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Modeling of flux, binding and substitution of urea molecules in the urea transporter dvUT

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Abstract

Urea transporters (UTs) are transmembrane proteins that transport urea molecules across cell membranes and play a crucial role in urea excretion and water balance. Modeling the functional characteristics of UTs helps us understand how their structures accomplish the functions at the atomic level, and facilitates future therapeutic design targeting the UTs. This study was based on the crystal structure of *Desulfovibrio vulgaris* urea transporter (dvUT). To model the binding behavior of urea molecules in dvUT, we constructed a cooperative binding model. To model the substitution of urea by the urea analogue N,N'-dimethylurea (DMU) in dvUT, we calculated the occupation probability of DMU along the urea pore and the ratio of the occupation probabilities of DMU at the external (S_{ext}) and internal (S_{int}) binding sites, and we established the mutual substitution rule for binding and substitution of urea and DMU. Based on these calculations and modelings, together with the use of the Monte Carlo (MC) method, we further modeled the urea flux in dvUT, equilibrium urea binding to dvUT, and the substitution of urea by DMU in the dvUT. Our modeling results are in good agreement with the existing experimental functional data. Furthermore, the modelings have discovered the microscopic process and mechanisms of those functional characteristics. The methods and the results would help our future understanding of the underlying mechanisms of the diseases associated with impaired UT functions and rational drug design for the treatment of these diseases.

Keywords

Molecular modeling; molecular dynamics simulations; Monte Carlo methods; membrane proteins; urea transporter

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1. Introduction

Urea is a common organic molecule in urine and other body fluids as a product of protein metabolism in mammals, and it is also a nitrogen form available to bacteria, fungi, and plants [1]. The urea molecule is uncharged but strongly polar, and therefore its permeation across biological membranes is slow [2,3]. So far, many urea transporters (UTs) have been identified. There are two major urea transporters in mammals, UT-A (with 6 isoforms) and UT-B (with 2 isoforms) [4–6]. These UTs have many homologs in bacteria, such as ApUT (*Actinobacillus pleuropneumoniae* urea transporter) [7] and dvUT (*Desulfovibrio vulgaris* urea transporter) [8]. UTs are channel-like proteins, which facilitate the diffusion of urea along their concentration gradients in the channel without costing extra energy. To understand how UTs coordinate and stabilize urea to achieve selectivity and facilitate permeation, the investigations of the structures and functional characteristics of UTs have been carried out extensively.

Two crystal structures of the UTs, dvUT [8] and UT-B [9], and one crystal structure of the proton-gated urea channel from *Helicobacter pylori*, HpUreI, [10] have been solved so far. These three-dimensional atomic structures have provided invaluable tools for us to understand the functional characteristics of UTs. Specifically, dvUT and UT-B are homologous. Both of them are trimer, and each monomer can transport urea or analogues individually.

In parallel to the structural studies, numerous investigations have been devoted to functional studies of UTs. Zhou and co-workers measured the urea flux passing through dvUT, equilibrium binding of urea to dvUT, and the substitution of the non-labeled urea and the urea analogue, N,N'-dimethylurea (DMU), for the bound labeled ^{14}C -urea [8]. Many groups have estimated the urea transport rates [11]. For example, based on the urea fluxes measured in the inner medullary collecting duct (IMCD) cells and the measured number of copies of vasopressin-regulated UT (vrUT or UT-A1), Kishore et al. estimated that the vrUT has a turnover number between 3×10^4 and 10^5 urea molecules/s [12]. MacIver et al. obtained transport rates of 4.6×10^4 and 5.9×10^4 urea molecules/s for mouse UT-A2 and UT-A3 expressed in purified *Xenopus laevis* oocyte plasma membranes, respectively [13]. Mannuzzu et al. obtained a turnover number between 2×10^6 and 6×10^6 urea molecules/s for the human UT-B1 transporter [14]; this urea transport rate is consistent with the value, 5×10^6 urea molecules/s, that was estimated by Mayrad and Levitt for UT-B1 [11,15].

Combined with the above experimental studies, we modeled the flux, binding and substitution of urea molecules in the urea transporter dvUT. Through these atomic-level modelings, we have revealed how urea molecules flow through the pore of dvUT, how urea molecules are assigned to the binding sites, and how urea molecules are replaced by DMU. When one of these functional characteristics is impaired, diseases may occur. Therefore, the modelings in the present study could help us understand the underlying mechanisms of these diseases and find out new cures for these diseases.

2. The simulation system and the methods

2.1. The urea-permeation pore

The simulation system is a urea-permeable pore, with two solution pools containing urea (or a mixture of urea and DMU) at both ends of the pore. The urea pore was prepared with the HOLE software [16,17], based on the equilibrated dvUT channel–membrane–solution system that was constructed in our previous study [18] by using molecular dynamics (MD) simulations. The HOLE software is widely used for searching pores of ion channel proteins. To test the applicability of HOLE to the present study, we ran the software 10 times for independent pore searches. The results reveal that in the wide area outside the two orifices the pore sizes and conformations being obtained from individual searches were more or less different. However, in the narrow area between the two orifices the pore sizes and conformations being obtained from individual searches were almost identical among these individual searches. Because the present study focuses on the transmission and binding of urea in the dvUT pore, and the substitution of non-labeled urea/DMU for ^{14}C -urea bound to dvUT, only the segment of the pore between the two orifices is relevant. Therefore, HOLE is applicable to search the pore of dvUT for this study.

