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70 Years of Crystalline Urease: What Have We Learned?

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Introduction

Sumner's Legacy. In 1917, as an Assistant Professor at the Cornell Medical College, James B. Sumner adopted as his research focus the preparation of a pure, crystalline enzyme in order to define the chemical makeup of these biological catalysts. For this project he chose to focus on the enzyme urease from jack bean seeds. In 1926, Sumner prepared crystalline urease,[1] and summarized his results in terms of seven discrete positive supporting arguments for the assertion that the protein crystals prepared were identical to urease (Figure 1). This result was slow to find acceptance. Even a year later, the leading enzymologist Wilstätter lectured that "a far and distant object of our investigations is the isolation of enzymes in the pure state."[2]

Ureases: A Nickel-Dependent Enzyme Family. During the 1950s to 1970s, when the principles of enzyme structure, mechanism, and catalysis were being worked out, jack bean urease was shown to be a very proficient, stable, and highly specific enzyme.[3−5] The second best substrate, semicarbazide, is a poorer substrate by a factor of more than 10^3.[6] In 1975, jack bean urease was shown to contain two nickel ions per active site.[7] Further studies by Zerner and colleagues allowed them to propose a urease mechanism (Figure 2)[8] in which one nickel ion activates water, the second activates urea, and the protein provides a nearby carboxylate to stabilize a urea resonance form, as well as a catalytic base (unidentified) and a general acid (thought to be a cysteine).

Urease enzymes have now been isolated and the urease genes cloned from additional plants, bacteria, and fungi.[9,10]

> "I undertook the task of isolating urease in the fall of 1917 with the idea that it might be found to be a crystallizable globulin, in which case the proof of its isolation would be greatly simplified. Other reasons for choosing urease were that the quantitative estimation of urease is both rapid and accurate, that urease can be reasonably expected to be an individual enzyme, rather than a mixture of enzymes, and that the jack bean appears to contain a very large amount of urease....
>
> I present below a list of reasons why I believe the octahedral crystals to be identical with the enzyme urease.
>
> 1. The fact that the crystals can be seen by the microscope to be practically uncontaminated by any other material.
>
> 2. The great activity of solutions of the crystals.
>
> 3. The fact that solvents which do not dissolve the crystals extract little or no urease and that to obtain solutions of urease one must dissolve the crystals.
>
> 4. The fact that the other crystallizable jack bean globulins, concanavalin A and B, carry with them very little urease when they are formed from solutions that are comparatively rich in urease.
>
> 5. The unique crystalline habit of the octahedra and their ready denaturation by acid.
>
> 6. The fact the the crystals are purely protein in so far as can be determined from chemical tests, combined with evidence from previous work to the effect that urease behaves like a protein in its reactions toward heavy metals, alkaloid reagents, alcohol, and acids.
>
> 7. The fact that the crystals are nearly free from ash and the fact that we have previously prepared solutions of urease that contained neither iron, manganese, nor phosphorous."

FIGURE 1. Sumner's evidence that the enzyme urease was a protein. The quote is from Sumner's original paper reporting crystals of urease.[1] In addition to crystallizing urease and providing much supporting evidence that it was a protein, Sumner carried out many other fundamental studies in biochemistry, published an important monograph on proteins, and shared the 1946 Nobel Prize in Chemistry with Northrup and Kunitz, two other pioneers of enzyme crystallization.

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(1) Sumner, J. B. *J. Biol. Chem.* **1926**, *69*, 435−441.
(2) Willstätter, R. *Problems and Methods in Enzyme Research*; Cornell University: Ithaca, NY, 1927; p 13.
(3) Varner, J. E. *The Enzymes*, 2nd ed.; Academic Press: New York, 1959; Vol. 4, pp 247−256.
(4) Reithel, F. J. *The Enzymes*, 3rd ed.; Academic Press: New York, 1970; Vol. 4, pp 1−21.
(5) Sumner, J. B.; Somers, G. E. *Chemistry and Methods of Enzymes*, 3rd ed.; Academic Press: New York, 1953; p 156.
(6) Gazzola, C.; Blakeley, R. L.; Zerner, B. *Can. J. Biochem.* **1973**, *51*, 1325−1330.
(7) Dixon, N. E.; Gazzola, C.; Blakeley, R. L.; Zerner, B. *J. Am. Chem. Soc.* **1975**, *97*, 4131−4133.
(8) Dixon, N. E.; Riddles, P. W.; Gazzola, C.; Blakeley, R. L.; Zerner, B. *Can. J. Biochem.* **1980**, *58*, 1335−1344.
(9) Mobley, H. L. T.; Island, M. D.; Hausinger, R. P. *Microbiol. Rev.* **1995**, *59*, 451−480.

