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The structure of the elusive urease-urea complex unveils a paradigmatic case of metallo-enzyme catalysis

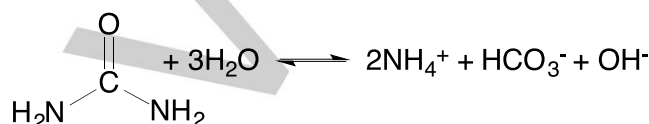
Luca Mazzei,^[a] Michele Cianci,^[b] Stefano Benini^[c] and Stefano Ciurli^{*[a]}

Abstract: Urease, the most efficient enzyme known, contains an essential dinuclear Ni(II) cluster in the active site. It catalyses the hydrolysis of urea, inducing a rapid pH increase that has negative effects on human health and agriculture. Thus, control of urease activity is of utmost importance in medical, pharmaceutical, and agro-environmental applications. All known urease inhibitors are either toxic or inefficient. The development of new and efficient chemicals able to inhibit urease relies on the knowledge of all steps of the catalytic mechanism. The short (microseconds) lifetime of the urease-urea complex has hampered the obtainment of its structure. The present study uses fluoride to substitute the hydroxide acting as the co-substrate in the reaction, preventing the occurrence of the catalytic steps that follow substrate binding. The 1.42-Å crystal structure of the urease-urea complex, here reported, resolves the enduring debate on the mechanism of this paradigmatic metallo-enzyme.

Urease (urea amidohydrolase; E.C. 3.5.1.5) has made the history of biological chemistry: it was the first enzyme crystallized and proven to have a proteinaceous nature^[1]; it was also the first enzyme established to depend on the presence of Ni as an essential element^[2], paving the way to the discovery of a group of important Ni-depending enzymes that play key roles in the bio-geo-chemical cycles of carbon, nitrogen and oxygen^[3]; finally, urease is the most efficient enzyme known, with a rate enhancement of ca. 10^{15} with respect to the rate of the uncatalyzed reaction^[4].

Urease is found in a large variety of organisms such as plants, algae, fungi, and prokaryotes and catalyses the hydrolytic decomposition of urea (Scheme 1)^[5]. This reaction causes a pH increase with negative consequences on both human health and agriculture. Urease is the main virulence factor for many human pathogens^[6] such as *Mycobacterium tuberculosis*^[7] and *Helicobacter pylori*^[8]. Moreover, ten among the twelve high-priority antibiotic-resistant strains of bacterial human pathogens indicated by the World Health Organization rely on urease activity

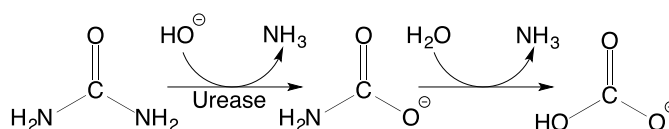
to infect the host organism^[9]. On the other hand, urease present in soil produces negative economic and environmental consequences for urea-based soil nitrogen fertilization^[10]: the ammonia volatilization resulting from too rapid urea hydrolysis triggers large N soil losses that decrease the absorption of ammonium by plant roots and cause formation of airborne particulate matter, contributing to atmospheric pollution^[11].



Scheme 1. Overall urea hydrolysis reaction.

All currently known urease inhibitors are either toxic or inefficient. The most used drug against urease, aceto-hydroxamic acid (AHA)^[12], shows severe side effects such as teratogenicity and toxicity^[13], while N-(n-butyl)-thiophosphoric triamide (NBPT), a urease inhibitor^[14] widely used in agriculture^[15], has negative effects on the healthy growth of crop plants^[16]. These aspects render urease a critical target to develop more efficient inhibitors for medical and agricultural applications^[17]. The development of such molecules relies on the knowledge, at the molecular level, of all steps of the catalytic mechanism.

The hydrolysis of urea involves two stages: the first, strictly enzymatic, produces ammonia and carbamate, while the second involves the uncatalyzed spontaneous decomposition of carbamate to yield another molecule of ammonia and bicarbonate (Scheme 2)^[5f-h].



Scheme 2. Urease-catalysed urea hydrolysis reaction.

