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A Kinetic and Isotope Effect Investigation of the Urease-Catalyzed Hydrolysis of Hydroxyurea[†]

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Abstract

The urease-catalyzed hydrolysis of hydroxyurea is known to exhibit biphasic kinetics, showing a rapid burst phase followed by a slow plateau phase. Kinetic isotope effects for both phases of this reaction were measured at pH 6.0 and 25°C. The observed nitrogen isotope effects for the ammonia leaving group were $^{15}(V/K)_{\text{NH}_3} = 1.0016 \pm 0.0005$ during the burst phase and $^{15}(V/K)_{\text{NH}_3} = 1.0019 \pm 0.0007$ during the plateau phase while those for the hydroxylamine leaving group are $^{15}(V/K)_{\text{NH}_2\text{OH}} = 1.0013 \pm 0.0005$ for the burst phase and $^{15}(V/K)_{\text{NH}_2\text{OH}} = 1.0022 \pm 0.0003$ for the plateau phase. These isotope effects are consistent with a rate-determining step that occurs prior to breaking either of the two possible C-N bonds. The observed carbonyl carbon isotope effects are $^{13}(V/K) = 1.0135 \pm 0.0003$ during the burst phase and $^{13}(V/K) = 1.0178 \pm 0.0003$ during the plateau phase. The similarity of the magnitude of the carbon isotope effects argues for formation of a common intermediate during both phases.

Urease catalyzes the hydrolysis of urea to ammonia and CO₂. The enzyme has both historical and biochemical significance. From a health perspective urease from *C. pylori* has been implicated in gastroduodenal disease, including the formation of gastric ulcers (1). Environmentally, ureases from soil bacteria increase soil alkalinity via hydrolysis of urea-containing fertilizers (1). From a chemical standpoint urease is a formidable catalyst; it is capable of rapid hydrolysis of urea, which has an estimated $t_{1/2}$ for hydrolysis of 520 years at room temperature (2).

Urease from jack bean was the first enzyme to be crystallized (Sumner, 1926), but ironically the only reported crystal structure for the jack bean enzyme is one of poor resolution (3.8 Å), containing a bound antibody fragment (3,4). High-resolution crystal structures are available for ureases from other sources, particularly those from bacteria. The active site residues are highly conserved for all sources of the enzyme leading to the reasonable assumption that the mechanisms of hydrolysis are also very similar (5). The jack bean enzyme is hexameric and requires two Ni atoms per subunit. A catalytically vital active site histidine (His320) is indicated by both X-ray crystal structures and mutagenesis studies of bacterial ureases; the mutant enzymes are ~10⁵ times less active when His320 is replaced

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Supporting Information Available. A graph of the fraction of reaction versus time for the experimental conditions employed for measurement of the kinetic isotope effects is provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

with ala, asn or gln (5,6). The most widely accepted mechanism is one involving a tetrahedral intermediate in which: (a) one Ni serves as a Lewis acid to activate the carbonyl oxygen, (b) the other Ni coordinates to the nucleophilic water and (c) His320 serves as a general acid which donates a proton to the leaving nitrogen atom. The catalytic steps of the mechanism are summarized in Scheme 1 (5).

Nitrogen and carbon KIE experiments have been reported for the hydrolysis of urea. The magnitude of the carbonyl-C KIE and the leaving-N KIEs are $^{13}(V/K) = 1.0206$ and $^{15}(V/K) = 1.0075$, respectively (7). Because both nitrogen atoms of urea are included in measurement of the observed KIE, the actual $^{15}(V/K)$ is presumably twice that (1.015) for the initial C-N cleavage, assuming no appreciable secondary ^{15}N isotope effect at the other nitrogen. This assumes the bond to the second NH_3 is cleaved after the first irreversible step. The result is consistent with partially rate-determining C—N bond cleavage. We have previously reported KIE studies of formamide and semicarbazide at pH 6.0, which are alternate, slow substrates for jack bean urease (8,9). The rationale for these two studies was simple. Formamide has only one leaving-N and allowed investigation of all steps of the mechanism up to loss of the first nitrogen. In addition, formamide afforded measurement of five different KIEs: the carbonyl-C, $^{13}(V/K) = 1.0241$; the carbonyl-O, $^{18}(V/K) = 0.9960$; the formyl-H, $^D(V/K) = 0.95$; the leaving-N, $^{15}(V/K) = 1.0327$; and the nucleophile-O, $^{18}(V/K) = 0.9778$. The KIE experiments for semicarbazide allowed for independent measurement of the KIE for both leaving groups ($-\text{NH}_2$ and $-\text{NHNH}_2$), as well as that for the carbonyl-C. In this case $^{15}(V/K)$ for the leaving-N (1.0010 for $-\text{NH}_2$ and 1.0090 for $-\text{NHNH}_2$) indicate that $-\text{NHNH}_2$ is the first leaving group to depart and that cleavage of this C-N bond is partially rate determining. The results for formamide and semicarbazide indicate that better leaving groups ($-\text{NHNH}_2 > -\text{NH}_2$) lead to faster (or less rate determining) C-N cleavage step.

