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A Model of Glycosylated Human Butyrylcholinesterase

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Abstract

Human butyrylcholinesterase (BChE) and its mutants have shown great potential in treating cocaine overdose and addiction. In order to effectively suppress cocaine reward in the brain for a long period of time after an exogenous cocaine hydrolase administration, the therapeutic enzyme should have not only a high catalytic efficiency against cocaine, but also a sufficiently long circulation time. It has been known that PEGylation (covalent attachment of a polyethylene glycol) modification of a therapeutic protein can prolong the biological half-life of the protein without affecting its biological function. However, the asparagine-linked glycans on the surface of glycosylated BChE may interfere with the PEGylation modification. In this study, we built a three-dimensional (3D) model of glycosylated human BChE to investigate the influence of glycans on the PEGylation modification. Glycans did not change the overall stability of the BChE structure, but could increase the flexibility of some local structures. For further evaluating the accessibility of the PEGylation reaction sites, particularly lysine residues, on the protein surface, we calculated the Solvent Accessible Surface Areas (SASAs) of these residues. The results indicate that some lysine residues have a significant decrease in SASA due to the direct or indirect influence of their surrounding glycans. The results also indicate that PEGylation reaction agents with smaller functional groups could have a better chance to react with lysine residues. This investigation provides a structural basis for rational engineering of human BChE and its mutants as therapeutic candidates.

Introduction

Cocaine overdose and addiction have resulted in serious medical and social problems in modern society.¹ There is still no anti-cocaine medication approved by the FDA, which has made the development of an effective pharmacological treatment a high priority.^{2–3} Cocaine is highly addictive and causes its reinforcing and toxic effects mainly by binding with the dopamine reuptake transporter, thus blocking the neurotransmitter reuptake. The classical pharmacodynamic approach has failed to yield a truly useful small-molecule receptor antagonist. The alternative pharmacokinetic approach is to interfere with the delivery of cocaine to its sites of action and/or accelerate its metabolism in the body.^{4–9} Human butyrylcholinesterase (BChE) is an enzyme existing in plasma that belongs to the choline esterase family.¹⁰ Studies have shown that BChE can hydrolyze cocaine into non-toxic metabolites,¹¹ and thus to help reduce the reinforcement of cocaine, indicating its great potential in anti-cocaine medication.¹²

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Electronic Supplementary Information Available. Three pdb files for (1) the initial BChE structure without glycans, (2) the initial BChE structure with glycans, and (3) the finally energy-minimized BChE structure for the final snapshot of the MD simulation. These materials are free via the Internet <http://pubs.rsc.org>.

Naturally occurring cocaine exists as (-)-cocaine which is biologically active. Unfortunately, the catalytic efficiency of wild-type BChE against (-)-cocaine is very low ($k_{\text{cat}} = 4.1 \text{ min}^{-1}$ and $K_{\text{M}} = 4.5 \text{ }\mu\text{M}$).¹³ In order to improve the catalytic efficiency of BChE against (-)-cocaine, we have successfully designed BChE mutants with a considerably improved catalytic efficiency against (-)-cocaine based on our understanding of catalytic mechanism of the enzymatic cocaine hydrolysis using an integrated computational-experimental approach.¹⁴⁻¹⁸ One of our recently designed and discovered BChE mutants, *i.e.* the A199S/F227A/S287G/A328W/Y332G mutant which has been known as the most active cocaine hydrolase, exhibits a ~2000-fold improved catalytic efficiency against (-)-cocaine compared to the wild-type BChE, and the *in vivo* data demonstrated that the minimum effective dose to protect mice from cocaine-induced lethality is 0.01 mg per mouse.¹⁸

Furthermore, to effectively suppress cocaine reward in the brain for a long period of time after an exogenous cocaine hydrolase administration, the therapeutic enzyme should have not only a high catalytic efficiency against (-)-cocaine, but also a sufficiently long circulation time for cocaine addiction treatment. Unfortunately, studies have shown that recombinant BChE expressed in various expressing systems all have a short biological half-life in animal models ranging from minutes to hours.¹⁹ Success in PEGylation modification of peptides and proteins would be an ideal option to improve the biological half-life.²⁰ However, as the potential PEGylation sites are always located on the protein surface, some post-translational modification, such as glycosylation, may interfere with the PEGylation. So, the nine asparagine-linked glycans on the surface of BChE could hinder the PEGylation modification.²¹ It is essentially important for rational design of protein engineering, particularly the PEGylation, to know the 3D structure of the glycosylated human BChE.

