A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition GDCh Chemie

www.angewandte.org

Accepted Article

Title: Computational Design and Crystal Structure of a Highly Efficient Benzoylecgonine Hydrolase

Authors: Xiabin Chen, Xingyu Deng, Yun Zhang, Yanan Wu, Kang Yang, Qiang Li, Jiye Wang, Weixuan Yao, Junseng Tong, Tian Xie, Shurong Hou, and Jianzhuang Yao

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.202108559

Link to VoR: https://doi.org/10.1002/anie.202108559

WILEY-VCH

10.1002/anie.202108559

WILEY-VCH

RESEARCH ARTICLE

Computational Design and Crystal Structure of a Highly Efficient Benzoylecgonine Hydrolase

Xiabin Chen*[a]#, Xingyu Deng[a]#, Yun Zhang[a]#, Yanan Wu[a], Kang Yang[b], Qiang Li[b], Jiye Wang[c], Weixuan Yao[c], Junseng Tong[a], Tian Xie*[a], Shurong Hou*[a], and Jianzhuang Yao*[b]

[a] Prof. Dr. X. Chen, X. Deng, Y. Zhang, Y. Wu, Dr. J. Tong, Prof. Dr. T. Xie, and Dr. S. Hou
College of Pharmacy, School of Medicine, Hangzhou Normal University, Hangzhou, Zhejiang, 311121, China.
E-mail: xch226@hznu.edu.cn (X. Chen); xbs@hznu.edu.cn (T. Xie); houshurong@hznu.edu.cn (S. Hou)
[b] K. Yang, Q. Li, and Dr. J. Yao
School of Biological Science and Technology, University of Jinan, Jinan 250022, China
E-mail: bio_yaojz@ujn.edu.cn (J. Yao)
[c] J. Wang and W. Yao
Key Laboratory of Drug Prevention and Control Technology of Zhejiang Province, Zhejiang Police College, Hangzhou, Zhejiang 310053, China.

# These authors contributed equally to this work.

Supporting information for this article is given via a link at the end of the document.

Abstract: Benzoylecgonine (BZE) is the major toxic metabolite of cocaine, and is responsible for the long-term cocaine-induced toxicity due to its long residence time in humans. BZE is also the main contaminant following cocaine consumption, representing a risk to our environment and non-target organisms. Here, we identified the bacterial cocaine esterase (CocE) as a BZE-metabolizing enzyme (BZEase), which can degrade BZE into biological inactive metabolites (ecgonine and benzoic acid). CocE was redesigned by a reactant-state-based enzyme design theory. An encouraging mutant denoted as BZEase2, presented a >400-fold improved catalytic efficiency against BZE compared with wild-type (WT) CocE. *In vivo*, a single dose of BZEase2 (1 mg/kg, IV) could eliminate nearly all BZE within only two minutes, suggesting the enzyme have the potential for cocaine overdose treatment and BZE elimination in the environment by accelerating BZE clearance. The crystal structure of a designed BZEase was determined, providing additional insights in support of our simulation results.

Introduction

Cocaine is one of the most reinforcing and toxic drugs, accounting for the majority of illicit drug-related emergence department visits without an approved medication specifically for cocaine overdose.[1] Cocaine elicits its toxic effects primarily by binding to noradrenergic transporters and blocking several voltage-gated ion channels including sodium and potassium channels,[2] leading to increased heart rate, elevated blood pressure, and vasoconstriction seen in cocaine users.[3] A therapeutic approach using an efficient cocaine-metabolizing enzyme has been recognized as the most promising strategy for cocaine abuse treatment. Administration of an efficient cocaine-metabolizing enzyme can dramatically accelerate the elimination of cocaine in both the brain and blood, and therefore significantly reduce cocaine-induced physiological and toxic effects.[5] A potential concern is that in human approximately 50% of cocaine is metabolized into benzoylecgonine (BZE), which has been recognized as cocaine's major toxic metabolite.[6] Studies reveal that BZE is a more potent vasoconstrictor than cocaine itself,[7] and the incidence of seizure is highly related to the presence of BZE.[8] Moreover, BZE has a significantly longer residence time in the body and is commonly used as a marker to screen individuals with a history of cocaine use. Therefore, BZE is mainly responsible for the long-term toxicity of cocaine. In addition, as the main substance excreted from urine following cocaine metabolism, BZE is one of the common addictive drug contaminants in the environment and is often detected in urban water systems in western and central European cities.[1, 9] Studies reported that BZE exposure could cause DNA damage, enhanced cell mortality, long-term change of protein expression profile, thereby leading to decreased offspring cycle for some animals or developmental inhibition for certain plants.[10] The increasing consumption of cocaine worldwide has caused significant environmental problems, and represents a potential hazard to non-target organisms, especially on aquatic animals and plants.[9a]

Given the toxic effects of BZE on both humans and the environment, an ideal therapy for cocaine overdose should detoxify not only cocaine itself but also its main toxic metabolite BZE. Therefore, we aim to develop a highly active BZE-metabolizing enzyme (BZEase), which can efficiently degrade BZE to biologically inactive metabolites, and therefore eliminate the toxicity of BZE. Such a BZEase is valuable not only in developing new therapeutics for cocaine overdose treatment but also in developing eco-friendly methods for BZE elimination from the environment. Here we demonstrated for the first time that bacterial cocaine esterase (CocE) can hydrolyze the BZE into biologically inactive metabolites ecgonine and benzoic acid (Figure 1a), but its catalytic activity against BZE should be dramatically improved. Enzyme engineering to increase catalytic activity remains a challenge in the research fields of biochemistry and drug discovery. Computer-aided enzyme design (CAED) has proven to be an effective method for enzyme engineering, especially with the continuous improvement of supercomputer performance and the emergence of computational chemistry algorithms.[12] However, the prediction accuracy of CAED is not sufficient enough,[13] which limits its applications in enzyme engineering. Hybrid QM/MM molecular dynamics (MD) and free energy (FE) simulations are powerful and accurate tools for the computational study of enzymatic reaction mechanisms and