In this paper we report a KIE study on the urease-catalyzed hydrolysis of a third alternate substrate for urease—hydroxyurea (HU). The overall reaction is given in equation 1. HU exhibits biphasic kinetics with a rapid burst phase, followed by a very slow plateau phase. The plateau phase has been attributed to irreversible (or very slowly reversible) substrate inhibition. The product, hydroxylamine, has been shown to be a weak but reversible inhibitor of urease; it is not the cause of the plateau phase (10, 11). The KIE experiments reported here include the carbonyl-C, the leaving-N for NH_3 and the leaving-N for NH_2OH .

(1)

Experimental Procedures (Materials and Methods)

Materials and Methods

Urease (from jack bean), HU, MES, EPPS, TiCl_3 and LiOH were from Sigma-Aldrich.

Glutamate dehydrogenase kits for ammonia determination were from Raichem.

Ultrafiltration was accomplished using a Millipore YM series membrane with a 10,000 MW cutoff. Isotopic composition of the carbon and nitrogen were measured on an isotope ratio mass spectrometer (IRMS) and were expressed in δ (per mil) notation as shown in equation 2, where $R_{(\text{sample})}$ is the isotope ratio (heavy/light) of the sample and $R_{(\text{standard})}$ is the isotope ratio of a standard. Heavy-atom KIEs were calculated using equations 3 and 4, where $k/^*k$ is the observed isotope effect ($*k$ is the rate constant for the heavier isotope), f is

the fraction of reaction, R_0 is the isotope ratio (heavy/light) for the unreacted starting material or the product after complete reaction, R_s is that for substrate after partial reaction, and R_p is that for the product after partial reaction (12). Normally the notation $k/^*k$ is simply shortened to $*k$ (e.g. ^{13}k , ^{15}k , etc.). For enzyme-catalyzed reactions, observed KIEs that are measured via the competitive method are actually isotope effects on V/K and are reported in an analogous fashion as $*(V/K)$.

$$\delta = \left(\frac{R_{(sample)}}{R_{(standard)}} - 1 \right) \times 1000 \quad (2)$$

$$k / *k = \log(1 - f) / \log[(1 - f)(R_s/R_0)] \quad (3)$$

$$k / *k = \log(1 - f) / \log[1 - f(R_p/R_0)] \quad (4)$$

Determination of Fraction of Reaction

In a typical assay a 10 μ L aliquot of the quenched reaction mixture was added to 1990 μ L of water. A 200 μ L aliquot of this diluted sample was then added to 1.0 mL of a solution containing NADPH and buffer at pH 7.8 in a non-continuous assay. The initial absorbance was recorded at 340 nm. A 50 μ L sample of glutamate dehydrogenase was added and the absorbance was again recorded at 340 nm after 5 min. The concentration of ammonia was calculated from the difference in absorbance. Controls showed that hydroxylamine does not react under these conditions.

Enzyme Kinetics

The glutamate dehydrogenase method described above was used to determine the rates of reaction, except the aliquot from the urease reactions was 200 μ L and was not diluted. The pH of the glutamate dehydrogenase assay was maintained at 7.8. The urease reaction solutions contained 50 mM MES buffer at pH 6.0 and various concentrations of HU. The ionic strength was maintained at 0.20 M with KCl. Dialysis experiments contained one sample that contained 50 mM MES buffer, 4.0 mM HU and 0.25 mg/mL urease and a second control which contained all of the above except HU. Both were allowed to incubate for 15 min. and were then dialyzed against 1.0 L of 50 mM MES buffer for 30 min. Both samples were then supplied with enough HU to bring the concentration to 4.0 mM and assayed over time with the glutamate dehydrogenase method.