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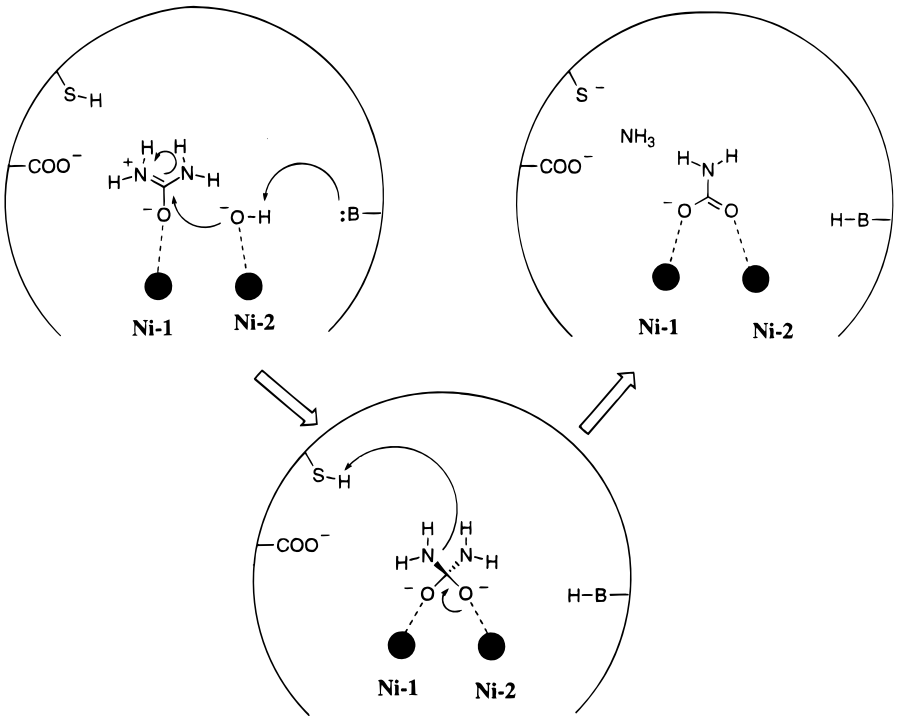
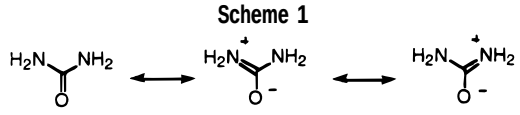


FIGURE 2. Urease mechanism as proposed in 1980.[8] One nickel ion (Ni-1) binds and activates urea, with one resonance structure stabilized by a nearby carboxylate, while the second (Ni-2) binds a hydroxide. The latter molecule is activated for attack on the urea carbon by a protein residue acting as a general base. The tetrahedral intermediate collapses, eliminating ammonia with the help of an active site thiol, which was proposed to act as a general acid.

Amino acid sequence alignments reveal that all known urease sequences are highly similar (≥50% identical), although the enzyme from different sources may be comprised of one, two, or three distinct polypeptide chains. On the basis of the similarities in sequence and reaction kinetics, it is reasonable to assume that the known ureases have a common structure and catalytic mechanism.

Enigmas of Urease Catalysis. Urea is highly stable in aqueous solutions ranging in pH from 2 to 12, with a half-life of 3.6 years at 38 °C.[11] The nonenzymatic breakdown proceeds via an elimination reaction to release ammonia and cyanic acid.[12] The stability of urea is understood to be due to its resonance energy (Scheme 1), which has been estimated at 30−40 kcal/mol.[13,14] The zwitterionic resonance forms donate electrons to the carbonyl carbon, causing it to be less reactive toward nucleophiles.

Scheme 1



In contrast to the elimination reaction undergone by urea in aqueous solution, urease carries out a true hydrolysis of urea to produce ammonia and carbamate.[15] The k_{cat}/K_M of urease is 10^{14}-fold higher than the rate of the uncatalyzed elimination reaction, implying the "proficiency" [16] of urease is $>10^{14}$, because the uncatalyzed hydrolysis reaction must be even slower. According to a common view of enzyme catalysis, the enzyme proficiency indicates how tightly the transition state is bound,[16,17] meaning that for urease the transition state binding involves >19 kcal/mol. Given that urea is a purely polar molecule with just six potential hydrogen bonding groups, what is the source of this binding energy? Furthermore, how is this binding energy focused to overcome the resonance stabilization of urea. This Account correlates the functional and structural characteristics of *Klebsiella aerogenes* urease to address these questions.

Properties of *K. aerogenes* Urease

General Properties. *K. aerogenes* urease is an oligomer with $(abg)_3$ stoichiometry ($\alpha = 60.3$ kDa, $\beta = 11.7$ kDa, $\gamma = 11.1$ kDa) and has three dinuclear active sites per molecule. The active sites behave independently according to all studies, and so enzyme kinetics are reported in terms of the activity per catalytic site. *K. aerogenes* urease has a K_M for urea of 2.5 mM and a k_{cat} of 3500 s^{-1} (corresponding to a specific activity of 2500 U/mg) at the optimal pH of 7.75.[18] The pH dependence of the enzyme kinetics reveals that the K_M is largely pH independent and the k_{cat} is strongly affected by apparent pK_a values near 6.5 and 9.0.[18] Other ureases show similar pH dependencies.[8,19,20]

(10) Hausinger, R. P. *Biochemistry of Nickel*; Plenum Press: New York, 1993; pp 23−57.
(11) Zerner, B. *Bioorg. Chem.* **1991**, *19*, 116−131.
(12) Blakeley, R. L.; Treston, A.; Andrews, R. K.; Zerner, B. *J. Am. Chem. Soc.* **1982**, *104*, 612−614.
(13) Wheland, G. W. *Resonance in Organic Chemistry*; Wiley: New York, 1955; p 100.
(14) Pauling, L. *The Nature of the Chemical Bond*; Cornell University: Ithaca, NY, 1948; p 138.
(15) Blakeley, R. L.; Hinds, J. A.; Kunze, H. E.; Webb, E. C.; Zerner, B. *Biochemistry* **1969**, *8*, 1991−2000.
(16) Radzicka, A.; Wolfenden, R. *Science* **1995**, *267*, 90−93.
(17) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 311−323.
(18) Todd, M. J.; Hausinger, R. P. *J. Biol. Chem.* **1987**, *262*, 5963−5967.
(19) Mobley, H. L. T.; Hausinger, R. P. *Microbiol. Rev.* **1989**, *53*, 85−108.
(20) Breitenbach, J. M.; Hausinger, R. P. *Biochem. J.* **1988**, *250*, 917−920.