In the present work, the center of mass of the C_α atoms in dvUT was set as the origin of the coordinate system ($z = 0$). The orifices connected to the extracellular solution and intracellular solution were found to locate at $z = -11 \text{ \AA}$ and $11 \text{ \AA}$, respectively.

2.2. The Monte Carlo method

To investigate the functional characteristics of the UTs, we designed Monte Carlo (MC) protocols for the calculations of the occupation probability, simulations of the urea flux, urea binding and substitution of urea/DMU for ^{14}C -urea in dvUT.

2.2.1. The MC method for the calculations of the occupation probabilities of DMU in the dvUT pore—With the help of the MC method, the occupation probability of DMU in the dvUT was calculated. The protocol is described as follows. The dvUT pore was divided into a three-dimensional grid with a grid spacing of $0.62 \text{ \AA}$. The level 0 and lever 35 of the grid corresponded to the orifices of the pore at the extracellular side and at the intracellular side, respectively. The DMU molecule moves on the grid from site i to its neighboring site j with a jumping probability

$$P_{ij} \propto \exp(-\Delta G_{ij}/N_A k_B T), \quad (1)$$

where $G_{ij} = G_j - G_i$, G_i and G_j are its free energies at site i and site j , respectively. N_A is the Avogadro constant, k_B is the Boltzmann constant, and T is the temperature. In this work, the absolute value of the free energy of the DMU molecule in dvUT is unknown, so G_{ij} is calculated by the difference between the variations in the free energy at site i and site j . The variations in the free energy of DMU along the pore axis in dvUT can be obtained by the adaptive biasing force approach used in our previous paper [18].

The MC simulation used for the calculation of the occupation probabilities of DMU in the dvUT pore are described as follows. At the beginning, a DMU molecule was placed at a randomly selected orifice of the pore. The molecule then started to randomly walk in the pore. When it reached any orifice, the molecule would leave the pore and enter the periplasm or the cytoplasm. During the random walk, the number of MC steps at each resident position along the pore was recorded. This simulation was repeated for 10^6 times, producing the average number of MC steps at each resident position. The ratio of the number of MC steps at a resident position to the total number of steps in the pore is the occupation probability of DMU at this position.

2.2.2. The MC method for the simulation of the urea flux through dvUT—To model and simulate the urea flux through dvUT we placed a dvUT pore between the extracellular and intracellular solutions, and denoted c_{es} and c_{is} as the concentrations in the extracellular and intracellular solutions, respectively. When the urea concentrations in the extracellular solution and in the intracellular solution are different, the osmotic pressure (or the chemical potentials) will also affect the jumping probability, and Eq. (1) can be rewritten as

$$P_{ij} \propto \exp(-[\Delta G_{ij} + \Delta \mu_{ij}]/N_A k_B T), \quad (2)$$

where $\mu_{ij} = \mu_j - \mu_i$, and μ_i and μ_j are the chemical potentials of the urea molecule at site i and site j , respectively. The variation in the free energy of urea, G_{ij} , has been presented in our previous paper [18].

For dilute solution and uniform gradient of the chemical potential along the pore axis (z-axis), the relationship between the chemical potentials and the concentration is [19]

$$\mu(c_{es}) - \mu(c_{is}) = RT \ln \left(\frac{c_{es}}{c_{is}} \right), \quad (3)$$

where R is the ideal gas constant. c_{es} and c_{is} are the concentrations of urea in the extracellular and intracellular solutions, respectively. $\mu(c_{es})$ and $\mu(c_{is})$ are the chemical potentials at the concentrations c_{es} and c_{is} , respectively.

In the simulation, c_{es} was fixed, and c_{is} increased with time as urea molecules flew into the cell. At the beginning of the simulation, c_{is}/c_{es} was set to 1/100. The first urea molecule entered the pore entrance (level 1) and then randomly walked in the pore. When the exit (i.e. level 35) was reached, the urea molecule entered the intracellular solution, thereby increasing the intracellular urea concentration by $c_{is} = c_{es} / 100$. Subsequently, the second urea molecule entered the pore, walked randomly, and then entered the cell. For each simulation, such operation continued until the 101-th urea molecule entered the cell. After each urea molecule entered the cell, the number of urea molecules in the cell, N_{in} , and the cumulative number of MC steps spent by the N_{in} molecules to go through the pore, N_{MC} ,

were recorded. Such MC simulations were performed 500 times. For each value of N_{in} , the average value of N_{MC} was calculated using 500 simulations. The curve of the number of urea molecules in the cell vs. the average cumulative number of MC steps, i.e., the N_{in} vs. N_{MC} curve, was obtained.

