Multiple Isotope Effect Study of the Hydrolysis of Formamide by Urease from Jack Bean (*Canavalia ensiformis*)†

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Received March 30, 2006; Revised Manuscript Received May 26, 2006

ABSTRACT: Multiple kinetic isotope effects have been measured for the urease-catalyzed hydrolysis of formamide at pH 6.0 and 25 °C. These kinetic isotope effects include the carbonyl-C ($^{13}k = 1.0241 \pm 0.0009$), the carbonyl-O ($^{18}k = 0.9960 \pm 0.0009$), the formyl-H ($^{D}k = 0.95 \pm 0.01$), the leaving-N ($^{15}k = 1.0327 \pm 0.0006$), and the nucleophile-O ($^{18}k = 0.9778 \pm 0.0005$). In addition, the enzyme does not catalyze the exchange of oxygen from the solvent into the carbonyl-O of formamide or the product, formate ion. The isotope effects are consistent with the rate-determining collapse of the tetrahedral intermediate (i.e., C−N bond cleavage). The pH optimum for formamide is at pH 5.3, whereas for urea, it is near 8.0. This is best accommodated by the mechanism proposed by Hausinger and Karplus, in which an active site cysteine binds to the nonleaving nitrogen in urea. For urea, the preference is for the anionic form of the sulfhydryl; for formamide, the neutral form is preferred, leading to the lower pH optimum.

Urease catalyzes a rather basic but difficult acyl group transfer reaction, namely, the hydrolysis of urea to ammonia and carbamate (*1*, *2*). Carbamate ultimately forms another ammonia plus carbon dioxide. Urea is hydrolyzed some 10^{12} times faster in the presence of the enzyme than in neutral aqueous media (*1*), making ureases among the most highly efficient catalysts known. The mechanism for enzyme catalysis differs strikingly from that for uncatalyzed hydrolysis. Enzymatic hydrolysis produces carbamate presumably via a tetrahedral intermediate (*2*). However, nonenzymatic hydrolysis produces cyanate via an elimination pathway (*1*). Semicabazide, hydroxyurea, formamide, and other related compounds are accepted by the enzyme but at a much reduced rate (*3*, *4*). High abundances of urease have been implicated in several animal disease states and in environmental problems due to the release of ammonia into the soil during nitrogen fertilization; Ciurli and Mangani have succinctly summarized these effects (*5*).

Urease has been isolated from several organisms, including plants, bacteria, and fungi (*5*, *6*). However, the enzyme from jack bean is somewhat of an enigma. It was the first enzyme ever crystallized (*7*), but to date, there has been no published crystal structure. Known ureases may be comprised of a different number of subunits, but all share a high degree of amino acid sequence alignment. Crystallographic data from ureases of different sources, including *Klebsiella aerogenes* (*6*) and *Bacillus pasteurii* (*5*), reveal structurally identical active sites. Regardless of the source of the enzyme, each urease subunit contains two nickels per active site; both are crucial for activity. The pH-rate profiles for the *K. aerogenes*, *B. pasteurii*, and jack bean enzymes show a pH-rate (V_m) maximum near 7.5−8, implicating in each case a role for active site residues with a pK_a value of 6.5, 9 and possibly 5.3 (*6*, *8*). All of the information supports the assumption that a common catalytic mechanism is operating for all ureases (*5*, *6*).

Chemical mutagenesis and crystallographic studies have led to two proposed mechanisms (*5*, *6*). These mechanisms share several features, including a Ni-coordinated hydroxide as the nucleophile for the hydrolysis and the formation of a tetrahedral intermediate, which breaks down to ammonia plus carbamic acid (or its anion). Each mechanism involves the coordination of at least the carbonyl-O of urea with one Ni. The mechanisms differ in the remaining types of coordination to each Ni and the role of various side chain residues used in hydrogen bonding and chemical catalysis.

The first mechanism is a refinement of an earlier proposal by Zerner that predated crystallographic data (*3*). It is based on crystallographic data (*5*, *6*), chemical modification (*9*), and site-directed mutagenesis and pH-rate profiles for the *K. aerogenes* enzyme (*10*, *11*). In this mechanism (Scheme 1), Hausinger, Karplus, and co-workers proposed histidine (His320) as the active site residue required to protonate the leaving nitrogen. When this residue is replaced by alanine, the rate is decreased by a factor 30 000 over that of the wild type (*10*, *11*). These workers proposed that no general base is needed to deprotonate Ni-coordinated water. Reverse protonation was proposed to explain how histidine (p$K_a \approx$ 6.5) could behave as a proton donor, and how a Ni-coordinated water (p$K_a \approx 9$) could act as a nucleophile in an enzyme with a pH maximum of 7.5−8. Although reverse protonation predicts the shape of the pH-rate profile, it suffers from having only 0.3% of the enzyme in the active form for

† J.F.M. thanks Professor W.W.Cleland for summer financial support in his laboratory (NIH Grant GM18938 to W.W.C.).

