Wide-Open Flaps Are Key to Urease Activity

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S *Supporting Information*

ABSTRACT: Substrate ingress and product egress from the active site of urease is tightly controlled by an active-site flap. Molecular dynamics simulations of urease have revealed a previously unobserved wide-open flap state that, unlike the well-characterized closed and open states, allows ready access to the metal cluster in the active site. This state is easily reached from the open state via low free energy barriers. Additionally, we have found that even when the flap is closed, a region of the binding pocket is solvent-exposed, leading to the hypothesis that it may act as a substrate/product reservoir. The newly identified wide-open state offers further opportunities for small-molecule drug discovery by defining a more extensive active-site pocket than has been previously described.

Helicobacter pylori, a bacteria found in the stomach, causes stomach ulcers, adenocarcinoma of the distal stomach, and lymphoma of the gastric mucosa[1−3] and has a 2−4% mortality rate among infected humans.[4] According to the Centers for Disease Control, two-thirds of people worldwide are infected with *H. pylori*, with the highest percentage from regions of western Africa, South America, and northern China.[5] Currently, *H. pylori* infection cannot be cured with a single drug. Instead, a combination of medications that cause side effects in approximately half of patients is often used.[6] This highlights the need for novel antimicrobials that target *H. pylori*, and urease is one such target.[7] However, the urease active-site region in the available crystal structures is highly constrained around the metal ion,[3] thereby allowing only small-molecule inhibitors such as acetohydroxamic acid,[3] boric acid,[8] or phosphate[9] that can both bind the metal cluster and satisfy the constraints of the active site. Herein we report our identification of a novel wide-open state of the urease active site that offers new opportunities for small-molecule drug discovery by defining a more extensive binding pocket that may or may not require a metal-binding warhead.

The ureases are a group of closely related enzymes found in certain plants, bacteria, and fungi.[10,11] Notably associated with *H. pylori* and other pathogenic species, ureases offer attractive targets for drug design because of their role in protecting the pathogen from the highly acidic pH of the gut.[12] Catalyzing the breakdown of urea into ammonia and carbamate,[13] they are extremely efficient enzymes, speeding up this reaction by at least 14 orders of magnitude and turning over several thousand substrate molecules per second.[14−16]

The ureases are multimeric, with each active site containing a dinickel cluster.[17] The precise mechanism of the enzyme-catalyzed reaction is not yet fully understood,[13,15,18−22] but in addition to breaking down urea, the catalytic cycle appears to facilitate large-scale protein motion such as diffusion of urease enzymes.[23] Each active site is capped by a 33-residue flap that governs access to and egress from the dinickel cluster.[15] In this study, we used classical molecular dynamics (MD) simulations to study the motion of these flaps.

We chose the urease from the bacterium *Klebsiella aerogenes* (KA) for study (see Figure 1) rather than *H. pylori* urease
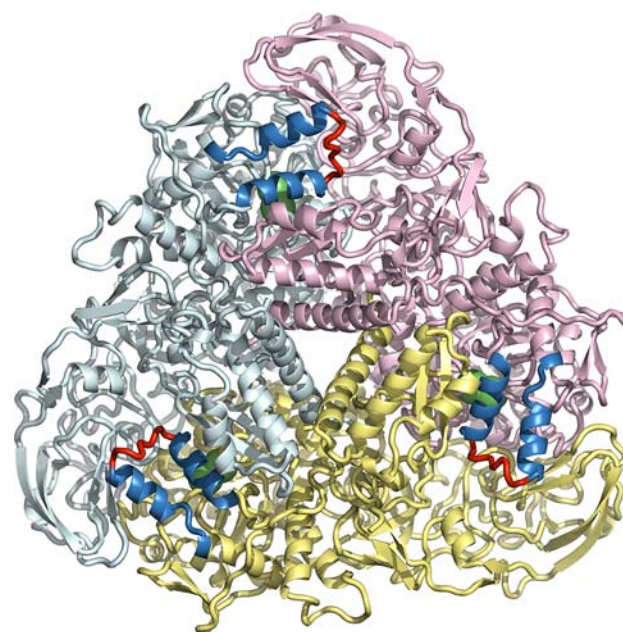

