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Computational Design of a Human Butyrylcholinesterase Mutant for Accelerating Cocaine Hydrolysis Based on the Transition-State Simulation**

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Cocaine is recognized as the most reinforcing of all drugs of abuse.[1–3] There is no available anticocaine medication. The disastrous medical and social consequences of cocaine addiction have made the development of an effective pharmacological treatment a high priority.[4–6] An ideal anticocaine medication would accelerate cocaine metabolism, thereby producing biologically inactive metabolites by a route similar to the primary cocaine-metabolizing pathway, that is, cocaine hydrolysis catalyzed by plasma enzyme butyrylcholinesterase (BChE).[5,7–11] However, the native BChE has a low catalytic efficiency against naturally occurring (−)-cocaine.[12–15] (−)-Cocaine has a plasma half-life of ≈45–90 min, long enough for manifestation of the central nervous system (CNS) effects, which peak in minutes.[13,16] Here we

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report an unconventional computational design that has led to the discovery of a human BChE mutant with a ≈151-fold improved catalytic efficiency; this mutant can be used as an exogenous enzyme in humans to prevent (−)-cocaine from reaching the CNS. The encouraging outcome not only provides a potential anticocaine medication but also demonstrates that a novel general approach of studying enzymatic mechanisms and computational drug design is promising.