2.2.3. The MC method for the simulation of binding of urea to dvUT—To model and simulate the equilibrium binding of urea to dvUT, N_{UT} dvUT proteins and N_{enter}^{lab} ^{14}C -labeled urea (abbreviated as ^{14}C -urea) molecules being able to enter a dvUT and bind to a binding site were placed in the solution. At the beginning of each simulation, N_{UT} dvUT proteins were placed in the solution, and then N_{enter}^{lab} ^{14}C -urea molecules were added to the solution one by one. Each newly added ^{14}C -urea molecule entered a randomly selected dvUT, and occupied a binding site according to the cooperative binding model (see Subsection 3.2.1). If the site to be bound was occupied, the former occupant was replaced and returned to the solution. Whenever a ^{14}C -urea molecule was added, the number of bound ^{14}C -urea (N_{bound}^{lab}) and the number of ^{14}C -urea entering a binding site of dvUT (N_{enter}^{lab}) were recorded. The N_{bound}^{lab} vs. N_{enter}^{lab} relation was then obtained for one MC simulation. Such MC simulations were performed 1000 times. For each value of N_{enter}^{lab} , the average of N_{bound}^{lab} was calculated for 1000 simulations. The average N_{bound}^{lab} vs. N_{enter}^{lab} relation was then achieved.

2.2.4. The MC method for the substitution of urea/DMU for ^{14}C -urea—In the presence of dvUT proteins, ^{14}C -urea molecules and non-labeled urea molecules, the urea molecules entered the dvUTs and occupied the binding sites of dvUTs. At this time, the labeled ^{14}C -urea molecules bound to the binding sites can be replaced by the non-labeled urea molecules. When the concentration of the labeled ^{14}C -urea molecules was fixed, the concentration of the replaced labeled ^{14}C -urea molecules varied with the concentration of the non-labeled urea molecules. To simulate the substitution of non-labeled urea for bound labeled ^{14}C -urea in dvUT, the following procedure was designed. In each simulation, N_{UT} dvUT proteins were placed in the solution, and then N_{enter}^{lab} ^{14}C -urea molecules and N_{enter}^{non} non-labeled urea molecules were added to the solution, one by one. The added urea molecule, either ^{14}C -labeled or non-labeled, was randomly selected according to the ratio, $N_{enter}^{lab}/N_{enter}^{non}$. This selected urea molecule entered a randomly chosen dvUT, and then occupied a binding site of dvUT according to the cooperative binding rule (see Part Two of Subsection 3.2.1). These steps were repeated until all of the N_{enter}^{lab} ^{14}C -labeled urea molecules and N_{enter}^{non} non-labeled urea molecules entered the dvUTs. After the simulation was completed, the number of bound ^{14}C -urea was recorded, denoted as N_{bound}^{lab} . Corresponding to each value of N_{enter}^{non} , the average of N_{bound}^{lab} was achieved by using the values of N_{bound}^{lab} in 1000 simulations. N_{enter}^{non} was varied from 0 to $(N_{enter}^{non})_{max}$ continuously,

and the above simulation was repeated for each $N_{\text{enter}}^{\text{non}}$. Thus, the average $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{non}}$ curve was generated.

3. Results and discussions

The function of dvUT has been thoroughly characterized by Zhou and co-workers in a series of biochemical assays [8]. In the present study, using the calculated variations in the free energy of urea [18] and DMU in dvUT and computer modeling, we reproduced their experimental findings, and analyzed the microscopic process and the underlying mechanisms.

3.1. Modeling the urea flux through dvUT

In their first biochemical experiment, Zhou and co-workers measured the urea flux through dvUT in a tracer uptake assay, and obtained the time course data for the uptake of ^{14}C -urea in oocytes injected with dvUT cRNA [8]. With the aid of the MC method, we modeled and simulated the experimental process and obtained a curve of the number of urea molecules in the cell (N_{in}) vs. the average cumulative number of MC steps (N_{MC}), as described in Subsection 2.2.2. The cumulative number of MC steps, N_{MC} , is the average value of 500 MC simulations. Therefore, N_{MC} can be regarded as the average MC time for the N_{in} molecules to go through the pore. The N_{in} vs. N_{MC} curve is plotted as the red dashed line in Figure 1. Because the number of urea molecules in the cell is proportional to the amount of ^{14}C -urea uptake per oocyte, and the average cumulative number of MC steps is proportional to the real time, the simulated N_{in} vs. N_{MC} curve is similar in shape to the experimental ^{14}C -urea uptake vs. time curve. A comparison of the horizontal coordinate at the starting saturation point between the simulation N_{in} vs. N_{MC} curve and the experimental ^{14}C -urea uptake vs. time curve shows that the mean MC step corresponds to 4.9×10^{-9} min (295 ns). Analogously, at the starting saturation point, the number of urea molecules in an oocyte cell (100) in the simulation corresponds to the concentration of ^{14}C -urea molecules absorbed by an oocyte (53 pmol) in the experiment. According to this proportion, the abscissa of the N_{in} vs. N_{MC} curve is expanded by 4.9×10^{-9} times, and the ordinate is expanded by 0.53 times. In this way, the simulated N_{in} vs. N_{MC} curve is translated into a simulated ^{14}C -urea uptake vs. time curve, which is plotted as the solid black line in Figure 1.

It can be seen from Figure 1 that our simulated ^{14}C -urea uptake-time curve is in good agreement with the experimental data points. It should be pointed out that our simulation results are insensitive to the initial intracellular and extracellular concentrations of urea, c_{in} and c_{ex} . As long as the initial ratio $c_{\text{in}} / c_{\text{ex}}$ is sufficiently small (e.g., 1/100 or 1/500), the simulation results remain unchanged.