Urease contains an essential dinuclear cluster of Ni(II) ions in the active site (Figure 1A)^[5e, 5g, 5h]. Each metal ion is bound to an imidazole N atom of two histidine residues and to a terminal solvent molecule in the form of neutral water, considering the estimated value for the first pK_a (10.6) of a Ni(II)-OH₂ moiety^[18]. One of the ions [Ni(1)] is less coordinatively saturated than the other, with Ni(2) additionally binding the carboxylate group of an aspartate residue. The two Ni(II) ions are bridged by a carbamylated lysine residue and by a solvent-derived ligand in

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the form of a hydroxide ion, considering the estimated very acidic first pK_a and a second pK_a (~ 9–10) of a $Ni(II)-(H_2O)-Ni(II)$ moiety [18]. In this scenario, the presence of a dinuclear $Ni(II)$ cluster causes the formation of a hydroxide ion that acts as the co-substrate necessary for the hydrolytic reaction of urea, at near neutral pH, where the enzyme has its maximal activity.

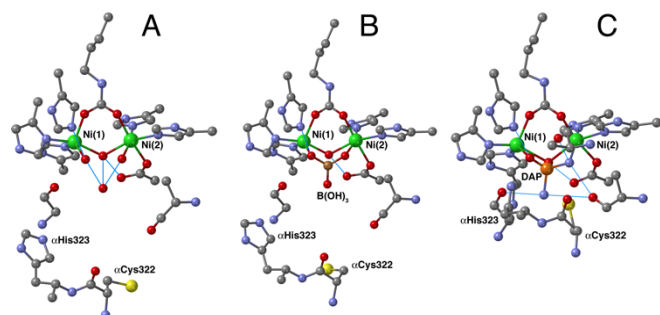
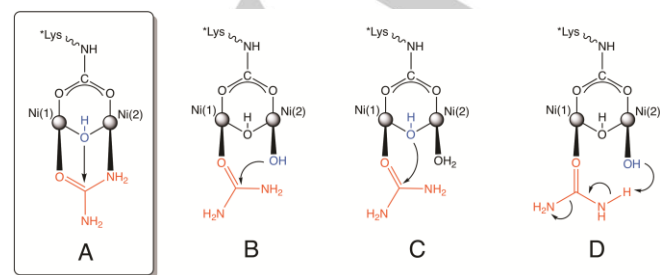


Figure 1. Crystallographic models of the coordination environment of the $Ni(II)$ ions in the active site of SPU in the resting state (A) (PDB code 4CEU [19d]), in complex with boric acid (B) (PDB code 1S3T [20c]) and in complex with DAP (C) (PDB code 3UBP [19c]). C, N, O, S and Ni atoms are coloured grey, blue, red, yellow and green, respectively. H-bonds are shown as thin blue lines. Only the amino acid residues side chains are shown.

The crystallographic structure determination of the $Ni(II)$ coordination environment in native urease revealed the molecular framework for the catalytic process [19], while the structures of several enzyme-inhibitor complexes uncovered the reactivity of the enzyme towards competitive, uncompetitive and non-competitive inhibitors [5e, 14, 19c, 20]. In particular, the structure of the complex between boric acid, a molecule acting as unreactive analogue of urea, and urease from *Sporosarcina pasteurii* (SPU), a widespread and highly ureolytic soil bacterium, shows a trigonal $B(OH)_3$ moiety symmetrically chelating the Ni ions using two O atoms, with the third O atom pointing away from the Ni ions [20c]; here, the bridging hydroxide is left in place and perpendicular to the plane of the $B(OH)_3$ molecule (Figure 1B). Moreover, the structure of the complex between SPU and diamidophosphate (DAP), a transition state analogue generated in situ by enzymatic catalysis of phenylphosphorodiamidate (PPD) or *N*-(*n*-butyl)-phosphoric triamide (NBPTO) [19c, 21], reveals the presence of DAP bound to the two $Ni(II)$ ions through three atoms: one O atom in a symmetric bridging position, one O atom is bound to $Ni(1)$, one N atom is bound to $Ni(2)$ and one N atom points away into the active site cavity (Figure 1C).



Scheme 3. Proposed mechanisms for urea hydrolysis catalysed by urease.

