# A Heavy-Atom Isotope Effect and Kinetic Investigation of the Hydrolysis of Semicarbazide by Urease from Jack Bean (*Canavalia ensiformis*)[†]

John F. Marlier,*[,‡] Emily J. Fogle,[‡] and W. W. Cleland[§]

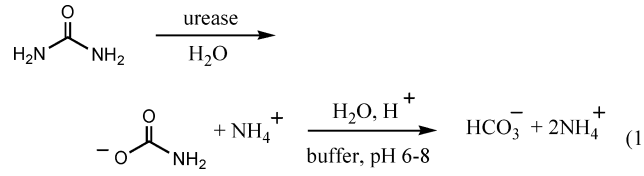
*Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, California 93407, and Institute for Enzyme Research, Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53726*

*Received July 15, 2008; Revised Manuscript Received August 13, 2008*

ABSTRACT: A kinetic investigation of the hydrolysis of semicarbazide by urease gives a relatively flat log *V*/*K* versus pH plot between pH 5 and 8. A log $V_m$ versus pH plot shows a shift of the optimum $V_m$ toward lower pH when compared to urea. These results are explained in terms of the binding of the outer N of the $NHNH_2$ group in semicarbazide to an active site residue with a relatively low p$K_a$ (∼6). Heavy-atom isotope effects for both leaving groups have been determined. For the $NHNH_2$ side, $^{15}k_{obs} = 1.0045$, whereas for the $NH_2$ side, $^{15}k_{obs} = 1.0010$. This is evidence that the $NHNH_2$ group leaves prior to the $NH_2$ group. Using previously published data from the urease-catalyzed hydrolysis of formamide, the commitment factors for semicarbazide and urea hydrolysis are estimated to be 2.7 and 1.2, respectively. The carbonyl-C isotope effect ($^{13}k_{obs}$) equals 1.0357, which is consistent with the transition state occurring during either formation or breakdown of the tetrahedral intermediate.

Urease catalyzes the hydrolysis of urea to carbon dioxide and ammonia. This enzyme and the more than 80 years of accumulated research are important to enzymologists for historical, biological, and chemical reasons (*1*). Urease from jack bean was the first enzyme to be crystallized (1926), but to date, only a low-resolution crystal structure is available (*2, 3*). Fortunately, ureases from other sources have yielded high-resolution crystal structures (*4*). The primary amino acid sequence is highly conserved for all sources of the enzyme, and the known crystal structures show virtually the same amino acids present at the active site (*1*). From a biological perspective, urease has been implicated in formation of gastric ulcers and in increasing soil alkalinity (*4*). From a chemical standpoint, urease has been cited as a model of extreme catalytic power because of its ability to rapidly hydrolyze a molecule as stable as urea; this catalytic prowess has been debated recently (*5, 6*). The overall reaction is given in eq 1. The first step is enzyme-catalyzed; the second is spontaneous and irreversible (*7*).

Urease from jack bean is a hexameric enzyme containing two Ni atoms per subunit; both Ni atoms are essential for catalysis (*3, 8*). X-ray crystal structures from bacterial sources of the enzyme, together with mutagenesis studies, implicate an active site His320 as a catalytically vital residue (*1, 9*). Mutant enzymes with Ala, Asn, or Gln replacing His320 are $\sim 10^5$ times less active than the wild-type enzyme. Mutants in which Cys319 is replaced with Ser and Asp lead to a much smaller loss of activity, indicating the requirement for Cys319 is not absolute (*10*). These Cys319 mutants also served to shift the pH optimum of $V_m$ toward lower pH.




(1)

The most widely accepted urease-catalyzed hydrolysis mechanism (Scheme 1) involves the utilization of one Ni to coordinate the carbonyl O of urea, while the other delivers a Ni-coordinated water as the nucleophile to the carbonyl C of urea (*1, 11, 12*). In this mechanism, His320 serves as a general acid to protonate the leaving N of urea while Cys319 coordinates to the nonleaving N of urea. A reverse protonation mechanism is required to explain how a protonated His (p$K_a$ ∼ 6) and a deprotonated, Ni-coordinated water (p$K_a$ ∼ 9) can function to bring about catalysis by an enzyme with a pH optimum near neutrality.

Kinetic isotope effects (KIEs)[1] on urea and a slow substrate, formamide, have been employed to further study the details of the mechanism. Schmidt measured both the carbonyl C KIE ($^{13}k_{obs} = 1.0206$) and the leaving N KIE ($^{15}k_{obs} = 1.0075$) for hydrolysis of urea (*13*). The determination of the leaving N KIE involves simultaneous determination of the isotopic composition of both nitrogen atoms of urea. Since cleavage of the second C−N bond occurs after the first irreversible step, the actual leaving N KIE is assumed

[†] Financial support (to W.W.C.) from NIH Grant GM18938. J.F.M. thanks Professor W. W. Cleland (University of Wisconsin) for summer financial support in his laboratory (NIH Grant GM18938).