3.2. Modeling equilibrium binding of urea to dvUT

In their second biochemical experiment, Zhou and co-workers measured equilibrium binding of urea to dvUT with a scintillation proximity assay (SPA) [8]. To model and simulate this experiment and analyze the microscopic process and the underlying mechanisms, a cooperative binding model was established as follows.

3.2.1. A cooperative binding model—Cooperativity is common in ligand binding. For example, in the case of positive cooperativity, if ligand molecules bind to multimeric proteins, binding of additional ligand molecules to the multimeric proteins can often be enhanced [20]. To describe quantitatively the cooperative behavior, we set up a cooperative binding model. The model includes two key ideas. (i) There are two basic ways for the binding of ligand molecules and multimeric proteins, non-cooperative binding and the strongest cooperative binding. Common cooperative binding can be considered as a mixture of these two approaches, and the strength of cooperativity is determined by the proportion of the strongest cooperative binding. (ii) In the case of the strongest positive cooperativity, a ligand molecule entering into a multimeric protein must surely bind to the protein if the protein has one or more empty binding sites.

Our proposed cooperative binding model is composed of two parts:

1. A cooperativity probability p was introduced, which represents the strength of the positive cooperativity. In the case of non-cooperative binding ($p = 0$), the binding of a ligand at any site is not influenced by the presence or absence of a ligand at any other site, i.e., the binding of a ligand at any site is an independent event. In the cases of positive cooperativity ($0 < p < 1$), the bound ligands at any site facilitate the binding of subsequent ligands to the unoccupied sites. For simplicity, we assume that the cooperativity increases linearly with the occupying ratio of the binding sites. In other words, $p = kr$, where k represents the degree of cooperativity, referred to as the cooperativity parameter, and r is the occupying ratio of the binding sites in the protein. k ranges from 0 to 1. $k = 0$ means non-cooperative binding, and $k = 1$ means the strongest positive cooperativity. For urea binding to dvUT, $r = i/3$. Here, $i = 0, 1$ or 2 , which correspond to occupation of zero, one or two sites of dvUT by urea molecules, respectively. When $i = 3$, the three binding sites of dvUT are fully occupied by urea molecules, not allowing the binding of any additional urea molecule, so $i = 3$ is not considered.
2. A cooperative binding rule was established for the assignment of a urea molecule to a specific binding site. Specifically, when a urea molecule enters the pore from the solution, the site to be bound will be determined by the ratio of binding probability. From our previous paper [18], the ratio of the binding probability of urea (i.e., the ratio of the occupation probability) for the external (S_{ext}), middle (S_{mid}), and internal (S_{int}) binding sites is $0.63 : 0.31 : 0.06$. In the case of non-cooperative binding ($p = 0$), the urea molecule will occupy one of the three binding sites according to the ratio of the occupation probability of urea, no matter whether the site has been occupied or not. If the site has been occupied, the former occupant will be replaced by the newcomer, and the former occupant will return to solution. In the case of the strongest positive cooperativity ($p = 1$) the urea molecule enters one of the unoccupied sites according to the ratio of occupation probability of those unoccupied sites. For general cases in which $0 < p < 1$, urea's occupancy of the binding sites will follow the $p = 0$ case at a probability of $1 - p$, and follow the $p = 1$ case at a probability of p .

3.2.2. The relationship of the concentration of ^{14}C -urea binding to dvUT vs. the concentration of ^{14}C -urea in solution

In addition to the established cooperative binding model, we set up a MC protocol to simulate the equilibrium binding of urea to dvUT as described in Subsection 2.2.3. In the simulation, the number of dvUT proteins N_{UT} was set to 100, and the number of ^{14}C -urea molecules that could enter a dvUT and bind to a binding site, $N_{\text{enter}}^{\text{lab}}$, was set to 30000. With the MC method, the average $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{lab}}$ relation (data not shown) was generated. Here, $N_{\text{bound}}^{\text{lab}}$ is the number of bound ^{14}C -urea. The $N_{\text{bound}}^{\text{lab}}$ vs. $\log_{10} N_{\text{enter}}^{\text{lab}}$ curve was plotted (not shown). Because the number of the ^{14}C -urea molecules entering the pore is proportional to the concentration of free ^{14}C -urea in solution (i.e., $N_{\text{enter}}^{\text{lab}} \propto [\text{}^{14}\text{C-urea}]_{\text{free}}$), and because the number of the bound ^{14}C -urea is proportional to the concentration of the bound ^{14}C -urea (i.e., $N_{\text{bound}}^{\text{lab}} \propto [\text{}^{14}\text{C-urea}]_{\text{bound}}$), the simulated $N_{\text{bound}}^{\text{lab}}$ vs. $\log_{10} N_{\text{enter}}^{\text{lab}}$ curve is similar in shape to the experimental $[\text{}^{14}\text{C-urea}]_{\text{bound}}$ vs. $\log_{10} [\text{}^{14}\text{C-urea}]_{\text{free}}$ curve. By superimposing the half-saturation point of the simulated $[\text{}^{14}\text{C-urea}]_{\text{bound}}$ vs. $\log_{10} [\text{}^{14}\text{C-urea}]_{\text{free}}$ curve with the half-saturation point of the experimental $[\text{}^{14}\text{C-urea}]_{\text{bound}}$ vs. $\log_{10} [\text{}^{14}\text{C-urea}]_{\text{free}}$ curve, the simulated $N_{\text{bound}}^{\text{lab}}$ vs. $\log_{10} N_{\text{enter}}^{\text{lab}}$ curve is converted into a simulated $[\text{}^{14}\text{C-urea}]_{\text{bound}}$ vs. $\log_{10} [\text{}^{14}\text{C-urea}]_{\text{free}}$ curve. The latter curve is shown as the solid black line in Figure 2a. In our simulations, the cooperativity parameter k of 0.3 yields the best agreement with the experimental data points.