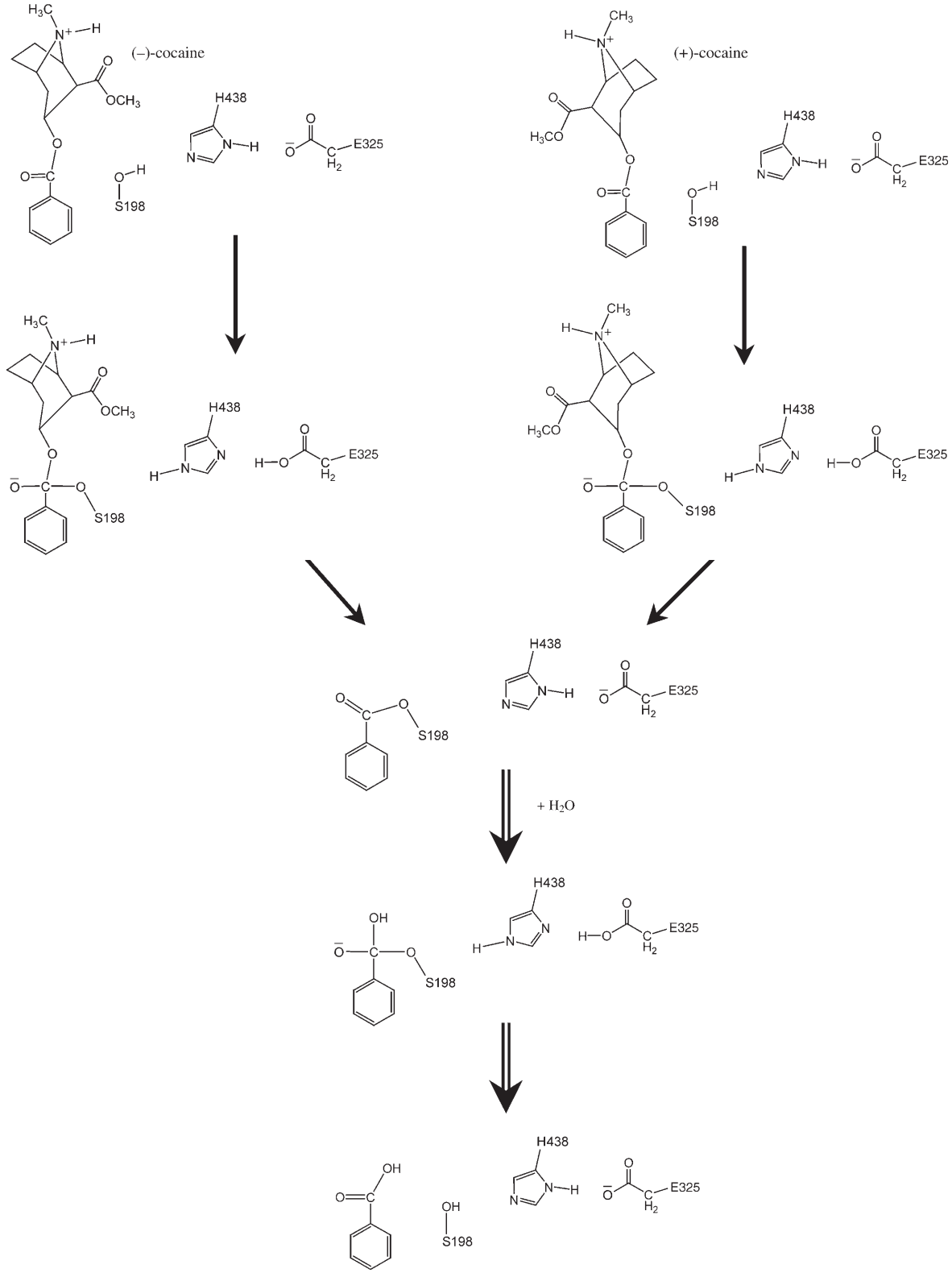
For rational design of a mutant enzyme with a higher catalytic activity for a given substrate, in general, one needs to design a mutation that can accelerate the rate-determining step of the entire catalytic reaction process while the other steps are not slowed down by the mutation. Reported computational modeling and experimental data indicated that the formation of the prereactive BChE–(−)-cocaine complex (ES) is the rate-determining step of (−)-cocaine hydrolysis catalyzed by wild-type BChE,[17–23] whereas the rate-determining step for the faster hydrolysis of the biologically inactive (+)-cocaine enantiomer is the chemical reaction process consisting of four individual reaction steps (Scheme 1).[18] This mechanistic understanding is consistent with the experimental observation[17] that the catalytic rate constant of wild-type BChE against (+)-cocaine is pH-dependent, whereas that of the same enzyme against (−)-cocaine is independent of the pH value. The pH-dependence of the rate constant for (+)-cocaine hydrolysis is clearly associated with the protonation of the H438 residue in the catalytic triad (S198, H438, and E325). For the first and third steps of the reaction process, when H438 is protonated, the catalytic triad cannot function and, therefore, the enzyme becomes inactive. The lower the pH value of the reaction solution, the higher the concentration of protonated H438 and the lower the concentration of the active enzyme. Hence, the rate constant was found to decrease with a decreasing pH value of the reaction solution for the enzymatic hydrolysis of (+)-cocaine.[17] Based on the above mechanistic understanding, the previously reported efforts for rational design of BChE mutants have focused on how to improve the ES formation process.[18,20,24]

Experimental observation[18] also indicated that the catalytic rate constant of A328W/Y332A BChE is pH-dependent for both (−)- and (+)-cocaine. The pH-dependence reveals that, for both (−)- and (+)-cocaine, the rate-determining step of the hydrolysis catalyzed by A328W/Y332A BChE should be either the first or the third step of the reaction process. Further, if the third step were rate determining, then the catalytic efficiency of the A328W/Y332A mutant against (−)-cocaine should be as high as that of the same mutant against (+)-cocaine because the (−)- and (+)-cocaine hydrolyses share the same third and fourth steps (see Scheme 1). However, it has been observed that the A328W/Y332A mutant only has a ≈9-fold improved catalytic efficiency against (−)-cocaine, whereas the A328W/Y332A mutation does not change the high catalytic activity against (+)-cocaine.[18] This analysis of the experimental and computational data available in the literature clearly shows that the rate-determining step of (−)-cocaine hydrolysis catalyzed by the A328W/Y332A mutant should be the first step of the chemical reaction process. Further, recently reported computational modeling also suggests that the formation of the prereactive BChE–(−)-cocaine complex (ES) is hindered mainly by the bulky side chain of the Y332 residue in wild-type BChE, but the hindering can be removed by the Y332A or Y332G mutation.[20] Therefore, by starting from the A328W/Y332A and A328W/Y332G mutants, our current study for improving the catalytic efficiency of BChE against (−)-cocaine aimed to decrease the energy barrier for the first reaction step without significantly affecting the ES formation and other chemical reaction steps. To achieve this aim, molecular dynamics (MD) simulations[25] were performed to simulate the structures of the first transition state (TS1) for (−)-cocaine hydrolysis catalyzed by wild-type BChE and its various mutants.