These structures led to the mechanistic proposal that the Ni-bridging hydroxide acts as the nucleophile in the reaction, attacking the carbonyl carbon of a urea molecule proposed to chelate the two $Ni(II)$ ions (Scheme 3A) [5e, 5h, 19c, 22]. This proposal is alternative to previous, and still lingering, hypotheses for the catalytic mechanism: these involve a urea molecule terminally bound to $Ni(1)$ through its carbonyl O atom attacked either by a hydroxide ion terminally bound to $Ni(2)$ [5b] (Scheme 3B) or by the bridging hydroxide (Scheme 3C) [19b, 23]; a third alternative mechanism, based on biomimetic chemistry [24] or computational methods [25], proposed the occurrence of an elimination reaction to form cyanate (Scheme 3D), an intermediate that, however, has never been observed in urease-catalysed reactions [5a, 26].

This controversy can be solved only through the determination of the structure of the enzyme-substrate complex: however, the very short lifetime of the urease-urea adduct (ca. 20 μs) [4] has hampered so far the determination of its structure. The present study reports on the determination of the high-resolution (1.42 Å, Table 1-SI) structure of the urease-urea complex, in which the hydroxide acting as the co-substrate for the reaction has been substituted with an unreactive fluoride, known to inhibit the enzyme by substituting Ni-bound solvent-derived O atoms [19d], thus preventing all subsequent catalytic steps from occurring.

The structure shows the typical $(\alpha\beta\gamma)_3$ assembly of SPU, closely matching that of the native enzyme (PDB code 4CEU; Figure 1-SI displays a structural comparison using the pairwise root mean square deviation (RMSD) of C α atoms) [19d]. The electron density around the $Ni(II)$ ions in the active site region is clearly defined (Figure 2), and the overall scaffold of the protein residues directly involved in Ni-binding is globally unchanged with respect to all previous structures of SPU. A spherical electron density bridging the two $Ni(II)$ ions, distinct from a triangle-shaped density in close proximity to the $Ni(II)$ ions, were revealed by the omit electron density map (Figure 2).

Our prior knowledge of the structural details of native and fluoride-inhibited SPU [19d], together with crystallographic analysis of the structural parameters for the current structure, allowed us to model a fluoride ion into the spherical density in the metal-bridging position (see Table 2-SI for a comparative structural evaluation). A urea molecule was thus successfully modeled in the triangle-shaped density (Figure 2). In the resulting model, the carbonyl O and one amide N atom of urea bind to $Ni(1)$ and $Ni(2)$, respectively, while the other amide N atom points away from the $Ni(II)$ ions. The urea O atom receives a hydrogen bond from the protonated $\alpha His222$ Ne2 [19a, 19c], a residue known to be involved in the formation of the enzyme-substrate complex [27]. The $Ni(1)$ -O distance (2.16 Å) is shorter than the $Ni(2)$ -N distance (2.27 Å), an effect possibly caused by the lower coordination number of $Ni(1)$ that renders this ion more positively charged. The bond between $Ni(2)$ and the urea N atom is stabilized by a network of H-bonds involving the backbone carbonyl O atoms of $\alpha Ala170$ and $\alpha Ala366$. This network supports the presence of an amide NH_2 group at this position. The orientation of the backbone carbonyl O atoms of $\alpha Gly280$ and $\alpha Ala366$ towards the distal urea N atom indicates the presence of two H-bonds, consistent with the second NH_2 amide group of urea at this position. In the established coordination mode of urea to the dinuclear center in urease, the reactivity of the substrate is impeded by the presence of the

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unreactive fluoride ion, supporting the role of the bridging hydroxide as the nucleophile in the hydrolytic reaction.