10.1002/anie.202108559

relative catalytic efficiency (k_{cat}/K_M).[14] However, the QM/MM-based relevant activation FE barriers calculation has not been extensively applied in enzyme design (ED),[15] because it is very computationally expensive.[12b]

To make QM/MM MD and FE simulations suitable for ED, in the present study, we combined the traditional enzymatic catalysis theories[16] and QM/MM tools to develop a computing-time-affordable reactant state (**R**S) based **ED** methodology (RED, see supporting information). Near attack conformers (NACs) in the nucleophilic attack and total hydrogen bonding energy (tHBE) in the oxyanion hole were calculated for evaluation of relative activation FE barriers of computer-engineered mutants. Thus, the computing-time-consuming reaction pathway (including transition state, TS) simulation[13c] can be avoided for the enzyme design. In addition, a CHARMM rotamer force field (named CHARMMr) containing all sets of internal coordinates for residues' sidechains was created for mutagenesis modeling and computational ED by CHARMM. This quickly identified the energy-favorable sidechain conformations of the mutant residues, minimizing MD simulation times. Starting from T172R/G173Q/L196C/I301C mutant of CocE (denoted as CocE-4M for convenience), a thermostable mutant without a significant change in its catalytic activity reported in our previous study,[17] the computationally designed mutant (*i.e*. the A51L/V116K/T172R/G173Q/L196C/I301C mutant of CocE, denoted as enzyme BZEase2 for convenience) has a 427-fold improved catalytic efficiency against BZE compared with wild-type (WT) CocE. A single dose (1 mg/kg, IV) of BZEase2 could degrade nearly all cocaine toxic metabolite BZE in rats within only two minutes, indicating it might be a very promising medicine for cocaine overdose treatment. A highly efficient BZEase is also valuable for BZE elimination in the environment.

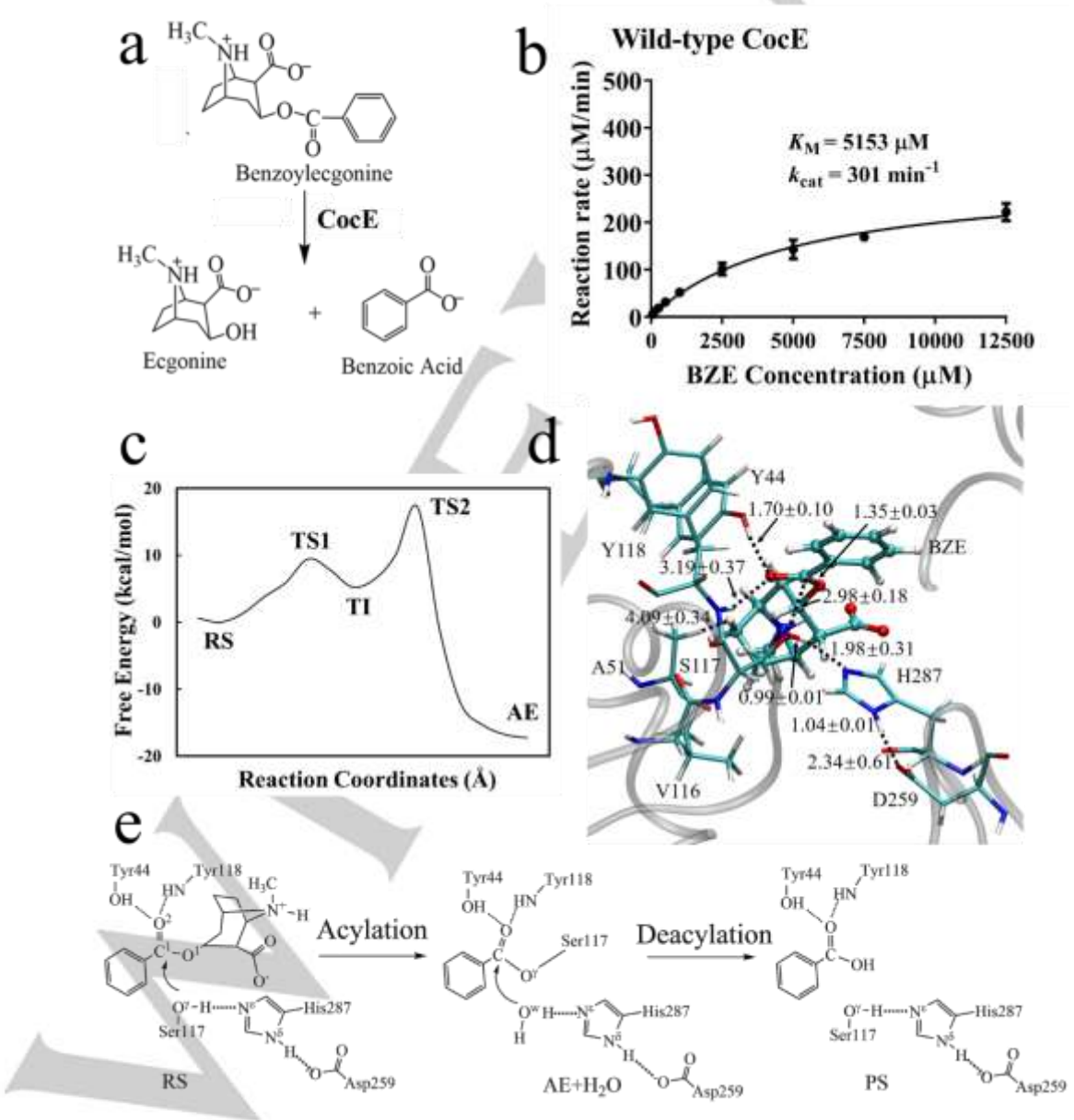


Figure 1. Identification of a potential BZEase. (a) Hydrolysis reaction of BZE catalyzed by CocE. (b) Kinetic data for WT CocE against BZE, with the reaction rate represented in μM min^{-1} per μM enzyme. (c) QM/MM free energy profile of the acylation process of the BZE hydrolysis catalyzed by the WT CocE. (d) Average structure of the CocE-BZE Michaelis-Menten complex, obtained by QM/MM MD simulations. (e) BZE hydrolysis mechanism catalyzed by the CocE.