Chemical modification and mutagenesis studies have strongly implicated two histidines as being critical for catalysis: His219 with a role in substrate binding and His320 acting as a general base with a pK_a of 6.5.[21] A His219Ala variant had a markedly increased K_M of 1100 mM and a decreased $k_{cat} = 60\ s^{-1}$, for a 10^4-fold decrease in k_{cat}/K_M. The His320Ala variant exhibited only moderate changes in K_M but possessed a nearly 10^5-fold reduction in k_{cat}. A third residue, Cys319, had originally been assigned as an essential residue because its modification inactivates urease, but the activity of a Cys319Ala variant has shown it is not critical.[22,23]

Three-Dimensional Structures. The crystal structure of *K. aerogenes* urease revealed a globular, 3-fold symmetric "trimer of trimers" structure.[24,25] The enzyme is a flattened sphere with a diameter of 110 Å and a height of 60 Å. The β and γ subunits each form single structural domains, and the large α subunit is composed of two structural domains, the larger of which is an $(\alpha/\beta)_8$ barrel and contains the metallocenter. The dinuclear active sites of the urease trimer are roughly 70 Å apart and appear to be completely independent. All residues in the protein are well-ordered except for residues 317−334 on the α chain which forms a mobile flap that covers the active site. This flap contains the key residue His320, as well as Cys319.

The metallocenter of urease contains two nickel ions, designated Ni-1 and Ni-2, which are ~3.5 Å apart and liganded by three and four protein atoms, respectively. One unusual feature is that the metal ligands include a carbamylated lysine. The presence of such a ligand was anticipated by biochemical studies showing that CO_2 was required for productive nickel ion binding to the urease apoenzyme.[26] The native enzyme structure reveals that the water−nickel interactions are somewhat heterogeneous; the electron density was originally interpreted as a single dominant hydration site on Ni-2 (Wat-502) but has now been extended to include a Ni-1 (Wat-501) and a bridging (Wat-500) hydration site with weaker occupancy.[24,27] Adjacent to the nickel ions is a small, solvent-filled pocket which is roughly the size of urea and lined by the side chains of His219, Cys319, His320, Arg336, Met364, and the main chain carbonyl oxygens of residues Ala167, Gly277, and Ala363. With the exception of Cys319, which is replaced by Thr in one urease sequence,[28] the side chains listed are conserved among known urease sequences.

Currently, we have solved 14 urease structures at 2.5 Å resolution or better. These include wild-type urease at ambient and cryogenic temperatures, the urease apoenzyme, and many mutants. An overlay of all of these structures allows three clear conclusions to be drawn (Figure 3): first, the bulk of the protein including all of the metal ligands and most of the groups lining the active site are well superimposable in all of the structures; second, the active site flap varies in terms of conformation and mobility among the structures, in some mutants being better fixed and in others being so disordered as to be invisible in the electron density map; and third, the hydration pattern of the nickel ions varies greatly between the structures. Despite the variability in the flap, all but one of the structures with an ordered His320 place it in roughly the same position; the exception is the inactive C319Y variant.

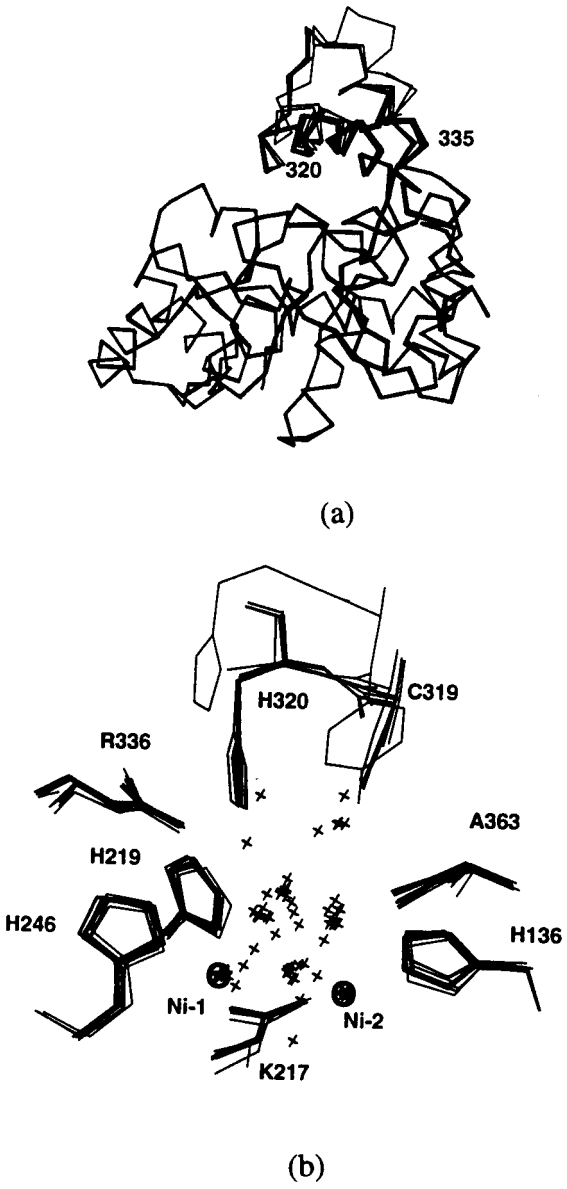


FIGURE 3. Structural variation in the active site flap and active site solvent molecules among 14 refined *K. aerogenes* urease structures. (a) Overlay of Ca atoms in the large $(a/b)_8$ barrel domain of the a subunit, which contains the active site. There is no variation in the structure of the barrel itself; the only obvious structural variation is in residues 317−335, the flexible active site flap which covers one end of the barrel. (b) Overlay of the active site residues, nickel ions (circled crosses), and water molecules (crosses). The conformations of the nickel ligands are consistent, even in structures that have lost one or both metal ions, indicating that the nickel binding site is highly preorganized. However, the active site solvent structure is highly variable, reflecting its sensitivity to mutations of active site residues and changes in temperature.