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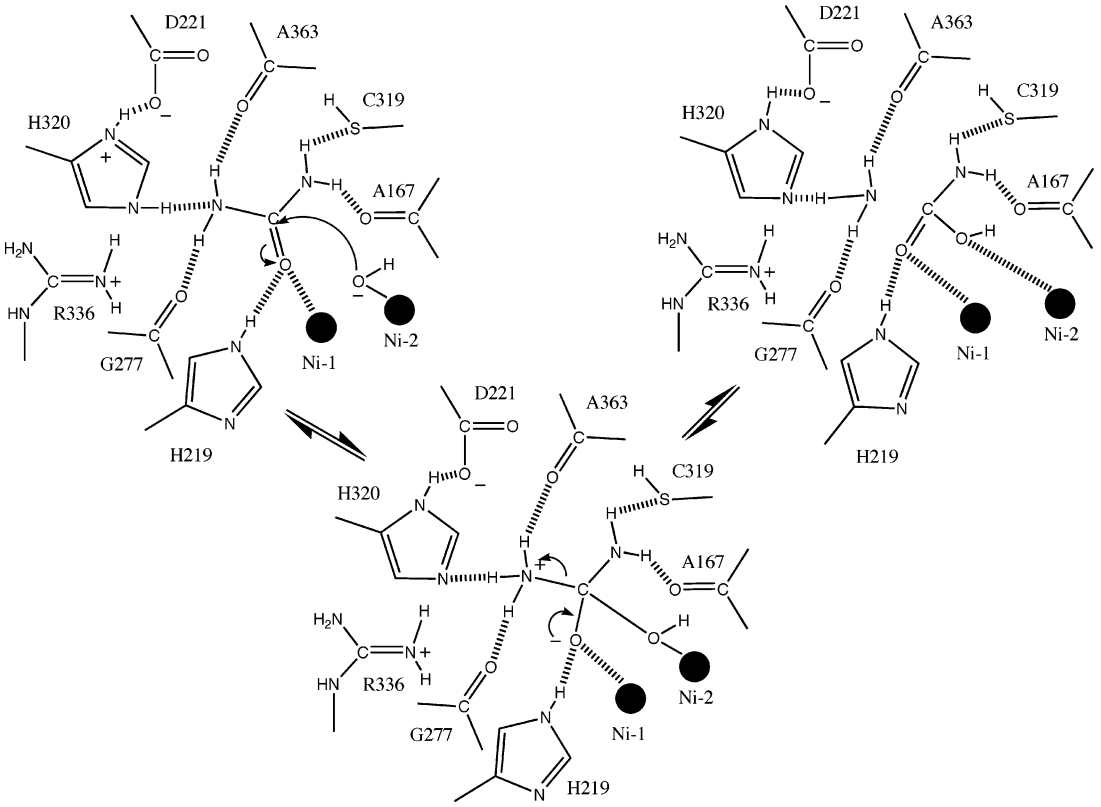
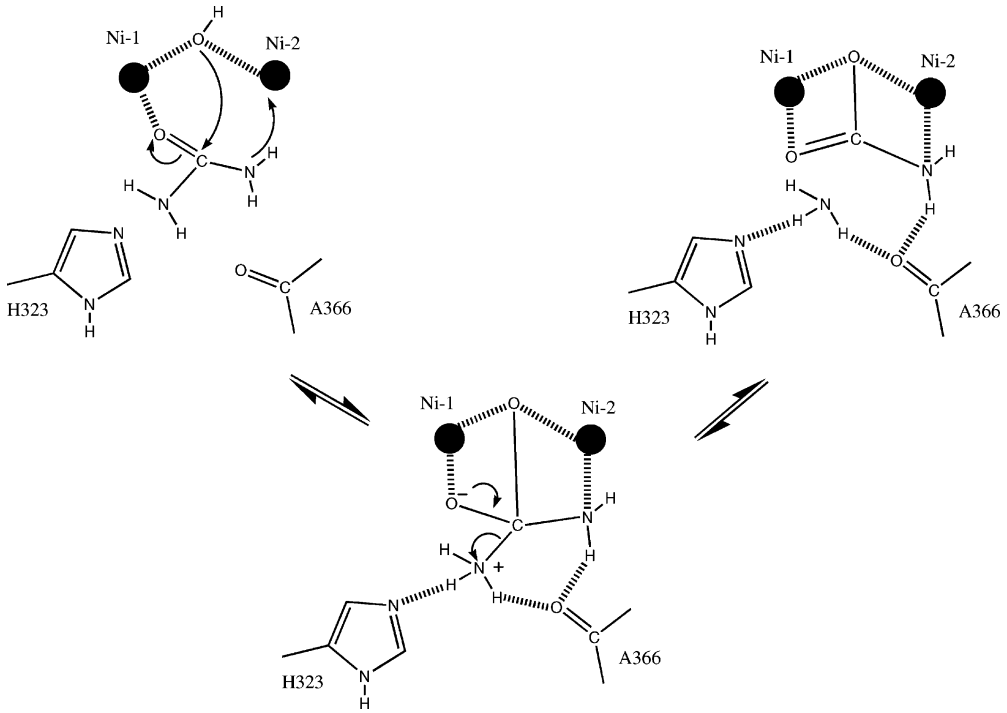
10.1021/bi060630z CCC: $33.50
Published on Web 07/19/2006

Scheme 1



Scheme 2



catalysis. An active site cysteine (cys319) is proposed to play a role in binding the nonleaving amino group of urea (*9*, *10*).

The second mechanism (Scheme 2), proposed by Ciurli, Mangani, and co-workers (*5*, *12*, *13*) is mainly based on crystallographic data (with bound inhibitors) for the *B. pasteurii* enzyme. This proposed mechanism involves the coordination of the carbonyl-O of urea to Ni-1 and the nonleaving-N of urea to Ni-2. The problem of reverse protonation is avoided by proposing that the nucleophilic hydroxyl is coordinated to both Ni atoms. The pK_a of this bridging water is lowered compared to that of a singly coordinated hydroxide, and it also becomes the proton source for the leaving-N, perhaps via an active site aspartate. The authors ascribe a minor role to His320 of stabilizing the positive charge of the leaving-N in the transition state but are vague on the mechanism of proton transfer to the leaving-N.

Several organometallic compounds containing two nickels have been synthesized in an attempt to model urease catalysis and to explore the mode of the binding of water to the Ni center (i.e., bridging or not) (*14*). Several of these models will bind urea via the carbonyl-O, but none show much ability to catalyze hydrolysis. One model that contains a bridging hydroxide similar to that in Scheme 2 binds urea but shows only a very slight ability to decompose urea (500-fold increase when heated to 60 °C in acetonitrile) (*15*). However, this reaction forms a cyanate rather than a carbamate. As mentioned earlier, cyanate is formed in the nonenzymatic hydrolysis of urea, but urease catalysis has been shown to produce carbamate (*1*). This model compound is offered as evidence for the role of a bridging hydroxide for urease catalysis (similar to that shown in Scheme 2), but the lack of any impressive rate acceleration makes it equally possible that the key missing element of the mechanism is a general acid (histidine) required to provide the proton for the leaving-N. This, in turn, would argue against the bridging hydroxyl as the proton donor in the enzymatic reaction.