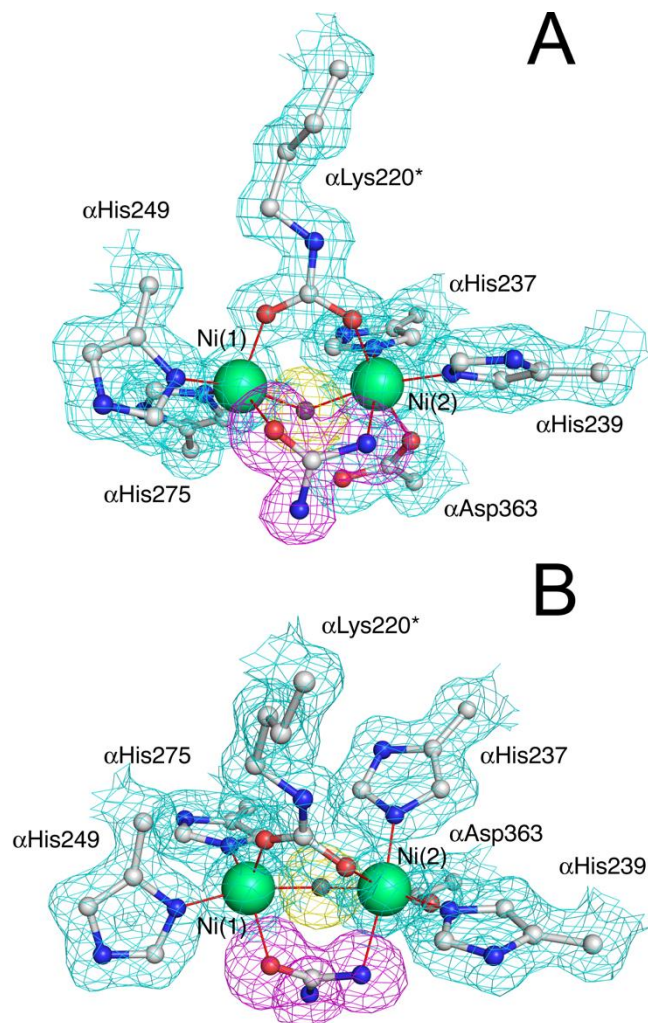


Figure 2. (A) Atomic model of the active site of SPU crystallized in the presence of fluoride and urea. (B) The same atomic model and Ni(II) coordination environment are shown rotated by ca. 90° along the x-axis. The Ni(II) coordination environment is shown superimposed on the final $2F_o - F_c$ electron density map contoured at 1 σ (cyan), while the unbiased $F_o - F_c$ omit map, corresponding to the ligands, is shown contoured at 3.0 σ and coloured in magenta and yellow for the triangle-shaped ligand (urea) and spherical shaped ligand (fluoride), respectively. C, N, O, F and Ni atoms are coloured grey, blue, red, dark green and green, respectively. Only the amino acid residues side chains are shown.

The entrance and the size of the active site cavity of urease is regulated by a mobile helix-turn-helix motif. In native SPU and its form inhibited by boric acid or fluoride [19d, 20c] (as well as in other urease-inhibitor complexes [5g, 5h]), the mobile flap is observed in an open conformation, while in the DAP-bound SPU the flap is closed [19c]. This conformational change was proposed to gate the passage of substrate and products [19c]. Additionally, this flap was proposed to stabilize the substrate and/or the intermediate of the reaction during catalysis [19c]. In the present structure of urease inhibited by fluoride, but in complex with urea, the flap is observed in the closed conformation (Figure 2-SI). This conformational

change moves α Cys322 and α His323 in close proximity to the urea molecule in the active site (Figures 2, 3 and 2-SI); these residues have been implicated in a catalytic proton transfer step [19b, 23b, 27-28]. A network of hydrogen bonds, involving α His323 N δ 1 and α Asp224 O δ 2, as well as α His323 NH ϵ 2 and α Arg339 NH η 2, locks α His323 in the observed position, consistent with site-directed mutagenesis studies of these conserved residues [23b]. This network imposes that α His323 must be neutral at the optimal pH for catalysis (8.0 for SPU [29]), supporting the hypothesis that α His323 is required to stabilize a nascent ammonia molecule formed upon proton transfer from the bridging hydroxide to the distal amide group of a Ni-bound urea, prior to the breaking of the C-N bond and release of ammonia [5e, 5g, 5h, 19c, 22].