On the other hand, it is extremely challenging to obtain a single crystal of a fully glycosylated protein and determine the crystal structure, particularly for human BChE. In order to prepare a single crystal of human BChE, one usually had to make certain mutations on the glycosylation sites.¹⁰ In fact, available X-ray crystal structures of human BChE or its mutant do not include all structural information about the glycans of the native protein.^{10,22}

In the present study, we modeled and simulated a 3D model of the glycosylated human BChE starting from the available X-ray crystal structure of the unglycosylated BChE (mutant). According to the simulated results, glycans did not change the overall stability of the BChE structure, but could increase the flexibility of some local structures. The Solvent Accessible Surface Area (SASA) analysis revealed that some lysine residues have a significant decrease in SASA due to direct or indirect effects of their surrounding glycans. The modeling and simulation provide valuable insights concerning how the glycans affect the BChE structure and how the glycans influence the PEGylation modification of BChE. These insights will be useful in guiding future rational design of BChE engineering.

Materials and Methods

Construction of the glycosylated BChE model

The starting (unglycosylated) BChE structure was taken from the X-ray crystal structure deposited in Protein Data Bank at 2.0 Å resolution (PDB ID: 1P0M),¹⁰ as this crystal structure has the highest resolution within all of the X-ray crystal structures available for BChE. A few modifications were made to fix this structure. First, all small molecules and the carbohydrate chains were removed, except the crystal water molecules. Second, the missing residues (Asp2, Asp3, Asp378, Asp379, and Gln455) were added manually by TLEAP module in Amber 9 package.²³ Further, the mutated residues Gln17, Gln455, Gln481, and Gln486 were changed back to Asn17, Asn455, Asn481, and Asn486, respectively. Finally, the protonation states of histidine residues were modified after

manually inspecting their local environments. His126, His214, His372, and His438 were protonated on their N δ atoms, whereas His77, His207, and His423 were protonated on their N ϵ atoms. This initial structure was then optimized using the SANDER module of the Amber 9 package²³⁻²⁴ for constructing the glycosylated BChE model.

The initial model of the glycosylated human BChE was constructed based on the above optimized BChE structure using the Glycam Biomolecule Builder.²⁵ Nine biantennary glycans are located on the surface of BChE. Each glycan is covalently linked to the N δ atom of asparagine (Asn17, Asn57, Asn106, Asn241, Asn256, Asn341, Asn455, Asn481, and Asn486).²¹ Each of the simulated glycans was composed of 11 carbohydrate residues (see Figure 1), according to the experimental results based on serum human BChE from Kolarich *et al.*²⁶ We checked and made sure that the conformation about the covalent linkage between the N δ atom and glycan was consistent with that found in the available X-ray crystal structure 4AQD (which has a lower resolution of 2.5 Å, but with a few incomplete glycans containing 1-2 GlcNAc residues).²² For convenience, the nine glycans are named in accordance with their linkage residue number, *i.e.* Glycan17, Glycan57, Glycan106, Glycan241, Glycan256, Glycan341, Glycan455, Glycan481, and Glycan486.

