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

John F. Marlier,*,‡ Lori I. Robins,‡ Kathryn A. Tucker,‡ Jill Rawlings,§ Mark A. Anderson,∥ and W. W. Cleland∥

‡*Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, California 93407,* §*Auburn University at Montgomery, Montgomery, Alabama 36124, and* ∥*Institute for Enzyme Research, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53726*

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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 [$^{15}(V/K)_{NH_3}$] were 1.0016 ± 0.0005 during the burst phase and 1.0019 ± 0.0007 during the plateau phase, while those for the hydroxylamine leaving group [$^{15}(V/K)_{NH_2OH}$] were 1.0013 ± 0.0005 for the burst phase and 1.0022 ± 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 [$^{13}(V/K)$] were 1.0135 ± 0.0003 during the burst phase and 1.0178 ± 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 CO_2. The enzyme has both historical and biochemical significance. From a health perspective, urease from *Campylobacter 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 (*3*), 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 (*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 ~10^5 times less active when His320 is replaced 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 that donates a proton to the leaving nitrogen atom. The catalytic steps of the mechanism are summarized in Scheme 1 (*5*).

Nitrogen and carbon KIE[1] experiments have been reported for the hydrolysis of urea. The magnitudes of the carbonyl C KIE [$^{13}(V/K)$] and the leaving N KIE [$^{15}(V/K)$] are 1.0206 and 1.0075, respectively (*7*). Because both nitrogen atoms of urea are included in the measurement of the observed KIE, the actual $^{15}(V/K)$ is presumably twice that (1.015) for the initial C−N bond cleavage, assuming no appreciable secondary ^{15}N isotope effect at the other nitrogen. This assumes the bond to the second NH_2 group 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 (NH_2 and $NHNH_2$), as well as that for the carbonyl C. In this case, $^{15}(V/K)$ values for the leaving N (1.0010 for NH_2 and 1.0090 for $NHNH_2$) indicate that the $NHNH_2$ group 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

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*To whom correspondence should be addressed. Phone: (805) 756-1327. Fax: (805) 756-5500. E-mail: jmarlier@calpoly.edu.

[1]Abbreviations: AMPSO, *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; EPPS, 4-(2-hydroxyethyl)piperazine-1-propanesulfonic 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, *N*-tris(hydroxymethyl)methylglycine.

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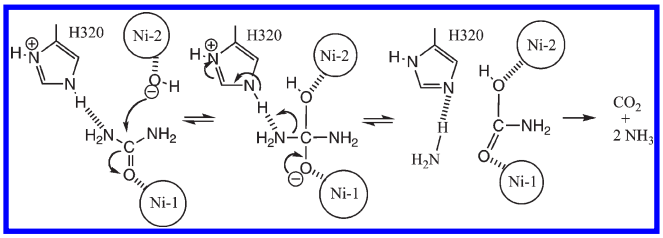
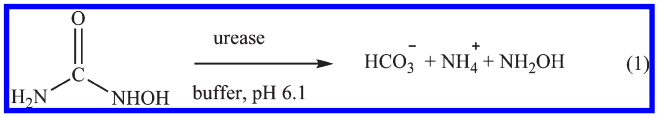
Scheme 1



better leaving groups ($NHNH_2$ > NH_2) lead to the faster (or less rate determining) C−N bond 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 eq 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_2, and the leaving N for NHOH.

EXPERIMENTAL PROCEDURES

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 molecular weight cutoff. The isotopic compositions of the carbon and nitrogen were measured on an isotope ratio mass spectrometer and were expressed in δ (per mil) notation as shown in eq 2

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

where $R_{(\text{sample})}$ is the isotope ratio (heavy to light) of the sample and $R_{(\text{standard})}$ is the isotope ratio of a standard. Heavy-atom KIEs were calculated using eqs 3 and 4

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

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

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 to light) for the unreacted starting material or the product after complete reaction, R_s is that for the 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 $^*(V/K)$.

