Explaining urease specificity towards nickel: a re-analysis of its proposed mechanism

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Abstract

Urease is a binuclear metalloenzyme extremely selective towards nickel, exhibiting a remarkable rate enhancement of the catalytic reaction. The accepted mechanism proposal for urease describes the coordination of urea to both nickel centers in an O, N bridged mode, enabling the attack of the carbonyl by a bridged hydroxide present between the metallic centers. However, the substitution of nickel by other metals (Fe^{2+}, Co^{2+}, Mn^{2+}, Zn^{2+}, and Cu^{2+}) significantly reduces urease´s catalytic efficiency. The proposed mechanism cannot explain this difference in activity since it does not follow a rational nucleophilicity scale. After a careful analysis of the literature data on thermodynamics, kinetics, inhibition, and mutations, we verified that by analyzing the mechanism from a different angle, another pathway is most likely occurring. This mechanism can explain urease´s selectivity towards nickel and all the data present in the literature, gathering almost a century of study about this enzyme.

Keywords: urease, mechanism, metal selectivity, water exchange rate

Introduction

Urease, an enzyme responsible for the hydrolysis of urea into ammonia and carbon dioxide, has a strict selectivity towards nickel. Metal selectivity/promiscuity of a metalloenzyme is determined by an orchestration of multiple factors such as hard/soft interaction, Irving-Williams series, and the environmental and intracellular concentration of the metal.[1] Also, the catalytic role of the metal is highly conserved in an enzyme mechanism within the same superfamily,[2] contributing to the design of mechanism proposals of different enzymes.

Owing to the classification of urease as a member of the TIM barrel amidohydrolase superfamily, its mechanism proposal can be compared to other enzymes of this superfamily.[3] The best comparison within enzymes in this family can be performed between urease and dihydroorotase (DHO), due to the similarity of their active sites. For instance, the active site of urease accommodates one pseudo octahedral (Ni2) and a five coordinated (Ni1) nickel center coordinated to two histidine residues, plus a terminal water or an asparagine residue (Figure 1A).[4,5] The nickel centers are also bridged by a carbamylated lysine and a hydroxide. In DHO, its active site bears two zinc ions similarly coordinated to the nickel centers in urease active site. The crystallization of DHO in a pH that enables the direct and reverse reactions, allowed the simultaneous visualization of the interaction between the protein, substrate, and product at the same time.[6] In the crystal structure, the substrate dihydroorotate interacts through the amide carbonyl to the tetracoordinate metal center, aiding the amide bond polarization. The carbonyl attack was observed to occur by the bridged hydroxide owing to its favorable orientation.[6] Hence, in the urease mechanism, it is proposed that three water molecules dissociate from the active site before urea coordination,[7] allowing an O, N bridged coordination mode between both nickel centers (Figure 1B),[8] as shown

in the urea-urease crystal structure.[9] However, despite the notion that urease´s mechanism is already determined,[10] it is evident that it still must be debated since it cannot explain urease´s catalytic behavior. For instance, when the nickel centers in urease are exchanged by Fe^{2+},[11] Co^{2+} or Mn^{2+}, only 2% of the restored Ni^{2+}-enzyme activity is observed, whereas the exchange by Zn^{2+} or Cu^{2+} completely inactivates it.[12] Therefore, if the hydroxyl bridging both metal centers is the nucleophile, the change in activity should follow the order of nucleophilicity.[13] In the case of DHO, the substitution of Zn^{2+} by Co^{2+} reduces the k_{cat} of DHO to 15% of its original k_{cat}, related to a change in the pK_a of the bridged hydroxyl from 5.8 to 6.9, decreasing the nucleophilicity of the bridged hydroxyl of the Co^{2+}-substituted DHO at the same pH, and increasing its activity at a higher pH.[14] However, the observed decay in k_{cat} by metal substitution in urease does not follow a nucleophilic order since the determined pK_{as} of the nucleophile (Mn^{2+}-urease:pK_{a2} of 9.2 and Co^{2+}-urease: pK_{a2} of 9.1) are similar to the one observed in the native enzyme (pK_{a2} of 8.9).[15] Therefore, another factor rather than just nucleophilicity is governing urease catalysis.