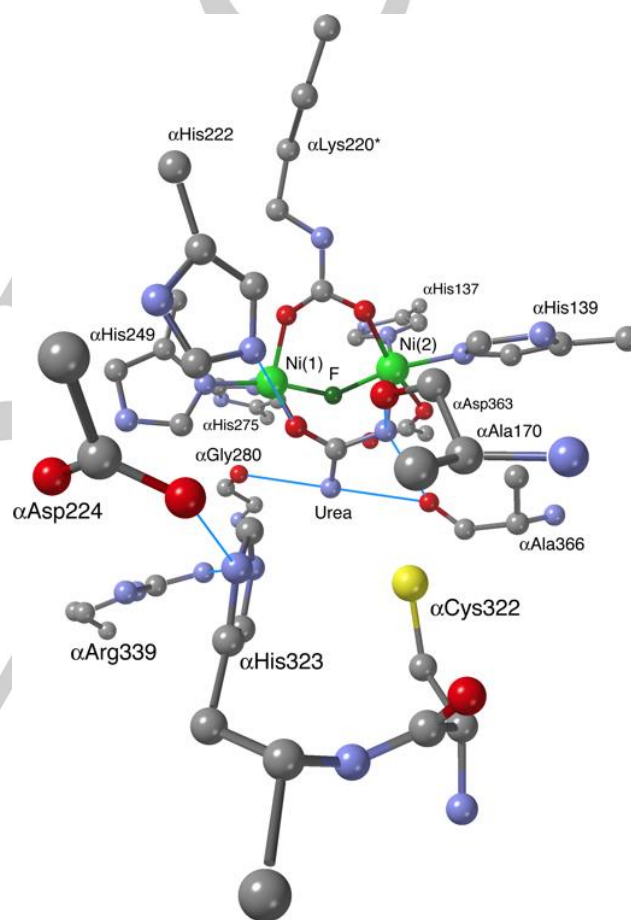


Figure 3. Perspective view of the structural model of the active site of SPU in complex with fluoride and urea. C, N, O, S, F and Ni atoms are coloured grey, blue, red, yellow, dark green and green, respectively. H-bonds are shown as thin blue lines. Only the amino acid residues side chains are shown.

The chelating mode of binding urea to the two Ni(II) ions, using both the O atom and the NH $_2$ group, renders the central C atom of urea electron-deficient and poised to undergo the nucleophilic attack by the Ni-bridging hydroxide present in the resting state of the enzyme. The closing of the flap stabilizes the binding of the substrate urea to the Ni ions in the active site. It can be envisioned that, after hydrolysis, the flap swings open allowing release of the products and entrance of a new urea molecule to re-start the

catalytic cycle. The knowledge of the structural details of the enzyme-substrate complex in urease sheds light on the initial step of the catalytic mechanism of this paradigmatic metallo-enzyme, and resolves the long-standing debate concerning the mechanism of the metal-driven hydrolysis of urea.

Experimental Section

Crystals were obtained by incubating SPU with fluoride prior to addition of urea in the crystallization drop (details of protein purification, crystallization, X-ray diffraction data collection and analysis are provided in the Supplementary Information).

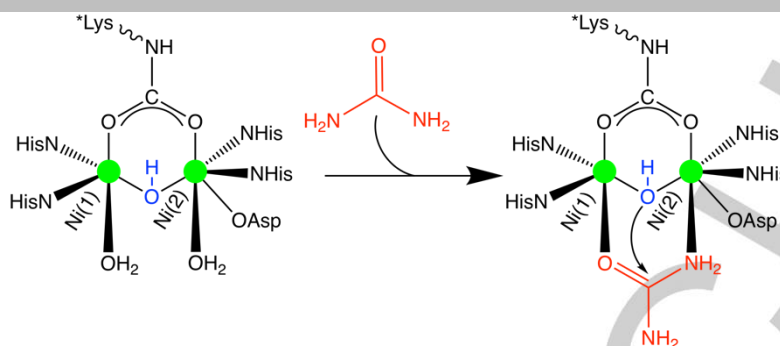
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Keywords: Urease • Nickel • Urea • Catalytic mechanism • X-ray diffraction