Molecular dynamic simulations

The general procedure for carrying out the molecular dynamic (MD) simulations is similar to that used in our previously reported computational studies.¹⁵⁻¹⁸ Briefly, all energy minimizations and MD simulations were carried out by using the AMBER 9 program package. The Amber force field (ff03) was used to establish the potentials of protein,²⁷ and Glycam06 force field was used to establish the potentials of glycans.²⁸ The system was immersed in an orthorhombic box of TIP3P water molecules with a minimum solute-wall distance of 10 Å.²⁹ The whole system was carefully equilibrated and fully energy-minimized. Specifically, the water molecules were first energy-minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method) with the enzyme atoms frozen. Then, the water molecules and side chains of the enzyme were energy-minimized for 1000 steps (including 500 steps using the steepest descent method and 500 steps using the conjugate gradient method) with the backbone of the enzyme frozen. Finally the whole system was energy-minimized for 5000 steps (including 2500 steps using the steepest descent method and 2500 steps using the conjugate gradient method). After that, the system was gradually heated in the NPT ensemble from 10 K to 300 K over 60 ps. Then, MD simulation was performed for 10 ns under the normally adopted temperature (300 K). During the MD simulation, Particle Mesh Ewald (PME) method was employed to deal with the long-range electrostatic interactions.³⁰ The SHAKE procedure was employed to constrain the lengths of all covalent bonds involving hydrogen atoms,³¹ with a time step of 2.0 fs. The atomic coordinates were saved every 1 ps for subsequent sampling and analysis.

The initial model of the glycosylated human BChE was built based on the fully energy-minimized BChE structure and, then, was further carefully equilibrated *via* MD simulation and energy-minimized again at the end. The same procedure used to simulate the unglycosylated human BChE structure was employed to simulate the glycosylated human BChE structure for 10 ns.

Results and Discussion

Structural features of the glycosylated BChE

Through molecular modeling and MD simulations, we obtained a dynamically stable 3D model of the glycosylated human BChE in comparison with that of the unglycosylated

human BChE. To explore dynamic stability of the models, we evaluated the time-dependent root-mean-square deviation (RMSD) of the atomic positions of the key atoms from their initial positions. Depicted in Figure 2 are the RMSD values of the protein backbone and the heavy atoms of glycans. As seen in Figure 2 (A), the RMSD values for the protein backbone in both models (*i.e.* the unglycosylated and glycosylated human BChE structures) reached the equilibrium after 1 ns during the MD simulations and kept stable in the remaining time of the simulations. The average RMSD value of the backbone of the unglycosylated BChE during the stable stage was 1.26 Å which is close to that of the glycosylated BChE, 1.24 Å, suggesting that the dynamic stability of BChE at room temperature was not changed significantly by the glycans.

As seen in Figure 2 (B) and (C), after 1 ns of the MD simulation, the RMSD values for the heavy atoms of the glycans all reached the dynamically equilibrium stage, with the RMSD values fluctuating between ~2 and ~6 Å. The average RMSD values of the heavy atoms of Glycan17, Glycan57, Glycan106, Glycan241, Glycan256, Glycan341, Glycan455, Glycan481, and Glycan486 were 3.37 Å, 4.65 Å, 4.38 Å, 3.52 Å, 3.55 Å, 4.09 Å, 3.79 Å, 4.33 Å, and 4.32 Å, respectively.

Depicted in Figure 3 is the MD-simulated structure (the last snapshot) of the glycosylated BChE. As seen in Figure 3 (in which the α -helix where Ser198 is located is at the center), nine glycans are located on the surface of the enzyme, including six (Glycan17, Glycan57, Glycan106, Glycan241, Glycan455, Glycan481, and Glycan486) on the left side and three (Glycan241, Glycan256, and Glycan341) on the right side. In addition, glycans are all far away from the active-site entrance of the enzyme. Hence, the glycosylation should have no significant influence on the catalytic function of human BChE.

It is interesting to know the potential hydrogen bonding between the glycans and BChE itself. Hence, hydrogen bond analysis was performed using PTRAJ module in AMBER 9 package based on the sampling snapshots (once every 20 ps after 1 ns) from the MD simulation. The hydrogen bond analysis was based on a simple criterion that the distance between the hydrogen and the hydrogen-bond acceptor (oxygen or nitrogen) was shorter than 3.0 Å. Hydrogen bonds were counted according to their overall appearance time during the simulation. For example, if a hydrogen bond existed in 50% of the collected snapshots, it was counted as half (0.5) of a hydrogen bond. According to the results, there were only 1.7 hydrogen bonds in average formed between each glycan and the BChE protein during the MD simulation, suggesting that the hydrogen bonding between the glycans and the BChE protein is moderate.