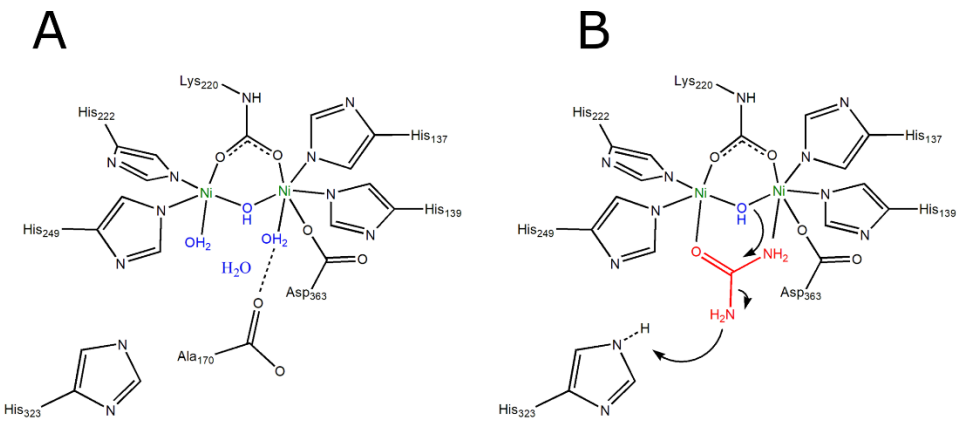

Figure 1. Active site of urease (A) and most accepted mechanism of urease enzyme (B).

Interestingly, the water exchange rate (k_{ex}) measures the speed of the reaction of ligand substitution at an aqueous metal.[16] Hence, all reactions involving the replacement of strongly bond hydration water from the first coordination sphere are governed by water exchange kinetics. It was found that there is flexibility in the hydration layer of Mg^{2+} when the water exchange time is greater than the folding of nucleic acids, enabling the bond waters to exchange with the bulk.[17] Moreover, the first hydration shell of a cation is affected by the chemical nature of the ion, with water exchange rates depending on the ion-water strength,[18] and stronger interactions determining the exchange dynamics by the hydration shell dynamics.[19] For instance, Remsing and co-workers have demonstrated that frustrated hydrated cations exhibit greater fluctuations concerning unfrustrated systems, playing a critical role in solvent-mediated processes.[20] Enzyme active sites can also be perceived as frustrated hydrated systems,[21] owing to their intrinsic pocket nature. It is known that different cations can influence the mean lifetime of water molecules in the first coordination shell, varying in more than 18 orders of magnitude,[22] but can this variance be related to the observed mechanism of many metalloenzymes? Here we will analyze the thermodynamics, kinetics, inhibition, and mutations data about urease and will demonstrate that by acknowledging the existence of competition between k_{cat} and k_{ex}, the mechanism can be redesigned to a proposal able to explain why urease required nickel to function properly.

Analysis of urease mechanism based on thermodynamic and kinetics

To understand the urease mechanism, we must analyze all factors governing the reaction. Urease has two nickel centers, each coordinated to two histidine residues, one water molecule, a carbamylated lysine, and a bridged hydroxide. Ni(2) is also coordinated to an aspartate, resulting in an asymmetric coordination number for the

nickel ions. Ni(1) is penta coordinated and Ni(2) is hexacoordinated. The overall charge of the active site complex is zero and partially, one, owing to the resonance structure of carbamate.[23] The charge of the active site complex and the entering substrate will govern kinetics, as will be explained further on.

It is known that any reaction of ligand substitution is always preceded by the entry of the ligand into the second coordination sphere of water molecules to form an encounter complex $M(L)_n \cdot X$, which is further converted to $M(L_{n-1})X$. The observed rate constant for the substitution reaction is given by equation 1, in which K_{os} is k_{12}/k_{21}.[24]

$$M(L)_n + X \rightleftharpoons M(L)_n \bullet X \rightarrow M(L_{n-1})X$$

$$k_{obs} = \{(k_{23}K_{os}[X])/(1+K_{os}[X])\} + k_{32} \qquad \text{equation 1}$$

When the stability of the encounter complex is low, $K_{os}[X]$ will be $<<1$ and $k_{obs} \approx k_{23}K_{os}[L^{x-}] + k_{32}$, whereas if $K_{os}[X]$ is $>> 1$, k_{obs} will be governed by the sum of k_{23} and k_{32}.[24] What determines the stability of the encounter complex is the charge of the ligand and $M(L)_n$. For instance, charged species form more stable encounter complexes than non-charged ones. Ligand substitution in $[Ni(H_2O)_6]^{2+}$ can be used as an example of the influence of the encounter complex stability in kinetics, since when several ligands are compared, it is possible to see that the variation in the encounter complex formation constant K_{os} is reflected on the rate constant (k_{obs}), revealing the role of the encounter complex in the kinetics of ligand exchange.