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

[‡] California Polytechnic State University.

[§] University of Wisconsin.

[1] 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, *N*-tris(hydroxymethyl)methylglycine.

10.1021/bi801338c CCC: $40.75 
Published on Web 09/26/2008

to be double the observed effect ($^{15}k_{obs} = 1.015$), and this is consistent with partially rate-determining C−N bond cleavage, since the maximum nitrogen KIE ($^{15}k$) is expected to be approximately 1.05 (*12*). A multiple-KIE study of formamide hydrolysis yielded more detailed mechanistic details (*12*). In this case, C−N bond cleavage is almost completely rate-determining ($^{15}k_{obs} = 1.0327$). The nucleophile O KIE ($^{18}k_{obs} = 0.9778$) and formyl H KIE ($^{D}k_{obs} = 0.95$) are consistent with nucleophile C−O bond formation that is at equilibrium, prior to the rate-determining C−N bond cleavage.

In this paper, another alternate substrate, semicarbazide, is investigated (*5, 11*). Semicarbazide contains a nitrogen atom on both sides of the carbonyl and closely resembles the structure of the natural substrate. With this substrate, it is possible to determine the KIEs for both the nitrogen that leaves during the breakdown of the tetrahedral intermediate (the leaving N) and the nitrogen that leaves spontaneously after catalysis (the nonleaving N). Measurement of both KIEs was not possible with other substrates studied to date.

## EXPERIMENTAL PROCEDURES

*Materials and Methods.* Urease (from jack bean), semicarbazide·HCl, MES, MOPS, Tricine, AMPSO, Nessler's reagent, and LiOH were from Sigma-Aldrich. Glutamate dehydrogenase kits for determination of ammonia concentration were from Raichem. Ultrafiltration was accomplished using a Millipore YM series membrane with a 10000 MW cutoff. The isotopic composition of carbon and nitrogen was measured on an isotope ratio mass spectrometer and expressed in $\delta$ (per mil) notation as shown in eq 2

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

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

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

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

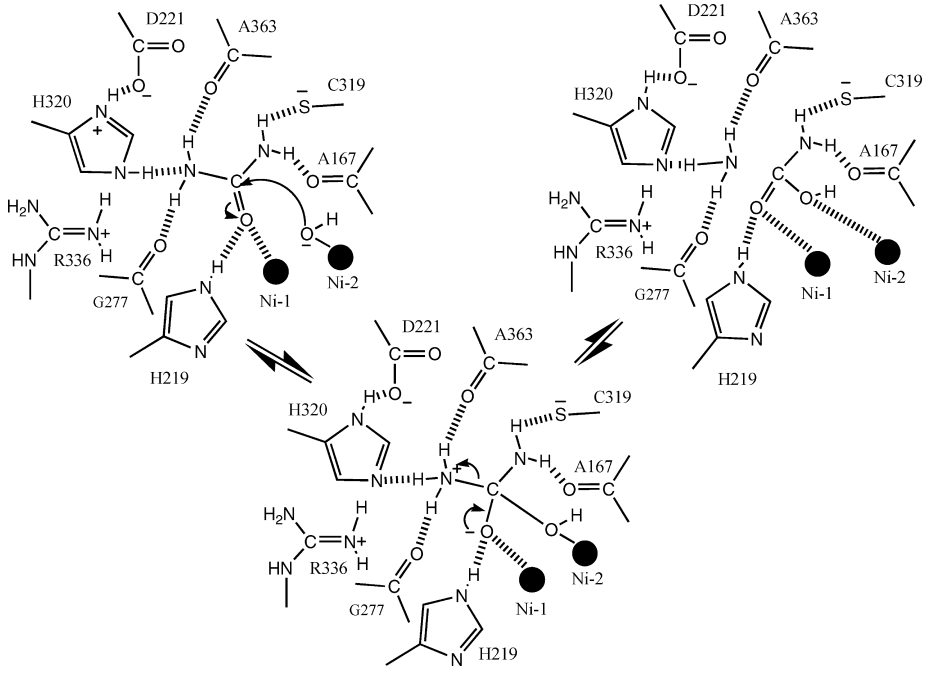
where $k/{}^*k$ is the observed isotope effect ($^*k$ is the rate constant for the heavier isotope), $f$ is the fraction of the reaction, $R_0$ is the isotope ratio (heavy to light) for the unreacted starting material or the product after complete reaction, $R_s$ is that for the substrate after partial reaction, and $R_p$ is that for the product after partial reaction (*14*).