Kinetic isotope effects (KIE[1]) are among the many physical techniques used to study the mechanism of enzyme-catalyzed urea hydrolysis. Isotope effects are one of the best available methods for determining both the rate-determining step of a reaction mechanism and the structure of the transition state. Early carbon KIEs were performed on enzyme preparations of uncertain purity and yielded inconsistent results. More recently, Schmidt (*16*) reliably measured the carbonyl-C ($^{13}k_{obs} = 1.0206$) and leaving-N ($^{15}k_{obs} = 1.0075$) KIEs for commercially available jack bean urease. The measured leaving-N KIE is only for the removal of the first nitrogen because this step is irreversible. Therefore, the isotopic composition of the second nitrogen released does not change as a function of the fraction of reaction (with the exception of a small secondary KIE), and the observed nitrogen KIE can be doubled to roughly determine the actual KIE on the first leaving-N ($^{15}k = 1.0150$). This is consistent with the partially rate-determining C−N bond cleavage. In the present work, isotope effects are measured for a slow substrate, formamide. This substrate is an excellent choice for the mechanistic investigations of urease catalysis for three reasons. First, there is no second nitrogen to contribute a secondary leaving-N KIE. Second, analytical procedures exist to determine the isotopic abundances of all of the atoms at the reactive center of the formamide and the product of the reaction, formate ion. Third, there is a large body of published isotope effects on the nonenzymatic hydrolysis of formamide under alkaline and acidic conditions to aid in the interpretation of the isotope effects for the enzymatic reaction (*17*, *18*).

EXPERIMENTAL SECTION

Materials and Methods. Anhydrous DMSO, 1-d-formamide, 1-h-formamide, anhydrous ethyl acetate, anhydrous sodium carbonate, Nessler's reagent, MES, HEPES, NAD^+, sublimed I_2, formate dehydrogenase, and urease (from jack bean) were obtained from Sigma-Aldrich. Ultrafiltration was accomplished using a Millipore YM series membrane with a 10 000 MW cutoff. The isotopic composition of the carbonyl oxygen of formamide used in the carbonyl-O and carbonyl-C KIEs ($^{18}\delta = -6.3$) was determined previously (*17*). Isotope ratios for carbon, nitrogen, and oxygen in heavy-atom isotope effects were measured on an isotope ratio mass spectrometer and were expressed in δ (per mil) notation as shown in eq 1, where $R_{(sample)}$ is the isotope ratio (heavy/light) of the sample, and $R_{(standard)}$ is the isotope ratio of a standard. For oxygen, these ratios are atomic ratios of $^{18}O/^{16}O$, not molecular ratios of $^{18}O{=}C{=}O^{16}/^{16}O{=}C{=}O^{16}$ for molecular CO_2. Heavy-atom KIEs were calculated using the equation of Bigeleisen and Wolfsberg (*19*). This equation requires the determination of δ for either the residual substrate or the product formed (at quenching) and a comparison to the δ of the unreacted substrate or the product formed after complete hydrolysis. The *m*/*z* 46/45 and 47/45 of formamide for the measurement of the formyl-H KIE and carbonyl-O exchange were determined directly on a GC-MS with an XT-1 nonpolar column.

$$\delta = \left(\frac{R_{(sample)}}{R_{(standard)}} - 1\right)x1000 \qquad (1)$$

Determination of the Fraction of Reaction. The fraction of reaction for the formyl-H, carbonyl-C, carbonyl-O, leaving-N, and nucleophile-O KIEs was determined via a formate dehydrogenase assay. Typically, a 25 μL aliquot of a quenched reaction mixture was added to 1000 μL of 0.10 M phosphate buffer at pH 7.4. A 50 μL aliquot of this diluted solution was added to a solution composed of 100 μL of 15 mM NAD^+ and 650 μL of the phosphate buffer at pH 7.4 in a 1 mL quartz glass cuvette. The reaction was initiated by the addition of 200 μL of a formate dehydrogenase solution (~50 U/mL in the phosphate buffer), and the increase in absorbance at 340 nm was determined. Formamide was not reactive under these conditions. From this assay, the amount of formate (not including unreacted formamide) present at the time of quenching was determined. To determine the total initial formamide present, a 50 μL aliquot of the quenched reaction mixture was mixed with 50 μL of 1.0 M NaOH and allowed to react for 1 h. Then a 50 μL aliquot of this reaction mixture was added to 2000 μL of the phosphate buffer. A 50 μL aliquot of this solution was then assayed via formate dehydrogenase as described above. The fraction of reaction was then calculated as the [formate] at quenching divided by the initial [formamide].

Carbonyl-C, Carbonyl-O, and Nucleophile-O Isotope Effect Procedures. These isotope effects employed the formamide of known isotopic composition at the carbonyl-O (*17*). A solution containing 250 μL of 0.10 M MES at pH 6.0, 750 μL of water, and about 1 mg of crystalline urease was incubated at 25 °C. Then, 16 μL of formamide was added via a syringe with gentle mixing. At a designated time (between 20 and 40 min), the reaction was rapidly cooled to 4 °C and immediately subjected to ultrafiltration at 4 °C (taking ~1 min.). Control experiments show that the amount of further reaction during this quenching was negligible (1−2 μmol). A 1.0 mL sample of 0.78 M MES at pH 6.0 was then passed through the membrane and added to the original filtrate. This is the quenched solution used in the formate dehydrogenase assay described above to determine the fraction of reaction. The remainder of the quenched reaction

[1] Abbreviations: DMSO, methylsulfoxide; KIE, kinetic isotope effect; MES, 4-morpholineethanesulfonic acid; NAD^+, β-nicotinamide adenine dinucleotide.

mixture (slightly under 2 mL) was placed in a round-bottom flask equipped with two stopcocks. One stopcock was on a sidearm that was capped with a septum. The second stopcock was for the attachment to the high vacuum line. The solution was evaporated to dryness at 70 °C overnight under high vacuum. While still under vacuum, 2 mL of anhydrous DMSO containing 250 mg of I_2 was added through the sidearm to the dried formate, and the resulting CO_2 was collected into a liquid nitrogen trap as previously described (*17*). Isotope ratio mass spectrometry gave the δ for oxygen and carbon, ultimately from formate after partial hydrolysis.

In separate experiments, formamide was completely hydrolyzed. A solution containing 250 μL of 0.10 M MES, 750 μL of water, 16 μL of formamide, and 2.3 mg of urease was incubated at 25 °C overnight. This solution was subjected to ultrafiltration, drying, and oxidation as described above. Isotope ratio mass spectrometry gave the δ for both the oxygen and carbon atoms for the formate produced after complete hydrolysis by urease.