Such analysis can be extended to an enzymatic reaction and substrate bonding and the encounter complex can be called $ES^{\neq}$. Hence, urease catalysis can be divided into four different steps: (1) formation of the $ES^{\neq}$ encounter complex, (2) formation of the ES complex from $ES^{\neq}$, (3) catalysis and formation of EP, and (4) release of products and regenerated enzyme (E+P).

$$E + S \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} ES^{\#} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} ES$$

In the urease system, urea is a non-charged ligand that will substitute at the mostly non charged urease active site, resulting in extremely low stability of $ES^{\neq}$. Therefore, the analysis of thermodynamic data for the formation of ES must account that it covers two steps of the reaction covering kinetics of $ES^{\neq}$ and ES formation. Krajewska *et al* have determined the ΔS_0, ΔG_0, ΔH_0 for urease and different ligands: urea, phosphate, boric acid, and mercaptoethanol.[7,25] Besides, the ΔV_b was also calculated for urea-urease complex formation. These results are shown in Table 1. All reactions are spontaneous and exergonic with a fairly constant value (between -14 and -23 kJ/mol), but the entropy of formation of the ES (or EI) complex is quite different from urea and urease inhibitors, indicating a substantial enthalpy–entropy compensation in a process that is largely enthalpy-driven. For instance, all urease inhibitors tested have a larger negative ΔS_0 than the urea-urease complex, indicating that the EI is more ordered than E + I. Also, observing table 1, it is possible to see that different values of entropy for the ES formation are obtained at different pHs, which would indicate a possible disordering of the initial state when protonation or deprotonation takes place at a group with pK_a near 6.8. From the ΔS_0 values of ES formation, it is possible to see that this value is very close to zero, which could be due to the weak bond between urea and the active site, reflecting an almost unchanged system due to the low stability of the encounter complex. In contrast, the inhibitors should form stronger bonds that are compensated by an increase in the entropy of the system, possibly by water release.[26,27] The number of released waters can only be speculated, but interestingly, in the crystal structure of the inhibited urease, no water molecule can be found coordinated to the nickel centers. The negative entropy obtained for the EI complex also indicates an

associative mechanism of ligand substitution, meaning that they first coordinate to nickel, and then water is displaced, which goes in contrast to the proposal of water dissociation before urea coordination.[7] However, ES is indeed positive (at pH 6.85), and water dissociation should occur before urea coordination.

Table 1. Thermodynamic parameters for ES formation ($E + S \rightarrow ES^{\neq} \rightarrow ES$).

Ligand	**ΔS^0 (J/mol)**	**ΔH^0 (kJ/mol)**	**ΔG^0 (kJ/mol)**	**ΔV_b (mL/mol)**
Urea at pH 6.84	7±3	-12±1	-14±2	-2 ±2
Urea pH 7	0,4±3	-14±1	-14±1	-
Urea at pH 6.45	-0,5±3	-14±1	-14±1	-
Phosphate at pH 6.45	-12±14	-17±4	-13±4	-
Boric acid	-63±21	-42±6	-23±6	-
Mercaptoethanol	-9±9	-20±3	-17±3	-

An important data in urea-urease interaction is the difference in volume between E+S and ES, which although close to zero could indicate the release of the structural water,[7] forming an outer sphere encounter complex. Once urea is on the outer sphere encounter complex, it can bind to Ni(1) since it is pentacoordinate and can still receive an electron pair from urea. The flap closure is dictated by αHis323(SPU numbering),[28] ordering the system and resulting in a large negative entropy of activation (-80 J /mol).[29] This also enables the concomitant removal of the Ni(1) coordinated water, resulting in an activation volume of 5 mL/mol, in agreement with the activation volume of dissociation of one water molecule from $[Ni(H_2O)_6]^{2+}$ (7.2 mL/mol). At this stage, the amide nitrogen from urea can coordinate to Ni(2) forming a bridge between both nickel centers, as proposed by Benini *et al.*[8] or one of the coordinated waters (W2 or WB) could attack the carbonyl as proposed by Zerner and Hausinger.[30] The acknowledgment of this competition guides to a simple thought: the step that happens faster (catalysis or

ligand substitution at Ni(2)) will lead the reaction. In other words, if the catalytic reaction occurs with the attack of the bridged hydroxide (or W2) faster than the rate of ligand exchange, this would indicate that Ni(2) would be hydrated during the whole process.