(21) Park, I.-S.; Hausinger, R. P. *Protein Sci.* **1993**, *2*, 1034−1041.
(22) Todd, M. J.; Hausinger, R. P. *J. Biol. Chem.* **1991**, *266*, 24327−24331.
(23) Martin, P. R.; Hausinger, R. P. *J. Biol. Chem.* **1992**, *267*, 20024−20027.
(24) Jabri, E.; Carr, M. B.; Hausinger, R. P.; Karplus, P. A. *Science* **1995**, *268*, 998−1004.
(25) Jabri, E.; Karplus, P. A. *Biochemistry* **1996**, *35*, 10616−10626.
(26) Park, I.-S.; Hausinger, R. P. *Science* **1995**, *267*, 1156−1158.
(27) Pearson. M. A.; Michel, L. O.; Hausinger, R. P.; Karplus, P. A. *Biochemistry* **1997**, *36*, 8164−8172.
(28) Jose, J.; Schäfer, U. K.; Kaltwasser, H. *Arch. Microbiol.* **1994**, *161*, 384−392.

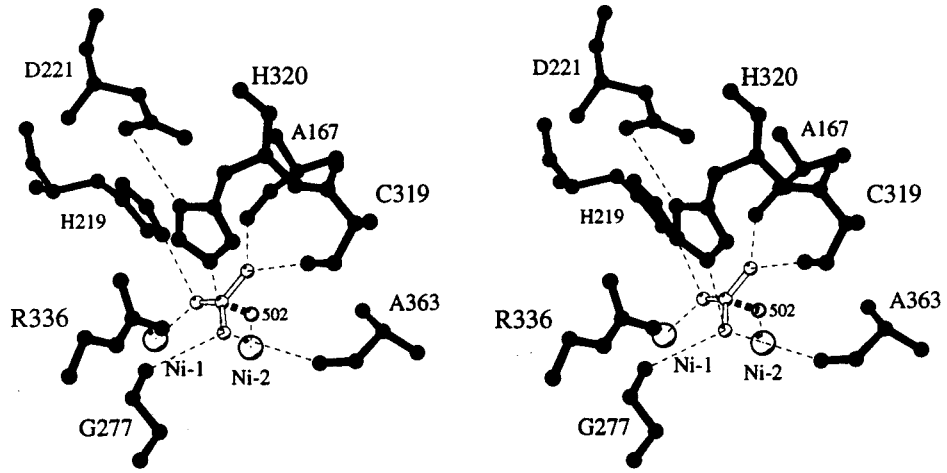


FIGURE 4. Proposed binding mode for urea. A stereoview is shown of a urea molecule modeled into the urease active site. The positions of Asp221, His320, and Wat-502 have been altered slightly (<0.5 Å) to allow better alignment of the hydrogen bonds, but no other atoms have been moved from their positions in the structure of the unligated active site[24] (PDB entry code 1FWJ). The thin dashed lines indicate hydrogen bonds to urea, and the thick dashed lines indicate the bonds formed in making the tetrahedral intermediate (see also Figure 6).

In addition to structural information on the native enzyme and selected variant proteins, we have been able to solve the structure of a complex of the Cys319Ala species with acetohydroxamic acid (AHA) bound.[27] AHA is a slow-binding competitive inhibitor with $K_i^* = 2.6\ \mu$M.[29] The carbonyl oxygen of AHA is seen to coordinate Ni-1, near the position of Wat-501, and is hydrogen bonded to His219 in the manner predicted for urea binding[24] (see below). The AHA hydroxyl group displaces Wat-500 to yield a chelating/bridging binding mode, which is nearly identical to that seen for a small molecule binickel complex containing two hydroxamate moieties.[30]

Proposed Mechanism for Urease Catalysis

Model for Urea Binding. Under the assumption that the protein does not undergo major conformational changes upon urea binding, Jabri et al.[24] proposed that urea coordinates via its carbonyl oxygen to Ni-1 and that the Ni-2 hydration site (Wat-502) is the hydrolytic water. This proposal is supported by our subsequent results noted above, which indicate a low structural plasticity for most of the active site, a consistency in the positions of the flap residues 319 and 320 when they are ordered, and an AHA binding mode which involves coordination to Ni-1 via its carbonyl. Assuming urea ligation to Ni-1 and Wat-502 as the hydrolytic water, it turns out that the small, fully enclosed active site pocket leads to a rather unique binding mode for urea (Figure 4). In this binding mode, all four urea protons make hydrogen bonds to the protein. Three protons are hydrogen bonded to well-fixed backbone oxygen atoms and the fourth interaction involves Cys319-Sγ as a hydrogen bond acceptor. Since Cys319 is on the mobile flap and hydrogen bonds with sulfur are rather rare and weak,[31] this proposed interaction is less certain than the others. Other notable features of the complex are the close approach of Wat-502 poised to attack urea, His219 hydrogen bonding to the urea oxygen, and His320 situated far away from Wat-502 but close to one of the urea nitrogen atoms.

His320 as a General Acid in a Reverse Protonation Scheme. The proposed mode for urea binding is reasonable according to steric and hydrogen bonding considerations, but it is in conflict with two aspects of the accepted mechanism: first, the functional studies had implicated His320 as the catalytic base, but it is located on the opposite side of urea from the hydrolytic water; and second, there is no appropriately positioned group which could act as a base to deprotonate Wat-502. Interestingly, the position of His320 appears to be ideal for a role as a catalytic acid which could protonate the urea nitrogen. To function in this role, however, His320 would have to be protonated. This raises the dilemma of how an enzyme with a pH optimum near 8 could require the protonated form of a group with pK_a near 6.5.