- [1] J. B. Sumner, *J. Biol. Chem.* **1926**, 69, 435-441.
- [2] N. E. Dixon, C. Gazzola, R. Blakeley, B. Zerner, *J. Am. Chem. Soc.* **1975**, 97, 4131-4132.
- [3] D. Zamble, M. Rowińska-Żyrek, H. Kozłowski, in *Metallobiology*, Vol. 10 (Ed.: C. D. Garner), Royal Society of Chemistry, Cambridge (UK), **2017**.
- [4] B. P. Callahan, Y. Yuan, R. Wolfenden, *J. Am. Chem. Soc.* **2005**, 127, 10828-10829.
- [5] a R. L. Blakeley, J. A. Hinds, H. E. Kunze, E. C. Webb, B. Zerner, *Biochemistry* **1969**, 8, 1991-2000; b N. E. Dixon, P. W. Riddles, C. Gazzola, R. L. Blakeley, B. Zerner, *Can. J. Biochem.* **1980**, 58, 1335-1344; c R. P. Hausinger, *Microbiol. Rev.* **1987**, 51, 22-42; d H. L. Mobley, R. P. Hausinger, *Microbiol. Rev.* **1989**, 53, 85-108; e S. Ciurli, S. Benini, W. R. Rypniewski, K. S. Wilson, S. Miletto, S. Mangani, *Coord. Chem. Rev.* **1999**, 190-192, 331-355; f B. Zambelli, F. Musiani, S. Benini, S. Ciurli, *Acc. Chem. Res.* **2011**, 44, 520-530; g M. J. Maroney, S. Ciurli, *Chem. Rev.* **2014**, 114, 4206-4228; h L. Mazzei, F. Musiani, S. Ciurli, in *The Biological Chemistry of Nickel* (Eds.: D. Zamble, M. Rowińska-Żyrek, H. Kozłowski), Royal Society of Chemistry, **2017**, pp. 60-97.
- [6] a J. C. Rutherford, *PLoS Pathog.* **2014**, 10, e1004062; b D. Mora, S. Arioli, *PLoS Pathog.* **2014**, 10, e1004472-e1004472.
- [7] W. Lin, V. Mathys, E. L. Y. Ang, V. H. Q. Koh, J. M. Martínez Gómez, M. L. T. Ang, S. Z. Zainul Rahim, M. P. Tan, K. Pethe, S. Alonso, *Infect. Immun.* **2012**, 80, 2771-2779.
- [8] a B. Marshall, J. R. Warren, *The Lancet* **1984**, 323, 1311-1315; b K. A. Eaton, C. L. Brooks, D. R. Morgan, S. Krakowka, *Infect. Immun.* **1991**, 59, 2470-2475.
- [9] WHO, **2017**.
- [10] S. Kiss, M. Simihaian, *Improving efficiency of urea fertilizers by inhibition of soil urease activity*, Kluwer Academic Publishers, Dordrecht, The Netherlands, **2002**.
- [11] F. Paulot, D. J. Jacob, *Environ. Sci. Technol.* **2014**, 48, 903-908.
- [12] a K. Phillips, D. J. Munster, R. A. Allardyce, P.-F. Bagshaw, *J. Clin. Pathol.* **1993**, 46, 372-373; b D. P. Griffith, M. J. Gleeson, H. Lee, R. Longuet, E. Deman, N. Earle, *Eur. Urol.* **1991**, 20, 243-247.
- [13] N. C. Bailie, C. A. Osborne, J. R. Leininger, T. F. Fletcher, S. D. Johnston, P. N. Ogburn, D. P. Griffith, *Am. J. Vet. Res.* **1986**, 47, 2604-2611.
- [14] L. Mazzei, M. Cianci, U. Contaldo, F. Musiani, S. Ciurli, *Biochemistry* **2017**, 56, 5391-5404.
- [15] C. J. Watson, International Fertiliser Society, York, UK, **2000**, pp. 1-40.
- [16] a J. M. Bremner, *Fert. Res.* **1995**, 42, 321-329; b L. Zanin, S. Venuti, N. Tomasi, A. Zamboni, R. M. De Brito Francisco, Z. Varanini, R. Pinton, *Front. Plant Sci.* **2016**, 7, 845-861; c L. Zanin, N. Tomasi, A. Zamboni, Z. Varanini, R. Pinton, *Front. Plant Sci.* **2015**, 6, 1007-1018.