Results and Discussion

Discovery of a novel BZE-metabolizing enzyme and reaction mechanism study. We first focus on identifying a suitable naturally evolved or engineered esterase that could efficiently hydrolyze the BZE analog (for example cocaine). Ideally, such a cocaine hydrolase should exhibit high safety and stability for humans, under hematic conditions. CocE was selected as a starting point for our QM/MM-based ED efforts because its safety and stability in humans have been proved by the clinical study.[5b] CocE contains a classical catalytic triad (Ser117, His287, and Asp259) and an oxyanion hole (Tyr44 and Tyr118).[18] We then built the initial coordinates of the CocE-BZE complex by using the X-Ray crystal structure of WT CocE (PDB ID: 1JU3)[19] and the energy-minimized cocaine structure obtained in our previous study.[20] As shown in Figure S1a, BZE fits the active site of CocE very well, and no steric hindrance is observed. The BZE phenyl ester carbonyl oxygen is located in the oxyanion hole and forms two hydrogen bonds with Tyr44 and Tyr118. The hydrogen bond network is observed in the catalytic triad and the distance between Ser117 nucleophile oxygen atom and BZE carbonyl carbon is 2.78 Å, suggesting CocE can catalyze the hydrolysis of BZE. CocE was then cloned, expressed, purified, and characterized for its activity against BZE. Interestingly, CocE indeed hydrolyzes the BZE into biological inactive metabolites ecgonine and benzoic acid (Figure 1a and Figure S3). According to the kinetic data (Figure 1b), WT CocE has a catalytic rate constant (*i.e.* turnover number, k_{cat}) of 301 min^{-1}, but a large Michalis-Menten constant value (K_M = 5153 μM) against BZE. Compared to its catalytic efficiency (k_{cat}/K_M) against cocaine ($k_{cat}/K_M = 1.97 \times 10^8\ M^{-1}\ min^{-1}$, Figure S4 & Table S3), WT CocE has a ~3000 fold lower catalytic efficiency against BZE, which is too low to be effective for BZE detoxification.

The reaction mechanism of the CocE-BZE complex was also revealed by QM/MM MD and FE (potential of mean force, PMF) simulations. We proposed that the reaction mechanism of the CocE-BZE complex follows the classical esterase catalytic mechanism, including two reaction processes, including acylation and deacylation. As shown in Figure 1e and Figure S2, the acylation process follows the classical mechanism of esterases.[20-21] It starts from the nucleophilic attack by Ser117 and ends with the ecgonine leaving the active site. One tetrahedral intermediate (TI) is sandwiched by TS1 and TS2. TI and TSs are stabilized by the electrostatic effect of hydrogen bondings in the oxyanion hole (Tyr44 and Tyr118). Particularly, the proton of Ser117 transfers to His287 during the nucleophilic attack. The positively charged His287 sidechain stabilizes the developing negative charge in oxygen atoms of the nucleophile and leaving group. The negatively charged Asp259 sidechain stabilizes the positively charged His287. The activation FE profile is plotted by QM/MM MD and FE simulations, and the corresponding FE barrier is about 17.2 kcal/mol (Figure 1d). Besides, the FE barrier of the deacylation process of the CocE-BZE complex is about 15.7 kcal/mol (corresponding to a k_{cat} value of ~1080 min^{-1}),[22] according to the conventional transition state theory (CTST). Taken together, our computational results predict that the activation FE barrier of CocE-catalyzed hydrolysis of BZE is about 17.2 kcal/mol, implicating that CocE can degrade BZE efficiently in terms of the turnover number. Indeed, the experimental-derived turnover number is 301 min^{-1} (Figure 1b). According to CTST, the activation FE barrier is 16.5 kcal/mol, which agrees well with our computational prediction. The agreement between the computer prediction and the subsequent experimental data suggests our computational obtained mechanism is reasonable.

However, the initial model (Figure S1a) shows that the carboxyl group of BZE is quite close to a hydrophobic residue Val116 (the closest distance between heavy atoms of BZE and Val116 is 4.66 Å). Indeed, the BZE carboxyl group moves away from Val116 in the equilibrated structure (Figure 1d) obtained by QM/MM MD simulation. No additional force is observed to stabilize the negatively charged carboxyl group of BZE during the equilibrated QM/MM MD simulation, which may contribute (at least partly) to the high K_M value. Thus, our strategy for developing a highly efficient BEZase focuses on improving the catalytic efficiency (k_{cat}/K_M, especially the binding affinity of CocE-BZE) by QM/MM MD and FE simulations.

Development of the RS-based ED method (RED). To make QM/MM MD and FE simulations suitable for the CocE redesign, RED was created by the integration of CHARMMr, NACs, and tHBE (Figure 2a). NACs proposed by Thomas Bruice are some conformations of the enzyme-substrate complex that belonged to RS[23]. NACs resemble TS and thus they can be used to estimate the relative TS stabilization of various computational-designed mutants, and it is a potential catalysis theory for the development of RS-based ED methods for enzymes such as esterases.

