Electronic Supplementary Information

(11 pages)

Smart Urea Ionic Co-crystals with Enhanced Urease Inhibition Activity for Improved Nitrogen Cycle Management

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1. Experimental Procedures

All reagents were purchased from Sigma-Aldrich or Alfa Aesar and used without further purification.

1.1 Solution Synthesis. Equimolar quantities of the starting materials (urea, $ZnCl_2$ and KCl) were dissolved in 5 mL of water at room temperature. The solution was divided in two portions: metastable form 1 was obtained by heating the solution to 80°C and leaving the solvent to evaporate at this temperature, while stable form 2 was obtained by slow solvent evaporation at room temperature.

1.2 Solid state synthesis. Pure form 2 was obtained by ball-milling urea (1 mmol) with $ZnCl_2$ (1 mmol) and KCl (1 mmol) in an agate jar for 60 min in dry conditions or with the addition of a drop of water. Form 2 was also obtained during pellets preparation of form 1, i.e. under hydrostatic pressure.

1.3 Slurry. Slurry experiments were performed in water at room temperature for 10 days. Form 2 appeared to be the only stable phase in the suspension, regardless of the of Urea:$ZnCl_2$:KCl stoichiometric ratio (1:1:1, 1:1:2 and 2:1:1) or the initial presence of only pure form 1.

1.4 Solubility tests. A qualitative analysis was performed for urea and ZnKU as described in the following. Solubility of urea at room temperature ranges from 1 to 1.2 g mL^{-1},[1] therefore a control experiment was conducted in which 1 g of urea was added to a vial and dissolved in 1mL of bidistilled water. In a second vial an amount of ZnKU form 2 (4.5 g) containing 1 g of urea and 1 mL of bidistilled water were then added: the dissolution was not complete, as can be seen in Figure ESI-1a. The solid not dissolved was filtered and weighed, resulting in ca. 900 mg of powder material, which corresponds to a reduction in the solubility of urea in ZnKU with respect to pure urea of ca. 20%. The undissolved powder was analysed via X-ray powder diffraction (see below in the X-ray powder diffraction section) and found to be ZnKU form 2. The experiment was repeated three times and in all cases the same behaviour and amount of undissolved substance was observed. In a fourth experiment, the addition of 1 mL of bidistilled water to the vial containing the undissolved form 2 caused complete dissolution of the solid residue (see Figure ESI-1b).

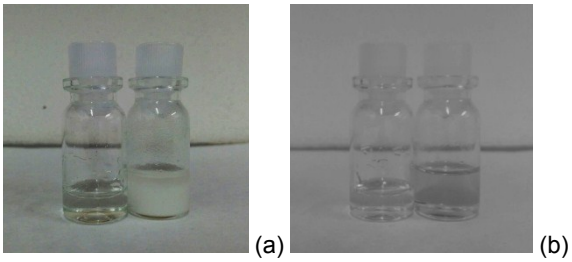

(a) (b)

Fig. ESI-1. Dissolution of the same quantity (1g) of urea as pure substance (left vial) and in ZnKU (right vial), added to 1 (a) and 2 (b) mL of water.

1.5 Thermogravimetric analysis. TGA measurements were performed with a PerkinElmer TGA7 in the temperature range 30-300 ºC and 30-450°C for urea and ZnKU, respectively, under N_2 gas flow at a heating rate of 5.00 ºC min^{-1}.

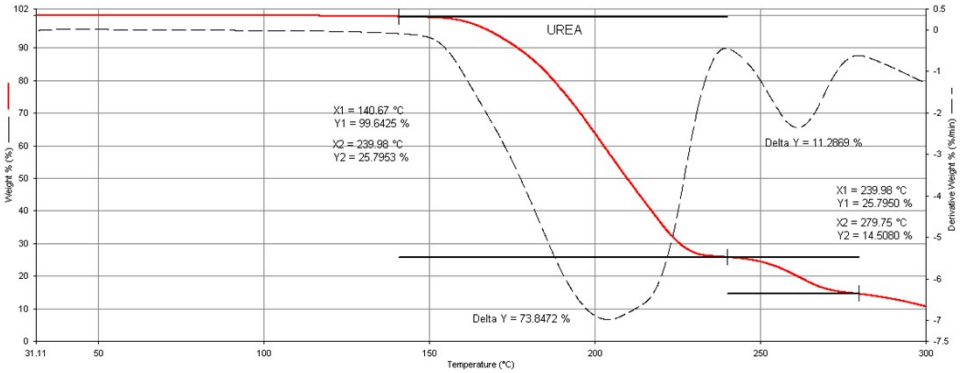


Fig. ESI-2. TGA trace for the solid urea used in all experiments.