- [17] P. Kosikowska, L. Berlicki, *Expert Opin. Ther. Pat.* **2011**, 21, 945-957.
- [18] F. Basolo, R. G. Pearson, *Mechanisms of Inorganic Reactions*, 2nd ed., Wiley, New York, **1967**.
- [19] a E. Jabri, M. B. Carr, R. P. Hausinger, P. A. Karplus, *Science* **1995**, 268, 998-1004; b P. A. Karplus, M. A. Pearson, R. P. Hausinger, *Acc. Chem. Res.* **1997**, 30, 330-337; c S. Benini, W. R. Rypniewski, K. S. Wilson, S. Miletto, S. Ciurli, S. Mangani, *Structure* **1999**, 7, 205-216; d S. Benini, M. Cianci, L. Mazzei, S. Ciurli, *J. Biol. Inorg. Chem.* **2014**, 19, 1243-1261.
- [20] a S. Benini, W. R. Rypniewski, K. S. Wilson, S. Ciurli, S. Mangani, *J. Biol. Inorg. Chem.* **1998**, 3, 268-273; b S. Benini, W. R. Rypniewski, K. S. Wilson, S. Ciurli, S. Mangani, *J. Biol. Inorg. Chem.* **2001**, 6, 778-790; c S. Benini, W. R. Rypniewski, K. S. Wilson, S. Mangani, S. Ciurli, *J. Am. Chem. Soc.* **2004**, 126, 3714-3715; d S. Benini, W. R. Rypniewski, K. S. Wilson, S. Miletto, S. Ciurli, S. Mangani, *J. Biol. Inorg. Chem.* **2000**, 5, 110-118; e L. Mazzei, M. Cianci, S. Benini, L. Bertini, F. Musiani, S. Ciurli, *J. Inorg. Biochem.* **2016**, 154, 42-49; f L. Mazzei, M. Cianci, A. Gonzalez Vara, S. Ciurli, *Dalton Trans.* **2018**, 47, 8240-8247; g L. Mazzei, M. Cianci, F. Musiani, S. Ciurli, *Dalton Trans.* **2016**, 45, 5455-5459; h L. Mazzei, M. Cianci, F. Musiani, G. Lente, M. Palombo, S. Ciurli, *J. Inorg. Biochem.* **2017**, 166, 182-189.
- [21] L. Mazzei, M. Cianci, U. Contaldo, S. Ciurli, *J. Agric. Food Chem.* **2019**, 67, 2127-2138.
- [22] F. Musiani, E. Arnoffi, R. Casadio, S. Ciurli, *J. Biol. Inorg. Chem.* **2001**, 6, 300-314.
- [23] a M. J. Todd, R. P. Hausinger, *Biochemistry* **2000**, 39, 5389-5396; b M. A. Pearson, I. S. Park, R. A. Schaller, L. O. Michel, P. A. Karplus, R. P. Hausinger, *Biochemistry* **2000**, 39, 8575-8584.
- [24] A. M. Barrios, S. J. Lippard, *J. Am. Chem. Soc.* **2000**, 122, 9172-9177.
- [25] G. Estiu, K. M. Merz, Jr., *J. Phys. Chem. B* **2007**, 111, 10263-10274.
- [26] J. B. Sumner, D. B. Hand, R. G. Holloway, *J. Biol. Chem.* **1931**, 91, 333-341.
- [27] I.-S. Park, R. P. Hausinger, *Protein Sci.* **1993**, 2, 1034-1041.
- [28] M. A. Pearson, L. Overbye Michel, R. P. Hausinger, P. A. Karplus, *Biochemistry* **1997**, 36, 8164-8172.
- [29] S. Ciurli, C. Marzadori, S. Benini, S. Deiana, C. Gessa, *Soil Biol. Biochem.* **1996**, 28, 811-817.

The crystal structure of the urease-urea complex, revealing the unprecedented chelating binding mode of urea to the di-nickel cluster in the active site, is a definitive evidence for the initial step of the catalytic mechanism of urea hydrolysis.



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The structure of the elusive urease-urea complex: a paradigmatic case of metallo-enzyme catalysis