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New Family of Biuret Hydrolases Involved in *s*-Triazine Ring Metabolism

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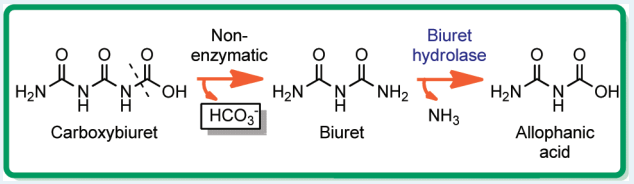
Supporting Information

ABSTRACT: Biuret is an intermediate in the bacterial metabolism of *s*-triazine ring compounds and is occasionally used as a ruminant feed supplement. We used bioinformatics to identify a biuret hydrolase, an enzyme that has previously resisted efforts to stabilize, purify, and characterize. This newly discovered enzyme is a member of the cysteine hydrolase superfamily, a family of enzymes previously not found to be involved in *s*-triazine metabolism. The gene from *Rhizobium leguminosarum* bv. *viciae* strain 3841 encoding biuret hydrolase was synthesized, transformed into *Escherichia coli*, and expressed. The enzyme was purified and found to be stable. Biuret hydrolase catalyzed the hydrolysis of biuret to allophanate and ammonia. The k_{cat}/K_M of 1.7×10^5 M^{-1} $s^{-1}$and the relatively low K_M of 23 ± 4 μM together suggested that this enzyme acts uniquely on biuret physiologically. This is supported by the fact that of the 34 substrate analogs of biuret tested, only two demonstrated reactivity, both at less than 5% of the rate determined for biuret. Biuret hydrolase does not react with carboxybiuret, the product of the enzyme immediately preceding biuret hydrolase in the metabolic pathway for cyanuric acid. This suggests an unusual metabolic strategy of an enzymatically produced intermediate undergoing nonenzymatic decarboxylation to produce the substrate for the next enzyme in the pathway.



KEYWORDS: biuret, biuret hydrolase, allophanate, enzyme, carboxybiuret, kinetics, ^{13}C NMR

INTRODUCTION

In 1776, Carl Scheele obtained cyanuric acid from the pyrolysis of uric acid.[1] The correct structure, however, was only elucidated in 1830 by Liebig and Wohler.[1] Since that time, hundreds of *s*-triazine compounds have been produced on industrial scales, including herbicides, dyes, and resin intermediates, such as melamine.[2] The agriculturally significant *s*-triazines, mainly herbicides and insecticides, are designed for dispersal in the environment, where they become growth substrates for a variety of soil microorganisms. For example, the herbicide atrazine is known to be metabolized by bacteria from the genera *Pseudomonas*,[3,4] *Arthrobacter*,[5,6] and *Nocardioides*[7] to use as a source of nitrogen for growth. Atrazine and other *s*-triazine compounds are metabolized via the intermediate cyanuric acid (Scheme 1). Cyanuric acid is the substrate for a ring-cleaving hydrolase; biuret is an intermediate and further metabolized.[8]

Of the enzymes involved in the microbial metabolism of *s*-triazines, only biuret hydrolase has resisted characterization. A biuret hydrolysis activity was demonstrated in protein fractions on numerous occasions,[9−12] but the catalytic activity has proven to be unstable upon fractionation and storage. Even gene cloning methodologies have failed to remedy this problem. For example, Martinez, et al.[12] in 2001 identified a gene cluster within *Pseudomonas* sp. ADP that encoded the enzymes catalyzing transformation of cyanuric acid and were denoted as *atzD, atzE,* and *atzF* (Figure 1A). The *atzE* gene, encoding a biuret hydrolase, was cloned and expressed in *E. coli* and catalytic activity with biuret was demonstrated with crude protein extracts from the cell. The enzyme, however, proved to be unstable, like previous preparations of biuret hydrolase.