Solvent Accessible Surface Area (SASA)

It has been known that PEGylation can significantly prolong the biological half-life of a therapeutic protein without affecting its biological functions. BChE can be PEGylated through lysine linkage chemistry, as there are lysine residues on the BChE surface. However, asparagine-linked glycans on the surface of glycosylated BChE may interfere with the PEGylation modification of the enzyme. In this study, we tried to evaluate the influence of the steric hindrance of glycans on the potential PEGylation reaction sites, specifically the N_ϵ atoms of lysine residues. To achieve this, the SASA values of the N_ϵ atoms of all the 31 lysine residues on the enzyme surface were calculated and analyzed by using the MSMS program³² for both the unglycosylated and glycosylated BChE models using the sampled snapshots of the MD-simulated structures (one snapshot for every 20 ps after 1 ns). Three probes with different radii were chosen to represent the approximate sizes of different functional groups of different PEGylation reagents.³³ A probe with a radius of 1.4 Å represents a small functional group, such as carbonyl group. A probe with a radius of 4 Å

represents a medium functional group, such as epoxide group. A probe with a radius of 8 Å represents a large functional group, such as benzotriazole group (see Figure 4).

The relative SASA values of the Nε atoms of lysine residues calculated using different probe radii in the unglycosylated and glycosylated BChE models are listed in Table 1. In the unglycosylated BChE, the relative SASA values of the Nε atoms calculated using the radii of 1.4, 4, and 8 Å range from 4.79% to 45.99%, 0.01% to 25.83%, and 0.0% to 16.97% with the average values of 30.12%, 11.34%, and 5.77%, respectively. In the glycosylated BChE model, the relative SASA values of the Nε atoms of lysine residues calculated using probe radii of 1.4, 4, and 8 Å range from 6.17% to 42.06%, 0.01% to 20.23%, and 0.0% to 10.67% with the average values of 25.98%, 7.84%, and 3.06%, respectively. In general, the relative SASA values of the Nε atoms of lysine residues on the enzyme surface decrease as the probe radius increases in both the unglycosylated and glycosylated BChE models, demonstrating that a larger probe radius or a larger PEGylation reagent has less chance to contact with the reaction sites (the Nε atoms of lysine residues) and, thus, is expected to have a lower PEGylation reaction rate.

To further analyze the steric hindrance of the glycosylation on the surface lysine residues, those lysine residues that showed a significant decrease in SASA (when the Percent of the Remain (POR) of SASA is less than 80%) are selected for further discussion, and those residues are listed in Table 2. When the probe radius was 1.4 Å, nine lysine residues were found to have a large decrease in SASA: Lys44, Lys45, Lys190, Lys248, Lys314, Lys 339, Lys355, Lys458, and Lys476. This group of residues, denoted as Group I for convenience, has the POR values ranging from 19.67% to 78.57%. When the probe radius was increased to 4 Å, four more lysine residues (Lys105, Lys348, Lys494, and Lys499) were added to the Group I, resulting in Group II (Lys44, Lys45, Lys105, Lys190, Lys248, Lys314, Lys 339, Lys355, Lys348, Lys458, Lys476, Lys494, and Lys499), with the POR values ranging from 1.31% to 76.13%. When the probe radius was further increased to 8 Å, 15 lysine residues (*i.e.* all the lysine residues listed in Table 2, denoted as Group III for convenience) showed a large decrease in SASA with the POR values ranging from 0.22% to 73.35%. Clearly, as the probe radius increases, the number of lysine residues with larger POR values increases, suggesting that the effects of the glycans on the PEGylation modification reactions are more significant when a larger reagent is used.