Determination of the Fraction of Reaction. In a typical assay, a 10 μL aliquot of the quenched reaction mixture was added to 1990 μL of water. A 200 μL 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 noncontinuous assay. The initial absorbance was recorded at 340 nm. A 50 μL 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 μL 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 6.1 and various concentrations of HU. The ionic strength was maintained at 0.20 M with KCl. Dialysis experiments included one sample that contained 50 mM MES buffer, 4.0 mM HU, and 0.25 mg/mL urease and a second control that 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 sufficient 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 the 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), and 0.50 M MES buffer (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 μL of 1.0 M H_2SO_4. The burst phase KIE experiments were allowed to run for 7.5−10.5 min before quenching, whereas the plateau phase KIE experiments were allowed to run for 5.25−10 h 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 μL 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 Bio-Rad AG 50W X8 strong cation exchange resin (Li form). The column was eluted with 100 mL of 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 N_2 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 $TiCl_3$ (in 20−30 wt % HCl) to induce a permanent faint pink color. This usually required ~0.6−0.7 mL of the $TiCl_3$ solution (*14*). After reduction to ammonia, the solution was steam-distilled and oxidized to N_2 with NaOBr as described above. Control experiments show that $^{15}\delta$ of N_2 from combustion of hydroxylamine was the same as that from ammonia that was produced by reduction of the same hydroxylamine with Ti^{3+}.

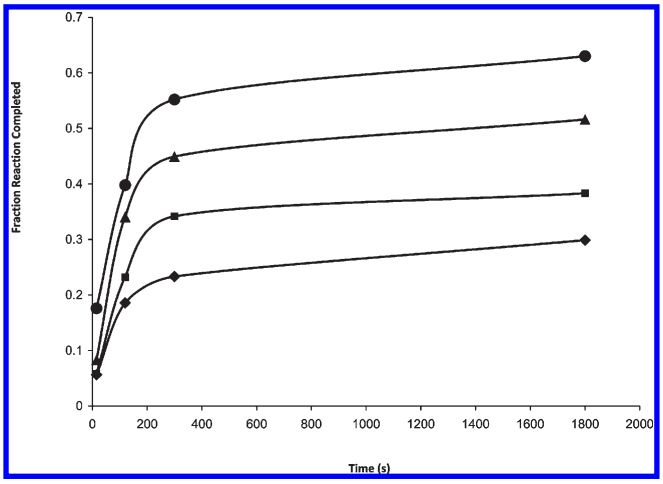


FIGURE 1: Fraction of HU hydrolysis vs time as a function of HU concentration at 0.25 mg/mL urease and pH 6.1 in MES buffer: (●) 0.75, (▲) 1.5, (■) 4.5, and (◆) 6.0 mM. The rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see Materials and Methods). Note that the *y*-axis is the fraction of total reaction, similar to plots in refs *10* and *11*. In this 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.

Complete hydrolysis of HU was accomplished in a 1.0 mL solution containing 0.50 M MES (pH 6.0), 0.10 M HU, and approximately 3 mg of urease. The reaction was allowed to proceed for 2 days at room temperature before workup as described above. As a control, the $^{15}\delta$ of N_2 from combustion of HU was shown to be the same as the average for N_2 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 CO_2 produced after partial reaction. The reaction mixture contained the same reagents as described above. The solution was sparged with CO_2-free N_2 in a 100 mL round-bottom flask equipped with a stopcock (to connect to the high-vacuum line) and a side arm containing a second stopcock. Urease was added with a syringe through a side arm that was capped with a septum. The reaction was quenched by addition of H_2SO_4 via this same side arm, and a 10 μL aliquot was withdrawn for determination of the fraction of reaction. The burst phase KIE experiments were allowed to run for 7.5−11 min before quenching, whereas the plateau phase KIE experiments were allowed to run for 8−10 h 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 CO_2 was collected through two dry ice/2-propanol traps and one liquid nitrogen trap. The details of this procedure have been published previously (*15*). The isolated CO_2 was then analyzed by IRMS. Isotopic analysis of unreacted hydoxyurea, which served as the R_0 in the determination of the KIE, was accomplished by combustion to CO_2 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*).