*Determination of the Fraction of the Reaction.* A 10 $\mu$L aliquot of the reaction mixture at a given reaction time was rapidly added to 990 $\mu$L of water. A 10 $\mu$L aliquot of this diluted sample was then added to 1.2 mL of a solution containing glutamate dehydrogenase, NADPH, and buffer. The concentration of ammonia was calculated from the absorbance change at 340 nm. Controls showed that hydrazine does not react under these conditions.

*Enzyme Kinetics.* The glutamate dehydrogenase method described above was used to determine the rates of reaction. Semicarbazide concentrations were 30, 40, 60, and 120 mM. Substrate concentrations above 120 mM gave ionic strength and pH control problems. The following buffers were employed (50 mM): acetate (pH 4.5 and 5.1), MES (pH 5.1, 5.5, and 6.1), MOPS (pH 6.1, 6.6, and 7.1), Tricine, (pH 7.1, 7.6, and 8.1), and AMPSO (pH 8.1, 8.5, and 9.0). The ionic strength was maintained at 0.20 M with KCl. Determination of $V/K$, $V$, and $K$ was accomplished by fitting the initial velocity data to the equation $v = VA/(K + A)$ using published statistical methods (*15*). Standard errors for determination of $V$ and $V/K$ averaged 7% (the highest was 18%). Similarly, the p$K_a$ values were determined by fitting log $V/K$ versus pH to the equation log $V/K$ = log $C/(1 + [H^+]/K_1 + K_2/[H^+])$.

*Nitrogen Isotope Effect Procedures.* The KIEs were measured using the competitive method via analysis of the product produced during partial and complete reaction. A 2.0 mL solution containing 0.15 M semicarbazide and 0.40 M MES buffer at pH 6 was added to approximately 2.5 mg

Scheme 1



of urease. After partial reaction, this mixture was rapidly cooled to 4 °C and an aliquot was assayed for the concentration of ammonia. While the assay was in progress, the remainder of the solution was subjected to ultrafiltration at 4 °C. Controls showed that a negligible amount of ammonia was produced during cold ultrafiltration (<1−2 $\mu$mol). 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 50 mL of 1 M AMPSO (pH 9); 5 mL fractions were collected. The hydrazine eluted in fractions 3−9. The column was then eluted with 50 mL of 0.1 M LiOH and the ammonia eluted in fractions 1−4. The fractions containing hydrazine were added to a second strong cation exchange column (Na form). The column was rinsed with water and eluted with 0.1 M NaOH. The second column was run to convert the $Li^+$ salt form of hydrazine to the $Na^+$ form which is more soluble in the NaOBr solution needed for subsequent oxidation (see below). The ammonia fractions from the first column were steam-distilled, rotary-evaporated to a volume of 2 mL, and oxidized to $N_2$ with NaOBr for analysis by IRMS (*16*). The $Na^+$ salt of hydrazine was rotary-evaporated to a volume of 2 mL and oxidized directly to $N_2$ with NaOBr for analysis by IRMS without steam distillation. As a control, semicarbazide was combusted to $CO_2$ and $N_2$. The $N_2$ was isolated on a high-vacuum line and analyzed by IRMS. The $^{15}\delta$ for the $N_2$ agreed with those determined from the hydrazine and ammonia samples after complete hydrolysis.

*Carbon Isotope Effect Procedure.* The KIEs were measured using the competitive method via analysis of the unreacted starting material and that remaining after partial reaction. After partial hydrolysis, semicarbazide was isolated by ion exchange chromatography as detailed above. The column was eluted with 0.10 M MES (pH 6), and 3 mL fractions were collected. Semicarbazide eluted in fractions 2−14. The combined fractions were placed in a 250 mL round-bottom flask equipped with a stopcock (to connect to the high-vacuum line) and with a sidearm containing 1.5 mL of 0.5 M chloramine-T. After freeze−thaw cycles that removed atmospheric $CO_2$, the chloramine-T solution was added to the semicarbazide solution and allowed to react for 15 min. The $CO_2$ was then collected through two dry ice/2-propanol traps and one liquid nitrogen trap, followed by IRMS analysis. Isotopic analysis of unreacted semicarbazide was accomplished by either combustion or direct oxidation with chloramine-T as described above. Both methods yielded identical isotope ratios.

## RESULTS

The leaving N KIEs for the hydrolysis of semicarbazide by urease at pH 6.0 (22−23 °C) were as follows: $^{15}k_{obs}$ = 1.0045 ± 0.0003 for the $NHNH_2$ leaving group and $^{15}k_{obs}$ = 1.0010 ± 0.0004 for the $NH_2$ leaving group. The integrity of the analytical procedures for isotopic analysis of the two sources of nitrogen was checked by comparing the $^{15}\delta$ of $N_2$ derived from hydrazine and that from ammonia (after complete hydrolysis of semicarbazide) to that of semicarbazide that had been combusted. The $^{15}\delta$ for $N_2$ derived from the hydrazine product was −2.2 ± 0.1; that from ammonia was +1.1 ± 0.8. This should give a $^{15}\delta$ for the total nitrogen of combusted semicarbazide of (−2.2)(0.67) + (1.0)(0.33) = −1.1. The measured $^{15}\delta$ for the total $N_2$ of semicarbazide after combustion was −1.0. The KIE data are given in Table 1.