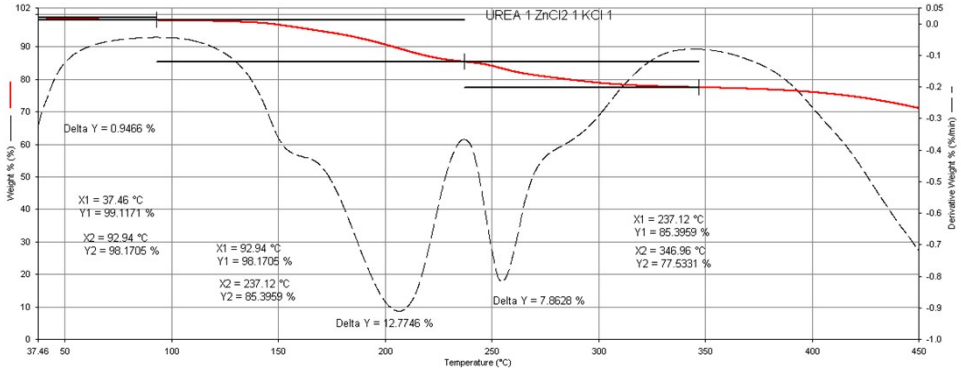


Fig. ESI-3. TGA trace for ZnKU form 1.

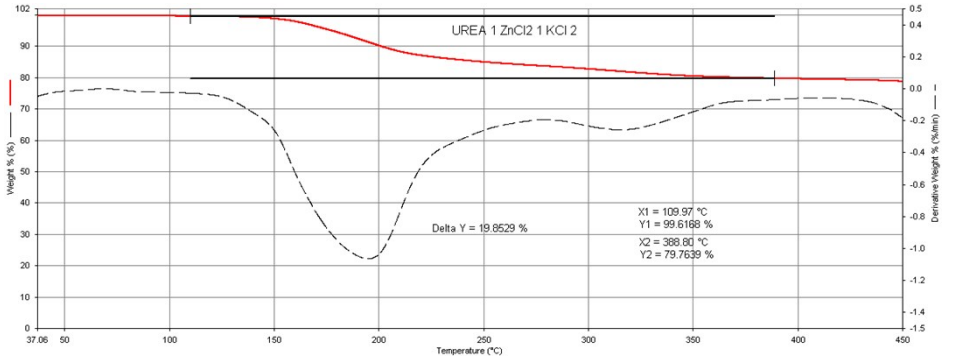


Fig. ESI-4. TGA trace for ZnKU form 2.

1.6 Differential Scanning Calorimetry. DSC traces were recorded using a Perkin-Elmer Diamond. The samples (1-3 mg range) were placed in open Al-pans. All measurements were conducted in the ranges 40-150/160/170 °C (for urea, ZnKU form 1 and ZnKU form 2, respectively), at a heating rate of 5ºC min^{-1}. Melting points for urea, ZnKU form 1 and ZnKU form 2 are 137, 135 and 142 °C, respectively (peak temperatures).