Figure 1. Initial structure of *K. aerogenes* urease in the open state, as used in our simulations. The trimeric subunits are shown in yellow, pink, and cyan. The flaps are shown as α-helices (blue) and loops (red). Nickel ions are shown as green spheres.

because of the immense size of the latter (150 000 atoms) relative to the former (35 000 atoms). Moreover, the flap and active-site regions are well-conserved between the two systems (see below), allowing us to extrapolate from KA to *H. pylori* realistically with regard to the dynamics in these regions. Nonetheless, simulations of *H. pylori* urease using modern graphics processing unit (GPU) technology are underway and will be reported in due course. KA urease is a homotrimer of heterotrimers and contains three active sites.[24] We ran two separate simulations, one starting from a structure in which all three flaps are closed (PDB entry 1FWJ) and the other from a
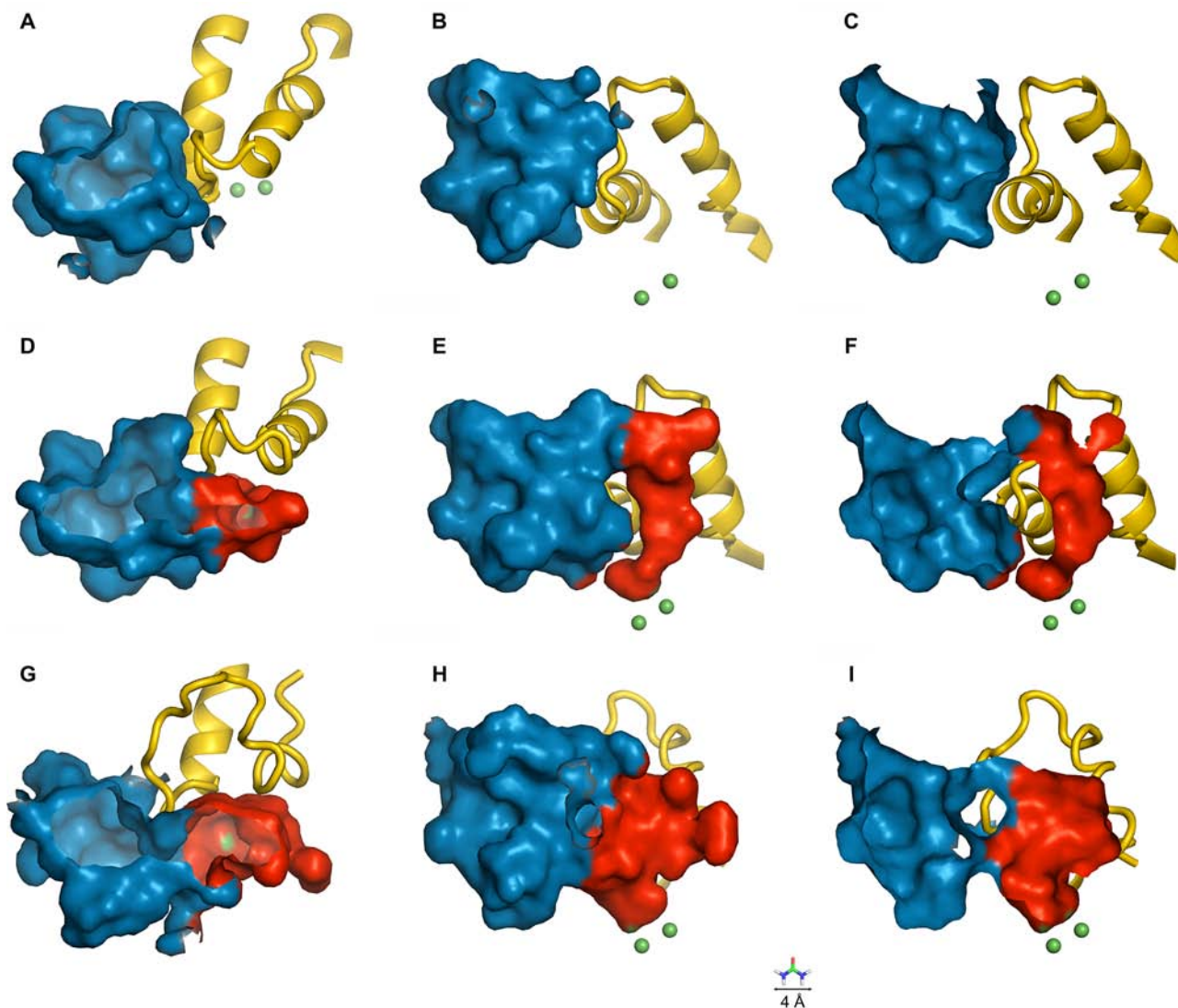
Figure 2. The flap, active site, and ancillary binding pocket of *K. aerogenes* urease. The flap is shown in yellow, the active site in red, and the ancillary binding pocket in blue. The nickel ions are shown as green spheres, and the exposed nickel surfaces in D−I are also shown in green. The three rows from top to bottom show the closed flap (A−C), the open flap (D−F), and the wide-open flap (G−I). In each row, three views are shown: from the top, looking down into the binding pockets (A, D, G); from the side (B, E, H); and from the side in a cutaway view (C, F, I). To show the scale, a urea molecule (4 Å across) is depicted at the bottom of the figure.