Table 1: Leaving N and Carbonyl C Kinetic Isotope Effects on the Urease-Catalyzed Hydrolysis of Semicarbazide at pH 6.0 and 22−23 °C

| atom | $f^a$ | $^{15}\delta$ or $^{13}\delta$ | | isotope effect ($k_{light}/k_{heavy}$)$^{c,d}$ |
|---|---|---|---|---|
| | | partial | substrate/product$^b$ | |
| $NHNH_2$ side (hydrazine) | 0.37 | −5.5 | −2.2 | 1.0042 |
| | 0.32 | −5.5 | −2.2 | 1.0040 |
| | 0.35 | −5.6 | −2.2 | 1.0043 |
| | 0.37 | −6.0 | −2.2 | 1.0049 |
| | 0.33 | −6.0 | −2.2 | 1.0047 |
| | 0.31 | −6.0 | −2.2 | 1.0046 |
| | | | average | 1.0045 ± 0.0003 |
| $NH_2$ side (ammonia) | 0.37 | +0.3 | +1.0 | 1.0010 |
| | 0.32 | +0.2 | +1.0 | 1.0011 |
| | 0.35 | +0.2 | +1.0 | 1.0011 |
| | 0.37 | +0.3 | +1.0 | 1.0010 |
| | 0.33 | +0.2 | +1.0 | 1.0011 |
| | 0.31 | +0.4 | +1.0 | 1.0008 |
| | | | average | 1.0010 ± 0.0004 |
| carbonyl C | 0.53 | −24.0 | −49.2 | 1.0362 |
| | 0.53 | −22.8 | −49.2 | 1.0377 |
| | 0.58 | −21.1 | −49.2 | 1.0345 |
| | 0.50 | −26.6 | −49.2 | 1.0352 |
| | 0.47 | −28.7 | −49.2 | 1.0351 |
| | 0.50 | −26.7 | −49.2 | 1.0353 |
| | | | average | $1.035_7 \pm 0.001_1$ |

[a] Determined via a glutamate dehydrogenase assay for ammonia. [b] The values are averages for five independent determinations: ±0.8 for ammonia, ±0.2 for hydrazine, and ±0.4 for $CO_2$. [c] Corrected for the fraction of the reaction. [d] Standard errors on the KIEs include errors in determination of $^{15}\delta$ or $^{13}\delta$ for unreacted substrate or for product after complete hydrolysis.

The carbonyl C KIE for semicarbazide under identical conditions ($^{13}k_{obs}$) is $1.035_7 \pm 0.001_1$. Control experiments showed that the $^{13}\delta$ from $CO_2$ generated by the chloramine-T oxidation of semicarbazide was identical to that from complete combustion of semicarbazide to $CO_2$. The KIE data are given in Table 1.

A log *V*/*K* versus pH rate profile for urease-catalyzed hydrolysis of semicarbazide was generated between pH 4.5 and 9.0. The results are plotted in Figure 1. This plot shows a rather flat log *V*/*K* profile between pH 5 and 8, similar to that for urea hydrolysis. Statistical analysis gave a p$K_1$ of 4.1 ± 0.3 and a p$K_2$ of 8.5 ± 0.1 (*15*). A log $V_m$ versus pH plot shows an optimum near pH 5.5 (Figure 2).

## DISCUSSION

*Mechanism.* Semicarbazide has been shown to be a slow substrate for urease; it has been reported to have a $K_m$ higher than that of urea (~60 mM vs 3 mM at pH 7) and a much lower $k_{cat}$ (by a factor of at least 200) at most pH values (*5, 11*). However, more detailed kinetic studies are lacking. In the literature, the pH optimum was assigned at pH 5.0, on the basis of investigations carried out only at pH 5 and 7 (*17*). Chemical catalysis of urea hydrolysis requires two important ionizable active site groups: (a) His320 (p$K_a$ ~ 6) which provides a proton to the leaving $NH_2$ group during hydrolysis and (b) Ni-OH (p$K_a$ ~ 9) which is the actual nucleophile. The widely accepted mechanism, given in Scheme 2, was postulated on the basis of crystal structures, mutagenesis, and, more recently, a multiple-heavy-atom KIE study of formamide hydrolysis (*12*).