Nitrogen Isotope Effect Procedures

The KIEs were measured using the competitive method via analysis of the product produced during partial and complete reactions. The KIE experiments during the burst and plateau phases had identical concentrations of all reagents. However, the total volume of solution for the burst phase was larger (6 mL) than that for the plateau phase (2 mL) to allow for an ample quantity of products for IRMS analysis. Each sample contained 0.15 M HU, urease (approximately 3.5 mg/mL), 0.50 M MES buffer at pH 6.0. Each was equilibrated to 25 °C and the reaction was initiated by addition of urease. The reaction was quenched by addition of 750 μ L of 1.0 M H_2SO_4 . The burst phase KIE experiments were allowed to run between 7.5–10.5 min. before quenching, whereas the plateau phase KIE experiments were allowed to run 5.25–10 hr. before quenching. The fraction of reaction at quench for burst phase KIEs was between 0.036 and 0.073; that for the plateau phase was between 0.31 and 0.36. Control

experiments show that no further hydrolysis occurs after addition of acid. A 10 μ L aliquot was then assayed to determine the concentration of ammonia by the glutamate dehydrogenase method described above. The remainder of the solution was subjected to ultrafiltration at 4°C.

The resulting solution was applied to a column containing 7 mL of Biorad AG 50W X8 strong cation exchange resin (Li-form). The column was eluted with 100 mL water, followed by 40 mL of a solution containing 0.20 M EPPS (pH 8.0) and 0.2 M LiCl. The hydroxylamine was quantitatively isolated by this elution. The column was then eluted with 40 mL of 0.1 M LiOH to quantitatively elute the ammonia. The ammonia was steam distilled, rotary evaporated to a volume of 2 mL and oxidized to N₂ with NaOBr for analysis by IRMS (13). The pooled hydroxylamine fractions were first quantitatively reduced to ammonia by addition of a 3 mL aliquot of 6 M HCl followed by addition of enough of a 10 wt. % solution of TiCl₃ (in 20–30 wt. % HCl) to induce a permanent faint pink color. This usually required about 0.6–0.7 mL of the TiCl₃ solution (14). After reduction to ammonia, the solution was steam distilled and oxidized to N₂ with NaOBr as described above. Control experiments show that $^{15}\delta$ of N₂ from combustion of hydroxylamine was the same as that from ammonia which was produced by reduction of the same hydroxylamine with Ti³⁺.

Complete hydrolysis of HU was accomplished in a 1.0 mL solution containing 0.50 M MES at pH 6.0, 0.10 M HU and approximately 3 mg of urease. The reaction was allowed to proceed for two days at room temperature before work up as described above. As a control the $^{15}\delta$ of N₂ from combustion of HU was shown to be the same as the average for N₂ obtained from hydroxylamine and ammonia after complete hydrolysis of hydroxyurea by urease.

Carbon Isotope Effect Procedure

The KIEs were measured using the competitive method via analysis of the CO₂ produced after partial reaction. The reaction mixture contained the same reagents as described above. The solution was sparged with CO₂-free N₂ in a 100 mL round bottom flask equipped with a stopcock (to connect to the high vacuum line) and a sidearm containing a second stopcock. Urease was added with a syringe through a sidearm that was capped with a septum. The reaction was quenched by addition of H₂SO₄ via this same sidearm and a 10 μ L aliquot was withdrawn for determination of fraction of reaction. The burst phase KIE experiments were allowed to run 7.5–11 min. before quenching, whereas the plateau phase KIE experiments were allowed to run 8–10 hr. before quenching. The fraction of reaction at quench for burst phase KIEs was between 0.036 and 0.058; that for the plateau phase was between 0.43 and 0.55. The flask was then attached to a high vacuum line and the CO₂ was collected through two dry ice-isopropanol traps and one liquid nitrogen trap. The details of this procedure have been published previously (15). The isolated CO₂ was then analyzed by IRMS. Isotopic analysis of unreacted hydroxyurea, which served as the R₀ in determination of the KIE, was accomplished by combustion to CO₂ and analysis by IRMS.

Results

Kinetics experiments

The rate of HU hydrolysis exhibits biphasic kinetics, which is dependent on both initial substrate and initial enzyme concentration (Figures 1 and 2). These results are similar to findings reported in the literature (10, 11).