To understand the relation between the lysine residues with the large decrease in SASA and their locations on the enzyme surface, we searched for the lysine residues that are located within a certain distance (4 or 8 Å) from each glycan. Specifically, the distance between a lysine residue and a glycan is defined as the internuclear distance between the Nε atom of the lysine and a glycan atom closest to the Nε atom. Based on the collected snapshots of the MD-simulated structure, 12 lysine residues were found to locate within 4 Å from a glycan: Lys44, Lys45, Lys60, Lys105, Lys190, Lys248, Lys313, Lys314, Lys348, Lys355, Lys458, and Lys476 (denoted as Group IV). 17 lysine residues were found to locate within 8 Å from a glycan: Lys44, Lys45, Lys60, Lys103, Lys105, Lys190, Lys248, Lys261, Lys313, Lys314, Lys348, Lys355, Lys366, Lys427, Lys458, Lys469, and Lys476 (denoted as Group V). Compared to Group IV, Group V, has five more residues (Lys103, Lys261, Lys366, Lys427, and Lys469). However, according to the data in Table 1, Lys103, Lys366, Lys427, and Lys469 all have very small SASA values calculated using all of the three probe radii in both the unglycosylated and glycosylated BChE models. This means that, even without glycans on enzyme surface, these lysine residues are hardly available to contact with the PEGylation reagents. So, ignoring these four lysine residues, Groups IV and V are almost the same, with Lys262 being the only additional lysine residue in Group V.

In comparison between Groups I and IV, it was noted that eight of the nine residues in Group I were included in Group IV, suggesting that glycans on BChE surface indeed mainly decrease the SASA of the N ϵ atoms of most lysine residues close to the glycans. However, Lys339 in Group I was not included in Group IV. After inspecting the location of Lys339, it was noted that Lys339 is located in an area surrounded by loop residues (Gln71-Glu80, Tyr332-Asn341, and Glu421-Glu432). The glycan closest to Lys339 is Glycan341, which is more than 10 Å away (see Figure 5 (A)). The large decrease in SASA of the N ϵ atom of Lys339 may be due to the amino-acid residues located in these highly flexible loops which may more significantly block the contact between Lys339 and the probe in the glycosylated BChE. In fact, the RMSD values of the backbone atoms of these loops (Gln71-Glu80, Tyr332-Asn341, and Glu421-Glu432) in the MD-simulated glycosylated BChE were 0.84 Å, 1.27 Å, and 0.71 Å, respectively, larger than the corresponding RMSD values of 0.77 Å, 1.19 Å, and 0.55 Å in the MD-simulated unglycosylated BChE. The glycans might not significantly change the overall stability of the BChE protein as mentioned above, but they may increase the flexibility of local protein structures. From this point of view, Lys339 is indirectly influenced by the glycosylation. In Group I, Lys314 and Lys355 are affected most by the glycosylation with the POR values being 32.20% and 19.67%, respectively. As seen in Figure 5 (B) and (C), Lys314 is very close to some carbohydrate residues of Glycan106, and Lys355 is almost fully blocked by several carbohydrate residues at the end of Glycan241.

Of the 13 lysine residues in Group II, 10 residues were included in Group IV. Besides the aforementioned Lys339, residues Lys94 and Lys499 were also not included in Group IV. These two residues were located in the same loop (Asn481-Lys499) and they were very close to each other. Similar to Lys339, the large decrease in SASA of the N ϵ atom of Lys494 and Lys499 was likely due to the reason that the amino-acid residues located in the highly flexible loop block the contact between Lys494/Lys499 and the probe. The RMSD value of the backbone atoms of the loop where these two residues are located in the MD-simulated glycosylated BChE was 1.22 Å, larger than that (0.90 Å) in the MD-simulated unglycosylated BChE. The larger RMSD in the MD-simulated glycosylated BChE was likely caused by Glycan481 and Glycan486 that are all connected to the same loop as Lys494 and Lys499 (see Figure 5 (D) for the detail). These computational data are consistent with previously reported experimental observation that Asn481 and Asn486 must be mutated to glutamine residues to avoid the glycosylation on these two sites and, thus, to obtain a single crystal for the X-ray diffraction.¹⁰ The probe radius also plays an important role here. According to the data in Table 2, a larger probe radius is associated with the smaller POR values, indicating that larger reagents would amplify the influence of the glycosylation. The POR value of Lys314 decreased from 32.20% associated with a probe radius of 1.4 Å to 11.28% associated with a probe radius of 4 Å.