Formyl-H Isotope Effect Procedure. The formyl-H KIE was measured by a GCMS method, using an ~1:1 mixture of 1-d-formamide to 1-h-formamide. The reaction with urease, quenching, and the determination of the fraction of reaction were performed as described above. The unreacted formamide in the quenched solution was isolated by repeated extraction with ethyl acetate. The ethyl acetate solution was dried over anhydrous sodium carbonate and filtered, and the ethyl acetate was removed by rotary evaporation. The isolated formamide was then dissolved in methanol (as a carrier), and the D/H ratio (*m*/*z* 46/45) was directly measured by GC-MS. The above plus the *m*/*z* 46/45 for unreacted formamide were used to calculate the KIE as described above.

Leaving-N Isotope Effect Procedure. This isotope effect employed natural abundance formamide. The reaction with urease, quenching, and the determination of the fraction of reaction were performed as described above. The quenched reaction mixture was applied to a column composed of 7 mL of strong cation-exchange resin (in the Na^+ form). The column was eluted first with water (8 fractions, 4 mL each), followed by 1 M NaCl (8 fractions, 8 mL each). The early fractions (assayed by UV absorbance at 240 nm) contained unreacted formamide; the later ones (assayed by Nessler's reagent) contained the product, ammonium ion. The unreacted formamide was hydrolyzed in 1 M NaOH for 2 h, followed by neutralization with H_2SO_4. This solution was then made basic with NaOH and steam distilled into 0.1 M H_2SO_4. The volume of the steam-distilled solution was reduced to about 2 mL by rotary evaporation and oxidized with NaOBr to N_2. The N_2 was analyzed by isotope ratio mass spectrometry and the KIE calculated as described above. The ammonium ion isolated by ion exchange chromatography was treated in a similar manner, except no alkaline hydrolysis was needed.

Oxygen Exchange from Water into Formamide. A solution containing 1070 μL of natural abundance water, 430 μL of ^{18}O-enriched water (96.7 atom % ^{18}O), 500 μL of 0.10 M MES at pH 6.0, and 1.2 mg of urease was incubated to 25 °C. Then 32 μL of formamide was added to start the reaction. The reaction was quenched by ultrafiltration at 4 °C, and the fraction of reaction was determined by the formate dehydrogenase assay as described above. The remaining quenched reaction mixture was extracted with ethyl acetate and analyzed for the $m/z = 47/45$ isotope ratio by GCMS as described for the formyl-H isotope effect procedure.

Table 1: Lack of Exchange of ^{18}O into Formate by Urease at 25 °C for 24 h

sample	$^{18}\delta$
formate, incubated in natural abundance water, no urease	−8.4
formate, incubated in enriched water, no urease	−7.9
formate, incubated in enriched water, with urease	−8.1
natural abundance water	−39.3[a]
enriched water	+51.0[b]

[a] Natural abundance water in Madison, WI measured after exchange into CO_2 and corrected for the fractionation factor between water and CO_2. [b] The enriched water used in this experiment measured after exchange into CO_2 and corrected for the fractionation factor between water and CO_2.

Oxygen Exchange from Water into Formate. A solution containing 1000 μL of 0.40 M sodium formate in 0.025 M MES at pH 6.0 and 20 μL of enriched ^{18}O-water (5 μL of 90 atom % ^{18}O in 1000 μL of natural abundance water) was prepared. A 250 μL portion of the above solution was incubated at 25 °C for 24 h. A second 250 μL portion was treated in an identical manner after first adding 0.8 mg of urease. Both solutions were quenched by ultrafiltration, dried under vacuum, oxidized to carbon dioxide, and analyzed by isotope ratio mass spectrometry. In a separate experiment, the isotopic composition of the ^{18}O-enriched water used in these isotope ratio mass spectrometry experiments was shown to have a much higher abundance of ^{18}O ($^{18}\delta = +51.0$) than natural abundance water (see below).

Determination of the $^{18}\delta$ for Water. A small sample of CO_2 (<100 μmol) was added to an evacuated round-bottom flask equipped with a stopcock containing 20 mL of degassed water and 1 mL of concentrated H_2SO_4. The mixture was stirred overnight, and the CO_2 was isolated and analyzed by isotope ratio mass spectrometry. The $^{18}\delta$ for Madison, Wisconsin water has not changed significantly over a ten-year period ($^{18}\delta = -39.3 \pm 0.3$).

RESULTS

Two experiments served to demonstrate that urease does not catalyze the exchange of oxygen from water into either the carbonyl-O of formamide or into the oxygen atoms of formate during hydrolysis. First, the incubation of natural abundance sodium formate with urease in both natural abundance and ^{18}O-enriched water showed no difference in the observed $^{18}\delta$ of formate (Table 1). In this experiment, the per mil difference between the natural abundance water (−39.3) and the enriched water (+51.0) was 89.3 per mil, easily discernible by isotope ratio mass spectrometry. The fact that the $^{18}\delta$ of the CO_2 derived from formate does not change whether it is incubated in natural abundance water or enriched water indicates the lack of exchange. Second, the partial hydrolysis of natural abundance formamide in very highly ^{18}O-enriched water (21.1 atom % ^{18}O) in the presence of urease failed to show the incorporation of any ^{18}O-label into formamide, within the limit of detection of the GC-MS method. It is estimated that the magnitude of k_h/k_{ex} is greater than 400.

Table 2: Heavy-Atom Isotope Effects for the Urease-Catalyzed Hydrolysis of Formamide at pH 6.0 and 25 °C

atom	isotope effect, $k_{(light)}/k_{(heavy)}$[a,b]
carbonyl-C	1.0241 ± 0.0009 (6)
carbonyl-O	0.9960 ± 0.0009 (6)
formyl-H	0.95 ± 0.01 (6)
leaving-N	1.0327 ± 0.0006 (6)
nucleophile-O	0.9778 ± 0.0005 (5)

[a] Corrected for the fraction of reaction. [b] The standard deviation is given for each isotope effect, and the number of determinations is shown in parentheses.