Substrate inhibition by HU appears to be very slowly reversible. When urease is pre-incubated with HU for 15 min and then dialyzed for 30 min, the activity of this dialyzed enzyme is somewhat less than for a control sample of urease that was dialyzed, but not pre-

incubated with HU (Figure 3). In both samples the biphasic kinetics are once again observed. It must be noted that this procedure is not the same as that reported in the literature, where the diluted samples that were pre-incubated with HU were assayed with the natural substrate, urea (11).

Isotope Effects

The nitrogen and carbon KIE procedures were subjected to the following controls: (a) The glutamate dehydrogenase assay for NH_3 did not produce an absorbance change at 340 nm in the absence of urease and after quenching the reaction mixture with H_2SO_4 . (b) The presence of NH_2OH did not produce any absorbance change at 340 nm in the above assay. (c) No CO_2 could be detected by manometric measurements after sparging with CO_2 -free N_2 or after quenching the reaction mixture with H_2SO_4 . (d) The $^{15}\delta$ for combustion of HU agreed with that generated by the complete hydrolysis of HU by urease followed by ion exchange chromatography, reduction to NH_3 (in the case of NH_2OH), steam distillation and oxidation to N_2 . A similar control showed no change in the $^{13}\delta$ for carbon (Table 1).

The KIEs were measured for both the $-\text{NH}_2$ and $-\text{NHOH}$ leaving groups, as well as for the carbonyl-C, during the burst and plateau phases of the hydrolysis of HU. The results for the burst phase are summarized in Table 2; those for the plateau phase are in Table 3. It must be noted that the plateau phase data are for the observed KIE, which means that a small part of the observed KIE is due to the burst phase. It must also be noted that the conditions for the KIE experiments are somewhat different than those for the kinetic experiments shown in Figures 1, 2 and 3. The major difference is in the concentration of HU and buffer. The KIE experiments required higher concentrations of HU (150 mM) than the kinetic experiments (0.75–6.0 mM) to produce enough CO_2 for IRMS analysis. This, in turn, required higher concentrations of buffer to maintain constant pH for the KIE experiments (500 mM) than for the kinetics experiments (50 mM). A graph of fraction of HU hydrolysis versus time under the experimental conditions for measurement of the KIEs can be found in the Supporting Information. It can be estimated that under the above conditions the burst phase was complete after 5–7% of total reaction. The reactions for the plateau phase KIEs for N and C were quenched at 31–36% and 43–55% total reaction, respectively. Assuming the burst phase is complete at approximately 6% total reaction, the observed KIEs given in Table 3 must then be composed of 81–83% and 86–89% of KIE from the plateau phase for N and C, respectively (with the remainder from the burst phase).

Discussion

HU is known to be an unusual alternate substrate for urease; the reaction is biphasic with a rapid, burst phase, followed by a slow plateau phase (10,11). The burst phase appears to follow Michaelis-Menten kinetics with a reported $K_m = 1.25\text{--}1.60$ mM and a $k_{\text{cat}} = 12\text{ s}^{-1}$ at pH 7.0 and 20 °C. By comparison urea hydrolysis follows Michaelis-Menten kinetics throughout the reaction and has a reported $K_m = 3$ mM and a $k_{\text{cat}} = 11,000\text{ s}^{-1}$ under the same conditions (16). These kinetic experiments with HU as the substrate have been repeated in the present work and the results show the same biphasic pattern (Figures 1 & 2). However, a reliable K_m and k_{cat} could not be obtained because the burst phase was found to be too rapid for our kinetic methods. Nevertheless, our crude estimates point to kinetic constants of the same order of magnitude as those reported.

Pre-incubation of urease with HU was shown to inhibit urea hydrolysis; the amount of inhibition depended directly on the length of time of pre-incubation, with maximum inhibition occurring after 20–30 min (11). Consequently, the plateau phase observed for HU hydrolysis was initially assumed to be the result of either inhibition by the substrate (HU) or by a product, presumably NH_2OH . In these studies urease was pre-incubated with HU for

various amounts of time and then diluted 100-fold with buffer. This resulted in urease which had greatly reduced urea hydrolysis activity as compared to the initial enzyme. It is claimed that no recovery of urease activity is observed when the diluted samples were allowed to equilibrate for varying amounts of time before assaying the rate of urea hydrolysis. This led the authors to conclude that the observed inhibition of urea hydrolysis by HU is largely irreversible (11). Further, in these studies the product, NH_2OH , was shown to be a rapidly reversible inhibitor and not likely to be a major cause of the observed inhibition. Close inspection of the data in reference 11 (Figure 2, left graph) suggests a slow recovery of urease activity when the diluted enzyme is allowed to sit for longer times (30 min.) prior to assaying the rate of urea hydrolysis. The current work also opens the door to the possibility that inhibition may be somewhat slowly reversed by dialysis (Figure 3). However, because the dialyzed enzyme was assayed with HU instead of urea and because dialysis resulted in the loss of activity for urease that was not pre-incubated with HU, a quantitative treatment of the recovery of activity is not possible. Even so, it is clear that biphasic kinetics are re-established after the long dialysis time.