According to the classical esterase mechanism,[21a, 21c, 24] TS is not only characterized by the nucleophilic-attack-associated bond-forming-and-breaking processes, but also by the oxyanion hole stabilization of developing negative charge of carbonyl oxygen atom along with the nucleophilic attack. The FE of the oxyanion hole stabilization at TS can be approximately evaluated by the total hydrogen bonding energy (tHBE) formed in the oxyanion hole. Our previous computational-designed work[25] of the cocaine hydrolase has proved that calculations of tHBE at TS had been proved to be a useful tool for ED because it is an efficient tool for evaluation of relative activation FE barriers of computer-engineered mutants. The tHBE method was first applied to the enzyme design tasks by Prof. Chang-Guo Zhan at the University of Kentucky College of Pharmacy.[26] Since NACs resemble TS, we propose that the integration of NACs and tHBE concepts would produce a more efficient RS-based computational ED theory (RED) for the efficient estimation of relative activation FE barriers of various engineered mutants by QM/MM and MD simulations.

Although RED can help to efficiently evaluate TS stabilization of various mutants, obtaining average structures of various mutants by QM/MM MD simulations is time-consuming, because it needs at least several nanoseconds for the sidechain of mutated residues to get equilibrated. To largely shorten the simulation time of CHARMM for the mutagenesis modeling purpose, we created CHARMMr (as a part of RED, available in supporting information) by integrating the residues sidechains rotamer structural coordinates of the Penultimate Rotamer Library into the traditional CHARMM force field.[27]

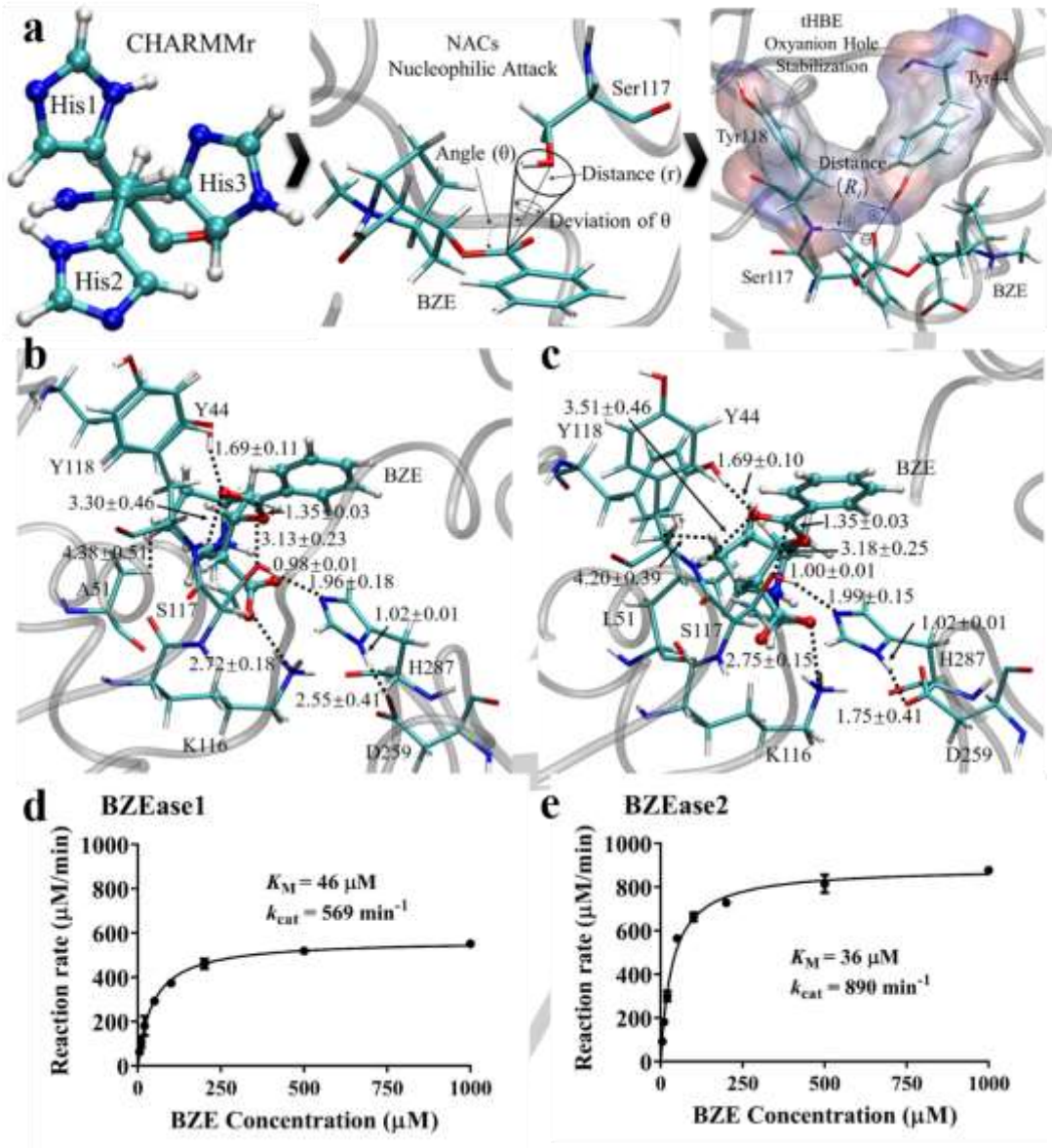


Figure 2. Computational design of highly efficient BZE hydrolases. (a) RS-based ED method (RED) development. Average RS structures of V116K-BZE (b), and A51L/V116K-BZE (c) complexes, obtained by QM/MM and MD simulations. Kinetic data for BZEase1 (d) and BZEase2 (e) against BZE. BZEase1 or BZEase2 is the thermostable mutant CocE-4M with extra V116K mutation or A51L/V116K mutations. The reaction rate is represented in μM min^{-1} per μM enzyme.

