Potential of Mean Force and Umbrella Sampling Simulation for the Transport of 5-Oxazolidinone in Heterotetrameric Sarcosine Oxidase

Shigetaka Yoneda,* Takami Saito, Daisuke Nakajima, and Go Watanabe

School of Science, Kitasato University, 1-15-1 Kitasato Minami-Ku, Sagamihara-Shi, Kanagawa-Ken 252-0373, Japan

*Correspondence to: Shigetaka Yoneda, School of Science, Kitasato University, 1-15-1 Kitasato, Minami-Ku, Sagamihara-Shi, Kanagawa-Ken 252-0373, Japan.

E-mail: syoneda@kitasato-u.ac.jp

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ABSTRACT

The structure of heterotetrameric sarcosine oxidase (HSO) contains a highly complex system composed of a large cavity and tunnels, which are essential for the reaction and migration of the reactants, products, and intermediates. Previous molecular dynamics (MD) simulation of HSO has identified the regions containing the water channels from the density distribution of water. The simulation is consistent with the selective transport hypothesis of the migration of the iminium intermediate, 5-oxazolidinone (5-OXA), of the enzyme reaction whereby tunnel T3 is the exit pathway of 5-OXA. In the present study, the potential of mean force (PMF) for the transport of 5-OXA through tunnels T1, T2, and T3 was calculated using umbrella sampling (US) MD simulations and the weighted histogram analysis method. The maximum errors of the calculated PMF were estimated by repeating the US simulations using different sets of initial positions. The PMF profiles for the three tunnels support the notion that tunnel T3 is the exit pathway of 5-OXA and that 5-OXA tends to stay at the middle of the tunnel. The PMF profile for the transport of glycine through tunnel T3 was also calculated to investigate where 5-OXA is converted into glycine, and how glycine is released to the outside of HSO was explained.

INTRODUCTION

A polyfunctional enzyme contains two or more active sites located in the different places of the molecule.[1] The product of the first active site of the enzyme is transferred to the second active site through tunnels connecting the sites, and becomes the substrate of the second active site. The tunnels in the enzyme play the role of channels for the reactants, products, and intermediates that enter into the enzyme from the outside, exit to the outside, and migrate between the active sites. Thus, investigating how molecules are transported in these enzymes will provide a basis for understanding their functions and mechanisms. A well-studied polyfunctional enzyme is the heterotetrameric sarcosine oxidase (HSO).

HSO was first purified from *Corynebacterium* sp. U96 in 1982[2] for the determination of creatinine.[3] HSO catalyzes sarcosine oxidation to generate glycine and hydrogen peroxide and either formaldehyde or 5,10-methylene-tetrahydrofolate, depending on the availability of tetrahydrofolate (THF). HSO consists of four non-identical subunits, α, β, γ, and δ, and contains nicotinamide adenine dinucleotide (NAD^+), Zn^{2+}, flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN).[4] As the X-ray structures clarified[5-7], the α-subunit of HSO is composed of the two half domains by which the β-, γ-, and δ-subunits are sandwiched (**Figure 1a**). The δ-subunit has a Cys_3His zinc finger motif to bind Zn^{2+}. The β-subunit is bound noncovalently with FAD and covalently with FMN. It is proposed that FAD is reduced by sarcosine, an electron is transferred from the reduced FAD to FMN, and the reduced FMN is oxidized by an oxygen molecule to yield an oxidized flavin. A sarcosine molecule is bound at the *re*-phase of the isoalloxazine ring of FAD near a large cavity found by X-ray analysis.[5] Several tunnels have been proposed for the transport of substrates and products,[7] as determined with the geometry-