Results from x-ray crystallography support the hypothesis that inhibition by HU causes the plateau phase. Acetohydroxamic acid (AHA) is a substance structurally similar to HU; it is an irreversible (or possibly very slowly reversible) inhibitor of urea hydrolysis but it is not a substrate for urease. *Klebsiella aerogenes* and *Bacillus pasteurii* ureases have been crystallized in the presence of AHA at 2.0 Å and 1.55 Å resolution, respectively (17,18). The results show that the $-\text{OH}$ of the hydroxamate group is within 2.6 Å from Ni-1 and 1.8 Å from Ni-2 in the *Klebsiella aerogenes* enzyme; it is within 2.6 Å of both Ni atoms in the *Bacillus pasteurii* enzyme (Figure 4). These results show the hydroxamate functional group to be in bonding distance of one or both of the Ni atoms at the active site. Therefore, it is clearly possible that coordination of the $-\text{OH}$ from the hydroxamate group of HU can displace the nucleophilic hydroxide and lead to the observed slowly reversible substrate inhibition.

A reasonable proposed chemical mechanism based on the above experimental results is given in Scheme 2. The top pathway shows breaking of the C—N bond to the $-\text{NHOH}$ leaving group occurring prior to the breaking of the C—N bond to the $-\text{NH}_2$ leaving group; the bottom pathway reverses the order of C—N bond breaking. The breaking of the second C—N bond occurs after the first irreversible step and does not affect the observed kinetics and isotope effects. Since the products are only bound to Ni by the carboxylate oxygen atoms, it is unlikely that product release would limit the rate. The structure at the top represents the proposed substrate inhibitor bound to Ni-2, as suggested by the x-ray data discussed above. Substrate inhibition like that for HU is not observed for the β -nitrogen of semicarbazide because the hetero atom bound to Ni-2 is replacing a hydroxide. The $\text{p}K_a$ of the hydroxamate oxygen is expected to be around 9 and can ionize to displace the anionic hydroxide. The β -nitrogen of semicarbazide has a much higher $\text{p}K_a$ and is not capable of facile ionization. The mechanism of scheme 2 is simplified; it shows only the two required Ni atoms and the chemically important His320 (6).

Isotope Effects

The leaving-N KIEs are of a very small magnitude and do not vary significantly with a change in leaving group from $-\text{NH}_2$ to $-\text{NHOH}$ or a change from the burst to the plateau phase (Tables 2 and 3). An inescapable conclusion from these results is that breakdown of the tetrahedral intermediate (the k_5 step, equation 5) is not appreciably rate determining under any of the experimental conditions used in this study. This means the rate determining step is either some non-chemical step or the formation of the tetrahedral intermediate. Observation of a carbonyl-C KIE in excess of 1% (see below) favors the latter explanation. Since all the leaving-N KIEs are so small, it is not possible to ascertain which C-N bond is

broken first during catalysis. This is in stark contrast to the aforementioned investigation of semicarbazide as an alternate substrate for urease. Semicarbazide does not show biphasic kinetics but has a larger $^{15}(V/K)$ for the $-NHNH_2$ side (1.0090) than for the $-NH_2$ side (1.0010), indicating that $-NHNH_2$ is the first C-N bond broken during catalysis (9).

