaine [35]. This latest virtual screening approach is based on the interaction energy calculation associated with the MD simulation and energy barrier prediction using QM/MM calculations, as depicted in Figure 4. The structure- and mechanism-based computational design was followed by wet experimental tests. A reliable, systematic virtual screening approach is valuable because the possible effects of mutations of different amino acid residues on the catalytic efficiency of the enzyme are not necessarily additive. Only an appropriate combination of various point mutations would make an enzyme more efficient. This novel virtual screening approach has led to a systematic search for the best possible combination of mutations on human BChE. The designed and discovered most efficient CocH, the A199S/F227A/S287G/A328W/Y332G mutant ($k_{\text{cat}} = 5700 \text{ min}^{-1}$; $K_{\text{M}} = 3.1 \text{ }\mu\text{M}$), has an approximately 2000-fold improved catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against (–)-cocaine compared with wild-type BChE [35]. The catalytic efficiency of the A199S/F227A/S287G/A328W/Y332G mutant of human BChE against (–)-cocaine is the highest of the currently known enzymes. The additional F227A mutation further increases the catalytic efficiency of the aforementioned A199S/S287G/A328W/Y332G mutant. Another quadruple BChE mutant (F227A/S287G/A328W/Y332M), reported by Pancook *et al.* in an abstract [39], also included the F227A mutation, but the F227A/S287G/A328W/Y332M mutant only has an approximately 34-fold improved catalytic efficiency ($k_{\text{cat}}/K_{\text{M}}$) against (–)-cocaine compared with the wild-type [31]. The high activity of the A199S/F227A/S287G/A328W/Y332G BChE mutant has been confirmed by *in vivo* tests on mice in Woods’ lab [35]. Pretreatment with the A199S/F227A/S287G/A328W/Y332G mutant (1 min prior to cocaine administration) dose-dependently protected mice against cocaine-induced convulsions and lethality [35]. In particular, the A199S/F227A/S287G/A328W/Y332G mutant 0.01 mg (per mouse) was able to fully protect mice from cocaine overdose induced by a lethal dose of cocaine of 180 mg/kg ($p < 0.05$) [35]. Clearly, these high-activity mutants of human BChE are promising for the therapeutic treatment of cocaine overdose and addiction.

Future perspective

Protein-based therapeutics have recently attracted considerable attention in the pharmaceutical industry. The relatively lower toxic (or nontoxic) and metabolic potential of protein-based therapeutics are their main attraction. Rational protein design through computational modeling can accelerate the protein drug-discovery process. In fact, computational protein design is the focus of many scientists, such as Ranganathan [40,41], Mayo [42,43], Hellinga [44–47], and others cited. One of the most challenging problems complicating the practical use of proteins as drugs is the issue of thermostability. The aforementioned success in generating more thermostable forms of a large enzyme suggests that the newly developed structure- and mechanism-based computational design approach is promising for the future rational design of thermostable enzymes and other proteins [22]. This computational approach will probably represent a valuable strategy for thermostabilization of other proteins and have dramatic implications on their therapeutic potential, as the similar strategy and approach can be used to design thermostable mutants of other proteins. In particular, to design thermostable mutants of a protein, one can first perform a MD simulation on the protein at an appropriately chosen high temperature to understand the protein inactivation pathway. Based on a detailed understanding of the protein structure and inactivation mechanism, one can carry out virtual screening of various possible mutants through the interaction energy calculations in order to predict the most likely thermostable mutants for wet experimental tests *in vitro* and *in vivo*. Hence, it is reasonable to expect that we will see more similar successes in rational design and discovery of thermostable mutants of many other proteins that have therapeutic potentials. Concerning future development of the computational design approach, one can expect to see further advances in more theoretical methods/scoring functions for the interaction energy calculations that can be used to more accurately predict the thermostable mutants of any protein with a known inactivation mechanism.

It should be pointed out that the *in vivo* half-life of a protein drug is determined by many factors. The above discussion has only considered the thermostability of the monomer. Some other factors, such as oligomerization, glycosylation and pegylation, could also affect the *in vivo* half-life of a protein drug. For example, it is known that tetramerization of BChE can considerably increase the *in vivo* half-life of BChE. The dimer has a significantly longer *in vivo* half-life than the monomer, and the tetramer has a significantly longer *in vivo* half-life than the dimer [48]. In addition, the *in vivo* half-life of an enzyme may also be extended through glycosylation, pegylation or fusion to another protein (e.g., human serum albumin) [38,49]. As the mechanisms of these protein stabilization processes can be explored using state-of-the-art computational techniques, one may also expect to see rational design of protein oligomerization, glycosylation or pegylation, and fusion to another protein in the future.

The structure- and mechanism-based design of the high-activity mutants of BChE against (–)-cocaine (i.e., CocHs) has been focused on the catalytic activity against cocaine. The designed mutants are not expected to improve the thermostability of BChE. Nevertheless, one can expect to see rational design and discovery of thermostable mutants of CocHs using a similar computational design strategy that has been used to design and discover thermostable mutants of CocE.

