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

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Abstract

A kinetic investigation of the hydrolysis of semicarbazide by urease gives a relatively flat log V/K vs. pH plot between pH 5 and 8. A log V_m vs. 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 pK_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 $^{15}k_{obs} = 1.0009$. This is evidence that $-NHNH_2$ leaves prior to $-NH_2$. Using previous data from the urease-catalyzed hydrolysis of formamide, the commitment factors for semicarbazide and urea hydrolysis are estimated as 2.7 and 1.2, respectively. The carbonyl-C isotope effect is $^{13}k_{obs} = 1.0357$ which is consistent with the transition state occurring either during 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 poor 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 due to 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).

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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 replacing Cys319 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.

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 non-leaving-N of urea. A reverse protonation mechanism is required to explain how a protonated His ($pK_a \sim 6$) and a deprotonated, Ni-coordinated water ($pK_a \sim 9$) can function to bring about catalysis by an enzyme with a pH optimum near neutrality.

Kinetic isotope effects (KIE) 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 ($^{13}k_{\text{obs}} = 1.0206$) and the leaving-N KIE ($^{15}k_{\text{obs}} = 1.0075$) for hydrolysis of urea (13). 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 to be double the observed effect ($^{15}k_{\text{obs}} = 1.015$) and this is consistent with partially rate-determining C—N bond cleavage, since the maximum nitrogen KIE is expected to be approximately $^{15}k = 1.05$ (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 ($^{15}k_{\text{obs}} = 1.0327$). The nucleophile-O KIE ($^{18}k_{\text{obs}} = 0.9778$) and formyl-H KIE ($^Dk_{\text{obs}} = 0.95$) 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 for the nitrogen that leaves spontaneously after catalysis (the non-leaving-N). Measurement of both KIEs was not possible with other substrates studied to date.

Experimental Procedures (Materials and Methods)

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 ammonia determination were from Raichem. Ultrafiltration was accomplished using a Millipore YM series membrane with a 10,000 MW cutoff. Isotopic composition of carbon and nitrogen were measured on an isotope ratio mass spectrometer (IRMS) and were expressed in δ (per mil) notation as shown in eq 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 eq 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 (14).

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

$$k/k_0 = \log(1-f)/\log[(1-f)(R_s/R_0)] \quad (3)$$

$$k/k_0 = \log(1-f)/\log[(1-f)(R_p/R_0)] \quad (4)$$

Determination of Fraction of Reaction

A 10 μL aliquot of the reaction mixture at a given reaction time was rapidly added to 990 μL of water. A 10 μL 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 buffers employed (50 mM) were: 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: $v = VA/(K + A)$ using published statistical methods (15). Standard errors for determination of V and V/K averaged 7% (the highest was 18%). Similarly the pK_a values were determined by fitting $\log V/K$ vs. 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 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 μmol). 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 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 side arm containing 1.5 mL of 0.5 M chloramine-T. After freeze-thaw cycles to remove atmospheric CO₂, the chloramine-T solution was added to the semicarbazide solution and allowed to react for 15 minutes. The CO₂ was then collected through two dry ice-isopropanol 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 $^{15}k_{\text{obs}} = 1.0045 \pm 0.0003$ for the $-\text{NHNH}_2$ leaving group and $^{15}k_{\text{obs}} = 1.0010 \pm 0.0004$ for the $-\text{NH}_2$ 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 N₂ derived from hydrazine and that from ammonia (after complete hydrolysis of semicarbazide) to that of semicarbazide that had been combusted. The $^{15}\delta$ for N₂ derived from the hydrazine product was -2.2 ± 0.1 ; that from ammonia was $+1.1 \pm 0.8$. This should give a $^{15}\delta$ for the total nitrogen of combusted semicarbazide of $[(-2.2)(.67) + (+1.0)(0.33)] = -1.1$. The measured $^{15}\delta$ for the total N₂ of semicarbazide after combustion was -1.0 . The KIE data are given in Table 1.

The carbonyl-C KIE for semicarbazide under identical conditions is $^{13}k_{\text{obs}} = 1.0357 \pm 0.0011$. Control experiments showed that the $^{13}\delta$ from CO₂ generated by the chloramine-T oxidation of semicarbazide was identical to that from complete combustion of semicarbazide to CO₂. The KIE data are given in Table 1.

A log V/K vs. 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 $pK_1 = 4.1 \pm 0.3$ and $pK_2 = 8.5 \pm 0.1$ (15). A log V_m vs. 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 higher K_m than 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 pH 7 (17). Chemical catalysis of urea hydrolysis requires two important ionizable active site groups: (a) His320 ($pK_a \sim 6$) which provides a proton to the leaving-NH₂ during hydrolysis and (b) Ni—OH ($pK_a \sim 9$) 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, on a multiple heavy-atom KIE study of formamide hydrolysis (12).