Computational design of the BZE-detoxifying hydrolase by RED-powered QM/MM MD and FE perturbation (FEP) simulations. Based on the analysis of the CocE-BZE initial model (Figure S1a) and the corresponding average structure (Figure 1d) obtained by QM/MM MD simulation, we modeled mutations of Val116 in the active site of CocE. Rationally, the best candidate residues to replace Val116 are positively charged residues such as Lysine and Arginine, because the positively charged sidechain may provide a strong electrostatic effect of the salt bridge to stabilize the negative charge of the BZE carboxyl group, thus improving the binding affinity of CocE-BZE. Arginine's sidechain is too large to fit the active site of CocE, so the Val116 is mutated to a Lysine residue. According to the initial coordinates of V116K modeled by CHARMM36mr force field (Figure S1c), the distance between the V116K sidechain nitrogen atom (N^Z) and BZE carboxyl oxygen atom (O^C) is only 2.27 Å, displaying a strong salt bridge between Lys116 and BZE, while the traditional CHARMM36m-constructed model presents no salt bridge formed between V116K and BZE (Figure S1b). The equilibrated RS average structure of the V116K-BZE complex supports the existence of the CHARMM36mr modeled salt bridge and the corresponding average distance is 2.72 Å (Figure 2b). The further experimental data showed that a single mutation V116K extensively decreases the K_M value to 46 μM, ~100 fold lower than that of WT CocE or CocE-4M mutant (Figure 2d). To further improve the binding affinity between the V116K mutant of CocE and the hydrophobic moiety of BZE at RS, we mutate the

ecgonine-surrounding Ala51 to a Leucine residue to form a double mutant A51L/V116K (Figure 2c).

To quantitatively compare the substrate (BZE) binding affinity difference between A51L/V116K and WT CocE, the state-of-the-art QM/MM FEP (alchemical) simulations were performed to calculate the binding FE difference by the PERT module implemented in CHARMM.[14b] The obtained relative substrate-binding FE $\Delta G_{WT\rightarrow A51L/V116K}$ = -4.79±1.51 kcal/mol. The calculated result predicts that BZE significantly prefers binding to the active site of A51L/V116K compared to the WT active site. In other words, the K_M value would be significantly decreased from WT to A51L/V116K. Indeed, the experimental-derived K_M value of A51L/V116K is 36 μM (Figure 2e), which is significantly lower compared to WT CocE K_M value of 5,157 μM. The agreement between the computational prediction and the experimental results proves that our mutagenesis modeling approaches including CHARMMr are valid in computational ED practice and would be an attractive start point for future ED methodology development.

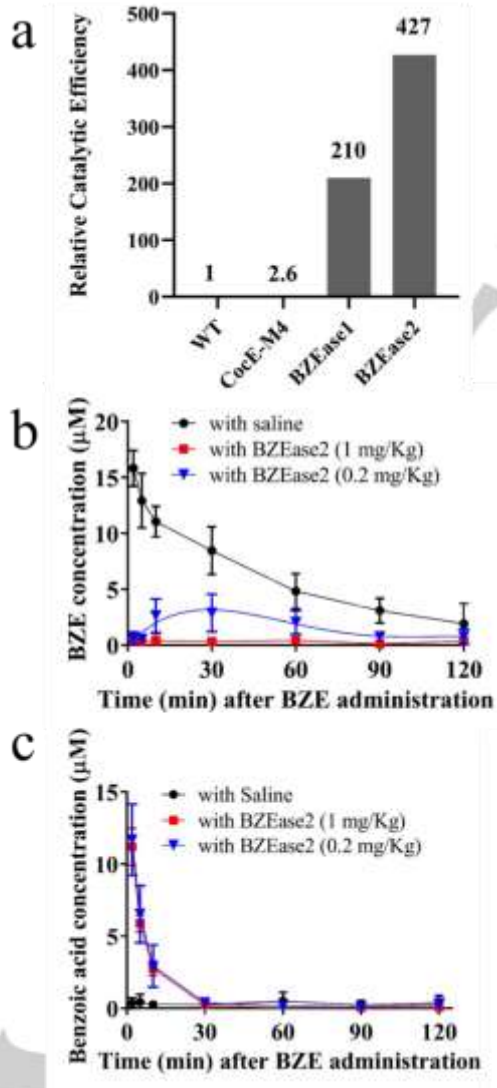


Figure 3. BZE clearance accelerated by the computational designed BZEase2. Saline or 0.2 mg/kg or 1 mg/kg BZEase2 was injected IV in rats (n = 5) followed by IV injection of 2 mg/kg BZE. (a) Highly efficient BZEases developed by CAED. (b) Time course of BZE concentration in rat blood. (c) Time course of benzoic acid (a metabolite of BZE) concentration in rat blood.