structure in which all three flaps are open (PDB entry 1EJX). We performed a symmetry expansion to generate the $(\alpha\beta\gamma)_3$ form. By running 180 ns of simulation on the closed-flap structure and 100 ns of simulation on the open-flap structure, we generated 840 ns (280 ns × 3 active sites) of flap dynamics.

The flap itself comprises three regions: two short α-helices and between them a flexible loop. The channel into the active site, protected by the flap, lies on the border between two trimeric subunits, with the third subunit lying on the other side of the channel from the flap. Each of the helices is able to tilt away from this other subunit, bringing the flap into one of two partially open states; when both α-helices do so at the same time, the flap enters the open state. Inspection of the crystal structures from the Protein Data Bank and our simulation trajectories revealed that neither the partially open state nor the open state allows ready access to the active-site region of urease, posing the fundamental question of how the substrate enters the dinickel active site.

Pertinent to this question, our simulations have revealed a new wide-open state. We propose that this state is important for substrate entry and product exit. It is distinguished from the open state by a loss of helical character in the α-helices with consequent extension of the loop into neighboring residues. Indeed, the loop itself appears to have characteristics of an intrinsically unstructured protein[25] that has multiple states it can access in the resting state, but it becomes ordered (forming the closed state) upon substrate or inhibitor binding. In this wide-open state, the extended loop moves away from the protein, opening up a wide pathway into the active site.

The closed, open, and wide-open states are shown in Figure 2. In the closed state (Figure 2A−C), the flap acts to seal off the dinickel cluster from the bulk solvent entirely. In the open state (Figure 2D−F), the flap moves slightly, enough to open only a very narrow pathway into the dinickel cluster. This pathway is too narrow to admit even a molecule of urea. Much of the obstruction of the active-site channel is due to a single residue, His 320 in the α subunit. This residue is thrust into the binding

pocket, dividing it into two parts and severely restricting access to the dinickel cluster. As part of the transition from the open state to the wide-open state, His 320 moves out of the way, opening up a wide pathway and allowing substrate molecules ready access to the dinickel cluster [Figure 2G–I; also see the Supporting Information (SI)]. In all three states, however, an interesting aspect of the binding pocket is revealed. An ancillary pocket remains open even while the flap is closed, although the function of this pocket is unknown. One hypothesis is that it may act as a substrate/product reservoir that is perfectly placed to take advantage of the increased access to the active site once the protein enters the wide-open state. However, it also may be a site for regulator binding or even simply a vestige of evolution.