The inhibitor shown at the top of scheme 2 is a dead end complex; it affects the overall rate, but not the partition ratios in the expression for V/K . Consequently, the chemical mechanism from scheme 2 can be reduced to the kinetic expression of equation 5. The expression that governs the relationship between the rate constants and magnitude of the KIEs of equation 5 is given in equation 6, where the superscript denotes an isotope effect (either equilibrium, K , or kinetic, k). Because HU is a 500-fold slower substrate than urea, $k_{off} \gg k_{cat}$ and it is reasonable to assume that $k_3 \ll k_2$ (16). With this assumption, equation 6 can be simplified to the expression in equation 7. Analysis of the leaving-N KIEs for the alternate substrate, semicarbazide, indicates that, given the choice of two different nitrogen leaving groups, urease chooses the one with the best leaving group ability (i.e. the lowest pK_a of the conjugate acid) as the first C-N bond to be cleaved (9). It must be emphasized again that all leaving-N KIEs are small for HU hydrolysis and it is not strictly possible to determine which is the first C-N bond broken. However, it is a reasonable assumption that the better of the two leaving groups is cleaved first and the following analysis will utilize this assumption.

$$^{15}(V/K) = \frac{(^{15}K_{eq}^{15}k_5) + ^{15}k_3(k_5/k_4) + (k_5/k_4)(k_3/k_2)}{1 + [k_5/k_4(1 + k_3/k_2)]} \quad (6)$$

$$^{15}(V/K) = \frac{(^{15}K_{eq}^{15}k_5) + ^{15}k_3(k_5/k_4)}{1 + (k_5/k_4)} \quad (7)$$

Calculation of the commitment factor, k_5/k_4 , is then possible during the burst and plateau phases using equation 7. This requires estimations of $^{15}K_{eq}$, $^{15}k_3$ and $^{15}k_5$. The rationale for this approach and the estimations of these intrinsic KIEs has been published (8, 9). $^{15}K_{eq}$ and $^{15}k_3$ were estimated from model systems to be 0.983 and 1.000, respectively. For the urease-catalyzed hydrolysis of formamide $^{15}(V/K) = 1.033$, leading to the conclusion that step 5 (k_5) is rate determining. In turn, this leads to simplification of equation 7 to: $^{15}(V/K) = ^{15}K_{eq} \times ^{15}k_5$ and allows calculation of the intrinsic KIE on step 5 of $^{15}k_5 = 1.050$ (8). Using the values of $^{15}K_{eq}$, $^{15}k_3$ and $^{15}k_5$ from above and $^{15}(V/K)$ for HU hydrolysis ($-NHOH$ side) in equation 7, the commitment factor (k_5/k_4) for the burst phase is 24 and for the plateau phase is 14. This result is within expectations, if it is assumed that $-NHOH$ is the first C-N bond broken. In this case the trend in k_5/k_4 for the burst phase would roughly follow the pK_a of the conjugate acid of the leaving group, where $-NHOH$ ($pK_a = 6.0$) has $k_5/k_4 = 24$, $-NHNH_2$ ($pK_a = 8.1$) has $k_5/k_4 = 2.7$ and $-NH_2$ ($pK_a = 9.6$) has $k_5/k_4 = 1.2$. This correlation is not strictly linear because the commitment factors do not just depend on the pK_a of the leaving groups, but also on the partitioning of the two tetrahedral intermediates in scheme 2. For example, if the commitment is low, then the two tetrahedral intermediates will be in equilibrium and the rate of breakdown to products might be determined by the pK_a of the leaving group. On the other hand, if the commitment is high, the two tetrahedral intermediates may not be at equilibrium and the influence of the pK_a of the leaving group

will be diminished. In addition, a lot of what is observed also depends on the geometry of the active site.

The carbonyl-C KIE gives a different result, where $^{13}(V/K)$ changes significantly from the burst phase (1.0135) to the plateau phase (1.0178). From a purely qualitative point of view the magnitude of these carbonyl-C KIEs is relatively small. Typical magnitudes for the reactions of esters and amides are in the range of 1.028–1.034 (19). Interpretation of carbonyl-C KIEs for acyl group transfers has been difficult in the past because both theoretical and empirical studies have shown that this KIE is not very sensitive to changes in transition state structure (20,21). In HU hydrolysis it is clear that the k_5 step is not rate determining. Therefore, $^{13}(V/K)$ arises largely from the KIE on the k_3 step. It is possible to quantitatively estimate this intrinsic KIE on step 3 ($^{13}k_3$) by substituting the commitment factors calculated above, an estimation of $^{13}K_{eq} \times ^{13}k_5$ (see below) and the observed $^{13}(V/K)$ for the carbonyl-C during the burst (1.0135) and plateau (1.0178) phases of HU hydrolysis into equation 8.

$$^{13}(V/K) = \frac{(^{13}K_{eq} \cdot ^{13}k_5) + ^{13}k_3 (k_5/k_4)}{1 + (k_5/k_4)} \quad (8)$$