Therefore, the straightforward analysis between k_{cat} and k_{ex} can lead to the answer. Taking for instance urease from Jack Bean with k_{cat} = 2.34 X 10^4 s^{-1} and the water exchange constant value for $[Ni(H_2O)_6]^{2+}$ of 3.2x10^4 s^{-1} it is clear that both of these constants are in the same order of magnitude and there is a competition between dehydration of Ni(2) and catalysis.[22,31] However, since the experimental thermodynamics does not present reasonable values of activation volumes for the release of two water molecules from the active site, it is most probable that Ni(2) is acting in its hydrated form. Moreover, if we substitute Ni^{2+} with any other metal, such as Fe^{2+}, to obtain an iron-based urease, the water exchange rate would be 4.4x10^6 s^{-1}, which is two orders of magnitude greater than k_{cat} (of a Ni^{2+} based urease). This faster water exchange suggests the removal of the competition between dehydration of Ni(2) and catalysis, and dehydration would take place, enabling the coordination of urea in a bidentate manner. The bidentate coordination mode is able of suffering the attack of the bridged hydroxide,[11] however, since the second metallic center is not hydrated, proton transfer and regeneration of the catalytic center is hindered, decreasing urease activity dramatically. Following this analysis, the substitution of nickel by the more labile metals Fe^{2+}, Co^{2+}, Mn^{2+},[15] Zn^{2+}, and Cu^{2+} would result in a urease enzyme able of coordinating to urea in a bridged mode and the difference between their activities could be explained by different pK_{as} of the bridged hydroxide influencing its nucleophilicity. This hypothesis can explain why nickel is the sole preferred metal in urease.

Now we should answer who is the nucleophile: WB or W2? Krajewska has determined that a group with pK_a of 8.8 is involved in the catalytic event, assigning it to the bridged hydroxide.[32] However, no single-bridged hydroxide has been demonstrated to bear a pK_a of 8.8. Also, the change in pH from 8.8 to 9.1 cannot explain why Mn^{2+}-urease has only 2% of the Ni^{2+} restored urease. The pK_a of other bridged hydroxo groups in bimetallic enzymes are much lower than 8.8. For instance, the Mn^{2+} dependent-phosphotriesterase was shown to have a pH-rate profile dependent on a single ionization of a group with pK_a 7.9 and 7.4.[33] Also, purple acid phosphatase based on Fe^{3+} and Mn^{2+} presents a terminal water group with pK_a of 3.7 and when a dinuclear Mn^{2+} active site is present, the pK_a raises to 6.80, indicating the presence of an μ-hydroxyl as a nucleophile.[34] Native Fe^{3+}−Fe^{2+} uteroferrin enzyme was also determined to have the terminal Fe^{3+}-bound H_2O/OH^- as the most likely nucleophile,[35] while for the Fe^{3+}−Ni^{2+} derivative, the pK_a of 4.6 has been assigned to the deprotonation of the μ-hydroxide.[13] In dihydroorotase, a dinuclear zinc enzyme, a pK_a of 6.0 was determined and when Zn^{2+} was replaced by Co^{2+}, the pK_a was raised to 7.8.[6] On the other hand, dinuclear nickel compounds were described to bear pK_{as} of 4.4 and 8.5 for each of the two bridging water ligands (and not hydroxide). Therefore, the hydroxide in urease should have a pK_a between 7.8 and 4, corresponding to the pK_{a1} observed in urease.[15]