3.2.3. The occupation rates at urea's three binding sites

Our modeling of equilibrium binding of urea not only reproduced the experiment of equilibrium binding of urea to dvUT, but also revealed the microscopic process of urea occupying the binding sites. Moreover, the modeling uncovered how the cooperativity affects occupation of urea on the binding sites. Figure 2b plots the simulated relationship between the occupation rate at the three binding sites and the concentration of ^{14}C -urea in the solution for three kinds of cooperativity ($k = 0, 0.3$ and 1). According to our cooperative binding model in the case of non-cooperativity ($k = 0$), urea molecules occupy S_{ext} , S_{mid} , and S_{int} with a ratio of occupation probability of $0.63 : 0.31 : 0.06$, respectively. It can be seen from Figure 2b that as the concentration of ^{14}C -urea in the solution increases, the external binding sites are quickly filled (black) , then the middle binding sites are filled (red) , and the internal sites are not fully filled until $[\text{}^{14}\text{C-urea}]_{\text{free}} = 178 \text{ mmol/l}$ (green) .

In the case of a positive cooperativity, a urea molecule occupying one binding site facilitates the binding of a subsequent urea molecule binding to the unoccupied sites in the same dvUT. Consequently, the previous rarely occupied sites get more chances of being occupied. Our simulations reveal that the cooperativity more significantly accelerates occupation of urea on the binding sites with smaller occupation probability. Specifically, in Figure 2b, the relationships between the occupation rate at the external sites (with the largest occupation probability) and the concentration of ^{14}C -urea in the solution are almost identical for both non-cooperativity ($k = 0$, represented by black) and the strongest cooperativity ($k = 1$, represented by black). However, when the internal sites (with smaller occupation probability) are filled, the concentration of $[\text{}^{14}\text{C-urea}]_{\text{free}}$ is 178 mmol/l for non-

cooperativity ($k = 0$), and 25 mmol/l for the strongest cooperativity ($k = 1$). Overall, the effect of cooperativity on the occupancy rates is largest at S_{int} , second largest at S_{mid} , and least at S_{ext} , which is consistent with the increasing order of the occupation probability of S_{int} , S_{mid} , and S_{ext} . This phenomenon is quantitatively analyzed by our simulations as follows. In the case of the strongest cooperativity ($k = 1$), among the 100 bound urea molecules, an average of 16.5, 44.6 and 84.6 urea molecules benefit from the cooperative effect on the binding sites S_{ext} , S_{mid} , and S_{int} , respectively. Namely, most internal sites are occupied by cooperative binding urea molecules. As aforementioned, even in the case of the strongest cooperativity, only 16.5% of the urea molecules bound to the external binding sites are cooperative. Therefore, the effect of cooperativity on the urea molecules bound to the external binding sites is minimal.

3.3. Modeling the substitution of non-labeled urea for bound labeled ^{14}C -urea

In their third biochemical experiment, Zhou and colleagues measured the substitution of non-labeled urea for bound labeled ^{14}C -urea in an SPA-based competition assay. They plotted the relationship of the specific luminescent counts per minute generated from ^{14}C -urea binding to dvUT vs. the concentration of non-labeled urea in the solution [8] (see Figure 3). To simulate the experimental process and reproduce the experimental curve in Figure 3, we designed the MC protocol as described in Subsection 2.2.4. In the simulations, the number of dvUT proteins (N_{UT}) was set to 8000. Accordingly, the number of ^{14}C -urea molecules that could enter a dvUT and bind to a binding site ($N_{\text{enter}}^{\text{lab}}$) was set to 1479, equivalent to 181 $\mu\text{mol/L}$ of ^{14}C -labeled urea. The maximum number of non-labeled urea molecules, $(N_{\text{enter}}^{\text{non}})_{\text{max}}$, was set to 4.09×10^5 , equivalent to 50 mmol/L of non-labeled urea.

The details of how to obtain the values of $N_{\text{enter}}^{\text{lab}}$ and $(N_{\text{enter}}^{\text{non}})_{\text{max}}$ can be found in Appendix:

Determination of $N_{\text{enter}}^{\text{lab}}$ and $(N_{\text{enter}}^{\text{non}})_{\text{max}}$. Using the MC simulations (see Subsection 2.2.4), we generated the relationship of the number between the bound ^{14}C -urea molecules and non-labeled urea molecules that entered dvUT and bound to the binding sites, i.e., the average $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{non}}$ relation (data not shown). Because the number of non-labeled urea molecules entering dvUTs and binding to a binding site of dvUT ($N_{\text{enter}}^{\text{non}}$) is proportional to the concentration of the non-labeled urea molecules in the solution ([non-labeled urea]), and because the number of ^{14}C -labeled urea molecules bound to dvUT ($N_{\text{bound}}^{\text{lab}}$) is proportional to the specific luminescent counts per minute generated by the binding of ^{14}C -urea to dvUT, the simulated $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{non}}$ curve is similar in shape to the experimental curve of the binding of ^{14}C -urea to dvUT vs. the concentrations of non-labeled urea in the solution (i.e., ^{14}C -urea binding vs. [non-labeled urea] curve). Using the maximum specific luminescent counts per minute (4000), which was extracted from the experimental ^{14}C -urea binding vs. [non-labeled urea] curve, to scale the simulated $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{non}}$ curve, the simulated $N_{\text{bound}}^{\text{lab}}$ vs. $N_{\text{enter}}^{\text{non}}$ curve was converted to the simulated ^{14}C -urea binding vs. [non-labeled urea] curve, which is plotted as the solid black line in Figure 3. Comparing our simulation

curve and the experimental data points, it can be seen that the modeling of the substitution of non-labeled urea for bound labeled ^{14}C -urea is successful.

3.4. Modeling the substitution of the urea analogue, DMU, for the bound labeled ^{14}C -urea

In addition to measuring the substitution of non-labeled urea for bound labeled ^{14}C -urea in dvUT, Zhou and co-workers also measured the substitution of DMU for bound labeled ^{14}C -urea [8]. Because the DMU molecule is significantly larger than urea, the process of the substitution of DMU for ^{14}C -urea is expected to be different from the substitution of non-labeled urea for ^{14}C -urea. To model and simulate the substitution of DMU for bound labeled ^{14}C -urea, it is necessary to determine the DMU-binding sites in dvUT and establish a urea-DMU substitution rule.

3.4.1. The binding sites and binding probabilities of DMU in dvUT—To find the most stable resident positions of DMU in dvUT, we calculated the variation in the free energy of DMU, $G(Z)$, along the pore axis Z by the adaptive biasing force approach used in our previous paper [18]. Figure 4a shows the profile of the variation in the free energy of DMU along the axis of the dvUT pore. The variation in the cross-sectional area of pore along the Z axis [18] is also plotted in the same figure. It can be seen from the curve of the cross-sectional area of pore and the axis of dvUT pore that the two orifices of the pore are located approximately at $-11 \text{ \AA}$ and $11 \text{ \AA}$, respectively. It can also be seen that between the two orifices of the pore there are three minima (energy valleys) in the curve of the variation in the free energy vs Z . They are located at $z = -5, 0$, and $10 \text{ \AA}$, respectively.

Based on the calculated variation in the free energy of DMU, we calculated the occupation probabilities of DMU and determined the DMU-resident sites in dvUT by using the MC method described in Subsection 2.2.1. The occupation probability of DMU vs the Z -coordinate along the whole pore was plotted in Figure 4b in both normal scale and logarithmic scale. The curve of the occupation probability of DMU and the axis of dvUT pore in logarithmic scale exhibits three maxima in the region between the two orifices. The calculation of the area under this curve shows that the ratio of the occupation probabilities was 0.917: 0.004: 0.079 for the three regions at $z \approx -5 \text{ \AA}$, 0 , and $10 \text{ \AA}$. In other words, the occupation probability at the middle region of the pore ($z \approx 0$) is 230-fold less than the occupation probability at the external region ($z \approx -5 \text{ \AA}$) and 20-fold less than the occupation probability at the internal region ($z \approx 10 \text{ \AA}$). This difference explains why the occupation probability at the middle region ($z \approx 0$) cannot be seen in the figure plotted in the normal scale.

We define the sites with the maximum occupation probabilities as the DMU-binding sites, at which DMU molecules bind strongly to the pore. It can be seen from Figure 3b that in dvUT the DMU binding sites locate approximately at $Z = -5.0 \text{ \AA}$ and $10.0 \text{ \AA}$, which are defined as the external (S_{ext}) and internal (S_{int}) resident sites, respectively. In the middle region of the pore ($z \approx 0$) the occupation probability has a tiny value. Our previous study [18] has shown that in the structure of the dvUT channel, $z = 0$ is the unstable equilibrium position of urea and DMU. In Figure 3a the curve of the variation in the free energy vs the axis of the dvUT pore shows that the minimum in the middle region of the pore ($z \approx 0$) is flanked by two high,

wide, and steep energy barriers. It is difficult for urea and DMU molecules to enter the middle region. Even if they enter the middle region, they jump out easily. Therefore, the middle region of the dvUT pore ($z \approx 0$) is not the binding site of the urea /DMU molecule.