In the present study, new types of biuret hydrolases were sought with the goal of obtaining a stable representative for detailed characterization. With the advent of widespread genomic DNA sequencing, it was possible to conduct a broad search for putative biuret hydrolase genes. The genes could be cloned and expressed in *E. coli* and tested for biuret hydrolase activity. In this manner, a novel biuret hydrolase from the cysteine hydrolase superfamily was identified. The enzyme was purified and characterized. It was determined that cyanuric acid hydrolase produces carboxybiuret, which decomposes nonenzymatically to release carbon dioxide and provide biuret for the novel biuret hydrolase characterized in this study. The kinetic parameters determined here suggested that the coupling of a nonenzymatic to an enzymatic step represents a viable cellular strategy for efficiently metabolizing *s*-triazine rings to obtain nitrogen for the cell.

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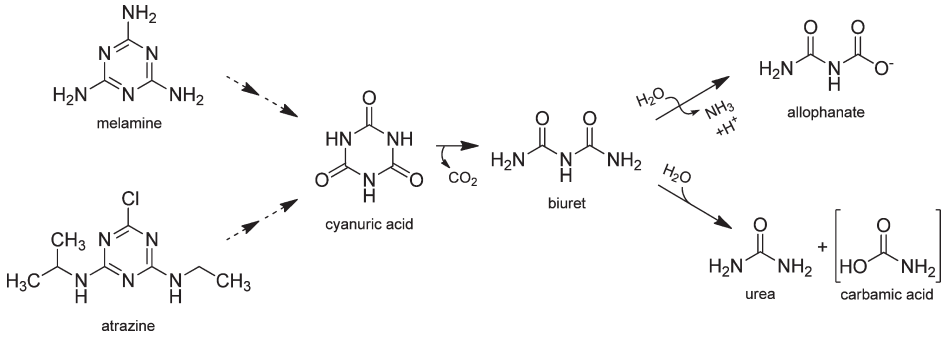
Scheme 1. Biuret Is an Intermediate in the Metabolism of *s*-Triazine Compounds and Could Be Metabolized to Allophanate or Urea



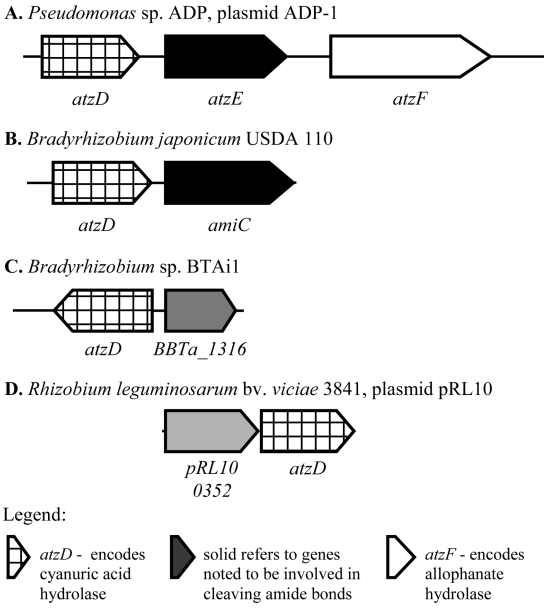


Figure 1. Alignments of gene regions encoding functional cyanuric acid hydrolases from various soil bacteria.

RESULTS AND DISCUSSION

Gene Identification, Cloning, and Expression. In *Pseudomonas* sp. ADP, the *atzE* gene encoding the biuret hydrolase protein is adjacent to *atzD*, encoding cyanuric acid hydrolase (Figure 1). Since bacterial enzymes that catalyze sequential reactions in pathways are often encoded by genes that are clustered together on the genomic DNA, bacterial genomic databases were searched for genes encoding putative amide-hydrolyzing proteins that were adjacent to ones encoding cyanuric acid hydrolases. Such a clustering was found among several members of the *Rhizobiaceae*. Those bacteria were tested and shown to grow on cyanuric acid or ammelide as sole sources of nitrogen (E. Reynolds, T. Dodge, J. Hall, unpublished results), indicating that ammonia might be released via a biuret hydrolase. Since the gene clustering within all of the microorganisms shown in Figure 1A−D differed, it was not possible to define gene function via bioinformatic approaches. In light of this, each gene was synthesized, inserted into an *E. coli* vector, transformed, expressed and the biuret hydrolase activity was measured.