Interestingly, a group with pK_a near 6.6 in jack bean urease has been ascribed to be involved in the binding of urea,[36] which is disturbed by phosphate. Phosphate is known to be a competitive inhibitor of urease, coordinating in a tridentate mode,[37] disturbing all coordinated waters, meaning that some of the coordinated waters could have this pK_a. We have shown in table 1 that different values of entropy were obtained at different pHs, possibly related to a group with pK_a near 6.8. Hence, the group with pK_a 6.6 could be the reason behind why urease has different entropy values (and signs)

at pHs lower, near, and higher than 6.6.[25] For instance, the protonation of the hydroxo bridge at pH 6.45 would reduce the ordering of the four centered hydrogen bonding arrangement of the active site water molecules (as shown in Figure 2),[38] and the displacement of the structural water by urea to form the encounter complex would result in a system with almost the same entropy as the initial state, with a ΔS near zero.[25] A similar trend would be observed if deprotonation takes place at pH 7, as shown in Figure 2. Therefore, we propose that the bridged hydroxide in urease has a pK_a of 6.6.

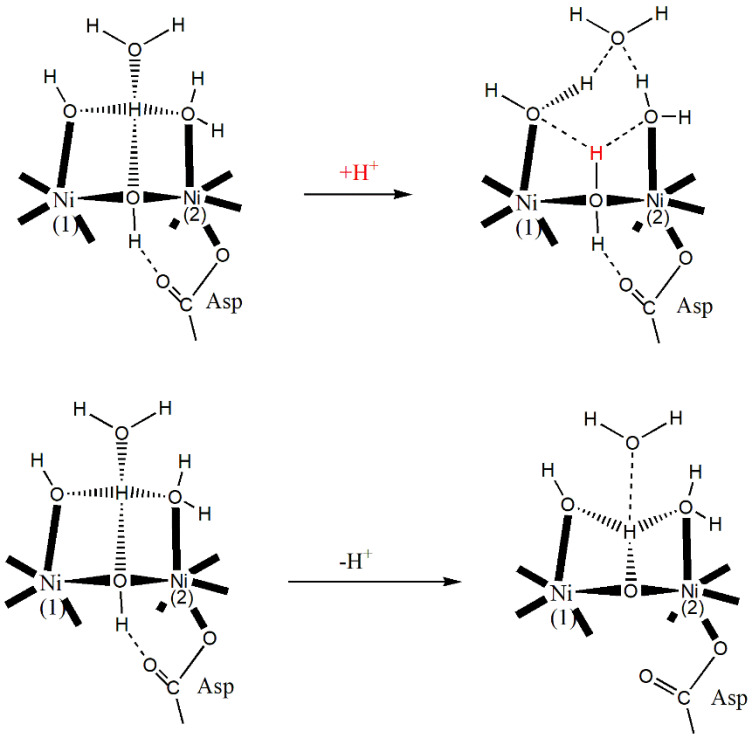

Figure 2. Scheme of the disordering of the system with changes in pH. Protonation and deprotonation of the hydroxide bridge enhances the entropy of the system.

Curiously, some dinuclear metalo-hydrolases exhibit an alkaline shift of the pK_a of the bridged hydroxide upon substrate binding and the bridging water ligand moves into a pseudomonodentate position.[39] Therefore, if upon urea coordination to Ni(1), the hydroxo bridge moves into a pseudomonodentate position, a shift from the pK_a of 6.6 to 8.8 would be observed. This would enable the nucleophilic attack of the hydroxo-

bridge to the carbonyl of urea. Proton exchange would be performed by the Ni(2) bond water, since its pK_a would be probably lower than the pK_a of $[Ni(H_2O)_6]^{2+}$ (9.8), owing to its proximity to the carboxyl group of alanine, making strong hydrogen bonds that weaken the O-H bond. [8] Proton exchange from W2 to the amide nitrogen facilitates the formation of ammonia (step 3, Figure 3). The resulting hydroxide should attack Ni1 to easily regenerate the active site (step 4, Figure 3). This mechanism also explains

fluoride acting as an uncompetitive inhibitor related to enzyme turnover.[40]