3.4.2. The urea-DMU substitution rule—In dvUT there exist three binding sites (S_{int} , S_{mid} and S_{ext}) for urea [18], and two binding sites (S_{int} and S_{ext}) for DMU. How do urea and DMU bind to dvUT in the simultaneous presence of urea and DMU in the dvUT? Let's make a rough estimation. Generally, the radii of the atoms in contact with each other of the adjacent molecules are represented by van der Waals (VDW) radii. In the NAMD software the VDW radius of the hydrogen atom of the DMU is 1.34 Å, and the corresponding value of the urea is 0.22 Å. This difference is caused by the difference between the terminal structure of DMU and that of urea. Therefore, when a urea molecule is in contact with a DMU, the distance between the centers of the two molecules is 6.6 Å. The distance from the common external binding site of urea and DMU to the middle binding site of urea is 9 Å, and the distance from the internal binding site of DMU to the middle binding site of urea is 6 Å. If a DMU molecule occupies the external binding site and a urea molecule occupies the middle binding site, there is a gap of 2.4 Å between the DMU and the urea, so this binding mode combination is feasible. In another case, if a DMU molecule occupies the internal binding site and a urea molecule occupies the middle binding site, there is an overlapping region of 0.6 Å between the DMU and the urea, so this binding mode combination is not feasible. In this way, we can illustrate all kinds of possible conformations with which urea and/or DMU can completely occupy the binding sites of dvUT (see Figure 5). It can be seen from Figure 5 when a DMU molecule occupies the internal binding site it not only occupies the region of the internal binding site of urea, but also partially occupies the region of the middle binding site of urea. Particularly, the DMU molecule is long enough to stretch to the region with higher potential energy at $z \approx 7$ Å. It is expected that a urea molecule entering the internal or middle site can not replace the DMU molecule previously bound to the internal site, because this kind of replacement would cost a big energy.

Based on the above analysis, a urea-DMU substitution rule was established as follows: When a DMU molecule enters a dvUT, if it attempts to bind the S_{ext} site, the urea molecule previously bound to the S_{ext} site will be replaced; if it attempts to bind the S_{int} site, the urea molecule(s) previously bound to the site(s) S_{mid} or/and S_{int} will be replaced. When a urea molecule enters a dvUT and attempts to bind the S_{ext} , S_{mid} or S_{int} site, if the S_{ext} site has been occupied by a DMU molecule, the urea will replace the DMU; however, if the S_{int} site has been occupied by a DMU molecule, the urea can bind neither the S_{mid} site nor the S_{int} site.

3.4.3. The relationship between the concentration of the bound and unsubstituted ^{14}C -urea and the concentration of DMU in solution—We performed MC simulations on the substitution of DMU for bound ^{14}C -urea, which were similar to the aforementioned simulations on the substitution of non-labeled urea for the bound ^{14}C -urea. Specifically, in the simulations of the substitution of DMU for ^{14}C -urea, the number of dvUTs (N_{UT}) was set to 8000, the number of ^{14}C -urea molecules entering one of the binding sites in dvUTs ($N_{\text{enter}}^{\text{lab}}$) was 1479, and the number of DMU molecules entering

one of the binding sites in dvUTs ($N_{\text{enter}}^{\text{DMU}}$) ranged from 0 to 4.09×10^5 . The details of the modeling (including how the above numbers were chosen) are provided in the Appendix A: Determination of $N_{\text{enter}}^{\text{lab}}$ and $(N_{\text{enter}}^{\text{non}})_{\text{max}}$. Here, $(N_{\text{enter}}^{\text{non}})_{\text{max}}$ is the maximum value of $N_{\text{enter}}^{\text{DMU}}$.

For each assigned $N_{\text{enter}}^{\text{DMU}}$, such simulations were performed 1000 times. Using the procedure similar to the procedure described in Subsection 3.3 and the urea-DMU substitution rule, the simulated ^{14}C -urea binding vs. [DMU] curve was obtained, which is plotted as the solid red line in Figure 3. Our simulated curve agrees with the experimental data points.

4. Conclusions

Urea transporters (UTs) play a key role in urea excretion and water balance for organisms. The flux, binding and substitution of urea are the basic functional characteristics of UTs. Based on the crystal structure of dvUT and with the use of the MC and MD methods, these functional characteristics were modeled and simulated. The modelings reveal the process of the flux, binding and substitution of urea in the dvUT at the molecular level. The results show that urea flow is the diffusion of urea molecules under the gradient of the urea concentration across the cell membrane and the motion is affected by the potential field of dvUT. The mutations of the residues along the pore have an impact on urea flow by changing the potential field and the pore size of dvUT. The modelings also show that equilibrium binding of urea to dvUT is cooperative. The proposed cooperative binding model can quantitatively describe the cooperative behaviors of the equilibrium binding of urea to dvUT. This model can also be applied to ligand binding to a receptor with positive cooperativity. In addition, the two binding sites of DMU in dvUT were determined by calculating the variation in the free energy of DMU in dvUT. Because there are two binding sites of DMU but three binding sites of urea in dvUT, a small amount of DMU can replace more urea molecules. Based on the analysis of the binding sites, and the sizes of urea and DMU molecules, the urea-DMU substitution rule has been established. With this rule, the substitution of urea by DMU in the dvUT was modeled. The present study shows that such biological processes can be reproduced at the molecular level by modeling and simulation. It leads to a deeper understanding of the functional characteristics of UTs and would help related rational drug design.