Kinetics

The current study extends the kinetic investigation between pH 4.5 and 9.0. Figs. 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 $\log V/K$ vs. pH data for semicarbazide hydrolysis yielded $pK_1 = 4.1 \pm 0.3$ and $pK_2 = 8.5 \pm 0.1$ (15). This compares to $pK_1 = 5.34 \pm 0.04$ and $pK_2 = 8.67 \pm 0.04$ reported for urea hydrolysis (18). However, the $\log V_m$ profile for semicarbazide has an optimum at about pH 5.5, which is lower than that for urea by 0.5-1 pH units. 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 $pK_a \sim 6$) to the non-leaving-N of urea versus the formyl-H of formamide (12). It was assumed that binding of the non-leaving-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 s listed above for His320, Ni-OH, Cys319, which showed 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 from semicarbazide, making $-\text{NH}_2$ the non-leaving-N. On this basis it is expected that the rate of hydrolysis for both semicarbazide and urea will have identical dependence on the pK_a s for His320, Ni—OH and Cys319, since both substrates have non-leaving $-\text{NH}_2$ groups. To shift the V_m optimum to 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), while 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 $pK_a \sim 6$) is at hydrogen bonding distance to 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 of the V_m optimum to lower pH will again be observed. Once again this preference need not be absolute.

In a similar manner to the formamide case described earlier it is possible to calculate the relative $\log V_m$ vs. pH for urea and semicarbazide hydrolysis using the pK_a s 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 of 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 $\log V_m$ vs. pH plot compared to the calculated one is due to the fact that the dependence of V_m on the pK_a s for His219 and Cys319 is not absolute.

Nitrogen KIEs

Formamide provided an opportunity to view urease-catalyzed hydrolysis from a unique perspective. The lack of a non-leaving-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 non-leaving-N did not

contribute to observed composite KIE. Although this is a good assumption, there is the possibility of small secondary KIEs on the non-leaving-N. Like the natural substrate, semicarbazide has nitrogen atoms on either side of the carbonyl, but these groups ($-\text{NHNH}_2$ and $-\text{NH}_2$) can be separated and analyzed independently after hydrolysis. Thus, this substrate allows the opportunity to independently measure the KIE on the non-leaving-N.

The nitrogen KIE (Table 1) for the $-\text{NHNH}_2$ side of semicarbazide is significantly larger than that for the $-\text{NH}_2$ side ($^{15}k_{\text{obs}} = 1.0045$ vs. 1.0009). The isotopic analysis of the $-\text{NHNH}_2$ side of semicarbazide includes both the inner-N (leaving-N) and outer-N. As an initial interpretation, the KIE for the inner-N of $-\text{NHNH}_2$ should be approximately $^{15}k = 1.0090$, if small secondary KIEs on the outer-N are ignored. Taken together these two KIEs are best explained by $-\text{NHNH}_2$, rather than $-\text{NH}_2$, 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 where either $-\text{NH}_2$ or $-\text{NHNH}_2$ can be the first groups 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 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 was determined to be $^{15}K_{\text{eq}} = 0.983$ (12,19). This allowed calculation of the intrinsic KIE ($^{15}k_5 = 1.050$) for C-N bond breaking (12).

$$^{15}k_{\text{obs}} = \frac{\left(^{15}K_{\text{eq}} ^{15}k_5\right) + ^{15}k_3 \left(\frac{k_5}{k_4}\right) + \left(\frac{k_5}{k_4}\right) \left(\frac{k_3}{k_2}\right)}{1 + \left[\frac{k_5}{k_4} \left(1 + \frac{k_3}{k_2}\right)\right]} \quad (5)$$

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

This intrinsic KIE ($^{15}k_5 = 1.050$) and the equilibrium isotope effect ($^{15}K_{\text{eq}} = 0.983$) 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 $-\text{NHNH}_2$ leaving group is so small that it can be ignored. If the overall estimate is good to about 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 on forming 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 cancel each other giving $^{15}k_3$ 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 plus $^{15}k_{obs}$ for semicarbazide or urea, the commitment becomes $k_5/k_4 = 2.7$ for semicarbazide hydrolysis and $k_5/k_4 = 1.2$ 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 magnitude 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 proton transfer 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 the present case ($^{13}k_{obs} = 1.0357$) is large enough to be consistent with a transition state occurring either during 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 ($^{15}k_{obs} = 1.0009$). Kinetic studies indicate the observed shift of the V_m optimum toward lower pH is consistent with preference of the outer-N of semicarbazide for binding to the protonated form of an active site residue with a pKa 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 analogs that are slow substrates, although the analog substrates show 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 pK of the leaving group, with hydrazine being a better leaving group than ammonia.

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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

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Figure 1. Log V/K vs. pH plot for the Urease-Catalyzed Hydrolysis of Semicarbazide at 25°C. The calculated line was fit to: $\log V/K = \log \{c/(1 + [H^+]/K_1 + K_2/[H^+])\}$. The best fit was obtained when $c = 0.0104$ (ref. 15). Error bars are included, but are generally smaller than data point 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 data point symbols.

Figure 3.
Calculated log relative V_m vs. pH for Urea (v) and semicarbazide (v) plot.

Scheme 1.

Scheme 2.

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 $^{13}\delta$ partial	$^{15}\delta$ or $^{13}\delta$ substrate/product ^b	Isotope Effect ($k_{\text{light}}/k_{\text{heavy}}$) ^{c,d}
-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: 1.0357 ± 0.0011

^a Determined via glutamate dehydrogenase assay for ammonia.

^b The values are averages for 5 independent determinations: ±0.8 for ammonia; ±0.2 for hydrazine; ±0.4 for CO₂.

^c Corrected for fraction of 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.