The nucleophile-O KIE can be determined from the $^{18}\delta_{(obs)}$ of CO_2 (from formate) produced after complete hydrolysis by urease. After complete hydrolysis, the observed $^{18}\delta_{(obs)}$ is composed of the $^{18}\delta$ for the carbonyl-O of unreacted formamide ($^{18}\delta_{(CO)}$) plus the $^{18}\delta$ for the nucleophile-O from water ($^{18}\delta_{(NU)}$). Because $^{18}\delta_{(obs)}$ from the isotope ratio mass spectrometer is the per mil abundance of ^{18}O atoms (not the per mil molecular abundance of the $m/z = 46$ in CO_2), the relationship between $^{18}\delta_{(obs)}$, $^{18}\delta_{(CO)}$, and $^{18}\delta_{(NU)}$ is given by eq 2.

$$^{18}\delta_{(obs)} = [(0.5)(^{18}\delta_{(CO)}) + (0.5)(^{18}\delta_{(NU)})] \quad (2)$$

At 100% reaction, the $^{18}\delta_{(CO)}$ must be the same as it was in formamide ($^{18}\delta = -6.3 \pm 0.3$) before the start of the reaction. This is true because the carbonyl-O does not exchange with the solvent during the reaction. Thus, it is possible to determine $^{18}\delta_{(NU)}$ from eq 2. The average $^{18}\delta_{(obs)}$ is -11.7 ± 0.3 at 100% reaction. The $^{18}\delta_{(NU)}$ is calculated as follows:

$$-11.7 = (0.5)(-6.3) + (0.5)(x) \quad (3)$$

$$^{18}\delta_{(NU)} = x = -17.1 \quad (4)$$

The nucleophile-O KIE is the difference between the per mil abundance of ^{18}O in the nucleophile-O contained in the resultant CO_2 ($^{18}\delta_{(NU)} = -17.1$) and that for water ($^{18}\delta_{(water)} = -39.3 \pm 0.3$). Therefore, the nucleophile-O KIE is $(-17.1) - (-39.3) = -22.2$ per mil. This is an inverse KIE of 2.22% or $^{18}k = 0.9778$ (Table 2). The Bigeleisen and Wolfsberg equation (*19*) is not necessary because the nucleophile (water) is present in great excess (56 M), and its isotopic composition does not change appreciably during the reaction.

The carbonyl-C and carbonyl-O KIEs were calculated from the measured $^{13}\delta$ and $^{18}\delta$ of CO_2 (from formate) present at quenching, the $^{13}\delta$ and $^{18}\delta$ of CO_2 (from formate) produced after complete hydrolysis by urease, and the known fraction of reaction (*f*) at quenching (*19*). The calculation of the carbonyl-C KIE is straightforward. However, the calculation of the carbonyl-O KIE is complicated because, as mentioned above, one of the oxygen atoms of formate is derived from the carbonyl-O of formamide, and the other is from the nucleophile. The calculation of the carbonyl-O KIE is simplified by two facts. First, there is no oxygen exchange from the solvent (water) into the carbonyl-O of formamide during urease-catalyzed hydrolysis. Second, urease does not catalyze the exchange of oxygen from water into the product formate during or after hydrolysis (Table 1). Thus, no corrections for exchange were required. The $^{18}\delta_{(CO)}$ at any fraction of reaction (*f*) can be calculated from eq 2, given the fact that $^{18}\delta_{(NU)}$ (−17.1) does not vary with *f* (eqs 5 and 6). The $^{18}\delta_{(NU)}$ (−17.1) is a constant in both the low conversion and 100% conversion samples. Because the carbonyl-O KIE is the difference between $^{18}\delta_{(CO)}$ at 100% conversion and $^{18}\delta_{(CO)}$ after the correction for *f*, this constant is eliminated in the calculation of the KIE. The results are summarized in Table 2; the original data are given in the Supporting Information.

$$(^{18}\delta_{(obs)})_{at\ f} = (0.5)(y) + (0.5)(-17.1) \quad (5)$$

$$(^{18}\delta_{(CO)})_{at\ f} = y = 17.1 + (2)(^{18}\delta_{(obs)})_{at\ f} \quad (6)$$

The leaving-N KIE experiments used natural abundance formamide. After the usual quenching, the residual formamide was separated from the product ammonium ion by ion exchange column chromatography. The residual formamide was quantitatively hydrolyzed to formate and ammonia. The $^{15}\delta$ for both sources of ammonia was determined after oxidation to N_2. The KIE was calculated from the $^{15}\delta$ mentioned above, the $^{15}\delta$ for ammonium ion generated by total hydrolysis, and the known fraction of reaction at quenching. The data are summarized in Table 2; the original data are given in the Supporting Information.

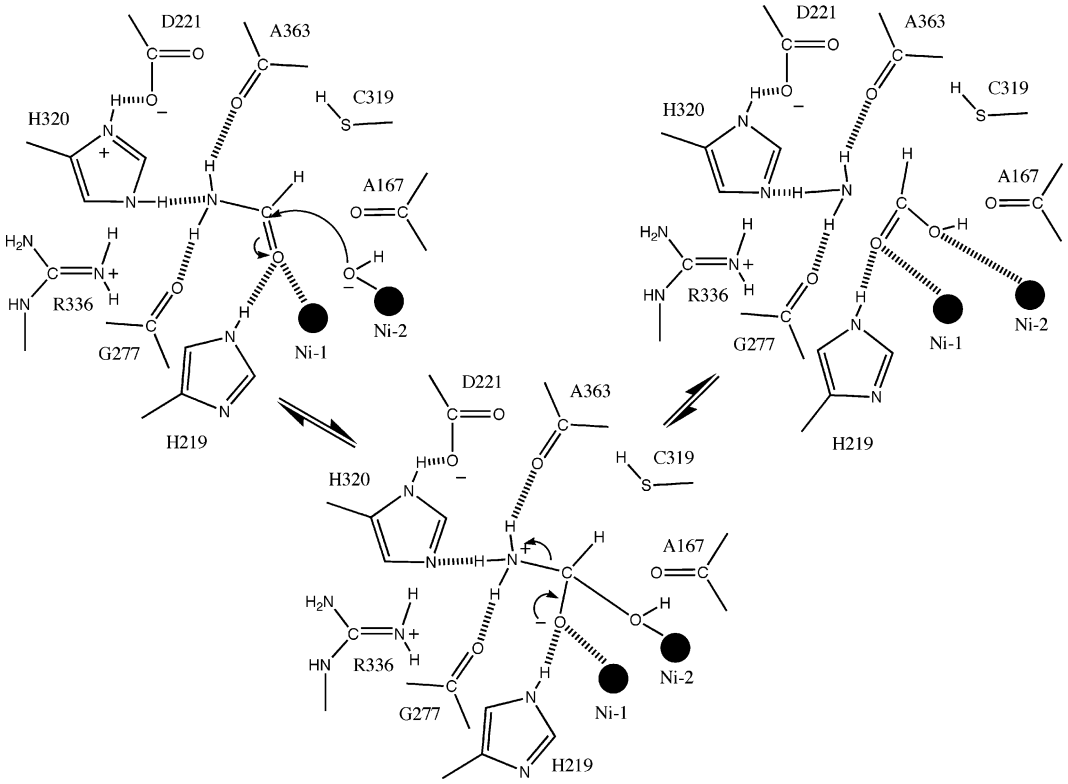
The formyl-H KIE was measured using an ~1:1 mixture of 1-h-formamide/1-d-formamide. After partial hydrolysis, the residual substrate was isolated by extraction with ethyl acetate, and the isotope ratio ($m/z = 46/45$) was determined by GCMS. These measured ratios, along with the isotope ratio for unreacted formamide and the known fraction of reaction at quenching were used to calculate the KIE. The results are summarized in Table 2; the original data are given in the Supporting Information.