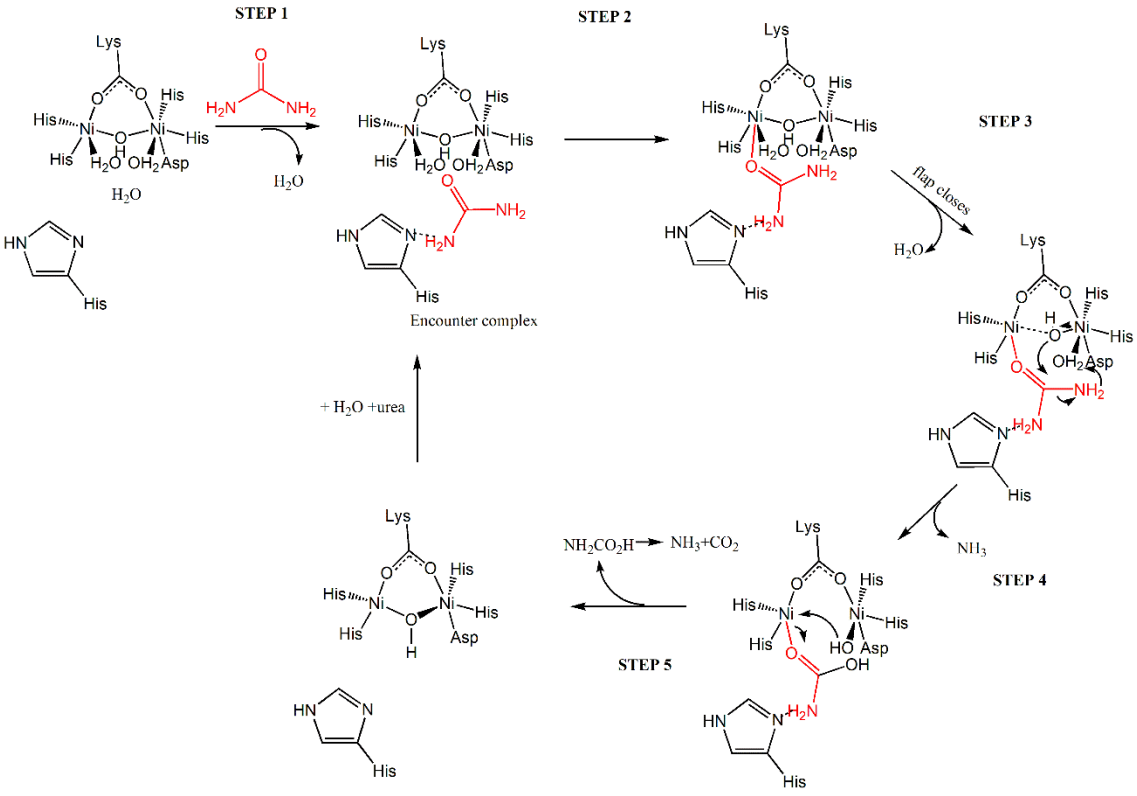

Figure 3. Mechanism proposal of urease. First urea forms a urea-urease encounter complex, then water from Ni(1) is removed concomitantly with flap closure. A shift from bridged to pseudo-monodentate coordination mode of the hydroxide explains the

high pK_a observed for this moiety. Proton exchange from Ni(2) bond water enables ammonia formation, whereas the Ni(2) hydroxide attacks Ni(1) to regenerate the active site.

This assumption contradicts the reverse protonation hypothesis, which proposes that His320 (in *Klebsiella aerogenes* numbering) is a general acid that enables catalysis in a reverse protonation. However, the reverse protonation relies on the probability of a fraction of the enzyme to be protonated in a manner opposite (or reverse) from what one would expect given the pK_{as} of the free amino acids.[41] It was previously thought that His320 had a pK_a of 6.5, which would result in only 0.3% of all urease molecules being in the optimal protonation state for catalysis.[41] However, a pK_a of 5.0 was assigned to this histidine residue, which should participate in substrate binding and catalytic reaction.[32] This smaller pK_a would almost prohibit the reverse protonation and the catalytic feature of histidine should most probably be acting as a general base. Moreover, His323 (SPU numbering) is essential for the flap movement,[42] therefore, without this residue, the catalytic activity is dramatically dropped.[43] Also, if the flap is open, the water displacement from Ni(1) probably does not occur, which would keep the pK_a of the bridged hydroxide at 6.6, explaining the pH profile of several mutants. For instance, mutant αH320A (KAU numbering) exhibited a significantly shifted pH optimum at around 6.75.[43] It is known that a constellation of groups with different pK_{as} is involved in catalysis and that our hypothesis could be a simplistic view and care should be taken.[44] However, the pK_a shift of the hydroxide from 6.6 to 8.8 would also explain the presence of two distinct active states in urease with different optimum pHs,[45] evidencing the competition between k_{cat} and k_{ex}, as shown in figure 4. For instance, the increase of ionic strength of the medium should affect negatively on k_{cat} for the high pH