based tunnel prediction tool CAVER2.[8] Tunnels T1, T2, and T3 that connect the cavity near FAD and the outer surface of HSO are associated with the selective transport hypothesis on the migration of the products as follows.[7,9] Sarcosine moves into the large cavity of HSO from the outer surface through tunnels T1, T2, or both, as shown in **Figure 2**. After the substrate reaches the FAD active site, dehydrogenation occurs and an iminium intermediate, 5-oxazolidinone (5-OXA), shown in **Figure 3**, is generated and released into the large cavity of HSO. Dynamic motion of Ala401 to His404 of the C-terminal region of the β-chain is associated with the migration of 5-OXA into the cavity.[7] From the cavity, 5-OXA further migrates into tunnel T3 to reach the binding site in the tunnel, and is converted to glycine and either 5,10-CH_2-THF or formaldehyde, depending on whether THF is bound to the site.[7] The final products are released from HSO through tunnel T3. To investigate the hypothesis, a 100-ns molecular dynamics (MD) simulation has been carried out for the HSO bound with the substrate analogue dimethylglycine.[9] The channel regions for the transport of water were determined from the water density distribution (**Figure 1b**). The detailed shape of the channel regions was different from that of tunnels determined using CAVER2, because the analysis of atomic motion clarified the invasion of water molecules into the fluctuating empty space within HSO. However, the overall shape and position of the channels were consistent with those of the tunnels by the CAVER2 analysis. The channels were therefore named CH1, CH2, and CH3 following the name of tunnels T1, T2, and T3, respectively. The narrowness of the water channels, the hydropathy indices of the residues, and the number of migration events of water molecules in the simulation have suggested that tunnel T3 (i.e., channel CH3) is the pathway for the exit of 5-OXA. Thus, in this study, the potentials of mean force (PMF) for the transport of 5-OXA through tunnels T1, T2, and T3 were calculated for a more quantitative investigation on the validity of the selected transport

hypothesis, focusing on which tunnel is selected for the transport, where 5-OXA is converted to glycine, and how glycine is released from HSO.

(Figure 1)

(Figure 2)

(Figure 3)

PMF is the free energy as a function of the reaction coordinate x, and is defined by the probability density $\rho(x)$ as follows.[10,11]

$$F(x) = -k_{\mathrm{B}}T \log \rho(x) \qquad (1)$$

where $F(x)$ is PMF, k_{B} the Boltzmann constant, T the temperature, and an arbitrary constant is omitted in the equation. In the present study, x is defined as the 1-dimensional coordinate of the center of mass of 5-OXA or glycine measured along the vector from entrance to exit of the tunnel. If the canonical ensemble is assumed, $\rho(x)$ is small for high potential energy positions. Therefore, enough sampling for the positions is difficult so that the statistical errors for $\rho(x)$ and thus $F(x)$ are large. As a traditional method to solve this deficient sampling problem, the constraint potential named "umbrella potential" is added to increase the apparent probability density $\rho^*(x)$ around the energy minima of the umbrella potential. The true PMF can be derived from $\rho^*(x)$ by the following umbrella sampling (US) equation.

$$F(x) = -k_{\mathrm{B}}T \log \rho^*(x) - U(x) + k_{\mathrm{B}}T \log < \exp(U(x)) >^* \qquad (2)$$

where $U(x)$ is the umbrella potential, $F(x)$ the true PMF, and $< >^*$ the average that is calculated from the simulation with the umbrella potential. In practice, $F(x)$ is calculated for the discrete bins of x that satisfy the inequality

$$k\delta x < x < (k+1)\delta x \qquad (3)$$

where k is an integer and δx the bin size. In the present study, a bin corresponds to a region sandwiched between two planes perpendicular to the vector from the entrance to the exit of the tunnel. PMF for a long tunnel is calculated with a series of US simulations, where the energy minima of their umbrella potentials are located at equal intervals along the tunnel and the calculated probability density distributions are overlapped. Because the PMF values from the US simulations are precise at the bins around the energy minimum of the umbrella potential, the precise values are connected to determine the entire PMF profile over the tunnel. The weighted histogram method (WHAM)[12,13] is widely used to minimize the statistical errors from the connection. In addition to the US method, in the Jarzynski equality (JE)[14] and the Crooks theorem,[15] it was recently shown that PMF can also be calculated by the path integral of the mean force, i.e., by the average of the integral of pulling force over numerous repeats of nonequilibrium steered molecular dynamics (SMD) simulations, whereby the center of mass of the molecule is moved to position x by a pulling force. The PMF calculation by pulling has intrigued many scientists for further developments and applications because of the clarity and possible applicability of the algorithm.[16-19] Both the US and SMD should determine the same true PMF in principle, if ideally large computer resources could be employed. The authors adopted the traditional US method for the present study on HSO based on viability, cost, and

computational efficiency.[20] The five PMF profiles in **Table 1** were calculated for the transport of 5-OXA and glycine through tunnels T1, T2, and T3.