We hoped to predict some possible mutations that can lower the energy of the TS1 structure and, therefore, lower the energy barrier for the first reaction step. Apparently, a mutant associated with stronger hydrogen bonding between the carbonyl oxygen atom of (−)-cocaine benzoyl ester and the oxyanion hole of the BChE mutant in the TS1 structure may potentially have a more stable TS1 structure and, therefore, a higher catalytic activity against (−)-cocaine. Hence, hydrogen bonding with the oxyanion hole in the TS1 structure is a crucial factor affecting the transition-state stabilization and the catalytic activity. The possible effects of some mutations on the hydrogen bonding were examined by performing molecular modeling and MD simulations on the TS1 structures for (−)-cocaine hydrolysis catalyzed by wild-type BChE and its various mutants. The initial candidate mutants were chosen by simple geometric consideration of the possible modification of the TS1 structure; only an energy minimization was carried out in the simple geometric consideration of each possible mutant. The MD simulations were then performed only for the candidate mutants whose energy-minimized TS1 structures clearly suggested possibly stronger hydrogen bonding between the carbonyl oxygen atom of (−)-cocaine and the oxyanion hole of the enzyme.

The MD simulation in water was performed for 1 ns or longer to make sure that we obtained a stable MD trajectory for each simulated TS1 structure with the wild-type or mutant BChE. The MD trajectories actually became stable quickly and so did the H···O distances involved in the potential hydrogen bonds between the carbonyl oxygen atom of (−)-cocaine and the oxyanion hole of BChE. The H···O distances in the simulated TS1 structures for wild-type BChE and its four mutants are summarized in Table 1 (see the Supporting Information for the key MD trajectory and MD-simulated TS1 structures).

As seen in Table 1, in the simulated TS1 structures for the wild-type, A328W/Y332A, A328W/Y332G, and F227A/S287G/A328W/Y332M BChEs, the carbonyl oxygen atom of (−)-cocaine can form up to two N−H···O hydrogen bonds with the peptidic NH hydrogen atoms of G117 and A199. The overall strength of the hydrogen bonding between the carbonyl oxygen atom of (−)-cocaine and the oxyanion hole of the enzyme only slightly increases when wild-type BChE is replaced by the A328W/Y332A, A328W/Y332G, or F227A/S287G/A328W/Y332M mutants, as seen from the estimated total hydrogen-binding energy (HBE) values in Table 1. In



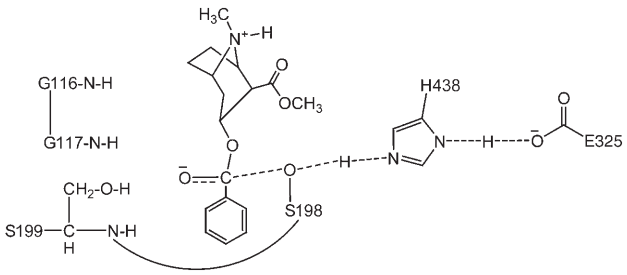
Scheme 1. Schematic representation of BChE-catalyzed hydrolysis of (−)- and (+)-cocaine.