Along with the rational design of CocE mutants (such as V116K and A51L/V116K), we also computationally evaluated their relative activation FE barriers using our newly developed RED, to make sure that the overall catalytic efficiencies of the mutants are largely increased compared to the WT CocE. RED is applied for the evaluation of relative TS stabilizations between WT and mutants of CocE-BZE complexes. According to the definition of NACs, NACs for the acylation of CocE-BZE reaction is defined as that the nucleophilic attack distance r(O^{γ}…C^{1}) within 2.8-3.2Å and the angle approach of angles θ(O^{γ}…C^{1}…O^{1}) deviates by ±15° of the θ (98.0±4.6°) at TS1 (Figure S2a). As shown in Table S1, the RS's average distances and angles of WT CocE and two mutants are all within the NACs range, showing that all of the three RS are NACs. Since these NACs (RS) resemble TS1, the calculation of NACs oxyanion hole's tHBE can be applied to the evaluation of TS1 stabilization of various mutants. Enhancement of tHBE at TS1 had been successfully applied in cocaine hydrolase design by our previous work.[25] According to the tHBE equation implemented in AutoDock software,[28] tHBE is correlated with hydrogen bonding distances, and is proportional to a descriptor, $\sum_i (1/R_i)^9$ in which R_i represents the *i*th hydrogen bonding distance in the oxyanion hole of the CocE-BZE complex, including the CocE mutants. As shown in Table S2, tHBE values are close to each other among WT CocE, V116K, and A51L/V116K mutants, indicating mutations V116K and A51L would not significantly decrease the k_{cat} values, compared to the WT CocE.

The close tHBE values predict that the k_{cat} values of V116K and A51L/V116K mutants of CocE toward BZE are slightly better than WT CocE. Indeed, the experimental-derived k_{cat} values (Figure 1b and Figure 2) prove the computational prediction that two mutants only show 1-2 fold improved k_{cat} values compared to WT CocE. The consistency between the RED prediction and experiments shows that RED is a simple, fast, and reliable ED method for the estimation of relative activation FE barriers for QM/MM MD and FE simulations. Overall, the successful prediction of the BZE hydrolase design suggests that the RED-powered QM/MM MD and FE simulations would be a promising and general computational ED method for not only future ED tasks but also ED methodology development.

BZE clearance accelerated by BZEase2. To test the effectiveness of BZEase2 (Figure 3a) in accelerating BZE hydrolysis *in vivo*, rats were injected intravenously (IV) with saline or 0.2 mg/kg or 1 mg/kg BZEase2, followed by IV injection of 2 mg/kg BZE one minute after the enzyme or saline injection. For each rat, blood samples were collected at 2, 5, 10, 30, 60, 90, and 120 min after the BZE injection. The collected blood samples were analyzed using an HPLC method to determine the blood concentrations of BZE and benzoic acid. The corresponding calibration curves of BZE and benzoic acid detection are shown in Figure S5. According to the *in vivo* data (Figure S3), BZEase2 dose-dependently accelerated BZE hydrolysis to benzoic acid and ecgonine. In the control rats, the average concentration of BZE in blood was 15.8 μM 2 min after the IV injection of BZE, while the average blood concentration of benzoic acid was ~0.3 μM. The injection of 0.2 mg/kg BZEase2 effectively enhanced the BZE metabolism *in vivo*, with a substantially lower concentration of BZE was detected (< 3 μM

10.1002/anie.202108559

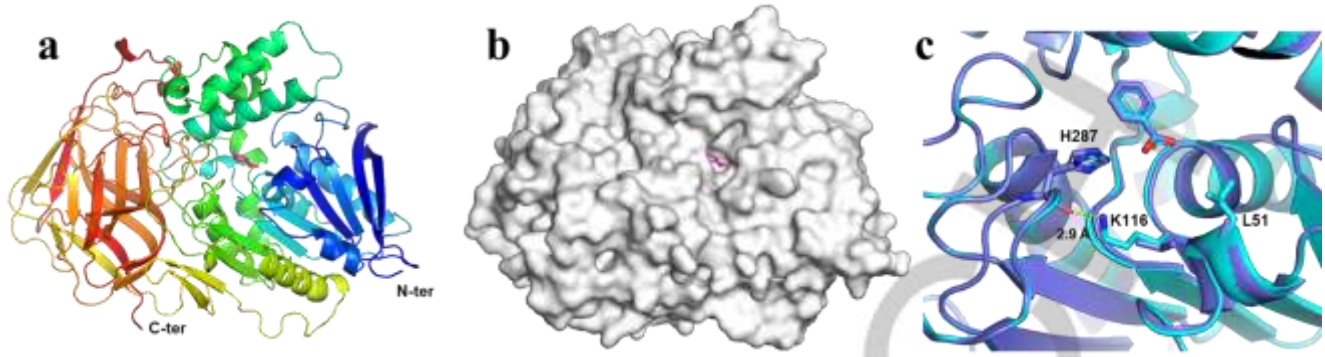


Figure 4. Crystal structures of an active CocE mutant. (a) Secondary structure elements of CocE mutant (T172R/G173Q/V116K/S117A/A51L) structure are shown as cartoon representations. (b) Surface representation of binding pocked with a BA product inside. (c) Structure comparison of CocE mutant (cyan) and WT (PDB ID: 1JU4, slate) at the substrate binding site (r.m.s.d.= 0.117 for 507 atoms). The new interaction between residue Lys116 and His287 has a distance of 2.9 Å.

vs. 15.8 μM in the control group). This enzyme-accelerated BZE metabolism (Figure 3b) is consistent with the increased production of BZE metabolite benzoic acid (Figure 3c), with the blood concentration at the first time point (2 min) of 11.2 μM. In the testing group with IV injection of 1 mg/kg BZEase2, nearly all BZE molecules were hydrolyzed into ecgonine and benzoic acid within 2 min after the injection of BZE. The average blood concentration of BZE at the first time point was ~0.3 μM which is lower than the limit of detection (LOD), while the concentration of benzoic acid is 11.7 μM (~0.3 μM in the control group).