(Table 1)

METHODS

The calculation conditions in the previous MD simulation[9] were followed in the present study, but the X-ray structure of the reduced form of HSO bound with no substrates (PDB-ID 3AD9[7]) was used for the initial structure. The 963, 404, 200, and 91 amino acid residues of the α-, β-, γ-, and δ-subunits, respectively, were included. After determining the protonation states of the amino acid residues,[21] adding the hydrogen atoms, and cleaning-up of the X-ray coordinates, the total number of atoms in the system was 124,646, including 25,009 atoms for the protein, 99,576 atoms for water molecules, and 61 Na^+ ions that were added to neutralize the entire system. GROMACS 4.6.7[22] was used for the simulations. The AMBER ff99SB-ILDN energy parameters[23] were used for HSO, Na+, and cofactors, TIP3P[24] for water molecules, and the parameters by Calimet and Simonson[25] for Zn^{2+}. The particle mesh Ewald method[26] was used for electrostatic interactions. HSO was located in a periodic boundary box with a size of 9.949 × 12.436 × 9.949 nm^3. After energy minimizations and a 20-ns MD simulation with positional constraints, a 100-ns equilibration run (**Figure 4**) with no constraints was performed at 300 K and 1 bar.[27] All bonds involving hydrogen atoms were restrained with LINCS[28] and the time step of 2 fs was adopted.

(Figure 4)

Five SMD simulations were performed using the final structure of the 100-ns equilibration run to prepare for the five series of US simulations that calculate the PMF profiles 1, 2, 3a, 3b, and G (**Table 1**). It should be noted that the SMD simulations in the present study did not calculate PMF, but were used only to model the initial structures of the US simulations. For starting the SMD simulations, the entrance and exit positions of the tunnels were determined

manually at the borders of the cavity and at the border to the outer surface of HSO, respectively (**Table S1**). 5-OXA or glycine were inserted at the entrance (profiles 1, 2, 3a, and G) or exit (profile 3b). Water molecules near 5-OXA were removed if necessary to prevent steric hindrance. The general AMBER force field (GAFF)[29] was used for 5-OXA, and the atomic charges of 5-OXA were determined by RESP[30,31] using the 6-31G* basis ab-initio calculations (**Table S2**). After energy minimization and a 1-ns MD simulation at 300 K with constant volume and with the positional constraints on the non-H atoms of HSO, 5-OXA or glycine was pulled towards a dummy atom at the other end of the tunnel in the SMD simulation. The pulling rate was 0.35 nm/ns for profile 1, 0.70 nm/ns for profile 2, and 0.60 nm/ns for profiles 3a, 3b, and G. The dummy atoms were constrained by a harmonic potential with a Hook's constant of 1000 kJ/mol/nm^2 (1 kcal/mol/Å^2 equals 418 kJ/mol/nm^2) to the initial positions. From the trajectory of each SMD simulation, a set of initial structures for the US simulations were selected at equal time intervals. A 0.1-ns MD simulation at 300 K and 1 bar with the positional constraints to the non-H atoms was firstly performed using the selected structures before a 2-ns US simulation with positional constraints to Cα atoms was started. The US simulation was composed of an equilibration run followed by a sampling of the time length from 1.5 ns to 2.0 ns. The following umbrella potential was adopted in the present study.

$$U(x) = K_{US} (x - x_0)^2 / 2 \qquad (4)$$

where K_{US} is the constant of 1000 kJ/mol/nm^2 and x_0 the position of the local minimum of the umbrella potential of the US simulation. The average distance between the minimum positions x_0 among the US simulations was 0.1 nm. The number of US simulations was 9, 18, 14, 13, and 16

for profiles 1, 2, 3a, 3b, and G, respectively. A short bin size δx of 0.01 nm was used for Equation 3. The PMF values from each set of US simulations were connected by WHAM.

RESULTS AND DISCUSSION

The root mean square deviations (RMSD) from the X-ray structure were 0.15 nm for the Cα atoms of the final structure of the 100-ns equilibration run with the eight flexible C-terminal residues of the δ-chain excluded in the RMSD. Thus, the HSO structure was highly stable, as described in a previous study.[9] The US simulations calculated the probability distributions of 5-OXA and glycine over the tunnels as shown in **Figure 5**. The probability distributions were well overlapped with each other. The PMF profiles were determined by connecting the PMF values by WHAM as shown in **Figure 6**. The bars depict the statistical uncertainty (standard deviations) by WHAM. **Figure 7** shows the initial positions of 5-OXA or glycine for the sets of US simulations, and **Figure 8** shows the amino acid residues surrounding tunnel 3. The PMF values at the exit of tunnels were approximately equal, indicating that the environments in the cavity and the bulk water are similar. Thus, although the entrance positions within the cavity were slightly separated for profiles 1, 2, 3a, and 3b as shown in Table S1, it is reasonable that the PMF values at the entrance were set to be equal (zero). The PMF profile for glycine (profile G) was not directly comparable with those for 5-OXA, because the chemical structures are different. However, the value of profile G at the tunnel entrance ($x = 0$) was also set to zero by adjusting the arbitrary constant, and the calculated value of profile G at the tunnel exit was approximately zero.