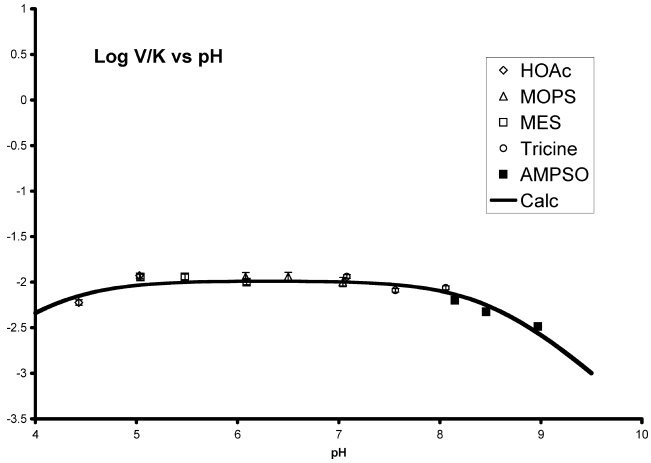



FIGURE 1: Log *V*/*K* vs pH plot for the urease-catalyzed hydrolysis of semicarbazide at 25 °C. The calculated line was fit to the equation log $V/K = \log[c/(1 + [H^+]/K_1 + K_2/[H^+])]$. The best fit was obtained when *c* equaled 0.0104 (*15*). Error bars are included but are generally smaller than the symbols.

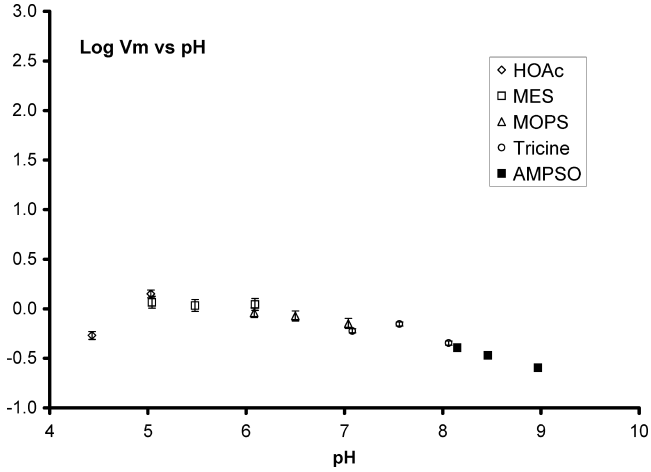



FIGURE 2: Log $V_m$ vs pH plot for the urease-catalyzed hydrolysis of semicarbazide at 25 °C. Error bars are included but are generally smaller than the symbols.

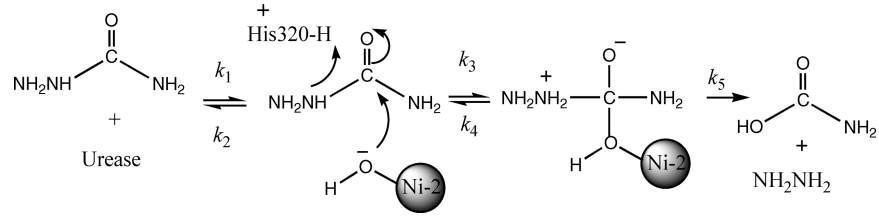
*Kinetics.* This study extends the kinetic investigation between pH 4.5 and 9.0. Figures 1 and 2 show the resulting pH profiles for log *V*/*K* and log $V_m$. The observed log *V*/*K* profile is flat between pH 5 and 8 and is similar to that from the most recent pH rate study of urea hydrolysis (*18*). Statistical analysis of the log *V*/*K* versus pH data for semicarbazide hydrolysis yielded a $pK_1$ of 4.1 ± 0.3 and a $pK_2$ of 8.5 ± 0.1 (*15*). This compares to a $pK_1$ of 5.34 ± 0.04 and a $pK_2$ of 8.67 ± 0.04 reported for urea hydrolysis (*18*). However, the log $V_m$ profile for semicarbazide has an optimum at pH ~5.5, which is lower than that for urea by 0.5−1 pH unit. This same lowering of the $V_m$ optimum was previously observed for formamide, another slow substrate of urease (*17*). For formamide, the shift of the $V_m$ optimum to lower pH was rationalized by the difference in the binding of Cys319 (assumed $pK_a$ of ~6) to the nonleaving N of urea versus the formyl H of formamide (*12*). It was assumed that binding of the nonleaving N of urea preferred the anionic form of Cys319, while the formyl H of formamide preferred the neutral form. A log $V_m$ profile was calculated on the basis of the three $pK_a$ values listed above for His320, Ni-OH, and Cys319, which exhibited the expected shift toward lower pH in the formamide case. However, the preference for the protonated or deprotonated forms of Cys319 appears not to be absolute for either substrate on the basis of both the mutagenesis studies and these pH profiles (*10*).