Crystal structure of CocE mutant. For the design of the next generation of BZEases, we solved the structure of CocE mutant (T172R/G173Q/V116K/S117A/A51L) in complex with benzoic acid at 2.20 Å resolution (Table S4). The disulfide bond (L196C and I301C) of BZEase2 was not included for the sake of crystal homogeneity. The overall monomeric structure of CocE mutant was composed of three domains, domain 1 (residues 1-144 and residues 241-354), domain 2 (residues 145-240), and domain 3 (residues 355-574), whose structures are a three-layered α/β sandwich, α-helical domain and jelly-roll β-barrel, respectively (Figure 4a). Initial efforts to obtain the structures bound with BZE failed (see details in Supporting Information) because mutation of one key residue Ser117 in catalytic triad to Ala was not enough to abolish the catalytic activity thoroughly (data not shown). Thus, the globular structure of CocE mutant was obtained in complex with its product benzoic acid (Figure 4b). Regarding the new mutation introduced into the active center, Val116 to Lys generated a new hydrogen bond with the oxygen of His287, which is consistent with our simulation result (Figure 4c). This new interaction introduced by the V116K mutation may also stabilize the active site conformation, contributing to the higher catalytic activity of designed BZEases. Overall, the structure of this quintuple mutant (T172R/G173Q/V116K/S117A/A51L) structure has no significant change compared with WT CocE (r.m.s.d = 0.117).

Conclusion

This is the first identification of an exogenous BZE-metabolizing enzyme, demonstrating that the bacterial CocE could catalyze the hydrolysis of BZE at the benzoyl ester group. To further improve the catalytic efficiency of CocE against BZE, we have developed a novel, generally applicable computational-aided enzyme design method (RED) based on the CHARMM-rotamer-force-field, near-attack-conformers, and total-hydrogen-bond-energy. The current method has led to the discovery of a potential therapeutic CocE mutant with a 427-fold improved catalytic efficiency against BZE (Figure 3c), sufficient for the effective metabolism of BZE to completely eliminate its toxicity. The successful design of a highly efficient BZEase demonstrates the RED theory is a promising ED method for the catalysis involving the nucleophilic attack and the oxyanion-hole stabilization.

Acknowledgments

Financial support from the National Natural Science Foundation of China (82073749 and 81803417 to X. Chen) We thank staffs from the BL17B1 beamline of the National Facility for Protein Science in Shanghai (NFPS) at Shanghai Synchrotron Radiation Facility, for assistance during data collection.

Keywords: cocaine detoxification • benzoylecgonine • enzyme therapy • CHARMM rotamer force field • protein design

[1] UNODC, *World Drug Report 2020, New York*, United Nations Publications, **2020**.

[2] a) K. Heard, R. Palmer, N. R. Zahniser, *The Open Pharmacology Journal* **2008**, *2*, 70-78; b) M. E. O'Leary, J. C. Hancox, *British Journal of Clinical Pharmacology* **2010**, *69*, 427-442.

[3] a) N. L. Benowitz, *Pharmacology & Toxicology* **1993**, *72*, 3-12; b) C.-E. Johanson, M. W. Fischman, *Pharmacological Reviews* **1989**, *41*, 3-52; c) G. Uhl, F. Hall, I. Sora, *Molecular Psychiatry* **2001**, *7*, 21-26; d) W. J. Crumb Jr, P. J. Kadowitz, Y.-Q. Xu, C. W. Clarkson, *Canadian Journal of Physiology and Pharmacology* **1990**, *68*, 622-625; e) D. Narasimhan, J. H. Woods, R. K. Sunahara, *Future Medicinal Chemistry* **2012**, *4*, 137-150.

[4] C. W. Schindler, Z. Justinova, D. Lafleur, D. Woods, V. Roschke, H. Hallak, L. Sklair-Tavron, G. H. Redhi, S. Yasar, J. Bergman, S. R. Goldberg, *Addiction biology* **2013**, *18*, 30-39.

[5] a) X. Chen, L. Xue, S. Hou, Z. Jin, T. Zhang, F. Zheng, C.-G. Zhan, *Proc. Natl. Acad. Sci.* **2016**, *113*, 422-427; b) A. F. Nasser, P. J. Fudala, B. Zheng, Y. Liu, C. Heidbreder,

J. Addict. Dis. **2014**, *33*, 289-302; c) C. W. Schindler, Z. Justinova, D. Lafleur, D. Woods, V. Roschke, H. Hallak, L. Sklair-Tavron, G. H. Redhi, S. Yasar, J. Bergman, S. R. Goldberg, *Addiction biology* **2013**, *18*, 30-39.

[6] W. Xie, C. V. Altamirano, C. F. Bartels, R. J. Speirs, J. R. Cashman, O. Lockridge, *Mol. Pharmacol.* **1999**, *55*, 83-91.

[7] N. J. Connors, R. S. Hoffman, *Journal of Pharmacology and Experimental Therapeutics* **2013**, *347*, 251-257.

[8] R. J. Konkol, B. A. Erickson, J. K. Doerr, R. G. Hoffman, J. A. Madden, *Epilepsia* **1992**, *33*, 420-427.

[9] a) R. Pal, M. Mallavarapu, P. Kirkbride, R. Naidu, *The Science of the Total Environment* **2012**, *463*, 1079-1092; b) S. Castiglioni, R. Bagnati, M. Melis, D. Panawennage, P. Chiarelli, R. Fanelli, E. Zuccato, *Water Research* **2011**, *45*, 5141-5150.

[10] a) M. Parolini, A. Pedriali, C. Riva, A. Binelli, *Science of The Total Environment* **2013**, *444*, 43-50; b) M. Parolini, A. Ghilardi, C. Della Torre, S. Magni, L. Prosperi, M. Calvagno, L. Del Giacco, A. Binelli, *Environmental Pollution* **2017**, *226*, 504-514; c) A. Binelli, I. Marisa, M. Fedorova, R. Hoffmann, C. Riva, *Aquatic Toxicology* **2013**, *140-141*, 268-278; d) M. Parolini, B. De Felice, C. Ferrario, N. Salgueiro-González, S. Castiglioni, A. Finizio, P. Tremolada, *Environmental Pollution* **2018**, *232*, 236-244; e) D. Spasiano, D. Russo, M. Vaccaro, A. Siciliano, R. Marotta, M. Guida, N. M. Reis, G. Li Puma, R. Andreozzi, *Journal of Hazardous Materials* **2016**, *318*, 515-525.