We investigated the possibility that the ancillary pocket acts as a substrate-specific binding site by comparing the PDB structure of KA urease with the structures of three other ureases, namely, those from *H. pylori*, *Bacillus pasteurii*, and jack bean. We found that the ancillary pocket exists in all four ureases and potentially fulfills the role of a reservoir; however, the amino acid residues that make a significant contribution to its surface are less conserved, on average, than in the protein at large. This may be contrasted with the flap itself, which is more highly conserved. On the basis of the structural and sequence analysis work, we hypothesize that it is unlikely that the ancillary pocket acts as a ligand-specific tight binding site.

From our simulation data, we computed a relative free energy map based on two separate distances between α-carbon atoms in residues across the flap opening from each other. One of these distances, r_{HG}, spans the gap between His 320 in the α subunit of trimer 1 and Gly 46 in the α subunit of trimer 3; the other, r_{IA}, spans the gap between Ile 326 in the α subunit of trimer 1 and Ala 68 in the β subunit of trimer 3. Values for r_{HG} and r_{IA} were also obtained from the other two flaps, at the trimer 3–trimer 2 and trimer 2–trimer 1 interfaces. A figure depicting r_{HG} and r_{IA} is included in the SI. The free energy map (Figure 3) shows that transition between the closed and open states is facile, with an activation barrier of ~1.5 kcal mol^{-1} and a ΔG of less than 1 kcal mol^{-1}. The wide-open state is less populated than either the closed state or the open state ($\Delta G \approx$ 2.5 kcal mol^{-1}). Nevertheless, it is readily accessible from the open state via two pathways, each with an activation barrier of no more than 4 kcal mol^{-1}.

The present simulations have revealed for the first time the entire ensemble of flap states available to urease and provide insight into how substrate gains access to the active site. We note that the flap itself appears to have the characteristics of an intrinsically unstructured protein[25] that becomes more ordered upon substrate binding to urease. Moreover, the computed free energy estimates are in accord with efficient catalysis, as is the secondary pocket, which we hypothesize may accelerate substrate/product ingress/egress in an assembly-line-like manner.

In conclusion, although the open structure is not open enough to allow substrate access to the active site, simulation data point to the existence and ready accessibility of the wide-open state, in which diffusion of substrate into the active site and departure of products should be rapid. This wide-open state reveals a more expansive active-site pocket that is more suitable for exploitation by small-molecule inhibitors. Protein dynamics have been important for characterizing conformational states for key drug discovery efforts, such as for the classical case of HIV-1 protease.[26]
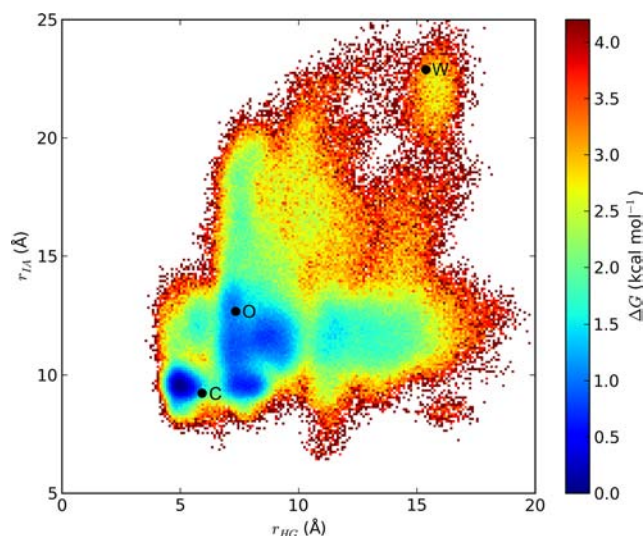
Figure 3. Relative free energy map for opening of the urease flap. The points labeled as "C", "O", and "W" respectively represent the closed, open, and wide-open structures shown in Figure 2. Points C and O represent the initial X-ray structures of the closed- and open-flap models (PDB entries 1FWJ and 1EJX, respectively); for the latter structure, the flap was added by homology modeling. Point W is a representative wide-open conformation selected from the simulation trajectory of 1FWJ.

■ ASSOCIATED CONTENT

⑤ Supporting Information

Details of computational methods and force field parameters, additional figures relating to our analysis of the simulations, and PDB text of KA urease with a flap in the wide-open conformation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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