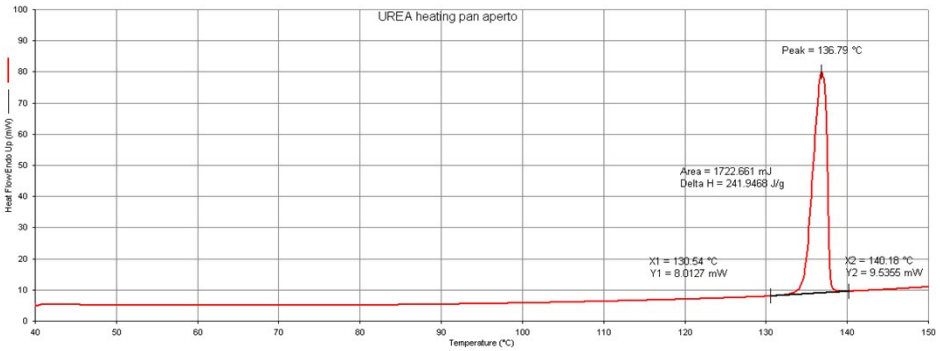


Fig. ESI-5. DSC trace for the solid urea used in all experiments.

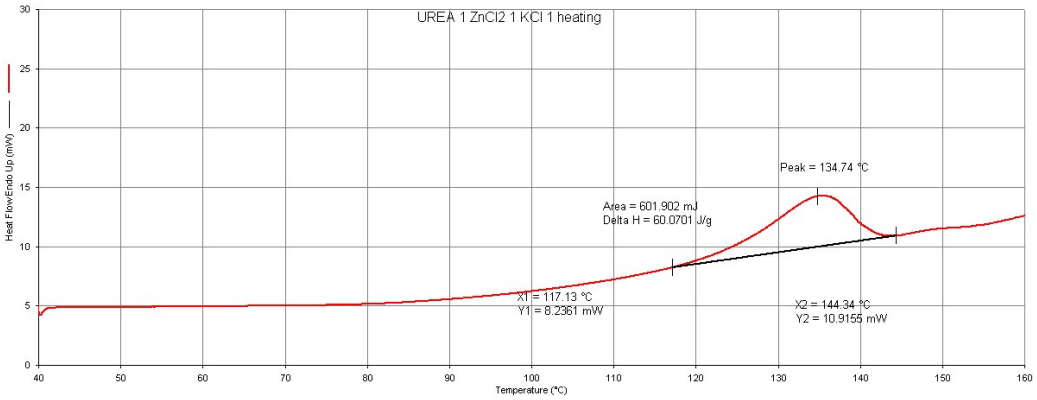


Fig. ESI-6. DSC trace for ZnKU form 1.

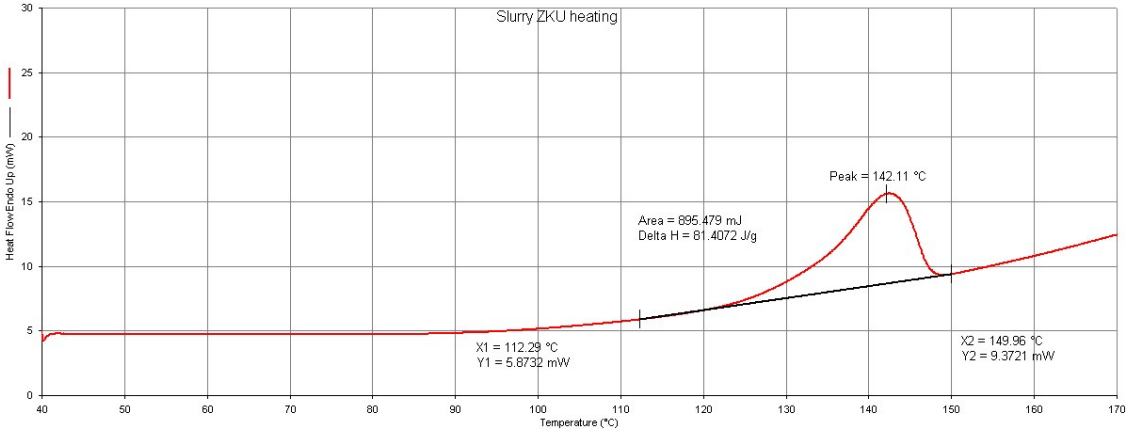


Fig. ESI-7. DSC trace for ZnKU form 2.

1.7 E-T diagram. Energy vs. Temperature (E-T) diagram for the dimorphic system ZnKU form 1 / ZnKU form 2. Form 2 has the higher melting point and the higher heat of fusion ($\Delta_m H_2$ 60 J/g vs. $\Delta_m H_1$ 81 J/g, for form 2 and form 1, respectively). According to the heat-of-fusion rule of Burger-Ramberger,[2] therefore, the system is monotropic.