[11] X. Chen, X. Zheng, M. Zhan, Z. Zhou, C.-G. Zhan, F. Zheng, *ACS Chem. Biol.* **2016**, *11*, 2186-2194.

[12] a) G. Kiss, N. Çelebi-Ölçüm, R. Moretti, D. Baker, K. N. Houk, *Angew. Chem. Int. Ed.* **2013**, *52*, 5700-5725; b) W. S. Mak, J. B. Siegel, *Curr. Opin. Struct. Biol.* **2014**, *27*, 87-94.

[13] a) F. Richter, A. Leaver-Fay, S. D. Khare, S. Bjelic, D. Baker, *PLoS One* **2011**, *6*, e19230; b) C. Wolf, J. B. Siegel, C. Tinberg, A. Camarca, C. Gianfrani, S. Paski, R. Guan, G. Montelione, D. Baker, I. S. Pultz, *J. Am. Chem. Soc.* **2015**, *137*, 13106-13113; c) X. Wang, R. Li, W. Cui, Q. Li, J. Yao, *Sci. Rep.* **2018**, *8*, 7042.

[14] a) Q. Cui, T. Pal, L. Xie, *J. Phys. Chem. B* **2021**, *125*, 689-702; b) X. Lu, D. Fang, S. Ito, Y. Okamoto, V. Ovchinnikov, Q. Cui, *Mol. Simul.* **2016**, *42*, 1056-1078.

[15] M. P. Frushicheva, M. J. L. Mills, P. Schopf, M. K. Singh, R. B. Prasad, A. Warshel, *Curr. Opin. Chem. Biol.* **2014**, *21*, 56-62.

[16] M. Garcia-Viloca, J. Gao, M. Karplus, D. G. Truhlar, *Science* **2004**, *303*, 186-195.

[17] L. Fang, K. M. Chow, S. Hou, L. Xue, X. Chen, D. W. Rodgers, F. Zheng, C.-G. Zhan, *ACS Chem. Biol.* **2014**, *9*, 1764-1772.

[18] J. Liu, A. Hamza, C.-G. Zhan, *J. Am. Chem. Soc.* **2009**, *131*, 11964-11975.

[19] N. A. Larsen, J. M. Turner, J. Stevens, S. J. Rosser, A. Basran, R. A. Lerner, N. C. Bruce, I. A. Wilson, *Nat. Struct. Biol.* **2002**, *9*, 17-21.

[20] J. Yao, X. Chen, F. Zheng, C.-G. Zhan, *Mol. Pharm.* **2018**, *15*, 3871-3880.

[21] a) J. Yao, H. Guo, M. Chaiprasongsuk, N. Zhao, F. Chen, X. Yang, H. Guo, *Biochemistry* **2015**, *54*, 5366-5375; b) J. Yao, H. Luo, X. Wang, *J. Chem. Inf. Model.* **2017**, *57*, 1179-1186; c) Y. Zhao, H. Dong, J. Ren, J. Song, J. Yao, J. Gao, C.-S. Jiang, X. Wang, *J. Phys. Chem. B* **2020**, *124*, 6699-6708.

[22] J. Liu, X. Zhao, W. Yang, C.-G. Zhan, *J. Phys. Chem. B* **2011**, *115*, 5017-5025.

[23] T. C. Bruice, *Chem. Rev.* **2006**, *106*, 3119-3139.

[24] a) J. Liu, C.-G. Zhan, *J. Chem. Theory Comput.* **2012**, *8*, 1426-1435; b) X. Wang, J. Yao, *Theor. Chem. Acc.* **2017**, *136*, 124.

[25] F. Zheng, L. Xue, S. Hou, J. Liu, M. Zhan, W. Yang, C.-G. Zhan, *Nature Communication* **2014**, *5*, 3457.

[26] F. Zheng, W. Yang, M.-C. Ko, J. Liu, H. Cho, D. Gao, M. Tong, H.-H. Tai, J. H. Woods, C.-G. Zhan, *J. Am. Chem. Soc.* **2008**, *130*, 12148-12155.

[27] S. C. Lovell, J. M. Word, J. S. Richardson, D. C. Richardson, *Proteins: Structure Function and Genetics* **2000**, *40*, 389-408.

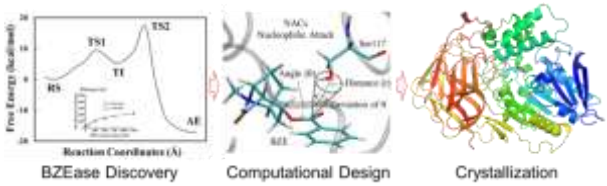
[28] G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew, A. J. Olson, *J. Comput. Chem.* **1998**, *19*, 1639-1662.

10.1002/anie.202108559

WILEY-VCH

RESEARCH ARTICLE

Entry for the Table of Contents



Benzoylecgonine (BZE) is the major toxic metabolite of cocaine and is responsible for cocaine-induced long-term toxicity due to its long residence time in humans. We computationally identified, re-designed, and crystalized a novel BZEase. The obtained BZEase mutant shows a 427-fold improved catalytic efficiency toward BZE compared to WT BZEase. *In vivo* tests prove that the BZEase mutant is a promising treatment for BZE detoxification.