the simulated TS1 structure for A199S/F227A/A328W/Y332G BChE, an O–H···O hydrogen bond is formed between the hydroxy group on the side chain of S199 and the carbonyl oxygen atom of (−)-cocaine, in addition to the two N–H···O hydrogen bonds with the peptidic NH of G117 and S199, as seen in Scheme 2 and Table 1. Due to the additional O–H···O hydrogen bond, the overall strength of the hydrogen bonding with the modified oxyanion hole of A199S/F227A/A328W/

Table 1: MD-simulated key distances (in Å) and the calculated total hydrogen-bonding energies (HBEs, in kcal mol^{-1}) between the oxyanion hole and the carbonyl oxygen atom of the (−)-cocaine benzoyl ester in the first transition state (TS1) with various BChEs.

Catalyst		Distance[a]				Total HBE value[b]
		D1	D2	D3	D4	
wild-type BChE	average	4.59	2.91	1.92		−5.5 (−4.6)
	maximum	5.73	4.14	2.35		
	minimum	3.35	1.97	1.61		
	fluctuation	0.35	0.35	0.12		
A328W/Y332A mutant	average	3.62	2.35	1.95		−6.2 (−4.9)
	maximum	4.35	3.37	3.02		
	minimum	2.92	1.78	1.61		
	fluctuation	0.23	0.27	0.17		
A328W/Y332G mutant	average	3.60	2.25	1.97		−6.4 (−5.0)
	maximum	4.24	3.17	2.76		
	minimum	2.89	1.77	1.62		
	fluctuation	0.23	0.24	0.17		
F227A/S287G/A328W/Y332M mutant	average	3.80	2.23	1.99		−6.1 (−4.8)
	maximum	4.61	3.08	2.88		
	minimum	3.16	1.75	1.67		
	fluctuation	0.25	0.23	0.18		
A199S/F227A/A328W/Y332G mutant	average	5.18	2.22	1.96	2.11	−9.8 (−7.4)
	maximum	5.94	3.08	2.44	3.30	
	minimum	4.28	1.68	1.65	1.56	
	fluctuation	0.23	0.21	0.13	0.28	

[a] D1, D2, and D3 represent the internuclear distances between the carbonyl oxygen atom of the cocaine benzoyl ester and the NH hydrogen atom of residues 116 (G116), 117 (G117), and 199 (A199 or S199) of BChE, respectively. D4 is the internuclear distance between the carbonyl oxygen atom of the cocaine benzoyl ester and the hydroxy hydrogen atom of the S199 side chain in the A199S/F227A/A328W/Y332G mutant. [b] Calculated by using the empirical HBE equation implemented in the AutoDock 3.0 program suite.[27] The total HBE value is the average of the HBE values calculated by using the instantaneous distances in all of the snapshots. The value in parenthesis is the total HBE value calculated by using the MD-simulated average distances.



Scheme 2. Schematic representation of the transition-state structure TS1 for the first reaction step of (−)-cocaine hydrolysis catalyzed by A199S/F227A/A328W/Y332G BChE.

Y332G BChE becomes significantly stronger than those of the wild-type, A328W/Y332A, A328W/Y332G, and F227A/S287G/A328W/Y332M BChEs, as seen from the estimated total HBE values in Table 1. These computational results suggest that the TS1 structure for (−)-cocaine hydrolysis catalyzed by A199S/F227A/A328W/Y332G BChE should be significantly more stable than that in the reaction catalyzed by the A328W/Y332A, A328W/Y332G, or F227A/S287G/A328W/Y332M mutants, due to the significant increase in the overall strength of hydrogen bonding between the carbonyl oxygen atom of (−)-cocaine and the oxyanion hole of the enzyme.

The aforementioned analysis of the literature[17,18,20] also indicates that the first chemical reaction step associated with TS1 should be the rate-determining step of (−)-cocaine hydrolysis catalyzed by a BChE mutant including a Y332A or Y332G mutation. Thus, the MD simulations predict that A199S/F227A/A328W/Y332G BChE should have a higher catalytic efficiency than the A328W/Y332A, A328W/Y332G, or F227A/S287G/A328W/Y332M BChEs against (−)-cocaine.