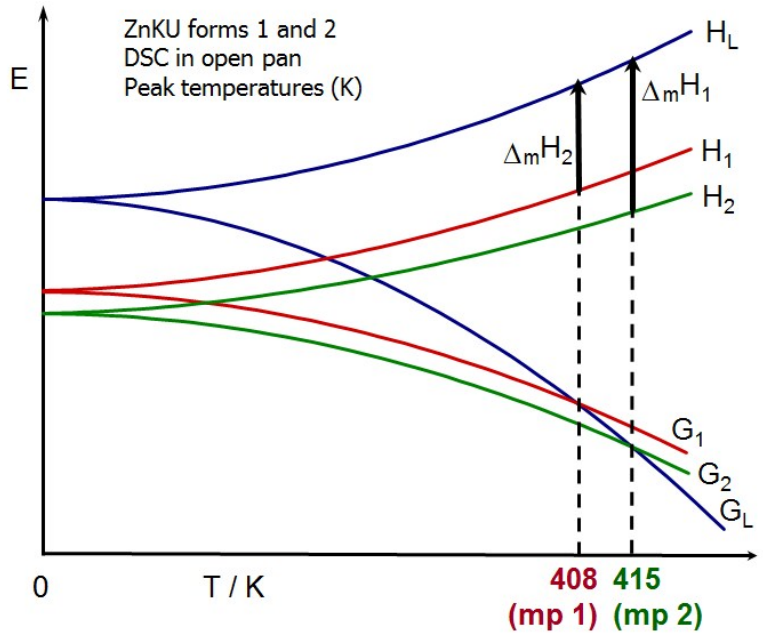


Fig. ESI-8. E-T diagram for the dimorphic system ZnKU form1 and ZnKU form 2.

2. X-ray diffraction analysis.

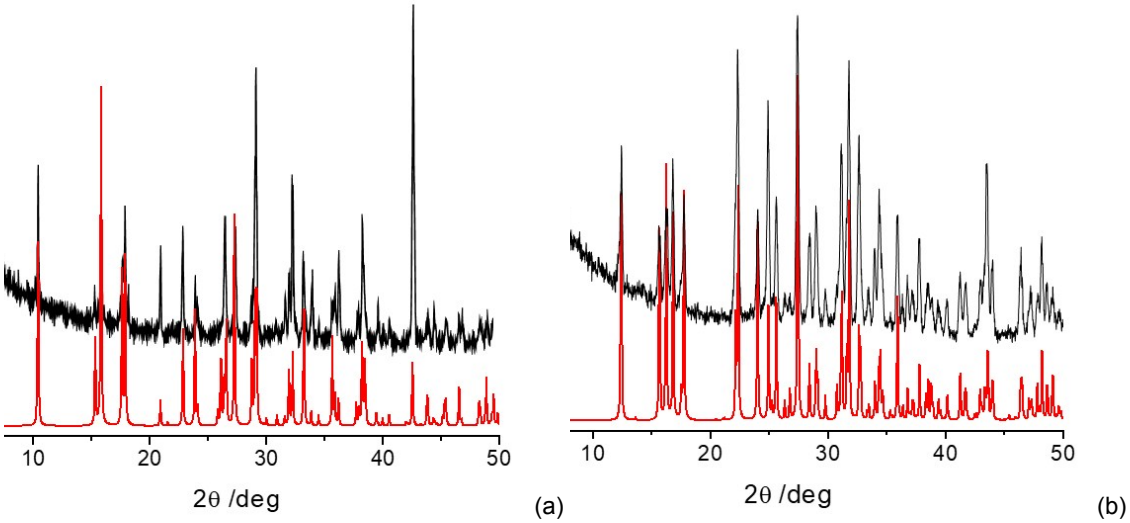
2.1 Single Crystal X-ray Diffraction. Single Crystal data were collected at room temperature with an Oxford Diffraction X´Calibur equipped with a graphite monochromator and a CCD detector. Mo-Kα radiation (γ=0.71073 Å) was used. Unit cell parameters for all compounds discussed herein are reported in Table ESI-1. The structure was solved by the Intrinsic Phasing methods and refined by least squares methods again F^2 using SHELXT-2014[3] and SHELXL-2014[4] with OLEX 2 interface.[5] Non-hydrogen atoms were refined anisotropically. Hydrogen atoms bound to nitrogen atoms were either located from a Fourier map or added in calculated positions, and their position was refined riding on their N atoms. In ZnKU form 1 the NH_2 moieties of urea are disordered over two equivalent positions, referred by a crystallographic mirror plane. The software Mercury 3.10.1[6] was used for graphical representations and to simulate the powder patterns based on single crystal data.