Concerning future anticocaine medication development itself, in theory, many medication strategies are possible, including classical receptor/transporter antagonists, monoclonal antibodies, vaccines, catalytic antibodies and metabolizing enzymes. A traditional approach to the development of a drug-abuse treatment has been to explore the classical receptor/transporter antagonists, such as DAT antagonists, that can be used to treat both overdose and addiction. Unfortunately, it has been and will continue to be very difficult to design a DAT antagonist that can potentially block DAT–cocaine binding without blocking DAT–dopamine

binding, because the binding sites for cocaine and dopamine in DAT overlap [7]. The use of a monoclonal antibody or vaccine could be valuable and practical in the treatment of cocaine addiction, although it is not expected to be efficient for the treatment of acute cocaine toxicity in the case of cocaine overdose. A major advantage of using a cocaine vaccine is that active immunization with a vaccine could lead to the cocaine antibody being in the body for a longer time period, which is important for the treatment of addiction. As a disadvantage, the active immunization would require an immune response to be effective and, thus, cannot be used to treat acute cocaine toxicity, which requires an immediate effect. An ideal approach to the treatment of acute cocaine toxicity, as in the case of cocaine overdose, is associated with the use of an efficient metabolizing enzyme (or catalytic antibody) that can catalyze the hydrolysis of (–)-cocaine at the benzoyl ester group. At the present time, only CocHs (i.e., high-activity mutants of human BChE) and CocE have demonstrated sufficiently high catalytic efficiency against (–)-cocaine. An additional advantage of using a cocaine-metabolizing enzyme is that one may expect to have a relatively lower chance of failure in the late development process of an enzyme therapy. It is well known that many small-molecule receptor antagonists are found to be highly effective in animal models of drug addiction but are not useful in humans because of species differences in their pharmacological actions and pharmacokinetics, or severe side effects. The species differences lead to structural differences in various receptors/transporters/proteins, thus, a small-molecule antagonist may interact with a receptor/transporter/protein in animals (or humans) but not in humans (or animals). Appropriate use of a the same exogenous cocaine-metabolizing enzyme for both animals and humans would not cause any structural differences in the drug–enzyme interactions between animals and humans. Thus, using an exogenous cocaine-metabolizing enzyme, one can reasonably expect to observe a similar cocaine metabolism in humans to that seen in animals.

A possible disadvantage of using an exogenous drug-metabolizing enzyme is that, in general, exogenous proteins, particularly bacterial enzymes, could induce strong immune responses. The potential immune responses will not only reduce the action of the enzyme, but could also produce pathological allergic responses, thus limiting the enzyme's potential use in humans. Fortunately, human BChE can be tolerated perfectly in humans, as discussed earlier. *In vivo* studies on CocE by Woods *et al.* also demonstrated that after a single prior CocE exposure (0.1–1 mg), CocE retained its potency against cocaine toxicity in mice, and these mice did not show an immune response [14]. After three prior CocE exposures (0.1–1 mg/week for 3 weeks), CocE still retained similar effectiveness in mice, although the mice displayed tenfold higher antibody titers. Thus, CocE may only be used to treat acute cocaine toxicity a few times [14]. Repeated use of CocE may increase its immunogenicity and partially reduce its protective ability [14]. In order to use CocE repeatedly, it is necessary to design further protein engineering techniques, with the aim of reducing the immunogenicity. Potentially valuable protein engineering approaches for this purpose include site-directed mutagenesis, pegylation and fusion to another protein (e.g., human serum albumin). Reducing the immunogenicity using these approaches will be another interesting study for further improving CocE-based medication.

Furthermore, the mechanisms of drug addiction or dependence are very complicated [50]. They are associated with acute cocaine-produced rewarding and psychostimulant effects, withdrawal syndromes, and craving and relapse to drug seeking during cocaine abstinence. The exogenous cocaine-metabolizing enzymes are designed to simply clean up cocaine in the body. However, the enzymes are not expected to directly repair any damage that has already been made by cocaine prior to the use of the exogenous enzymes, as with cocaine antibodies/vaccines or other cocaine antagonists. Nevertheless, this does not necessarily mean that an exogenous cocaine-metabolizing enzyme is not helpful for treatment of cocaine addiction. Studies by Brimijoin and associates demonstrated that a CocH designed by Zhan *et al.* [33] can block reinstatement of cocaine seeking in rats [38]. To effectively use a cocaine-metabolizing enzyme in the

treatment of cocaine addiction, one may try to further extend the circulatory half-life of the enzyme. One might also expect to see combined use of an efficient cocaine-metabolizing enzyme and a cocaine vaccine (or another addiction treatment strategy) for a possible simultaneous therapeutic treatment of both acute cocaine toxicity and cocaine addiction.