DISCUSSION

Formamide has been shown to be a slow substrate for urease with a high K_m (516 mM at pH 5.2) and a low k_{cat} (246 s^{-1} at pH 5.2) compared to those of the natural substrate ($K_m = 3$ mM, $k_{cat} = 11\,000\ s^{-1}$ at pH 6.8) (*2*−*4*). Formamide hydrolysis shows a different pH optimum than that of urea (5.3 vs 7.5−8 for urea); the K_m for both substrates does not vary significantly with pH (*4*). Formamide is also a competitive inhibitor ($K_i = 404$ mM) of the hydrolysis of urea. This is evidence that formamide binds at the active site of urease and that hydrolysis takes place at that same site.

Mechanism of Hydrolysis. The two mechanisms proposed for urease-catalyzed hydrolysis of urea are given in Schemes 1 and 2 (*5*−*6*). Any viable mechanism must be consistent with the following facts for urea and formamide hydrolysis. First the pH optimum of 7.5−8 for urea is lowered to 5.3 for formamide hydrolysis (*4*). It is the k_{cat} values of both substrates that is most sensitive to changing pH not the K_m value. Second, the leaving-N KIEs suggest that breaking the C−N bond changes from being partially rate-determining for urea hydrolysis (*16*) to fully rate-determining for formamide hydrolysis ($^{15}k = 1.0150$ for urea; $^{15}k = 1.0327$ for formamide). Each of these factors will be explored in the following paragraphs.

Scheme 3



Recent pH-rate correlations for jack bean urease have assigned a role to three active site residues with pK_as near 5.3, 6.5, and 9.0 (*8*). The precise assignment of the pK_a values is difficult, but what is clear is that there are two low pK_a groups (near 6) and one high pK_a group (near 9). The crystallographic data for the *K. aerogenes* and *B. pasteurii* enzymes suggests that one of the low pK_a groups is an active site histidine (*5*, *6*). This is supported by data from mutants where histidine is replaced by alanine, resulting in a k_{cat} that is decreased by a factor 30 000 (*10*, *11*). Karplus assigns the histidine a role as a general acid, which supplies a proton to the leaving-N; Ciurli and Mangani propose a charge stabilization role. The second low pK_a group has been proposed to be an active site sulfhydryl group such as cys319 in the *K. aerogenes* enzyme (*6*) or a carboxylate group, such as asp363 in the *B. pasteurii* enzyme (*5*). The group with a pK_a = 9.0 has been at the heart of the controversy between the two proposed mechanisms. Karplus assigns this to the deprotonation of the Ni-coordinated water at the active site, whereas Ciurli and Mangani leave this pK_a unassigned, opting instead for bridging water with a much lower pK_a (*5*, *6*).

Although the isotope effects can be made to fit the mechanism of Ciurli and Mangani, the pH-rate data best support the Karplus and Hausinger mechanism at the present time (*8*). More importantly, the lowering of the pH optimum for formamide can be reasonably accommodated by this mechanism. In formamide hydrolysis, His320 still serves as the proton source for the leaving-N, and a Ni-coordinated hydroxide is still the nucleophile. What changes the pH optimum is the protonation state of Cys319. For urea, the -SH prefers to be anionic in order to associate (or weakly H-bond) with the partial positive charge on the nonleaving-N. With formamide, this nonleaving-N of urea is replaced with the neutral formyl-H of formamide, and consequently, Cys319 is preferred in the neutral form. This change from anionic to protonated sulfhydryl causes the lowering of the pH optimum observed for formamide. The mechanism for formamide hydrolysis is shown in Scheme 3.

A simple calculation (plotted in Figure 1) of the fraction of the enzyme in the correct ionic form (plotted as log of the relative velocity) as a function of pH demonstrates that this hypothesis is reasonable. In these plots, the pK_a for both His320 and Cys319 is assumed to be 6 and that for the Ni-coordinated water is assumed to be 9. The solid line in Figure 1 represents a preference for Cys319 in the anionic form (urea hydrolysis); the dashed line represents a preference for Cys319 in the neutral form (formamide hydrolysis). The shift in the pH optimum is as expected and quite similar to what is observed (*4*). In addition, the actual profile for formamide is flatter at the high pH region, indicating that the preference for the neutral form of Cys319 is not absolute. As a result of the above discussion, the isotope effects of this present study are interpreted within the framework of the Hausinger and Karplus mechanism.

Oxygen Isotope Exchange. The carbonyl-O of formamide does not exchange with water in the presence of urease within the precision of the GC-MS method used. In addition, the product, formate ion, was also shown not to exchange its oxygen atoms with water in the presence of urease. This should not be a surprise. Although the exchange into the carbonyl-O has been commonly observed for the alkaline hydrolysis of formamide (*17*) and other acyl groups (*20*, *21*), it is rare for enzyme-catalyzed hydrolyses. This is due to stereochemical reasons (i.e., enzymes are chiral and can recognize prochiral groups during acyl transfer). Consequently, one cannot conclude that the lack of measurable oxygen exchange means that the tetrahedral intermediate