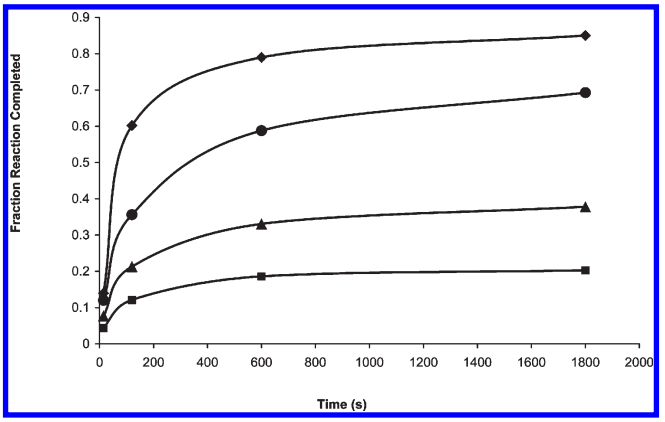


FIGURE 2: Fraction of HU hydrolysis vs time as a function of urease concentration at 1.3 mM HU and pH 6.1 in MES buffer: (■) 0.03, (▲) 0.06, (●) 0.13, and (◆) 0.25 mg/mL. Note that the rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see Materials and Methods). Note that the *y*-axis is the fraction of total reaction, similar to plots in ref *10* and *11*. In all these reactions, the initial substrate (HU) concentration was constant (1.3 mM).

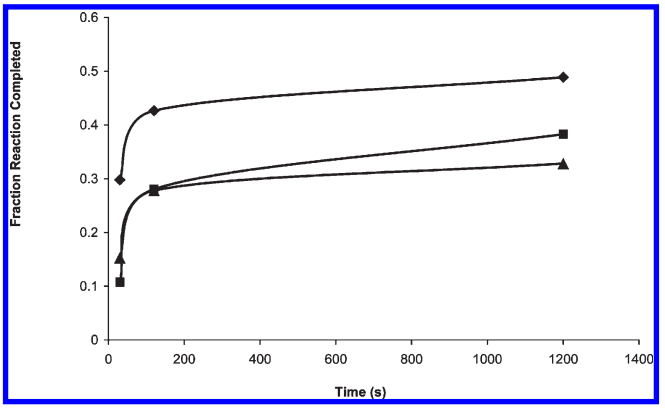


FIGURE 3: Effect of dialysis on the fraction of HU hydrolysis vs time: (◆) initial assay with HU, (■) 15 min incubation without HU followed by dialysis into MES buffer at pH 6.1, and (▲) 15 min incubation with HU followed by dialysis. The rate of reaction was determined by measuring the ammonia produced using the glutamate dehydrogenase assay (see Materials and Methods). Note that the *y*-axis is the fraction of total reaction, similar to plots in ref *10* and *11*. In all these reactions, the initial substrate (HU) concentration was constant (4.0 mM) and that of urease was 0.25 mg/mL.

Substrate inhibition by HU appears to be very slowly reversible. When urease is preincubated with HU for 15 min and then dialyzed for 30 min, the activity of this dialyzed enzyme is somewhat lower than that for a control sample of urease that was dialyzed, but not preincubated 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 preincubated 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 did not show further absorbance change after the reaction had been quenched with H_2SO_4. (b) The presence of NH_2OH did not produce any absorbance change at 340 nm in this assay. (c) No CO_2 could be detected by manometric measurements after sparging the reaction mixture (without urease) with CO_2-free N_2 or after

Table 1: Nitrogen and Carbon Isotopic Composition after Combustion and after Complete Hydrolysis of HU[a]

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 (calculated)	−23.7 (5)

[a]The number of determinations is given in parentheses.

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] ($^{13}\delta$ or $^{15}\delta$)	product[c] ($^{13}\delta$ or $^{15}\delta$)	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
			average	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
			average	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
			average	1.0135 ± 0.0003

[a]Fraction of reaction as determined by the glutamate dehydrogenase assay. [b]δ at quench. [c]Average of independent determinations from each batch of substrate. [d]Corrected for the fraction of total reaction. Uncertainties are expressed as the standard deviation.