The observed lower-pH optimum of $V_m$ for semicarbazide compared to urea can be explained with similar logic but requires an additional active site residue. Strong KIE evidence presented below is consistent with hydrazine being the first group to leave semicarbazide, making the $NH_2$ group the nonleaving N. On this basis, we expect that the rate of hydrolysis for both semicarbazide and urea will have an identical dependence on the $pK_a$ values for His320, Ni-OH, and Cys319, since both substrates have nonleaving $NH_2$ groups. Shifting the $V_m$ optimum to a lower pH would then require semicarbazide to prefer binding to the protonated form of some additional active site residue (one with a relatively low $pK_a$); urea prefers binding to the unprotonated form. The outer nitrogen of the hydrazine side of semicarbazide is capable of forming strong hydrogen bonds and might readily bind to a hydrogen bond donor at the active site. Are there any candidates based on the X-ray structure? His218 (assumed $pK_a$ of ~6) is within hydrogen bonding distance of the carbonyl O of the substrate (*1*).

If the outer nitrogen of semicarbazide prefers the protonated form of His219 (or any other residue with a similar $pK_a$ and hydrogen bonding ability) and urea prefers the neutral form, a shift in the $V_m$ optimum to lower pH will again be observed. Once again, this preference need not be absolute.

In a manner similar to the formamide case described earlier, it is possible to calculate the relative log $V_m$ versus pH for urea and semicarbazide hydrolysis using the $pK_a$ values for His320, His219, Cys319, and Ni-OH given above. The results of these calculations are plotted in Figure 3 and show, as expected, a shift in the $V_m$ optimum to lower pH. (Note that the plot for semicarbazide should be lower on the *y*-axis than that for urea; they are placed on the same scale for ease of viewing.) This calculation does not prove that the observed shift of the $V_m$ optimum is due to binding of the outer nitrogen to an active site residue but establishes it as a reasonable hypothesis. The shallowness of the observed log $V_m$ versus pH plot compared to the calculated one is due to the fact that the dependence of $V_m$ on the $pK_a$ values for His219 and Cys319 is not absolute.

Scheme 2



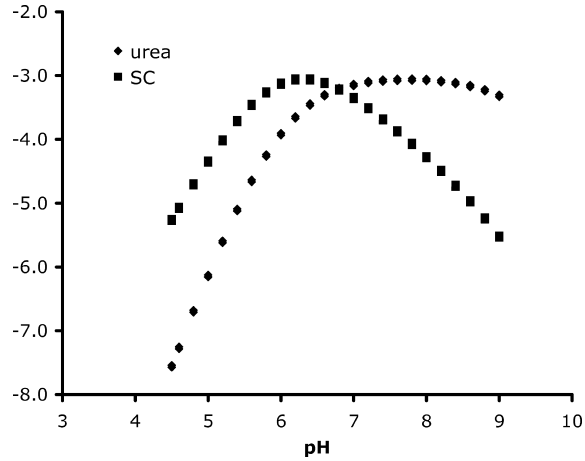



FIGURE 3: Calculated log relative $V_m$ vs pH for urea (◆) and semicarbazide (■).

*Nitrogen KIEs.* Formamide provided an opportunity to view urease-catalyzed hydrolysis from a unique perspective. The lack of a nonleaving N gave the opportunity to directly measure the KIE for cleavage of the C−N bond to the only possible leaving N. Recall that the leaving N KIE for urea gave a composite KIE for both nitrogen atoms. Since the bond to one of the nitrogen atoms was severed after the first irreversible step, it was assumed that this nonleaving N did not contribute to the observed composite KIE. Although this is a good assumption, there is the possibility of small secondary KIEs on the nonleaving N. Like the natural substrate, semicarbazide has nitrogen atoms on either side of the carbonyl, but these groups ($NHNH_2$ and $NH_2$) can be separated and analyzed independently after hydrolysis. Thus, this substrate allows an opportunity to independently measure the KIE on the nonleaving N.

The nitrogen KIE (Table 1) for the $NHNH_2$ side of semicarbazide is significantly larger than that for the $NH_2$ side ($^{15}k_{obs} = 1.0045$ and 1.0010, respectively). The isotopic analysis of the $NHNH_2$ side of semicarbazide includes both the inner N (leaving N) and outer N. As an initial interpretation, the KIE for the inner N of the $NHNH_2$ group ($^{15}k$) should be approximately 1.0090, if small secondary KIEs on the outer N are ignored. Taken together, these two KIEs are best explained by the $NHNH_2$ group, rather than the $NH_2$ group, as the first group to leave during hydrolysis because any KIE on the first C−N bond severed will be a large primary isotope effect while those on the second C−N bond cleaved will be smaller secondary ones. It is possible to construct another mechanism by which either the $NH_2$ or the $NHNH_2$ group can be the first group to leave. However, the very small magnitude of the nitrogen KIE for the ammonia side of semicarbazide is most consistent with a secondary KIE for formation of the tetrahedral intermediate. In addition, protonated hydrazine is a much better leaving group than protonated ammonia. To more fully explain the observed KIEs, one must also take into account the steps of the known mechanism as shown in Scheme 2.