state owing to the neutralization of the charges involved in the flap closure,[28] increasing the possibility of a bridged O, N urea bonding. In addition, taking as an example the substitution of His219 (KAU numbering), it is seen that charged residues, such as glutamine or asparagine, had a much higher impact on the pH profile than H219A mutant, with a described possible misorientation of His320 in these proteins, causing a shift on the flap. This flap shift could increase the bridged O, N urea bonding mode increasing the low pH profile of activity. Also, the mutation in aspartate, D221A, shows an open flap and more density associated with the bridging water, explaining the observed activity at lower pH.[44]

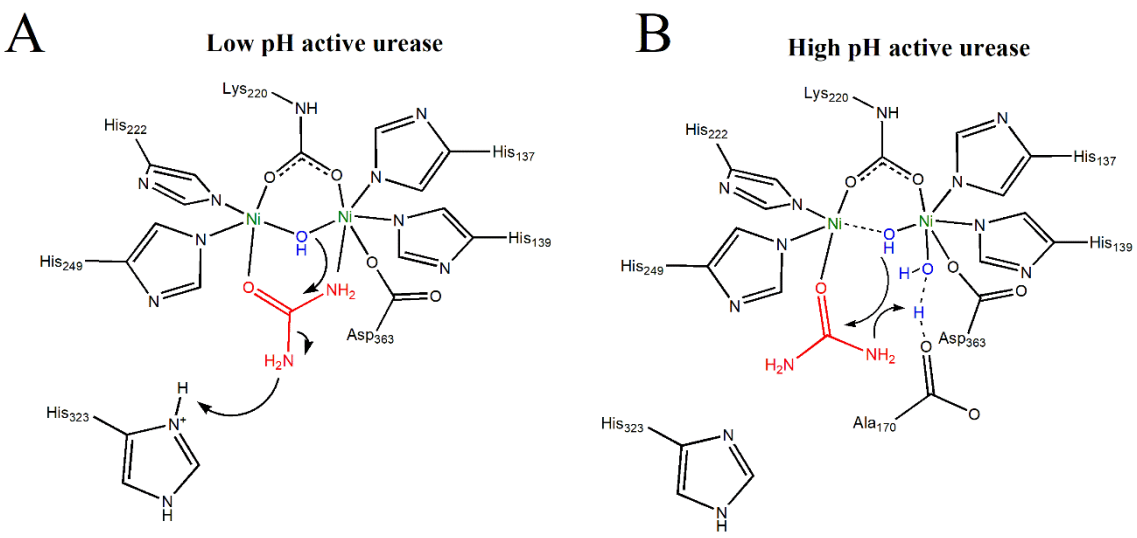

Figure 4. Two active states of urease. (A) The low pH active urease involves urea coordination in a bridged mode and the nucleophilic attack of the bridged hydroxyl with pKa 6.6. (B) The shift from bridged to pseudo-monodentate coordination mode of the hydroxide explains the high pK_a observed for this moiety. Proton exchange from Ni(2) bond water enables ammonia formation, whereas the Ni(2) hydroxide attacks Ni(1) to regenerate the active site.

These facts allowed us to propose a new model of urease activity based on a literature review, indicating that the competition between k_{cat} and k_{ex} might also be happening in other enzymes.

Conclusion

In this analysis, we verified the role of the competition between metal lability and reaction rate in the determination of a metalloenzyme mechanism. This competition is the basis for urea selectivity towards nickel since it disables (or enables) urea coordination in a bridged mode. We have also re-analyzed the data regarding pK_a to explain the role of histidine 320 and the bridged hydroxyl in the reaction pH. With this mechanism proposal, we can explain the thermodynamic data, the formation of ES, the competition between catalysis and dehydration of Ni(2), the pK_{as} involved in catalysis, the fast regeneration of the system, the fluoride action as an uncompetitive inhibitor related to the turnover of the enzyme and finally, why urease uses nickel instead of other metals.

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