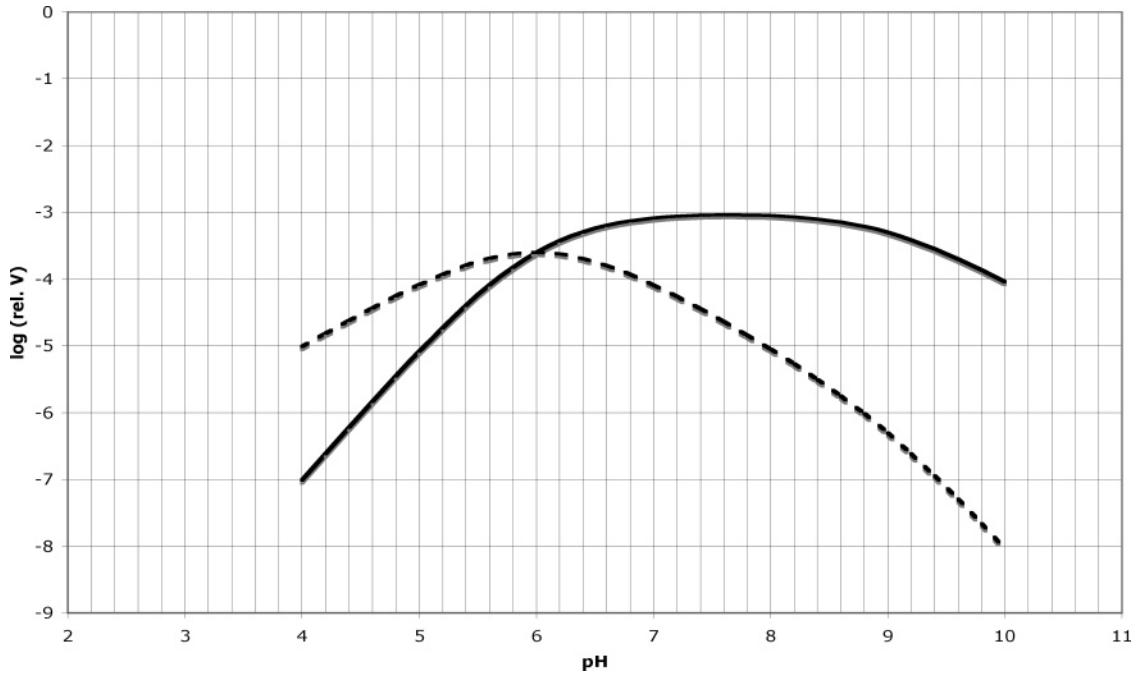


FIGURE 1: Calculated plot of the log of the relative velocity of urea hydrolysis (—) and formamide hydrolysis (---) versus pH. Urea hydrolysis prefers Cys319 to be in the anionic form, whereas formamide hydrolysis prefers the neutral form.

partitions much faster to the product than to the starting amide. Quite the opposite is true, and this is established on the basis of the leaving-N KIE (see below). Nevertheless, it was necessary to establish that no exchange occurs during urease-catalyzed hydrolysis to be sure this enzyme is not an exception to the general rule (of no exchange) and to show that the carbonyl-O KIE need not be corrected for such oxygen exchange.

Leaving-N Isotope Effect. The observed leaving-N KIE for formamide hydrolysis by urease ($^{15}k = 1.0327$) is much larger than those for the nonenzymatic hydrolysis of formamide ($^{15}k = 1.0040$, alkaline; $^{15}k = 1.0050$, acid-catalyzed) (*17*, *18*) and is also much larger than that measured by Schmidt for the natural substrate, urea ($^{15}k = 1.0150$) (*16*). In fact, this is one of the largest normal nitrogen KIEs measured for an enzyme-catalyzed hydrolysis. Clearly, the breaking of the C−N bond (during the collapse of the tetrahedral intermediate) is rate-determining for the urease-catalyzed hydrolysis of formamide. The assumed kinetic mechanism is shown in eq 7, where FA is formamide and TI is the tetrahedral intermediate.

$$\mathrm{E} + \mathrm{FA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{E{\cdot}FA} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \mathrm{E{\cdot}TI} \overset{k_5}{\rightleftharpoons} \mathrm{Products} \qquad (7)$$

The observed isotope effect for any reacting atom in eq 7 is related to the individual rate constants by eq 8, where $*k$ is a KIE (k_{light}/k_{heavy}) on a particular rate constant, and $*K_{eq}$ is the equilibrium isotope effect on formation of the TI (i.e., $*k_3/*k_4$). It is assumed that there is no KIE on the binding steps, k_1 or k_2. Because all KIEs were measured by the competitive method, all are on V/K.

$$*k_{obs} = \frac{(*K_{eq}*k_5) + *k_3(k_5/k_4) + (k_5/k_4)(k_3/k_2)}{1 + [k_5/k_4(1 + k_3/k_2)]} \qquad (8)$$

Because $^{15}k_{obs}$ is near the maximum expected for the rate-determining breaking of the C−N bond (*22*, *23*), one can assume that k_5 is much smaller than k_4, reducing eq 8 to that given in eq 9. This result is important because it establishes a framework for the interpretation of all the other isotope effects.

$$*k_{obs} = *K_{eq}*k_5 \qquad (9)$$

From the above equation and information in the literature, one can calculate a value for the intrinsic nitrogen KIE on k_5. The equilibrium isotope effect on the protonation of an amine is $^{15}K_{eq} = 0.9836$ (*24*). In the present case, the formation of the TI introduces another heavy atom (the nucleophile-O), which would stiffen the bonding to the nitrogen even further, leading to a even more inverse equilibrium isotope effect. However, the loss of resonance to the carbonyl would weaken the bonding to the nitrogen in the TI and would force the isotope effect to become more normal. Given these constraints, it is reasonable to expect the equilibrium isotope effect to be $^{15}K_{eq} = 0.980-0.985$. This leads (via eq 9) to an intrinsic KIE for $^{15}k_5$ close to 1.05, clearly within the expectations of theory (*25*).

Formyl-H Isotope Effect. The observed formyl-H KIE is small and inverse ($^{D}k_{obs} = 0.95$). As discussed above, the observed formyl-H KIE is composed of an equilibrium isotope effect on formation of the tetrahedral intermediate and a KIE on its breakdown. The formyl-H equilibrium isotope effect for the formation of the tetrahedral intermediate has been previously estimated to be approximately $^{D}K_{eq} = 0.69$ (*17*). This allows for the calculation of the KIE for the breakdown of the tetrahedral intermediate ($^{D}k_5 = 0.95/0.69 = 1.37$). This large normal KIE on k_5 is in agreement with the conclusion from the leaving-N KIE, namely, that the transition state of highest energy occurs during the breakdown of the TI and that this transition state is a late one (i.e., contains very little tetrahedral character).