The mechanism given in Scheme 2 assumes that protonation of the leaving N occurs simultaneously with attachment of the nucleophilic O to create a zwitterionic tetrahedral intermediate (*12*). Obviously, a stepwise process is possible. The relationship between the various rate constants and the observed KIE is given by eq 5. For the hydrolysis of formamide, the unusually large observed leaving N KIE simplified eq 5 to eq 6 (because $k_5 \ll k_4$ and $k_3 \ll k_2$). A reasonable estimate of the equilibrium isotope effect on formation of the tetrahedral intermediate ($^{15}K_{eq}$) was determined to be 0.983 (*12, 19*). This allowed calculation of the intrinsic KIE ($^{15}k_5 = 1.050$) for C−N bond breaking (*12*).

$$^{15}k_{obs} = \frac{(^{15}K_{eq}\,^{15}k_5) + {}^{15}k_3(k_5/k_4) + (k_5/k_4)(k_3/k_2)}{1 + (k_5/k_4)(1 + k_3/k_2)} \quad (5)$$

$$k_{obs} = (^{15}K_{eq})(^{15}k_5) \quad (6)$$

This intrinsic KIE ($^{15}k_5 = 1.050$) and the equilibrium isotope effect ($^{15}K_{eq} = 0.983$) can now be used to estimate the commitment factor ($k_5/k_4$) for the hydrolysis of semicarbazide and urea. Since the two nitrogen atoms of hydrazine are analyzed simultaneously on the IRMS, it is necessary to assume that the secondary KIE on the outer N of the $NHNH_2$ leaving group is so small that it can be ignored. If the overall estimate is good to approximately two significant figures, this is an acceptable assumption. The expression in eq 5 can be further simplified. Because semicarbazide is a slow substrate (at least 200 times slower than urea), it is reasonable that $k_3 \ll k_2$. The isotope sensitive $^{15}k_3$ can be estimated to be unity as follows. There will be a loss of resonance upon formation of the tetrahedral intermediate (small normal secondary KIE), but at the same time, there will be increased bending and torsional modes in the tetrahedral intermediate due to the addition of another heavy atom (small inverse KIE). These two effects will tend to offset each other, giving a $^{15}k_3$ close to unity. As a result, eq 5 will simplify to the expression given in eq 7. Using $^{15}k_5$ and $^{15}K_{eq}$ from above and $^{15}k_{obs}$ for semicarbazide or urea, the commitment ($k_5/k_4$) becomes 2.7 for semicarbazide hydrolysis and 1.2 for urea hydrolysis.

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

The magnitudes of the commitment factors for urea and semicarbazide are as expected from a chemical perspective. Protonated hydrazine is a better leaving group than protonated ammonia. This will make breakdown to products ($k_5$) relatively faster than formation of the tetrahedral intermediate in the case of semicarbazide, thereby increasing the commitment factor. Comparisons of both of these substrates to formamide are not so straightforward because of the greater differences in the chemical structure of the substrates.

*Carbonyl C KIE.* The carbonyl C KIE for most ester and amide hydrolyses has been shown both theoretically and empirically to be rather insensitive to changes in transition state structure (*20, 21*). There can be exceptions. For example, the aminolysis of methyl formate at pH 10 involves rate-determining transfer of a proton to and from heteroatoms within the tetrahedral species. In this case, the carbonyl C KIE reflects mostly an equilibrium isotope effect on formation of the tetrahedral intermediate. It is significantly smaller ($^{13}k_{obs} = 1.020$) than those for the typical formyl group transfers where formation or breakdown of the tetrahedral intermediate is rate-determining ($^{13}k_{obs} = 1.028-1.034$) (*22*). The carbonyl C KIE in this case ($^{13}k_{obs} = 1.0357$) is sufficiently large to be consistent with a transition state occurring during either formation or breakdown of the

tetrahedral intermediate. It does show that the chemistry is rate-limiting in the reaction.

## CONCLUSIONS

The heavy-atom isotope effect study of urease-catalyzed hydrolysis of semicarbazide is consistent with the reverse protonation mechanism in which hydrazine is the first group to leave. This conclusion is based on the measurement of the leaving N KIE for both the hydrazine group ($^{15}k_{obs}$ = 1.0090, ignoring the outer N) and the $NH_2$ group ($^{15}k_{obs}$ = 1.0010). Kinetic studies indicate the observed shift in the $V_m$ optimum toward lower pH is consistent with the preference of the outer N of semicarbazide for binding to the protonated form of an active site residue with a p$K_a$ of ~6, whereas urea, lacking this outer N, prefers the neutral form of this residue.