The mathematical product ($^{13}K_{eq} \times ^{13}k_5$) can again be estimated from the observed $^{13}(V/K)$ for the carbonyl-C (1.0241) of the urease-catalyzed hydrolysis of formamide, where breakdown of the tetrahedral intermediate (the k_5 step) is rate determining. In this case equation 8 once again reduces to: $^{13}(V/K) = ^{13}K_{eq} \times ^{13}k_5$ (8). Substituting this value and the values of the commitment factors (k_5/k_4) for the burst and plateau phases into equation 8 yields $^{13}k_3 = 1.0131$ for the burst phase and $^{13}k_3 = 1.0173$ for the plateau phase. These results are in remarkably close agreement (they should be the same), especially considering that the KIE for the plateau phase includes a partial contribution from the KIE of the burst phase.

A plausible explanation for the change in the magnitude of the carbonyl-C KIEs involves the -NHOH end of the substrate (HU) coordinating to the Ni-2, as seen in the x-ray structure for urease with structurally similar AHA bound in the active site (17,18). An alternative explanation might have the carbonyl-O coordinated to the Ni-2 and the hydroxyl group coordinated to Ni-1. However, this does not fit the above x-ray data and, as mentioned earlier, only the hydroxyl group of HU has the right pK_a to displace the anionic hydroxide that is bound to Ni-2. It is then most likely that coordination of the hydroxyl group to Ni-2 results in the observed inhibition, which is slowly reversible. At equilibrium most of subunits of this hexameric enzyme are in this inhibited form. However, if one of the monomers is still active, the commitments could change, leading to the observed change in the observed KIE.

Conclusion

HU is an alternative substrate for urease; the hydrolysis exhibits biphasic kinetics, where there is a burst phase followed by a plateau phase. The results of the heavy-atom KIE investigation of urease-catalyzed HU hydrolysis yield very small KIEs for both nitrogen leaving groups during the burst and plateau phases. Assuming (as seems likely) that urease employs the same basic mechanism for hydrolysis of HU as is used during urea hydrolysis, this result is consistent with formation of the tetrahedral intermediate, not its breakdown to products, as the rate determining step in hydrolysis. Since all leaving-N KIEs are small, it is not rigorously possible to conclude which of the two C-N bonds is broken first because bond

breaking occurs after the rate determining step. However, previous studies of semicarbazide hydrolysis show that the enzyme prefers to hydrolyze the better of the two leaving groups first (9).

The carbonyl-C KIE does change significantly on going from the burst to the plateau phase, due to a change in the commitment factor, k_5/k_4 . The most likely explanation for the onset of the plateau phase and for the change in commitment factor involves the coordination of the –OH of the hydroxylamine leaving group of HU to Ni-2 at the active site of urease. Once HU is coordinated to Ni-2, the coordinated nucleophilic hydroxide is released as water and inhibition ensues at that particular active site, thereby leading to the observed plateau phase. This inhibition appears to be slowly reversible. Because urease is a hexamer, the plateau phase is a result of at least a small number (perhaps only one) of the subunits remaining active.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

AMPSO	N-(1,1-Dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid
HOAc	acetic acid
IRMS	Isotope Ratio Mass Spectrometry
KIE	kinetic isotope effect
MES	4-morpholineethanesulfonic acid
MOPS	3-(N-morpholino)propanesulfonic acid
NADPH	β -nicotinamide adenine dinucleotide phosphate
tricine	<i>N</i> -tris(hydroxymethyl)methylglycine

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Figure 1.

Fraction of HU hydrolysis versus time as a function of [HU] at 0.25 mg/mL urease at pH 6.1 in MES buffer. • = 0.75 mM, ▲ = 1.5 mM, ■ = 4.5 mM, ◆ = 6.0 mM. The rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see: Materials and Methods). Note: The y-axis is fraction of total reaction, similar to plots in ref. 10 and 11. In the above experiment, samples containing a higher initial concentration of substrate (HU) actually produce a higher concentration of assayed product (NH_3) at a given time point, but this represents a lower fraction of the total possible reaction.

Figure 2.

Fraction of HU hydrolysis versus time as a function of [urease] at 1.3 mM HU at pH 6.1 in MES buffer. ■ = 0.03 mg/mL, ▲ = 0.06 mg/mL, ● = 0.13 mg/mL, ◆ = 0.25 mg/mL. Note: The rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see: Materials and Methods). Note: The y-axis is fraction of total reaction, similar to plots in ref. 10 and 11. In all these reactions the initial substrate (HU) concentration was constant at 1.3 mM.