Compared to the fact that Groups I and II only included some of the lysine residues in Table 2, Group III included all of the 15 lysine residues in Table 2, which further reflects the aforementioned observation that the steric hindrance of the glycosylation on the lysine residues is more significant when a larger probe is used. In comparison between Groups III and IV, 11 of the 14 lysine residues listed in Group III are also listed in Group IV; the remaining four residues are Lys262, Lys339, Lys494, and Lys499. Of these four exceptions, three (*i.e.* Lys339, Lys494, and Lys499) have been discussed above, and the only newly added one is Lys262. Notably, Lys262 is included in Group V, suggesting this residue is still significantly affected by the glycosylation when a larger PEGylation agent is used.

Conclusion

In this study, a 3D structural model of the glycosylated human BChE has been developed to examine the possible effects of the glycosylation on the possible PEGylation modification reactions. The calculated Solvent Accessible Surface Area (SASA) values revealed that most surface lysine residues located within 8 Å, or more precisely 4 Å, of glycans in the glycosylated BChE model have a significant decrease in SASA compared to the corresponding SASA values in the unglycosylated BChE, demonstrating the direct influence of the glycosylation. Three lysine residues (Lys399, Lys494, and Lys499) that are not very close to the glycans still have a significant decrease in SASA due to the indirect influence of the glycosylation. The structural information obtained from the present study could be useful in future rational design of protein engineering which aims to prolong the biological half-lives of recombinant human BChE and its mutants *via* PEGylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
The composition of the glycan in human BChE.

Figure 2.

Time-dependent RMSD values for the atomic positions of the protein backbone and glycan backbone in the MD-simulated unglycosylated and glycosylated human BChE structures.

(A) BChE (red) and Glycosylated BChE (black); (B) Glycan17 (black), Glycan57 (red), Glycan106 (green), Glycan241 (blue), and Glycan256 (pink); (C) Glycan341 (black), Glycan455 (red), Glycan481 (green), and Glycan486 (blue).

Figure 3. Overall structure of the glycosylated human BChE model derived from the MD simulation. The orange ribbons represent BChE, and the blue sticks refer to the nine glycans. The cyan sticks represent the nine linkage asparagine residues. The red ribbon refers to the α -helix where Ser198 is located.

Figure 4.
Representative PEGylation reactions.

Figure 5. Locations of representative lysine residues. The orange ribbons represent BChE. The blue sticks represent the glycans. The cyan sticks refer to the linkage residue asparagine. The green sticks represent the surrounding residues. The red ribbon refers to the α helix where the Ser198 located. The pink sticks represent the lysine residues: (A) Lys339; (B) Lys314; (C) Lys355; (D) Lys494 and Lys499.

Table 1

The relative SASA values of the Ne atoms of lysine residues on the surface of human BChE calculated by using different probe radii.