The ammonia detection method showed traces of ammonia with the original *E. coli* strain. However, all recombinant *E. coli* clones showed substantial biuret hydrolase activity. The highest activity was obtained by using the gene derived from *Rhizobium leguminosarum* bv. *viciae* strain 3841 and this clone was selected for larger-scale protein expression, purification, and characterization.

Purification and General Characteristics. The gene sequences were synthesized with a His_6 tag in the pJexpress401 vector and expressed in *E. coli* DH5α. The crude protein extract from this strain was purified in a single step using a Ni-chromatography column and shown to be homogeneous via SDS-PAGE (Supporting Information Figure 2). Unlike previous biuret hydrolases, the present enzyme proved to be stable during purification and storage (>1 year at −80 °C). Enzyme activity was tested in an array of buffers; potassium phosphate proved to yield optimal catalytic rates. The enzyme was active over the range of pH 4.5−11, with optimum activity at pH 8.6. The addition of $ZnSO_4$ or $CoSO_4$ (50 μM and 1 mM) to enzyme assays gave only nominal increases in activity. No transition metal cofactors were detected with ICP-MS; a stoichiometry of <0.1 metal per subunit was observed for V, Cr, Mn, Fe, Co, Ni, Cu, and Zn.

The properties of biuret hydrolase were compared to previously identified biuret hydrolases (Table 1).[9,12] Other enzymes were not fully purified or stabilized and thus data are incomplete. However, the comparisons that can be made all indicated that the other enzymes differ significantly from the biuret hydrolase described here. The enzyme AtzE was only studied in crude cell lysates.[12] However, sequencing of the *atzE* gene indicated that AtzE is a member of the amidase signature protein superfamily.[12] The biuretase sequence[9] had not been obtained so its protein family affiliation could not be ascertained. The biuret hydrolase described here is a member of the cysteine hydrolase superfamily (CDD classification cl00220) and cysteine hydrolase family (CDD classification cd00431).[14]

Biuret hydrolase showed highest relatedness with proteins annotated as isochorismatases. The isochorimatase protein family forms a cluster within the cysteine hydrolase superfamily. Biuret hydrolase showed only 23−30% sequence identity to superfamily members for which X-ray structures are available. However, a putative catalytic triad of D−K−C could be observed in multiple sequence alignments and the region of greatest relatedness surrounded the putative active-site cysteine (Figure 2). The region shown shows 11 out of 19 residues completely conserved or

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Table 1. Summary of Characteristics for Biuret Hydrolases

entry	enzyme	calculated subunit MW (Da)[a]	calculated pI[a]	pH optimum	specific activity (μmol/min·mg)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	product	ref
1	biuret hydrolase[b]	27,121	5.7	8.5	8.9	4.0 ± 0.2	23 ± 4	1.7×10^5	allophanate	this study
2	AtzE	48,120	6.3						allophanate	12
3	biuretase			9.5	1.6		610		urea	9

[a] Calculated with Expasy pI/MW tool.[13] [b] Modified enzyme.

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  BH   ...165IILTGITTDVCVSTTMREA183...
3o90   ...146VILTGVLTDICVLHTAIDA164...
3EEF   ...113VVLIGLDADICVRHTAADA131...
1IM5   ...123VYICGVATEYCVRATALDA141...
           : : *: :: **  *  :*
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Figure 2. Amino acid sequence alignment in region of superfamily active site cysteine for biuret hydrolase (BH) with: *Streptococcus pneumoniae* nicotinamidase (3o90),[16] *Thermoplasma acidophylum* N-carbamoyl-sarcosine amidase Ta0454 (3EEF),[17] and *Pyrococcus horikoshi* pyrazinamidase (1IM5),[18] for which X-ray structures are known. An * indicates a completely conserved residue and a : indicates a highly conserved residue. The active site cysteine is highlighted with a box.

with strongly similar properties by virtue of a score greater than 0.5 in the Gonnet PAM 250 matrix.[15]

Enzyme inhibition studies were also consistent with assignment of biuret hydrolase to the cysteine hydrolase superfamily. Treatment of biuret hydrolase with the sulfhydryl-modifying reagents *N*-ethylmaleimide or iodoacetamide at neutral pH resulted in a significant loss of enzyme activity. For example, a 5 min preincubation of 14.8 μM biuret hydrolase with 500 μM *N*-ethylmaleimide resulted in greater than 90% loss of activity. Iodoacetmide was even more potent as an inhibitor. A 5 min preincubation of 50 μM iodoacetamide with 14.8 μM biuret hydrolase gave greater than 90% inhibition of ammonia release from 3 mM biuret. Controls indicated neither inhibitor interfered with the activity assay.