(Figure 5)

(Figure 6)

(Figure 7)

(Figure 8)

The PMF profile was calculated twice for the transport of 5-OXA through tunnel 3 (profiles 3a and 3b) to estimate calculation errors. In principle, any MD simulation produces different trajectory and energetic properties if the calculation conditions, initial structures, or initial velocities adopted are different. Therefore, an SMD simulation (in profile 3b) was carried out in the inverse direction to obtain different initial structures for the US simulations. Any other procedure, including a manual placing of 5-OXA with computer graphics, could be adopted for this purpose. Because the reaction coordinate x (the vector from the entrance to the exit of tunnel T3) adopted was almost identical for profiles 3a and 3b, the comparison of PMF is straightforward. Profiles 3a and 3b were equal within statistical certainty everywhere except when x = 0.3 nm to 0.6 nm, with a difference of approximately 3 kcal/mol at maximum. When the simulation structures around x = 0.5 nm were compared for profiles 3a and 3b, the RMSD was small, approximately 0.07 nm for the Cα atoms of HSO. No clear difference was shown for the fluctuating tunnel structures, indicating that the calculations did not contain any systematic problems, but rather that the difference in PMF resulted from insufficient sampling. However, the overall feature of PMF was shared by profiles 3a and 3b, i.e., the PMF was negative over the entire tunnel and the minimum of approximately -4 kcal/mol was observed at the middle. This is clearly different from the features of profiles 1, 2, and G. In profile 2, PMF varied around zero, with a variation amplitude of about 2 kcal/mol. In profile 1, PMF was negative and the minimum was approximately -2.5 kcal/mol. Considering the thermal energy, 5-OXA can occasionally migrate into the deep regions of all the tunnels. Based on the PMF profiles, 5-OXA should most easily move into tunnel T3 from the cavity and stay in the region from approximately x = 0.5 nm to approximately x = 1.3 nm.

In a previous study,[7] the properties of tunnels were represented using the electrostatic potential. Because the electrostatic potential is an analytical function of position, the representation can have an atomic level resolution if necessary. In contrast, PMF is a coarser function of position, because it is calculated by sampling probability density from limited spatial regions, bins. Furthermore, the calculation of probability density is always accompanied with statistical errors. Therefore, the present PMF calculation does not have pinpoint accuracy, and a further investigation to identify the residue and atoms related to the selective transport hypothesis will need an exhaustive analysis using other calculational methods at the atomic level.

However, PMF profiles in the present study provided much information about the transport hypothesis. The profile G is the PMF for the translation of glycine through tunnel T3. The PMF was positive, approximately 4 kcal/mol, at the middle of tunnel T3. Thus, if glycine was placed within tunnel T3, it tended to escape from the tunnel towards the cavity or the outer surface of HSO. Considering the curve of profile G, i.e., with the maximum PMF value at the middle of the tunnel, glycine tended to escape towards the outside of HSO, if glycine was placed at a region farther from the middle of the tunnel. Thus, the PMF profiles in the present study support the selective transport hypothesis of 5-OXA, and indicating that the channel for the transport of 5-OXA is tunnel T3, the conversion site is at the middle of tunnel T3 (approximately $x > 1.0$ nm), and glycine exits from HSO by the free energy difference. The region at the middle of tunnel T3 is surrounded by the residues Tyr663, Ser775, Phe776, and Phe824 of the α-chain, as shown in **Figure 8**.