A fully satisfactory resolution to this dilemma is found in the phenomenon known as reverse protonation.[32,33] It is commonly assumed that a bell-shaped pH profile, like that seen for urease, implies that the lower pK_a belongs to a group which must be present in the deprotonated form for activity and the higher pK_a belongs to a group which must be present in the protonated form for activity. However, this need not be the case. The bell-shaped pH profile also can result from the opposite assignment of the pK_a values (Figure 5): the low-pK_a group must be protonated, and the high-pK_a group must be deprotonated. For urease, this would mean that His320 must be protonated for catalysis and another group with a pK_a near 9 must be deprotonated. We speculate that this second group is Wat-502 itself, as such a pK_a is quite reasonable for a metal-bound water.[34,35] This assignment also resolves the second conflict if a deprotonated Wat-502, present as a nickel-bound hydroxide, does not require further activation by a general base.

(29) Todd, M. J.; Hausinger, R. P. *J. Biol. Chem.* **1989**, *264*, 15835−15842.
(30) Stemmler, A. J.; Kampf, J. W.; Kirk, M. L.; Pecoraro, V. L. *J. Am. Chem. Soc.* **1995**, *117*, 6368−6369.
(31) Creighton, T. E. *Proteins*, 2nd ed.; W. H. Freeman and Co.: New York, 1993; p 148.
(32) Mock, W. L.; Aksamawati, M. *Biochem. J.* **1994**, *302*, 57−68.
(33) Mock, W. L.; Stanford, D. J. *Biochemistry* **1996**, *35*, 7369−7377.
(34) Basolo, F.; Pearson, R. G. *Mechanisms of Inorganic Reactions*, 2nd ed.; Wiley: New York, 1967; pp 31−33.
(35) Omburo, G. A.; Kuo, J. M.; Mullins, L. S.; Raushel, F. M. *J. Biol. Chem.* **1992**, *267*, 13278−13283.

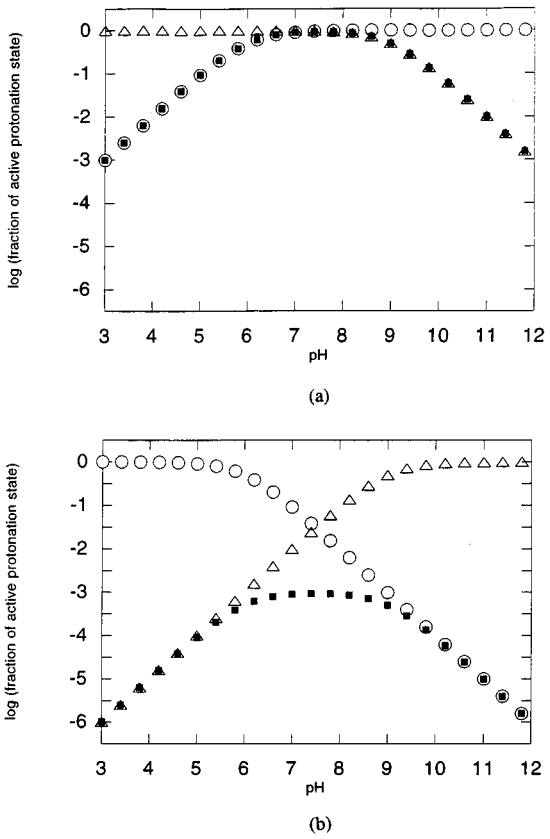


FIGURE 5. Analysis of pH dependence by standard and reverse protonation models. In each panel, the fraction of the active form of the enzyme (filled squares) is plotted on a logarithmic scale as a function of pH. The fraction of enzyme in the active form is equal to the fraction which has the low-pK_a residue in its active protonation state (open circles) multiplied by the fraction of enzyme having the high pK_a residue in its proper protonation state (open triangles). (a) Standard protonation model. A bell-shaped pH dependence is normally assumed to imply that a key residue with the low pK_a must be in its deprotonated form and a key residue with the high pK_a must be in its protonated form. At the optimal pH, nearly every enzyme molecule is in the active form. (b) Reverse protonation model. A bell-shaped pH dependence also results if a key residue with the low pK_a must be in its protonated form and a key residue with the high pK_a must be in its deprotonated form. This is called reverse protonation because for both residues the active protonation states are not the most populated states. In the reverse protonation model, the fraction of active enzyme at the optimal pH is equal to $10^{-\Delta pK_a}$, where ΔpK_a is the difference between the two relevant pK_a values. In kinetics texts, this phenomenon is also discussed as the "principle of kinetic equivalence".[58,59] In studies of enzyme mechanism, the possibility of reverse protonation appears to have been largely ignored, except for the papers of Mock and coworkers[32,33] that brought this possibility to our attention.

Since the difference in the two limiting pK_a values in urease is near 2.5, it follows that, at the optimal pH, only ~1/300 (i.e., 0.3%) of the urease molecules would exist in the active protonation state at a given time. Although this may seem terribly inefficient, the very low amount of active enzyme is partly counterbalanced by enhanced reactivity of the active species. It has been well documented that, when comparing nucleophilicity among a homologous series of compounds, the deprotonated form of the compound with the highest pK_a is the strongest nucleophile.[36] Thus, the putative active site hydroxide with a pK_a of 9 will be a much stronger nucleophile than would be a hydroxide with a pK_a of 6.5. Similarly, a protonated His320 with a pK_a of 6.5 serves as a much stronger acid than would a His with a pK_a of 9.