quenching this same reaction mixture with H_2SO_4 followed by addition of urease. (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 NH_2 and 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 listed in Table 3. It must be noted that the plateau phase data are for the observed KIEs, 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 from those for the kinetic experiments shown in Figures 1−3. The major difference is in the concentration of HU and buffer. The KIE experiments required higher concentrations of HU (150 mM) than the kinetics experiments (0.75−6.0 mM) to produce enough CO_2 for IRMS analysis. This, in turn, required higher concentrations of buffer to maintain a constant pH for the KIE experiments (500 mM) than for the kinetics experiments (50 mM). A graph of the 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 conditions described here the burst phase was complete after 5−7% of total reaction. The reactions

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] ($^{13}\delta$ or $^{15}\delta$)	product[c] ($^{13}\delta$ or $^{15}\delta$)	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
			average	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
			average	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
			average	1.0178 ± 0.0003

[a]Fraction of reaction as determined by the glutamate dehydrogenase assay. [b]δ at quench. [c]Average of independent determinations from each batch of substrate. [d]Corrected for the fraction of total reaction. Uncertainties are expressed as the standard deviation.

for the plateau phase KIEs for N and C were quenched at 31−36 and 43−55% total reaction, respectively. If we assume 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 of 1.25−1.60 mM and a k_{cat} of 12 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 of 3 mM and a k_{cat} of 11000 s^{-1} under the same conditions (*16*). These kinetic experiments with HU as the substrate have been repeated in this work, and the results show the same biphasic pattern (Figures 1 and 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 that are on the same order of magnitude as those reported.

Preincubation of urease with HU was shown to inhibit urea hydrolysis; the amount of inhibition depended directly on the duration of the preincubation, 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 inhibition either by the substrate (HU) or by a product, presumably NH_2OH. In these studies, urease was preincubated 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 that of 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 the rate of urea hydrolysis is assayed. 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 ref *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 the assay of the rate of urea hydrolysis. The work presented here 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 preincubated 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 and 1.55 Å resolution, respectively (*17*, *18*). The results show that the OH group of the hydroxamate group is within 2.6 Å of Ni-1 and 1.8 Å of Ni-2 in the *K. aerogenes* enzyme; it is within 2.6 Å of both Ni atoms in the *B. pasteurii* enzyme (Figure 4). These results show the hydroxamate functional group to be within bonding distance of one or both of the Ni atoms at the active site. Therefore, it is clearly possible that coordination of the OH group 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 experimental results described above is given in Scheme 2.

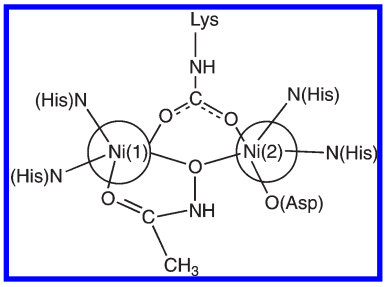


FIGURE 4: Crystal structure of urease (from *B. pasteurii*) active site with acetohydroxamic acid (AHA) from ref *14*.

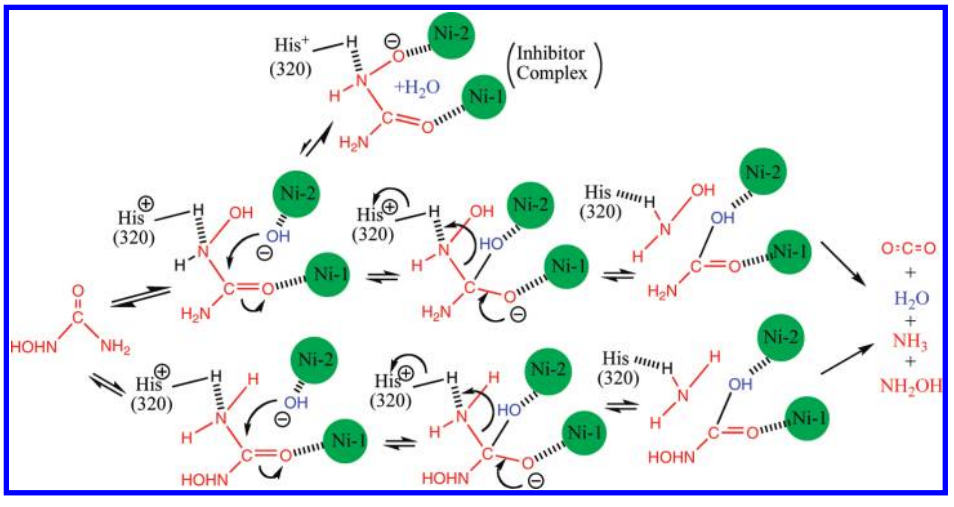
The top pathway shows breaking of the C−N bond to the NHOH leaving group occurring prior to the breaking of the C−N bond to the NH_2 leaving group; the bottom pathway reverses the order of C−N bond breaking. The breaking of the second C−N bond occurs after the first irreversible step and does not affect the observed kinetics and isotope effects. Because the products are 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 heteroatom bound to Ni-2 is replacing a hydroxide. The pK_a of the hydroxamate oxygen is expected to be ~9, and it can ionize to displace the anionic hydroxide. The β-nitrogen of semicarbazide has a much higher pK_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 very small in magnitude and do not vary significantly with a change in leaving group from NH_2 to 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, eq 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 nonchemical step or the formation of the tetrahedral intermediate. Observation of a carbonyl C KIE in excess of 1% (see below) favors the latter explanation. Because 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*).