CONCLUSION

The PMF profiles were calculated using US simulations and WHAM for the transport of 5-OXA through tunnels T1, T2, and T3 and for the transport of glycine through tunnel T3. The PMF of Profile 2 for the migration of 5-OXA through tunnel T2 was varied around zero. Profile 1 through tunnel T1 was not largely different from zero, with a minimum of approximately -2.5 kcal/mol at the middle of the tunnel. Profile 3a and 3b through tunnel T3 were approximately -4 kcal/mol, with the minimum occurring at the middle of the tunnel. Thus 5-OXA most easily enters into tunnel T3 from the cavity and probably resides therein. The PMF of glycine through tunnel T3 was positive, i.e., approximately 4 kcal/mol, at the middle of the tunnel, so that glycine should migrate towards the outer surface of HSO if it is located near the tunnel exit. Thus, the present study is in good agreement with the selective transport hypothesis whereby tunnel T3 is the exit channel of 5-OXA, 5-OXA is converted into glycine at the middle of tunnel T3, and the product goes out of HSO by the free energy difference.

REFERENCES

1. Suzuki H. *How Enzymes Work, 2nd Edition*. Singapore: Jenny Stanford; 2019.

2. Suzuki M. Purification and Some Properties of Sarcosine Oxidase from *Corynebacterium* sp. U-96. *J. Biochem.* 1981;89(2):599-607.

3. Suzuki H. Sarcosine oxidase: structure, function, and the application to creatinine determination. *Amino Acids*. 1994;7(1):27-43.

4. Jorns MS. Properties and Catalytic Function of the Two Nonequivalent Flavins in Sarconsine Oxidase. *Biochemistry*. 1985;24(13):3189-3194.

5. Ida K, Moriguchi T, Suzuki H. Crystal structure of heterotetrameric sarcosine oxidase from *Corynebacterium* sp. U-96. *Biochem. Biophys. Res. Commun.* 2005;333(2):359-366.

6. Chen Z, Hassan-Abdulah A, Zhao G, Jorns MS, Mathews FS. Heterotetrameric Sarcosine Oxidase: Structure of a Diflavin Metalloenzyme at 1.85 Å Resolution. *J. Mol. Biol.* 2006;360(5):1000-1018.

7. Moriguchi T, Ida K, Hikima T, Ueno G, Yamamoto M, Suzuki H. Channeling and conformational changes in the heterotetrameric sarcosine oxidase from *Corynebacterium* sp. U-96. *J. Biochem*. 2010;148(4):491-505.

8. Damborský J, Petřek M, Banáš P, Otyepka M. Identification of tunnels in proteins, nucleic acids, inorganic materials and molecular ensembles. *Biotechnology J.* 2007;2(1):62-67.

9. Watanabe G, Nakajima D, Hiroshima A, Suzuki H, Yoneda S. Analysis of water channels by molecular dynamics simulation of heterotetrameric sarcosine oxidase. *Biophys. Physicobiol.* 2015;12:131-138.

10. Torrie GM, Valleau JP. Nonphysical sampling distributions in Monte Carlo free-energy estimation: Umbrella sampling. *J. Comput. Phys.* 1977;23(2):187-199.

11. Roux B. The calculation of the potential of mean force using computer simulations. *Comput. Phys. Commun.* 1995;91:275-282.

12. Kumar S., Bouzida D, Swendsen RH, Kollman PA, Rosenberg JM. The weighted histogram method for free-energy calculations on biomolecules. I. The method. *J. Comput. Chem.* 1992;13(8):1011-1021.

13. Hub JS, de Groot BL, van der Spoel D. g_whams-A free weighted histogram analysis implementation including robust error and autocorrelation estimates. *J. Chem. Theory Comput.* 2010;6(12):3713–3720.

14. Jarzynski C. Nonequilibrium Equality for Free Energy Difference. *Phys. Rev. Lett.*, 1997;78(14):2690-2693.

15. Crooks GE. Path-ensemble averages in systems driven far from equilibrium. *Phys. Rev. E*. 2000;61:2361-2366,.

16. Xiong H, Crespo A, Marti M, Estrin D, Roitberg AE. Free energy calculationswith non-equilibrium methods: applications of the Jarzynski relationship. *Theor. Chem. Acc.* 2006;116: 338-346.

17. Giorgino T, De Fabritis G. A High-Throughput Steered Molecular Dynamics Study on the Free Energy Profile of Ion Permeation through Gramicidin A. *J. Chem. Theory Comput.* 2011;7(6):1943-1950.

18. De Vivo M, Masetti M, Bottegoni G, Cavalli A. Role of Molecular Dynamics and Related Methods in Drug Discovery. *J. Med. Chem.* 2016;59(9):4035-4061.

19. Park S, Schulten K. Calculating potentials of mean force from steered molecular

dynamics simulations. *J. Chem. Phys.* 2004;120(13):5946-5961.