Figure 3.

The effect of dialysis on the fraction of HU hydrolysis versus time. $\blacklozenge$ = initial assay with HU, $\blacksquare$ = 15 min. incubation without HU followed by dialysis into MES buffer at pH 6.1, $\blacktriangle$ = 15 min. incubation with HU followed by dialysis. The rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see: Materials and Methods). Note: The y-axis is fraction of total reaction, similar to plots in ref. 10 and 11. In all these reactions the initial substrate (HU) concentration was constant at 4.0 mM and urease was 0.25 mg/mL.

Figure 4.
Crystal Structure of Urease (from *Bacillus Parteurii*) Active Site with Acetohydroxamic Acid (AHA) from ref. 14.

Scheme 1.

Scheme 2.

Table 1

Nitrogen and Carbon Isotopic Composition after Combustion and after Complete Hydrolysis of HU.

Experiment	$^{15}\delta$ for NH_3	$^{15}\delta$ for NH_2OH	Average $^{15}\delta$ (both nitrogens)	$^{13}\delta$ for CO_2
Combustion of HU	--	--	-3.7 (2)	-23.7 (2)
100% Hydrolysis of HU	+6.1 (7)	-14.0 (7)	-4.0 (calc)	-23.7 (5)

Table 2

Kinetic Isotope Effects on the Burst Phase of Urease-Catalyzed Hydrolysis of Hydroxyurea in MES Buffer at pH 6 and 25 °C.

atom	f ^a	partial ^b (¹³ δ or ¹⁵ δ)	product ^c (¹³ δ or ¹⁵ δ)	isotope effect ^d
-NHOH side	0.071	-15.3	-14.0	1.0014
	0.043	-14.7	-14.0	1.0007
	0.051	-14.9	-14.0	1.0009
	0.036	-15.6	-14.0	1.0017
	0.073	-14.5	-12.8	1.0018
			AVE	1.0013 ± 0.0005
-NH ₂ side	0.071	4.5	6.1	1.0017
	0.043	4.7	6.1	1.0014
	0.051	5.0	6.1	1.0011
	0.036	4.8	6.1	1.0013
	0.073	5.0	7.3	1.0024
			AVE	1.0016 ± 0.0005
carbonyl-C	0.051	-36.7	-23.7	1.0139
	0.036	-36.3	-23.7	1.0133
	0.041	-36.2	-23.7	1.0132
	0.055	-36.2	-23.7	1.0133
	0.058	-36.5	-23.7	1.0137
			AVE	1.0135 ± 0.0003

^a Fraction of reaction as determined by glutamate dehydrogenase assay.

^b δ at quench.

^c Average of independent determinations from each batch of substrate.

^d Corrected for fraction of total reaction. Uncertainties are expressed as standard deviation.

Table 3

Kinetic Isotope Effects on the Plateau Phase of Urease-Catalyzed Hydrolysis of Hydroxyurea in MES Buffer at pH 6 and 25 °C.

atom	f ^a	partial ^b (¹³ δ or ¹⁵ δ)	product ^c (¹³ δ or ¹⁵ δ)	isotope effect ^d
-NHOH side	0.31	-16.2	-14.0	1.0027
	0.32	-15.8	-14.0	1.0022
	0.33	-15.5	-14.0	1.0019
	0.32	-14.6	-12.8	1.0022
	0.36	-14.3	-12.8	1.0019
			AVE	1.0022 ± 0.0003
-NH ₂ side	0.31	5.1	6.1	1.0012
	0.32	5.0	6.1	1.0013
	0.33	4.6	6.1	1.0018
	0.32	5.0	7.3	1.0028
	0.36	5.4	7.3	1.0024
			AVE	1.0019 ± 0.0007
carbonyl-C	0.45	-36.4	-23.7	1.0181
	0.43	-36.2	-23.7	1.0174
	0.55	-35.0	-23.7	1.0179
	0.55	-34.8	-23.7	1.0176
	0.53	-35.4	-23.7	1.0181
			AVE	1.0178 ± 0.0003

^a Fraction of reaction as determined by glutamate dehydrogenase assay.

^b δ at quench.

^c Average of independent determinations from each batch of substrate.

^d Corrected for fraction of total reaction. Uncertainties are expressed as standard deviation.