Table ESI-1. Crystal data and details of measurements for ZnKU form 1 and form 2.

	ZnKU form1	ZnKU form2
Chemical formula	$CH_4Cl_3KN_2O$ Zn	$CH_4Cl_3KN_2O$ Zn
M_r, g*mol^{-1}	270.88	270.88
T / K	293 (2)	293 (2)
Morphology, colour	Block, colourless	Prism, colourless
Crystal system	Monoclinic	Monoclinic
Space group	P 2_1/m	P 2_1/n
a / Å	6.8599(10)	7.4220(6)
b / Å	7.3530(12)	13.5530(10)
c / Å	8.4999(11)	8.4219(5)
α / °	90	90
β / °	99.069(13)	92.089(7)
γ / °	90	90
V / Å^3	423.38(11)	846.60(11)
Z	2	4
d / mg.cm^{-3}	2.125	2.125
μ / mm^{-1}	4.266	4.266
Reflections collected/unique	1624/804	9911/1867
R_{int}	0.0521	0.0301
Threshold expression	> 2σ(I)	> 2σ(I)
R_1 (obs)	0.0589	0.0305
wR_2 (all)	0.1006	0.0834

Crystal data can be obtained free of charge from the Cambridge Crystallographic Data Centre via https://www.ccdc.cam.ac.uk and have been allocated the accession numbers **CCDC 1841314** (ZnKU form 1) and **CCDC 1841315** (ZnKU form 2).

2.2 X-ray Diffraction from Powder. For phase identification purposes X-ray powder diffraction (XRPD) patterns were collected on a PANalytical X´Pert Pro Automated diffractometer equipped with an X´celerator detector in Bragg-Brentano geometry, using Cu-Kα radiation (γ=1.5418 Å) without monochromator in 2θ range between 3º and 50º (step size 0.033º; time/step: 20 s; Soller slit 0,04 rad, antiscatter slit: ½, divergence slit: ¼ ; 40 mA*40kV).