The general computational strategy and design protocols that have been used for the structure- and mechanism-based design of high-activity mutants of BChE, in combination with appropriate wet experiments *in vitro* and *in vivo*, may be used to rationally design and discover high-activity mutants of any other interesting enzyme or catalytic antibody [33–37]. In particular, the future development of catalytic antibodies will not necessarily be limited to the immunization with various stable analogues of the transition state structure for the nonenzymatic reaction of the corresponding small-molecule compound. It would also be interesting to design and discover high-activity mutants of the currently known catalytic antibodies, using a similar computational experimental approach to that used for design and discovery of high-activity mutants of BChE. It is also desirable to develop high-activity mutants of other metabolic enzymes for future therapeutic treatments of other drugs of abuse or metabolic diseases. A metabolic disease is a disorder caused by the accumulation of chemicals produced naturally in the body [51,52]. The metabolic diseases are usually serious, some are even life threatening. Using exogenous enzymes to metabolize the chemicals is clearly an ideal therapeutic strategy, particularly where the metabolic disease is caused by a genetic defect or the absence of certain metabolic enzymes. For each of the metabolic enzymes, design of the high-activity mutants against a specific compound (substrate) will first need the fundamental catalytic mechanism to be uncovered and structure- and mechanism-based design of mutations that can potentially stabilize the rate-determining transition state and lower the free energy barrier to be performed.

In addition to engineering existing enzymes with clear therapeutic potentials, rational design and discovery of a completely new therapeutic enzyme is also possible in the near future. Computational design of a completely new enzyme capable of catalyzing any desirable chemical reaction is a challenge, but it is certainly do-able, as proved recently by Baker and associates [53,54], who have successfully designed and discovered new enzymes for Retro–Aldol reaction and Kemp elimination. The new enzymes designed *de novo* have desirable catalytic activities, although they might not be sufficiently high for practical use. However, the aforementioned structure- and mechanism-based computational design approaches can be used to design high-activity mutants of the new enzymes [33–37]. Hence, one can also expect that completely new therapeutic enzymes will be designed and discovered in the near future through appropriately combined use of the *de novo* enzyme design approach and the aforementioned high-activity mutant enzyme design approaches based on the structures and mechanisms of the enzymes.

Executive summary

- In the design and discovery of novel protein drugs, it is important to engineer an existing nontoxic protein such that the engineered protein has the desirable biological functions and thermostability.
- State-of-the-art computational design of high-activity or thermostable mutants of enzymes using these new strategies/approaches has been based not only on the structure of the enzyme, but also on detailed catalytic/inactivation mechanisms.
- The structure- and mechanism-based computational design efforts have led to discovery of high-activity mutants of butyrylcholinesterase and thermostable mutants of cocaine esterase without decreasing the catalytic activity for anticocaine medication.

- The structure- and mechanism-based computational strategies and design approaches may be used to design high-activity and/or thermostable mutants of many other proteins that have therapeutic potential.
- It is reasonable to expect that we will see further similar successes in the rational design and discovery of thermostable mutants of many other proteins that have therapeutic potential.
- Future development of catalytic antibodies will not necessarily be limited to immunization with various stable analogues of the transition state structure for the nonenzymatic reaction of the corresponding small-molecule compound.
- The development of high-activity mutants of other metabolic enzymes for future treatments of other drugs of abuse or metabolic diseases is also desirable.
- One can also expect that completely new therapeutic enzymes will be designed and discovered in the near future through combined use of the *de novo* enzyme and high-activity mutant enzyme design approaches.

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Glossary

Enzyme Therapy	The use of exogenous enzymes to facilitate the digestive process and improve the body's ability to maintain balanced metabolism or clean-up toxic compounds in the body
Protein Engineering	Procedures by which protein structure and function are changed or created by altering existing or synthesizing new structural genes that direct the synthesis of proteins with certain sought-after properties
Transition state simulation	Modeling and simulating the detailed molecular structures of transition states for enzymatic reaction processes

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- ■ of considerable interest

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Figure 1. Butyrylcholinesterase-catalyzed hydrolysis reactions of (-)-cocaine and (+)-cocaine.

Figure 2. Cocaine esterase-(-)-cocaine binding structure modeled by molecular docking and molecular dynamics simulation at 298 K. Data from [17].

Figure 3.
First reaction step for (-)-cocaine hydrolysis catalyzed by a butyrylcholinesterase mutant including A199S mutation.

Figure 4. Stages in the structure- and mechanism-based computational design and discovery of high-activity mutants of human BChE

The approach depicted here can be used for any other enzyme, as TIE can be the total interaction energy between all (or some key) atoms of the substrate and all (or some key) atoms of the enzyme. TIE refers to the total interaction energy between the whole oxyanion hole of the enzyme and all (–)-cocaine atoms in the TS1 structure for each possible mutant. $\Delta E^\ddagger$ represents the energy barrier for the first step of the enzymatic hydrolysis of (–)-cocaine.

ΔE : Energy barrier; BChE: Butyrylcholinesterase; TIE: Total interaction energy.