Residue	SASA in unglyco-BChE ^a			SASA in glyco-BChE ^b		
	R = 1.4 Å ^c	R = 4 Å	R = 8 Å	R = 1.4 Å	R = 4 Å	R = 8 Å
Lys9	43.70% ^d	21.05%	11.32%	42.06%	18.03%	8.03%
Lys12	45.99%	19.92%	10.51%	37.94%	16.95%	9.11%
Lys44	29.58%	7.93%	2.26%	20.64%	3.71%	0.40%
Lys45	27.86%	7.23%	1.75%	19.55%	2.57%	0.21%
Lys51	41.39%	16.52%	8.29%	41.73%	19.68%	10.00%
Lys60	35.19%	15.91%	8.18%	34.97%	14.13%	6.00%
Lys103	25.57%	4.40%	0.56%	29.25%	7.46%	1.52%
Lys105	42.93%	25.82%	16.97%	42.24%	20.23%	10.67%
Lys131	20.87%	2.53%	0.15%	17.85%	1.16%	0.01%
Lys180	25.71%	5.40%	1.67%	29.63%	7.11%	2.68%
Lys190	42.47%	22.43%	13.43%	33.37%	9.80%	2.30%
Lys248	39.73%	19.92%	10.40%	26.50%	5.93%	1.35%
Lys262	35.38%	16.16%	9.14%	33.63%	14.00%	6.52%
Lys267	29.02%	11.45%	5.25%	28.89%	11.24%	5.27%
Lys313	6.75%	0.64%	0.00%	9.72%	0.94%	0.07%
Lys314	35.78%	17.37%	10.50%	11.52%	1.96%	0.23%
Lys323	23.53%	6.55%	0.63%	31.00%	6.49%	0.36%
Lys339	32.99%	8.78%	2.02%	24.28%	5.19%	0.59%
Lys348	38.05%	19.40%	12.67%	33.84%	14.77%	6.88%
Lys355	31.37%	10.70%	4.46%	6.17%	0.14%	0.01%
Lys366	4.79%	0.31%	0.03%	7.72%	0.01%	0.00%
Lys407	20.69%	0.61%	0.00%	22.80%	0.57%	0.00%
Lys408	10.88%	0.60%	0.00%	12.22%	0.80%	0.01%
Lys427	10.35%	0.01%	0.00%	7.93%	0.00%	0.00%
Lys458	39.02%	18.53%	11.65%	26.86%	4.15%	0.22%
Lys469	14.93%	1.57%	0.09%	14.85%	0.19%	0.00%
Lys476	28.91%	2.46%	0.11%	17.11%	0.53%	0.00%

Residue	SASA in unglyco-BChE ^a			SASA in glyco-BChE ^b		
	R = 1.4 Å ^c	R = 4 Å	R = 8 Å	R = 1.4 Å	R = 4 Å	R = 8 Å
Lys494	41.90%	18.84%	11.47%	37.19%	14.29%	5.91%
Lys499	37.96%	17.04%	8.60%	37.31%	12.27%	1.23%
Lys513	31.80%	13.12%	6.45%	28.10%	11.37%	5.38%
Lys528	38.61%	18.47%	10.23%	38.37%	17.29%	9.83%

^aSASA of the Ne atom of lysine residues in the unglycosylated BChE.

^bSASA of the Ne atom of lysine residues in the glycosylated BChE.

^cR refers to the probe radius (1.4, 4, or 8 Å).

^dThe relative SASA value refers to the calculated exposed percent of the surface area of the Ne atom (with a van der Waals radius of 1.55 Å)³⁴ is $4\pi(R+1.55)^2$ in which R is the probe radius.

Table 2

The Percent of Remain (POR) of SASA of the N ϵ atoms of lysine residues on BChE surface calculated using different probe radii

Residue	R = 1.4 Å ^a	R = 4 Å	R = 8 Å
Lys44	69.78% ^b	46.78%	17.70%
Lys45	70.17%	35.55%	12.00%
Lys60	99.37%	88.81%	73.35%
Lys105	98.39%	78.35%	62.88%
Lys190	78.57%	43.69%	17.13%
Lys248	66.70%	29.77%	12.98%
Lys262	95.05%	86.63%	71.33%
Lys314	32.20%	11.28%	2.19%
Lys339	73.60%	59.11%	29.21%
Lys348	88.94%	76.13%	54.30%
Lys355	19.67%	1.31%	0.22%
Lys458	68.84%	22.40%	1.89%
Lys476	59.18%	21.54%	0.00%
Lys494	88.76%	75.85%	51.53%
Lys499	98.29%	72.01%	14.30%

^aR represents the probe radius (1.4, 4, or 8 Å).

^bThe POR of SASA of the N ϵ atom of lysine residues in the glycosylated BChE compared to SASA of the N ϵ atom of lysine residues in the unglycosylated BChE. POR = SASA of the N ϵ in glycol-BChE / SASA of the N ϵ in (glycosylated) BChE.