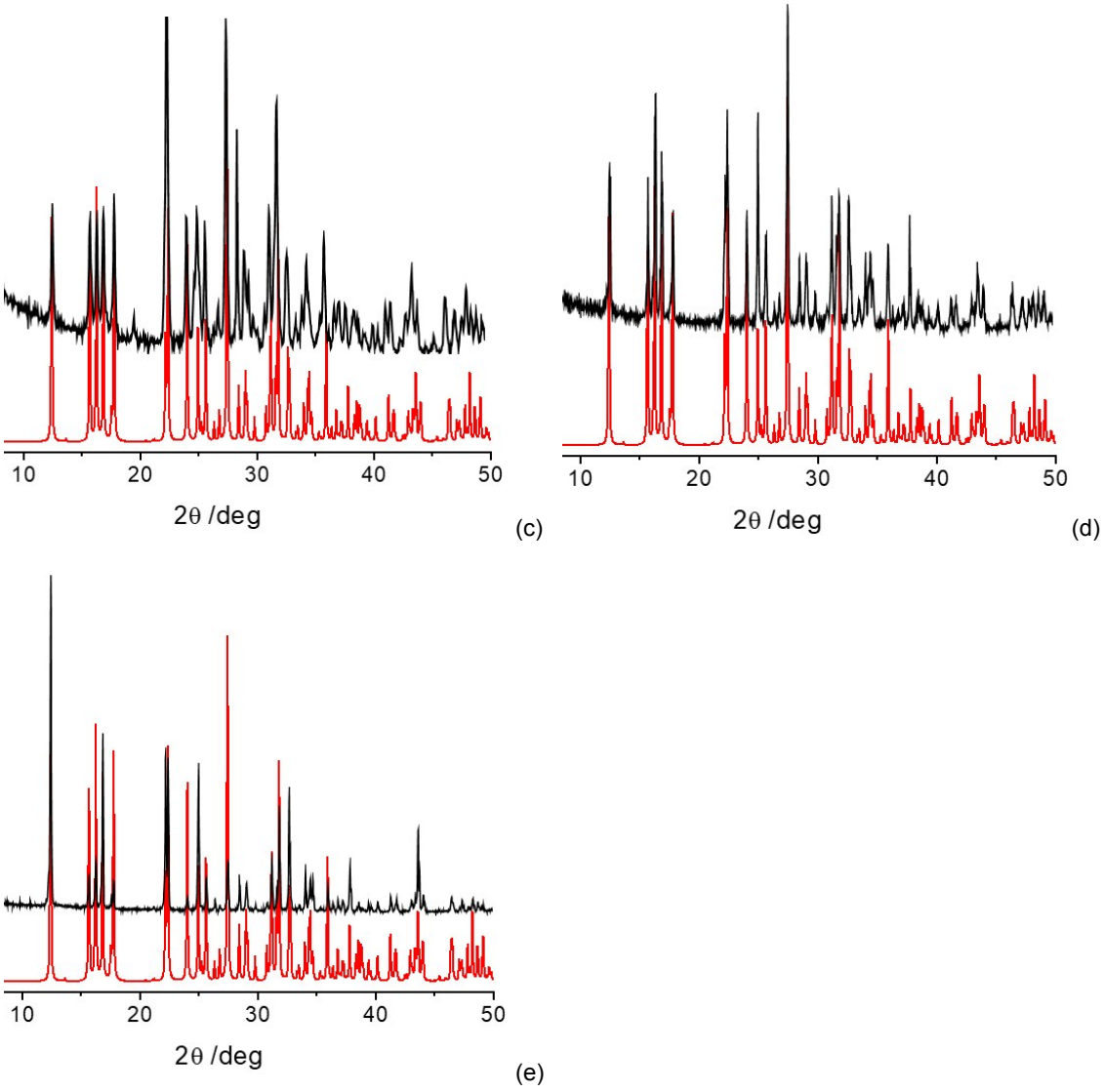


Fig. ESI-9 (a) Comparison of the experimental XRPD pattern for ZnKU form 1, as obtained from solution at 80°C, and the pattern calculated on the basis of single crystal data; (b) Comparison of the experimental XRPD pattern for ZnKU form 2, as obtained from solution, and the pattern calculated on the basis of single crystal data; (c) Comparison of the experimental XRPD pattern for ZnKU form 2, as obtained via ball milling, and the pattern calculated on the basis of single crystal data. (d) Comparison of the experimental XRPD pattern for the product of the 10 days slurry of ZnKU from 1; (e) Comparison of the experimental XRPD pattern for the residual, undissolved solid obtained in the solubility test, and the pattern calculated on the basis of single crystal data for ZnKU form 2. Black lines experimental, red lines calculated XRPD patterns.

3. Enzymatic assay using the pH-STAT method.

Urease activity was determined in triplicate using the pH-STAT method, as described by Blakeley et al.[7] In particular, a T1 pH-meter equipped with a 50-14 T electrode (Crison Instruments, SA), was used to record, every 0.5 min and for a 3 min reaction time, the volume of a 100 mmol L^{-1} HCl solution necessary to maintain the 10 mL solution containing urease and its substrate urea at the fixed pH value of 7.5. The measurement started 0.5 min after urea addition in order to allow time to reach uniform substrate concentration in the sample volume. One unit of enzyme is defined as the amount of urease required to hydrolyze 1 μmol urea min^{-1} of reaction.