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Appendix: Determination of Nenterlab and (Nenternon)max

To model and simulate the substitution of non-labeled urea and DMU molecules for ^{14}C -urea, the number of ^{14}C -labeled urea molecules bound to dvUTs, $N_{\text{bound}}^{\text{lab}}$, and the maximum number of non-labeled urea molecules entered and bound to dvUTs, $(N_{\text{enter}}^{\text{non}})_{\text{max}}$, were determined as follows. It can be seen from Figure 2a that when urea binding is saturated, the

concentration of bound ^{14}C -urea, $([^{14}\text{C}\text{-urea}]_{\text{bound}})_{\text{satur}}$, is $0.24 \mu\text{mol/L}$. Because each dvUT has three urea-binding sites, the concentration of dvUTs, $[\text{dvUT}]$, is therefore $0.24/3 = 0.08 \mu\text{mol/L}$. Corresponding to our simulation the number of dvUTs, N_{UT} , is 8000. On the other hand, the concentration of ^{14}C -urea in solution, $[^{14}\text{C}\text{-urea}]_{\text{free}}$, is $181 \mu\text{mol/L}$ (see “METHODS: SPA-based binding assay” in the Ref.8). Thus, the number of ^{14}C -labeled urea molecules in the solution, $N_{\text{free}}^{\text{lab}}$, is $181 N_{\text{UT}}/0.08 = 1.81 \times 10^7$. Moreover, the maximum concentration of non-labeled urea in solution, $[\text{Non-labeled urea}]_{\text{free}}$, is 50 mmol/L (see Figure 3), which corresponds to the maximum number of non-labeled urea molecules in the solution, $(N_{\text{free}}^{\text{non}})_{\text{max}} = 5 \times 10^4 N_{\text{UT}}/0.08 = 5 \times 10^9$. However, not all ^{14}C -labeled and non-labeled urea molecules in the solution have the opportunity to enter the dvUTs, instead, only a portion of them do. By fitting the half-saturation point of the simulation $N_{\text{bound}}^{\text{lab}}$ vs. $\log_{10} N_{\text{enter}}^{\text{lab}}$ curve to that of the experimental $[^{14}\text{C}\text{-urea}]_{\text{bound}}$ vs. $\log_{10} [^{14}\text{C}\text{-urea}]_{\text{free}}$ curve (see Figure 2a), the transformation factor between $N_{\text{free}}^{\text{lab}}$ and $N_{\text{enter}}^{\text{lab}}$ was evaluated, and a proportional coefficient A of 12241 was achieved. From the simulations, $N_{\text{enter}}^{\text{lab}}$ was found to be $1.81 \times 10^7/12241 = 1479$, and the maximum number of non-labeled urea that entered and bound to the dvUTs, $(N_{\text{enter}}^{\text{non}})_{\text{max}}$, was $5 \times 10^9/12241 = 4.09 \times 10^5$.

Highlights

- Proposed a cooperative binding model & Monte Carlo protocols for urea transporters.
- Modeled the urea flux in the urea transporter dvUT.
- Modeled equilibrium urea binding to dvUT.
- Modeled the substitution of urea by the urea analogue DMU in dvUT.
- The modeling results are in agreement with the experiments.

Figure 1.

The relationship between the urea flux and time. The solid black line shows the MC simulation results. The solid black squares are the experimental data points of the uptake of ^{14}C -labeled urea molecules in oocytes [8]. The red dashed line shows the simulated relationship between the number of urea molecules in an oocyte cell and the cumulative number of MC steps.

Figure 2.

Equilibrium binding of urea to dvUT. (a) Simulated (solid black line) and experimental [8] (solid black squares) relationship of the concentration of $^{14}\text{C-urea}$ binding to dvUT vs. the concentration of $^{14}\text{C-urea}$ in solution. In these simulations, the cooperativity model with a cooperativity degree k of 0.3 was used. (b) Simulated relationship between the occupation rate of the binding sites and the concentration of $^{14}\text{C-urea}$ in solution for the external (S_{ext} , black), middle (S_{mid} , red), and internal (S_{int} , green) binding sites, respectively. The simulated results for non-cooperative binding ($k = 0$), present cooperative binding ($k = 0.3$) and the strongest cooperative binding ($k = 1$) were denoted by symbol $\triangle$, solid line, and symbol $\bullet$, respectively.

Figure 3.

Substitution of the non-labeled urea (black) and DMU (red) for ^{14}C -urea binding to dvUT. [compound] represents the concentration of non-labeled urea ([non-labeled urea]) or the concentration of DMU ([DMU]) in the solution. The solid lines stand for the simulated results. The symbols show the experimental data points [8]. “sp.c.p.m” is the abbreviation of “specific counts per minute”.

Figure 4.

(a) Variation in the free energy of DMU along the axis of the dvUT pore. The red dashed line represents the cross-sectional area of the pore along the pore axis. (b) The occupation probability of DMU along the pore axis. The red dashed line shows the plot drawn in $\log_{10}$ -scale on the Y-axis.

Figure 5.

Binding modes of urea and its analogue DMU in dvUT. Top: The structures of urea (left) and DMU (right). Bottom: Various conformations with which the urea and/or DMU molecules completely occupy the binding sites of dvUT. (a) Three urea molecules occupy the external (S_{ext}), middle (S_{mid}), and internal (S_{int}) sites, respectively. (b) A urea molecule occupies the S_{ext} site, and a DMU molecule occupies the S_{int} site. (c) A DMU molecule occupies the S_{ext} site, and two urea molecules occupy the S_{mid} and S_{int} sites, respectively. (d) Two DMU molecules occupy the S_{ext} and S_{int} sites, respectively. Oxygen, carbon, nitrogen and hydrogen atoms are colored red, cyan, blue and white, respectively.