The catalytic efficiency (rate of catalysis/Michaelis constant, k_{cat}/K_M) of A328W/Y332A BChE against (−)-cocaine was reported to be $\approx 8.6\times10^6$ M min^{-1},[18] which is ≈9.4 times the k_{cat}/K_M value ($\approx 9.1\times10^5$ M min^{-1}) of wild-type BChE against (−)-cocaine. The catalytic efficiency of A328W/Y332G BChE was found to be slightly higher than that of A328W/Y332A BChE against (−)-cocaine.[20] To examine our theoretical prediction of the higher catalytic activity for A199S/F227A/A328W/Y332G BChE, we produced the A328W/Y332A and A199S/F227A/A328W/Y332G mutants of BChE through site-directed mutagenesis.[26] To minimize the possible systematic experimental errors of the kinetic data, we performed kinetic studies with the two mutants and wild-type BChE under the same conditions and compared the catalytic efficiency of the A328W/Y332A and A199S/F227A/A328W/Y332G BChEs with that of the wild-type for (−)-cocaine hydrolysis at the benzoyl ester group. Based on the kinetic analysis of the measured time-dependent radiometric data and the ELISA data, the ratio of the k_{cat}/K_M value of A328W/Y332A BChE to the k_{cat}/K_M value of wild-type BChE against (−)-cocaine was determined to be ≈8.6. The determined catalytic efficiency ratio of ≈8.6 is in good agreement with the ratio of ≈9.4 determined by Sun et al.[18] Further, by using the same experimental protocol, the ratio of the k_{cat}/K_M value of A199S/F227A/A328W/Y332G BChE to the k_{cat}/K_M value of A328W/Y332A BChE against (−)-cocaine was determined to be ≈16.8. These data indicate that A199S/F227A/A328W/Y332G BChE has a ≈(151 ± 14)-fold improved catalytic efficiency against (−)-cocaine compared to the wild-type; that is, A199S/F227A/A328W/Y332G BChE has a k_{cat}/K_M value of $\approx(1.37\pm0.13)\times10^8$ M min^{-1} against (−)-cocaine.

The catalytic efficiency of A199S/F227A/A328W/Y332G BChE against (−)-cocaine is significantly higher than that of AME-359 (that is, F227A/S287G/A328W/Y332M BChE, $k_{cat}/K_M = 3.1\times10^7$ M min^{-1}, whose catalytic efficiency against (−)-cocaine is the highest within all of the BChE mutants reported so far by other groups),[24] which has a ≈34-fold improved

catalytic efficiency against (−)-cocaine compared to wild-type BChE. By using our designed A199S/F227A/A328W/Y332G BChE as an exogenous enzyme in human, when the concentration of this mutant is kept the same as that of the wild-type BChE in plasma, the half-life time of (−)-cocaine in plasma should be reduced from the ≈45–90 min to only ≈18–36 s.

In summary, an enzyme mutant is designed in this study based on the transition-state simulation. The designed BChE mutant has a significantly improved catalytic efficiency against (−)-cocaine, thereby demonstrating that transition-state simulation is a promising approach for rational enzyme redesign and drug discovery.

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[25] See the Supporting Information concerning how classical MD can be employed in a way to simulate the transition state and how the MD simulations were performed.
[26] See the Supporting Information for the experimental materials and methods.
[27] a) 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; b) based on the general HBE equation, we have $HBE(r) \approx 5\varepsilon r_0^{12}/r^{12} - 6\varepsilon r_0^{10}/r^{10}$, in which r is the H···O distance in the considered hydrogen bond and r_0 is the minimum value of the H···O distance for which the HBE equation can be used. The ε value was determined by using the condition that $HBE(r) = -5.0$ kcal mol^{-1} when $r = 1.90$ Å.