Structurally Detailed Mechanism. The modeled binding mode of urea (Figure 4) combined with a reverse protonation scenario leads us to propose a new detailed mechanism which has many chemically reasonable details (Figure 6). In short, the single major kinetic barrier is due to a step in which the hydroxide form of Wat-502 attacks the carbonyl carbon of urea to form a tetrahedral hydrated urea intermediate. During formation of the hydrated urea, the pK_a of the conjugate acid of the urea nitrogen interacting with His320 will shift from a value near −2 in urea to a value near 6 in the hydrated urea and will be protonated by His320. This protonation event may be concerted with the attack or may occur subsequently, but in either case, the low activity of the His320Ala variant indicates that the putative hydrogen bond made by His320 to the π-orbital of the urea nitrogen plays a direct role in lowering the activation energy for the hydroxide attack. Once the N-protonated, tetrahedral intermediate has been formed, ammonia can eliminate and the bound carbamate can dissociate from the enzyme in non-rate-limiting steps.

Correlation of These Ideas with Other Urease Properties. The structure−function relations and proposed mechanism outlined above allow rationalization for some other properties of wild-type urease. The exquisite substrate specificity of urease[5,6] can now be understood in terms of the requirement for a closed flap to desolvate urea and to position His320 for interaction with the urea nitrogen. This necessity for flap closure places strict limits on the size of the substrate and on its hydrogen-bonding character. Similarly, the observations that selected competitive inhibitors actually enhance the rate of modification of Cys319 by iodoacetamide and other sulfhydryl reagents (leading to more rapid inactivation of the enzyme)[37] can be understood as a simple physical effect: inhibitors which are much larger than urea will bind to the active site nickel ions and not allow the flap to close properly, hence increasing the average accessibility of Cys319 to these reagents. Consistent with this concept, the only active site ligands that confer strong protection against these sulfhydryl reagents are very small compounds such as phosphate, borate, and urea itself.

An important feasibility check for a reverse protonation mechanism is that the observed rate of the reaction divided by the small fraction of active enzyme must not exceed the diffusion-controlled limit of near 10^{10} $M^{-1}s^{-1}$.[38] For urease, if only ~one in $10^{2.5}$ molecules have the correct protonation state, then the intrinsic k_{cat}/K_M for the active fraction of molecules is $10^{2.5}$ times the observed k_{cat}/K_M of 10^6 $M^{-1}s^{-1}$. This approaches but does not exceed the diffusion-controlled limit. Interestingly, the reverse protonation model also gives some insight into the proficiency of urease, by providing an explanation for the 10^{14}-fold rate enhancement over the uncatalyzed elimination reaction. Elimination of ammonia from urea can be viewed as a reaction requiring reverse protonation: it

(36) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 79−87.

(37) Todd, M. J.; Hausinger, R. P. *J. Biol. Chem.* **1991**, *266*, 10260−10267.

(38) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 147−148.

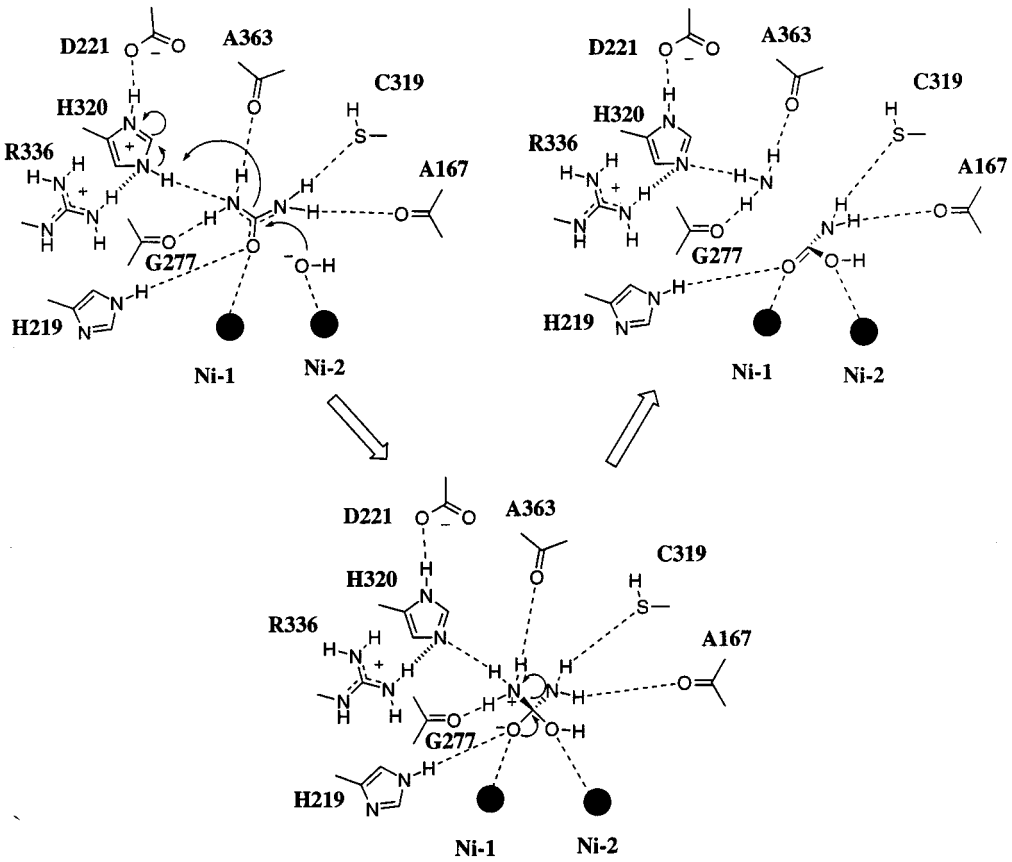
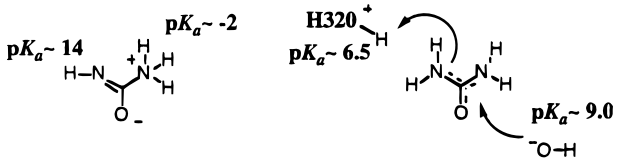