$$E + HU \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\cdot HU \underset{k_4}{\overset{k_3}{\rightleftharpoons}} TI \xrightarrow{k_5} CO_2 + H_2O + NH_2OH + NH_3 \quad (5)$$

The inhibitor shown at the top of Scheme 2 is a dead end inhibitor complex; it affects the overall rate, but not the partition ratios in the expression for V/K. Consequently, the chemical

Scheme 2



mechanism from Scheme 2 can be reduced to the kinetic expression of eq 5. The expression that governs the relationship between the rate constants and magnitude of the KIEs of eq 5 is given in eq 6

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

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, eq 6 can be simplified to the expression in eq 7.

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

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.

Calculation of the commitment factor, k_5/k_4, is then possible during the burst and plateau phases using eq 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 have 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 the k_5 step is rate-determining. In turn, this leads to the simplification of eq 7 to $^{15}(V/K) = ^{15}K_{eq}\,^{15}k_5$ and allows calculation of a value for the intrinsic KIE on the k_5 step of 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 eq 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 a k_5/k_4 of 24, $NHNH_2$ (pK_a = 8.1) has a k_5/k_4 of 2.7, and NH_2 (pK_a = 9.6) has a k_5/k_4 of 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, much 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 $^{13}K_{eq}\,^{13}k_5$ (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 eq 8.

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

The mathematical product ($^{13}K_{eq}\,^{13}k_5$) 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, eq 8 once again reduces to $^{13}(V/K) = ^{13}K_{eq}\,^{13}k_5$ (*8*). Substituting this value and the values of the commitment factors (k_5/k_4) for the burst and plateau phases into eq 8 yields a $^{13}k_3$ of 1.0131 for the burst phase and a $^{13}k_3$ of 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 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 Ni-2 and the hydroxyl group coordinated to Ni-1. However, this does not fit the X-ray data given above, 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 that 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. Because 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, because of 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 group 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.