20. Noh SY, Notman R. Comparison of Umbrella Sampling and Steered Molecular Dynamics Methods for Computing Free Energy Profiles of Toluene Molecules through Phospholipid Bilayers. *ChemRxiv.* 2019:9897710.

21. Labute P. Protonate3D: Assignment of ionization states and hydrogen coordinates to macromolecular structures. *Proteins*. 2009;75(1):187-205.

22. Pronk S, Páll S, Schulz R, Larsson P, Bjelkmar P, Apostolov R, Shirts MR, Smith JC, Kasson PM, van der Spoel D, Hess B, Lindahl E. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics.* 2013;29(7):845-854.

23. Lindorff-Larsen K, Piana S, Palmo K, Maragakis P, Klepeis JL, Dror, RO, Shaw DE. Improved side-chain torsion potentials for the Amber ff99SB protein force field. *Proteins.* 2010;78(8):1950-1958.

24. Jorgensen WL, Chandrasekhar J, Madura JD, Impey RW, Klein ML. Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* 1983;79(2):926–935.

25. Calimet N, Simonson T. Cys_xHis_y-Zn^{2+} interactions: Possibilities and limitations of a simple pairwise force field. *J. Mol. Graph. Mol.* 2006;24(5):404-411.

26. Darden T, York D, Pedersen L. Particle mesh Ewald: An N•log(N) method for Ewald sums in large systems. *J. Chem. Phys.* 1993;98(12):10089-10092.

27. Bussi G, Donadio D, Parrinello M. Canonical sampling through velocity rescaling. *J. Chem. Phys.* 2007;126(1):014101-014107.

28. Hess B, Bekker H, Berendsen HJC, Fraajie, JGEM. LINCS: a linear constraint solver for molecular simulations. *J. Comput. Chem.* 1997;18(12):1463-1472.

29. Wang J, Wolf RM, Caldwell JW, Kollman PA, Case DA. Development and testing of a general amber force field. *J. Comput. Chem.* 2004;25(9):1157-1174.

30. Bayly, CI, Cieplak P, Cornell W, Kollman PA. A well-behaved electrostatic potential based method using charge constraints for deriving atomic charges: the RESP model. *J. Phys. Chem.* 1993;97(40):10269-10280.

31. Frisch MJ, Trucks GW, Schlegel HB, Scuseria GE, Robb MA, Cheeseman JA, *et al.* Gaussian 03 Revision C. 02. (Gaussian Inc., Wallingford CT, 2004).

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FIGURE LEGENDS

Figure 1. X-Ray structure of HSO. (a) The surfaces by CAVER2 in cyan, light magenta, and magenta depict tunnels T1, T2, and T3, respectively. The cavity drawn in red is overlapped by tunnel T2. (b) Grids from the simulated water density in cyan, light magenta, and magenta depict the water channels CH1, CH2, and CH3, respectively.

Figure 2. Proposed transport pathways of 5-OXA and glycine. Sarcosine is denoted by "Sar". The both-directed arrow in cyan depicts the motion of the residues including Ala401 and His404 of the β-chain.

Figure 3. 5-Oxazolidinone (5-OXA).

Figure 4. Final structure of the 100-ns equilibration run. The α, β, γ, and δ-subunits of HSO are depicted in cyan, yellow, magenta, and yellow. Na^+ ions are depicted by blue balls.

Figure 5. Apparent probability density, $\rho^*(x)$. The curve of $\rho^*(x)$ calculated from each US simulation is depicted in different colors.

Figure 6. Calculated PMF profiles. The lengths of the curves are different among the profiles, because the lengths of the tunnels are different. The entrance of tunnel is at $x = 0$ nm.

Figure 7. Initial positions of the US simulations. The meshes in blue, pink, and magenta depict the tunnels T1, T2, and T3, respectively, calculated by CAVER2. The solid surface in orange, cyan, pink, and magenta depict the cavity and the water channels CH1, CH2, and CH3, respectively, calculated by the previous MD simulation. The small spheres in yellow green

depict the initial positions of 5-OXA or glycine for each US simulation. The cavity is shown on left side and the bulk water on the right side.

Figure 8. Residues surrounding tunnel T3. The labels for amino acids denote the residues of the α-chain. The same colors and structure as in Figure 7c are used.

SUPPLEMENTAL INFORMATION

S1.pdf The initial coordinates for the SMD simulations and the entrance and exit positions of the tunnels.

S2.pdf The energy parameters of 5-OXA and glycine in the GROMACS itp format.