FIGURE 6. Structurally detailed mechanism for urease catalysis. The key players in the mechanism are included in this drawing of our proposed Michaelis (ES) complex. The urea is drawn with three partial double bonds to avoid choosing one particular resonance form, and hydrogen bonds are shown as dashed lines. In this mechanism, many features work together to overcome the resonance energy of urea so as to activate the carbonyl carbon for attack. These features include the following: (1) O-coordination to Ni-1 to withdraw electrons along the carbonyl bond; (2) His219 hydrogen bonding to the oxygen, helping orient urea and causing further polarization of the carbonyl bond; (3) the replacement of urea solvation with neutral hydrogen bond acceptors for all four urea protons (more polar or charged acceptors would push electrons toward the urea carbon, tending to deactivate the carbon toward nucleophilic attack); (4) an enhanced nucleophilicity of the hydroxide form of Wat-502 compared to a nucleophilic water with a lower pK_a; (5) an enhanced acidity of the imidazolium form of His320 compared to a His with a higher pK_a (Asp221 and Arg336 presumably enhance the directionality and the acidity of His320); (6) a favorable antiperiplanar geometry[60] of the Wat-502 nucleophile and the protonation by His320; (7) a nickel separation such that optimal coordination by Wat-502 and urea places them at less than van der Waals contact, creating a local high-energy interaction which can be relieved at the transition state.

Scheme 2



requires protonation of a group which has a pK_a ~ −2 and deprotonation of a group which has a pK_a ~ 14 (Scheme 2). This formulation explains why the rate of elimination is independent of pH between values of 2 and 12,[12] as throughout that range a constant fraction of urea molecules (~one in 10^{16}) have the correct protonation state for elimination. Thus, the fraction of urease molecules which have the correct protonation state for urea hydrolysis (i.e., $\sim 10^{-2.5}$) is roughly $10^{13.5}$ times higher than the fraction of urea molecules which are configured to react in solution .

Suboptimal Interactions as an Important Source of Binding Energy. The $>10^{14}$-fold catalytic proficiency of urease corresponds to a stabilization energy of >19 kcal/mol. This stabilization free energy is equal to the difference in free energy between the solvated transition state plus the solvated (empty) enzyme versus the solvated enzyme-transition state complex represented by the equilibrium "$(E)_{aq} + (S^{\ddagger})_{aq} \leftrightarrow (ES^{\ddagger})_{aq}$". What is the origin of this stabilization energy? One possibility is that the enzyme−ligand interactions are unusually strong. An alternative, which we believe is important in the case of urease, is that the empty active site makes especially weak interactions with water, so that even "normal" hydrogen bonds to the ligand contribute significantly to binding.

The nickel solvation pattern first led us to consider that suboptimal interactions exist in the solvated empty active site. As described above, the nickel binding portion of the active site is rather rigid and has a constellation such that Ni-1 and Ni-2 cannot both be optimally hydrated: if Wat-501 is present then Wat-502 cannot be optimally situated (and vice versa) without incurring a large unfavorable steric penalty. In contrast, the true transition state (which probably has two oxygens about 2 Å apart) can optimally ligate both nickel ions simultaneously, so that even normal ligation energetics are favorable compared to the suboptimal ligation provided by water.

The mobility of the active site flap provides further evidence for suboptimal interactions in the empty active

site. Assuming that the flap is fully closed at the transition state of the reaction, one may ask the following relevant question: why is the flap not fully closed in the empty enzyme seen in the crystal? Two possibilities are that (i) the extended interactions involved in flap closure are unfavorable so that very favorable interactions made by urea are required to drive flap closure or (ii) that the extended interactions made by the flap are sufficient to favor flap closure, but side chains and water in the urea binding pocket make unfavorable interactions that destabilize the closed state of the empty enzyme. Although the first explanation has been commonly invoked for enzymes which undergo such induced fits,[39−41] the second explanation is more consistent with the view that the enzyme is designed for maximally effective binding of the transition state. Experimental support for the second explanation is provided by the observation that His219Ala and Cys319Ala mutations both cause an ordering of the flap: since these mutants do not affect the extended interactions of the flap, but simply remove interactions from the active site region, it suggests that the flap ordering is driven by the relief of unfavorable (i.e., suboptimal) interactions that were in the active site. A recent study of a cytochrome *c* peroxidase mutant provides a clear case in which loop mobility is caused by local unfavorable interactions overcoming extended favorable interactions.[42]

The use of suboptimal interactions in unliganded active sites to provide a driving force is likely to be of general relevance in enzymes. First, induced-fit conformational changes are common, and the above arguments suggest that disordered states that often precede induced fits may result from suboptimal interactions in the empty (solvent-filled) active site. If this is the case, then, contrary to the common paradigm, the enzyme is not unfavorably distorted during the induced fit but relaxes (i.e., in Figure 2 of Wolfenden,[41] the energy of the enzyme and its associated solvent will decrease rather than increase). A second argument for the widespread relevance of suboptimal interactions is simple deductive logic: given that (i) an enzyme's closed active site is optimally complementary to the reaction transition state and (ii) a transition state will not generally have a similar constellation of hydrogen bonding groups as water, one can conclude that (iii) water will in general not be a good mimic for a transition state, so that an empty active site cannot provide optimal complementarity to water. The idea that an unliganded enzyme is in a high-energy state due to suboptimal interactions is reminiscent of the "entatic state" concept proposed by Vallee and Williams nearly three decades ago.[43,44] It is also supported by evidence reviewed by Lemieux[45] that solvent reorganization upon ligand binding can make significant enthalpic contributions to binding.