SUPPORTING INFORMATION AVAILABLE

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

REFERENCES

1. Mobley, H. L. T., and Hausinger, R. P. (1989) Microbial Ureases: Significance, Regulation, and Molecular Characterization. *Microbiol. Rev. 53*, 85–108.
2. Callahan, B. P., Yuan, Y., and Wolfenden, R. (2005) The Burden Borne by Urease. *J. Am. Chem. Soc. 127*, 10828–10829.
3. Sumner, J. B. (1926) The Isolation and Crystallization of the Enzyme Urease. *J. Biol. Chem. 69*, 435–441.
4. Sheridan, L., Wilmot, C. M., Cromie, K. D., van der Logt, P., and Phillips, S. E. V. (2002) Crystallization and Preliminary X-ray Structure Determination of Jack Bean Urease with a Bound Antibody Fragment. *Acta Crystallogr. D58*, 374–376.
5. Karplus, P. A., Pearson, M. A., and Hausinger, R. P. (1997) 70 Years of Crystalline Urease: What Have We Learned? *Acc. Chem. Res. 30*, 330–337.
6. Pearson, M. A., Park, I. S., Schaller, R. A., Michel, L. O., Karplus, P. A., and Hausinger, R. P. (2000) Kinetic and Structural Characterization of Urease Active Site Variants. *Biochemistry 39*, 8575–8584.
7. Schmidt, H. L. (1982) Isotope Effect on Each, C- and N-Atoms, as a Tool for the Elucidation of Enzyme-Catalyzed Amide Hydrolysis. In Stable Isotopes, Analytical Chemistry Symposia series (Schmidt, H. L., Forstel, H., and Heinzinger, K., Eds.) Vol. 11, pp 77−82, Elsevier Scientific Publishing Co., Amsterdam.
8. Marlier, J. F., and Cleland, W. W. (2006) A Multiple Isotope Effect Study of the Hydrolysis of Formamide by Urease from Jack Bean (*Canavalia ensiformis*). *Biochemistry 45*, 9940–9948.
9. Marlier, J. F., Fogle, E. J., and Cleland, W. W. (2008) A Heavy-Atom Isotope Effect and Kinetic Investigation of the Hydrolysis of Semicarbazide by Urease from Jack Bean (*Canavalia ensiformis*). *Biochemistry 47*, 11158–11163.
10. Fishbein, W. N., Thorne, W. S., and Davidson, J. D. (1965) Urease Catalysis: Stoichiometry, Specificity, and Kinetics of a Second Substrate: Hydroxyurea. *J. Biol. Chem. 240*, 2402–2406.
11. Fishbein, W. N., and Carbone, P. P. (1965) Urease Catalysis: Inhibition of the Enzyme by Hydroxyurea, Hydroxylamine, and Acetohydroxamic Acid. *J. Biol. Chem. 240*, 2407–2414.
12. Bigeleisen, J., and Wolfsberg, M. (1958) Theoretical and Experimental Aspects of Isotope Effects in Chemical Kinetics. *Adv. Chem. Phys. 1*, 15–76.
13. (a) Bremner, J. M. (1965) Isotope-Ratio Analysis of Nitrogen in Nitrogen-15 Tracer Investigations. In Methods of Soil Analysis, Part 2 (Black, C. A., Ed.) pp 1256−1286, American Society of Agromomy, Madison, WI. (b) Weiss, P. M. (1991) Heavy-Atm Isotope Effects Using an Isotope Ratio Mass Spectrometer. In Enzyme Mechanisms from Isotope Effects (Cook, P. F., Ed.) pp 291−311, CRC Press, Boca Raton, FL.
14. Tomat, R., and Rigo, A. (1972) Effect of pH on the Kinetics of the Reduction of Hydroxylamine by Ti(III). *J. Electroanal. Chem. 35*, 21–26.
15. O'Leary, M. H. (1980) Detemination of Heavy-Atom Isotope Effects on Enzyme-Catalyzed Reactions. *Methods Enzymol. 64*, 83–104.
16. Fishbein, W. N. (1977) Formamide: The Minimum-Structure Substrate for Urease. *Biochim. Biophys. Acta 484*, 433–442.
17. Benini, S., Rypniewski, W. R., Wilson, K. S., Miletti, S., Cuirli, S., and Mangani, S. (2000) The Complex of *Bacillus pasteurii* Urease with Acetohydroxamate Anion from X-ray Data at 1.55 Å Resolution. *J. Biol. Inorg. Chem. 5*, 110–118.
18. Pearson, M. A., Michel, L. O., Hausinger, R. P., and Karplus, P. A. (1997) Structures of Cys319 Variants and Acetohydroxamate-Inhibited *Klebsiella aerogenes* Urease. *Biochemistry 36*, 8165–8172.
19. Marlier, J. F. (2001) Multiple Isotope Effects on the Acyl Group Transfer Reactions of Amides and Esters. *Acc. Chem. Res. 34*, 283–290.
20. Hogg, J. L., Rodgers, J., Kovach, I., and Schowen, R. L. (1980) Kinetic Isotope-Effect Probes of Transition-State Structure. Vibrational Analysis of Model Transition States for Carbonyl Addition. *J. Am. Chem. Soc. 102*, 79–85.
21. Marlier, J. F., and O'Leary, M. H. (1990) Carbon Kinetic Isotope Effects on the Hydrolysis of Aryl Carbonates. *J. Am. Chem. Soc. 112*, 5996–5998.