Urease appears to employ the same basic mechanism for the hydrolysis of urea and the analogues that are slow substrates, although the analogue substrates exhibit higher $K_m$ and lower $k_{cat}$ values. Presumably, the slower reactions result from a reduced population of near-attack conformations in the enzyme−substrate complex. Formamide has a hole where one $NH_2$ group of urea would bind, while semicarbazide has a larger leaving group, which may lead to steric hindrance. Once Ni-bound hydroxide does attack to form the tetrahedral intermediate, however, the partitioning of the intermediate reflects the p$K$ of the leaving group, with hydrazine being a better leaving group than ammonia.

## ACKNOWLEDGMENT

We thank Dr. Mark A. Anderson of the Institute for Enzyme Research for combusting a sample of semicarbazide for isotopic analysis.

## REFERENCES

1. 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.
2. Sumner, J. B. (1926) The Isolation and Crystallization of the Enzyme Urease. *J. Biol. Chem. 69*, 435–441.
3. 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.
4. Benini, S., Wojciech, R. R., Wilson, K. S., Miletti, S., Ciurli, S., and Mangani, S. (1999) A New Proposal for Urease Mechanism Based on the Crystal Structures of Native and Inhibited Enzyme from *Bacillus pasteurii*: Why Urea Costs Two Nickels. *Struct. Folding Des. 7*, 205–216.
5. Gazzola, C., Blakeley, R. L., and Zerner, B. (1973) On the Substrate Specificity of Jack Bean Urease (Urea Amidohydrolase, EC 3.5.1.5). *Can. J. Biochem. 51*, 1325–1330.
6. Callahan, B. P., Yuan, Y., and Wolfenden, R. (2005) The Burden Borne by Urease. *J. Am. Chem. Soc. 127*, 10828–10829.
7. Blakeley, R. L., Hinds, J. A., Kunze, H. E., Webb, E. C., and Zerner, B. (1969) Jack Bean Urease (EC 3.5.1.5). Demonstration of a Carbamoyl-Transfer Reaction and Inhibition by Hydroxamic Acids. *Biochemistry 8*, 1991–2000.
8. Dixon, N. E., Gazzola, C., Blakeley, R. L., and Zerner, B. (1975) Jack Bean Urease (EC 3.5.1.5). A Metalloenzyme. A Simple Role for Nickel? *J. Am. Chem. Soc. 97*, 4131–4133.
9. 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.
10. Martin, P. R., and Hausinger, R. P. (1992) Site-directed Mutagenesis of the Active Site Cysteine in *Klebsiella aerogenes* Urease. *J. Biol. Chem. 267*, 20024–20027.
11. Dixon, N. E., Riddles, P. W., Gazzola, C., Blakely, R. L., and Zerner, B. (1980) Jack Bean Urease (EC 3.5.1.5). V. On the Mechanism of Action of Urease on Urea, Formamide, Acetamide, N-methyl Urea, and Related Compounds. *Can. J. Biochem. 58*, 1335–1344.
12. 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.
13. 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, Volume 11* (Schmidt, H. L., Forstel, H., and HeinzingerK., Eds.) pp 77−82, Elsevier Scientific Publishing Co., Amsterdam.
14. Bigeleisen, J., and Wolfsberg, M. (1958) Theoretical and Experimental Aspects of Isotope Effects in Chemical Kinetics. *Adv. Chem. Phys. 1*, 15–76.
15. Cleland, W. W. (1979) Statistical Analysis of Enzyme Kinetic Data. *Methods Enzymol. 63*, 103–138.
16. O'Leary, M. H., and Kluetz, M. D. (1972) Nitrogen Isotope effects on the Chymotrypsin-Catalyzed Hydrolysis of *N*-Acetyl-L-Tryptophanamide. *J. Am. Chem. Soc. 94*, 3585–3589.
17. Fishbein, W. N. (1977) Formamide: The Minimum-Structure Substrate for Urease. *Biochim. Biophys. Acta 484*, 433–442.
18. Krajewska, B., and Ciurli, S. (2005) Jack Bean (*Canavalia ensiformis*) Urease. Probing Acid-Base Groups of the Active Site by pH Variation. *Plant Physiol. Biochem. 43*, 651–658.
19. Rishavy, M. A., and Cleland, W. W. (1999) $^{13}C$, $^{15}N$, and $^{18}O$ Equilibrium Isotope Effects and Fractionation Factors. *Can. J. Chem. 77*, 967–977.
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.
22. Marlier, J. F. (2001) Multiple Isotope Effects on the Acyl Group Transfer Reactions of Amides and Esters. *Acc. Chem. Res. 34*, 283–290.

BI801338C