Nucleophile-O Isotope Effect. Heavy-atom KIEs for solvent nucleophiles are usually quite difficult to

measure but can be of great value in determining the transition state structure for a reaction. The esters and amides of formic acid are ideal substrates for the measurement of these KIEs because an analytical methodology exists to measure the isotopic composition of the nucleophile-O, once this atom has been incorporated into the formate molecule. Such KIEs have been measured for the alkaline and acid-catalyzed hydrolysis of methyl formate and the alkaline hydrolysis of formamide (*17*, *26*, *27*). The magnitude of these KIEs is typically large and normal, but exceptions are possible.

Nucleophile-O KIEs, like all KIEs, depend on two commonly accepted factors (*25*). First is the temperature-independent factor (TIF), which involves the motion of the isotopes along the reaction coordinate; this is always normal. The second is the temperature-dependent factor (TDF), which is due to the creation of new vibrational modes in the transition state; these can be normal (looser bonding to the isotope) or inverse (tighter bonding to the isotope). Most nucleophile-O KIEs are expected to be normal because of the dominance of the TIF. However, there are two possible exceptions. The first exception is when the transition state for formation of the bond between the nucleophile and the substrate is a very late one (*28*). Then, the newly created bonding modes (TDF) will become more important and will decrease the magnitude of an expected normal contribution from the TIF. In extreme cases, this could lead to an observed inverse nucleophile KIE. The second exception is when the bond between the nucleophile and substrate is formed in an equilibrium step, prior to some later rate-determining step that does not significantly involve the nucleophilic atom. In this case, the observed isotope effect will be an equilibrium one on the formation of the nucleophile−substrate adduct and may very well be inverse.

The observed nucleophile-O KIE is large and inverse ($^{18}k = 0.9778$). On the basis of the previous conclusion that the breakdown of the TI is rate-determining, the nucleophile-O KIE best fits the second case described in the previous paragraph. This is true because changes in bonding to the nucleophile-O, which occur during the breakdown of the TI, are relatively minor and can be largely ignored. This means that $^{18}k_5 = 1.0$ and, consequently, $^{18}k_{obs} = {}^{18}K_{eq}$ (eq 9). Is $^{18}K_{eq} = 0.9778$ reasonable for this reaction? The best way to access this is through model equilibria. The fractionation factors between water and alcohols favor ^{18}O in the alcohol (*29*). For methanol−water, the fractionation factor is 2.2%, and for 2-propanol, it is 3.3%. The effect of the Ni atom is not clear, but it is likely to be small as seen in the case of the oxygen atoms of phosphate coordinated with the divalent Mg ion (*30*). Ni coordination to the O−H of the TI derived from the nucleophile would likely lead to stiffer bonding to the oxygen. Conversely, this Ni-coordination would be replacing one H bond to the solvent. Taken as a whole, the evidence from model compounds points to an equilibrium isotope effect for the formation of the TI which favors ^{18}O by about 2%, as observed.

Carbonyl-O KIE. The carbonyl-O KIE is small and inverse ($^{18}k_{obs} = 0.9960$); it is identical to that observed during the acid-catalyzed hydrolysis of formamide (*18*). The observed KIE is a secondary KIE because the connection to the carbonyl-O is not severed during the reaction. It is composed of an equilibrium isotope effect for the formation of the TI and a kinetic one for its decomposition of the TI. There are no good models for the equilibrium formation of the TI, making a calculation of $^{18}k_5$ impossible. However, a qualitative description is possible. The formation of the TI involves the breaking of the carbonyl π-bond. This bond is already weakened in the ground state by resonance with the leaving-N. As a result, breaking this weakened C−O bond will lead to only a small normal contribution to the equilibrium isotope effect. At the same time, the coordination to the Ni and the addition of the nucleophile will stiffen bonding to the TI, giving a small inverse contribution. On the basis of these arguments, it is reasonable to expect $^{18}K_{eq}$ to be near unity. The KIE for the breakdown of the TI will involve reforming the carbonyl-π bond (an inverse secondary KIE) and the loss of coordination to the Ni (a normal contribution), leading to a very small inverse KIE. These expectations, as crude as they are, argue that a small inverse $^{18}k_{obs}$ is reasonable.

Carbonyl-C Isotope Effect. All measured carbonyl-C KIEs for nonenzymatic hydrolysis (acidic or basic) of the esters and amides of formic acid fall in a narrow range ($^{13}k_{obs} = 1.028-1.031$) (*17*, *18*, *20*, *26*, *27*). The observed carbonyl-C KIE for the urease-catalyzed hydrolysis of formamide falls close to this observed range at $^{13}k_{obs} = 1.0241$. It has been well documented, both experimentally (*27*, *31*) and theoretically, (*28*) that the carbonyl-C KIE does not change appreciably with changes in transition state structure. In fact, all of the carbonyl-C KIEs measured for ester and amide hydrolysis vary in the narrow range of 1.028−1.043, despite such varied structures as methyl formate, methyl benzoate, substituted and unsubstituted diphenyl carbonates, *p*-nitrophenyl acetate, and formamide (*18*, *20*, *27*, *31*).

CONCLUSIONS

A multiple isotope effect study of the urease-catalyzed hydrolysis of formamide is consistent with the rate-determining breakdown of the tetrahedral intermediate, involving C−N bond cleavage ($^{15}k = 1.0327$). Under these conditions, the tetrahedral intermediate is in equilibrium with the starting materials as evidenced by a nucleophile-O KIE of $^{18}k = 0.9778$. The pH-rate profile for formamide hydrolysis best fit the mechanism proposed by Karplus and Hausinger (*6*) in which a Ni-coordinated hydroxide serves as the nucleophile, and an active site histidine serves as a general base to donate a proton to the leaving-N. The shift to a lower pH optimum in the case of formamide hydrolysis is accommodated by formamide preferring a neutral -SH instead of the anionic form, which is preferred by urea.

SUPPORTING INFORMATION AVAILABLE

Original isotopic abundances and fractions of reaction used to calculate the carbonyl-C, carbonyl-O, leaving-N, and formyl-H KIEs and the logic and equations used to determine the carbonyl-O KIE. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI060630Z