Urease/Phosphotriesterase/Adenosine Deaminase Superfamily. Protein structural comparisons reveal extensive similarity between the nickel-containing domain of urease and the zinc-dependent enzymes phosphotriesterase and adenosine deaminase.[24,46−49] Phosphotriesterase possesses a dinuclear active site and appears to be more closely related to urease than adenosine deaminase which has a mononuclear active site. Given the presumed homologies among these proteins, it is interesting to compare the proposed urease mechanism with the mechanisms of these enzymes.

The carbamate-bridged dinuclear metal center of phosphotriesterase is very similar to that found in urease; however, the other key residues in the urease active site are not present in this enzyme, leading to marked differences in activity. The pH dependence of phosphotriesterase activity is not bell-shaped but shows increased activity at high pH and suggests that activity requires the deprotonated state of a group with p$K \sim 6$.[50,51] The most recent proposal assigns this to a metal-bound nucleophilic water, mechanistically (and structurally) equivalent to Wat-502 of urease, with no additional enzymic general base. This proposed mechanism is analogous to our proposed urease mechanism, with the exception that no general acid catalysis is present. Given that phosphotriesterase only cleaves phosphotriesters having very good leaving groups such as fluoride or activated phenolates,[52] that the uncatalyzed rate of hydrolysis of such phosphotriesters is rather high,[50,51] and that small organometallic complexes can catalyze the hydrolysis of such phosphoesters,[53] the lack of general acid catalysis in phosphotriesterase is easily understood.

In adenosine deaminase, structures of inhibitor-bound forms of the murine enzyme clearly show that the single zinc ion coordinates the hydrolytic water molecule.[48,49] The lack of a second, substrate-binding metal ion correlates with the substrate's lack of an appropriately positioned carbonyl oxygen. Instead, adenosine deaminase uses an acid (Glu217) to protonate the purine N-1 atom and withdraw electrons from C6 to activate it for attack.[48,54,55] Adenosine deaminase, like urease, exhibits a bell-shaped activity dependence on pH with effective limiting pK_a values near 5 and 9 for the murine enzyme[56] and 6.5 and 7.5 for the yeast enzyme.[57] Despite the structural information, there has been difficulty in assigning the groups

(39) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; pp 289−291.
(40) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 331−333.
(41) Wolfenden, R. *Mol. Cell. Biochem.* **1974**, *3*, 207−211.
(42) Fitzgerald, M. M.; Musah, R. A.; McRee, D. E.; Goodin, D. B. *Nat. Struct. Biol.* **1996**, *3*, 626−631.
(43) Vallee, B. L.; Williams, R. J. P. *Proc. Natl. Acad. Sci. U.S.A.* **1968**, *59*, 498−505.
(44) Williams, R. J. P. *Eur. J. Biochem.* **1995**, *234*, 363−381.
(45) Lemieux, R. U. *Acc. Chem. Res.* **1996**, *29*, 373−380.
(46) Benning, M. M.; Kuo, J. M.; Raushel, F. M.; Holden, H. M. *Biochemistry* **1995**, *34*, 7973−7978.
(47) Vanhooke, J. L.; Benning, M. M.; Raushel, F. M.; Holden, H. M. *Biochemistry* **1996**, *35*, 6020−6025.
(48) Wilson, D. K.; Rudolph, F. B.; Quiocho, F. A. *Science* **1991**, *252*, 1278−1284.
(49) Wilson, D. K.; Quiocho, F. A. *Biochemistry* **1993**, *32*, 1689−1694.
(50) Donarski, W. J.; Dumas, D. P.; Heitmeyer, D. P.; Lewis, V. E.; Raushel, F. M. *Biochemistry* **1989**, *28*, 4650−4655.
(51) Hong, S.-B.; Raushel, F. M. *Biochemistry* **1996**, *35*, 10904−10912.
(52) Dumas, D. P.; Caldwell, S. R.; Wild, J. R.; Raushel, F. M. *J. Biol. Chem.* **1989**, *264*, 19659−19665.
(53) Volkmer, D.; Hommerich, B.; Griesar, K.; Haase, W.; Krebs, B. *Inorg. Chem.* **1996**, *35*, 3792−3803.
(54) Mohamedali, K. A.; Kurz, L. C.; Rudolph, F. B. *Biochemistry* **1996**, *35*, 1672−1680.
(55) Bhaumik, D.; Medin, J.; Gathy, K.; Coleman, M. S. *J. Biol. Chem.* **1993**, *268*, 5464−5470.
(56) Sharff, A. J.; Wilson, D. K.; Chang, Z.; Quiocho, F. A. *J. Mol. Biol.* **1992**, *226*, 917−921.
(57) Merkler, D. J.; Schramm, V. L. *Biochemistry* **1993**, *32*, 5792−5799.
(58) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; W. H. Freeman and Co.: New York, 1985; pp 90−91.

associated with the limiting pK_a values using a standard protonation scenario.[57] Although insufficient data are present to allow sure conclusions, a reverse protonation scenario would resolve some of the difficulties and should be considered.

Concluding Remarks

We have combined crystallographic and functional studies of wild-type urease and some site-directed variants to propose a detailed catalytic mechanism for urease which accounts for its kinetic properties and its structure. This mechanism assigns the limiting pK_a's of 6.5 and 9.0 to the general acid His320 and the hydrolytic water, respectively. This assignment implies that only a small fraction of the urease molecules are present in the correct protonation state for activity. Observed disorder of the solvation in the active site and the comparison of multiple structures provide evidence for suboptimal interactions in the water-filled active site. We propose that these suboptimal interactions lead to an enthalpy-driven free energy gain when this water is released into the bulk solvent upon urea binding. As such, the relaxation of the high-energy state could provide a large amount of binding energy required for catalysis.

(59) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; pp 182−187.
(60) Deslongchamps, P. *Stereoelectronic Effects in Organic Chemistry*; Pergamon Press: Oxford, U.K., 1983; p 102.

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