3.1 Determination of the kinetic parameters for urea hydrolysis by urease

The 10 mL reaction mixture was composed of 9.9 mL of 2 mmol L^{-1} 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer at pH 7.5, containing increasing concentrations of urea in the range 1 - 64 mM. The reaction was started upon the addition of a concentrated solution (0.1 mL) of urease from *Canavalia ensiformis* (Jack Bean urease, JBU) (Sigma-Aldrich) to the reaction mixture. The resulting values for the enzyme activity measured at each concentration of urea were plotted as a function of substrate concentration and fitted by using the Michaelis-Menten equation (Equation S1) in order to derive the maximal velocity (V_{max}) for the enzymatic hydrolysis of urea, as well as the Michaelis constant (K_M) for the urease - urea couple.

$$reaction\ rate = \frac{V_{max}[S]}{K_M + [S]} \qquad \textbf{Eq. S1}$$

3.2 Determination of urease inhibition by ZnKU

The inhibition strengths of ZnKU form 1 and form 2 on urease were determined with the same experimental protocol of that described above for the enzyme in the absence of ZnKU. In this case, the reaction mixture consisted of 9.9 mL of 2 mM HEPES buffer at pH 7.50, also containing 64 mM of urea and increasing concentrations of ZnKU (0.54, 1.08 and 2.16 μg mL^{-1} in the case of ZnKU form 1, 0.69, 1.38 and 2.76 μg mL^{-1} in the case of ZnKU form 2). The slightly dissimilar concentrations used for the two ZnKU forms reflect the different stoichiometry of Zn(II) inside the two crystal forms and have been chosen in order to work at the same Zn^{2+} concentration. The experimental results were normalized with respect to the activity measured in the same conditions in the absence of ZnKU (control experiment) and plotted, as a percentage, as a function of the amount of inhibitor tested.

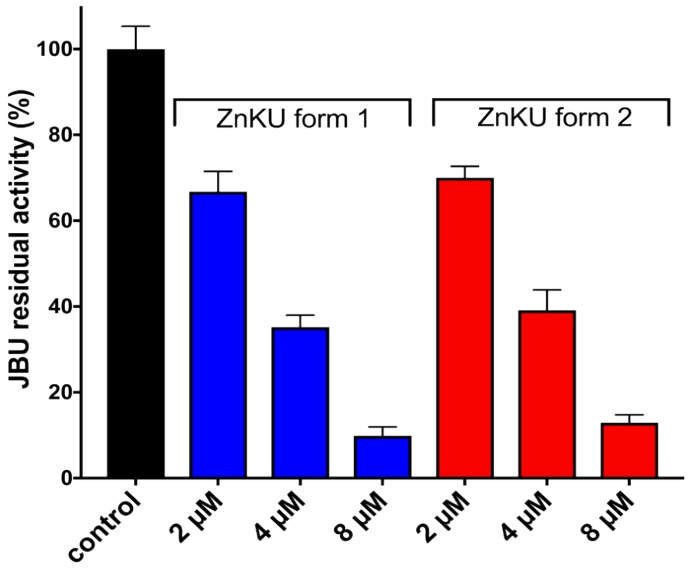


Fig. ESI-S10: Residual percentage activity of jack bean urease (JBU), referred to 100% (control, black bar) in the presence of increasing concentrations of the two polymorphic ZnKU compounds, at pH 7.5. The blue bars represent the residual activity of urease in the presence of 2, 4 and 8 μM of ZnKU form 1, while the red bars represent the residual activity of urease in the presence of the same concentrations of ZnKU form 2, as already described in the main text.

The data in Fig. ESI-S10 are consistent with the known value of Ki for the inhibition of urease with Zn(II) (0.76 microM – ref. 32 in the main text). The mode of action for this inhibition likely resembles that elucidated recently by the 1.91 Å resolution structure of *Sporosarcina pasteurii* urease inhibited by Ag(I),[8] which revealed the presence of two metal ions bound to the largely conserved triad αCys322/αHis323/αMet367: the first two residues are located on a mobile structural motif (flap) that is essential for modulating the size of the active site cavity and the position of key residues for enzyme catalysis, while αMet367 is on a loop facing the flap at the entrance of the active site cavity. The binding of the Ag(I) ions at the edge of the active site channel appears to block the movement of the flap, inhibiting the catalytic activity of urease, and so should also occur for Zn(II).

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