Reaction Products. The biuret hydrolase reaction released ammonia from biuret; reactions allowed to go to completion yielded 0.77 ± 0.01 mol of ammonia per mole of biuret. As shown in Scheme 1, ammonia could be liberated directly or via the intermediacy of carbamate, which would rapidly decompose to ammonia. These alternative routes could be differentiated by analyzing the second product of the biuret hydrolase reaction with the use of specific enzymes. Allophanate hydrolase or urease will only release ammonia from allophanate or urea, respectively. If allophanate was the product, then supplementation of the reaction mixture with allophanate hydrolase would release two additional moles of ammonia. Previously, allophanate hydrolase was shown to produce two moles of ammonia from one mole of allophanate.[19] If urea was the product, then no additional ammonia would be generated since allophanate hydrolase is not reactive with urea. In the experiments conducted here, incubation of biuret with biuret hydrolase and allophanate hydrolase yielded 2.49 ± 0.11 mol ammonia. In a parallel experiment, the addition of urease to reaction mixtures containing biuret hydrolase and biuret only increased the ammonia released by 5% compared to reactions without urease. Together, these data are consistent with allophanate being produced by biuret hydrolase.

Since the enzyme-based methods used here only provided indirect evidence for the product, ^{13}C NMR experiments were conducted using uniformly labeled [U−^{13}C]-biuret. ^{13}C-compounds used or detected in this study, and their chemical shifts, are provided in Table 2.

Incubation with biuret hydrolase yielded clearly discernible allophanate and bicarbonate with no urea observed (Figure 3A). Confirmation that the compound in Figure 3A was allophanate was obtained by allowing for its decomposition. Under the buffer conditions of the experiment, allophanate is known to decarboxylate to urea overnight and this was observed (Figure 3B). Confirmation that this product was urea was obtained by incubating the material in Figure 3B with urease and observing its rapid and stoichiometric transformation to bicarbonate (Figure 3C).

Biuret Hydrolase Reactivity. The finding that one mole of biuret was transformed to one mole each of allophanate and ammonia allowed the use of the Berthelot reaction for ammonia to follow the reaction course. The steady-state kinetic parameters determined here were a k_{cat} of 4.0 ± 0.2 s^{-1} and a K_M of 23 ± 4 μM with a k_{cat}/K_M of 1.7×10^5 M^{-1} s^{-1}. This is the first report of a k_{cat}/K_M for a biuret hydrolase and thus cannot be compared to other biuret hydrolases. However, these values are comparable to the values determined for cyanuric acid hydrolase ($k_{cat}/K_M = 1.2 \times 10^5$ M^{-1} s^{-1})[22] and allophanate hydrolase ($k_{cat}/K_M = 1.1 \times 10^4$ M^{-1} s^{-1})[19] purified from *Pseudomonas* sp. ADP. These similar values are expected because cyanuric acid hydrolase, biuret hydrolase and allophanate hydrolase catalyze consecutive reactions in a metabolic pathway.

To further ascertain that the biuret hydrolase purified here acts physiologically on biuret (Table 3, entry 1), a range of alternative compounds were tested for reactivity (Table 3). In all, 34 additional compounds were tested for reactivity with biuret hydrolase. Each bore some structural analogy with biuret, with most being amides and some thioamides. As shown in Table 3, only two of the 34 showed reactivity with biuret hydrolase (entries 2 and 3). These were both substituted urea compounds. *N*-formylurea and *N*-acetylurea showed approximately 3% and 1% of the specific activity of biuret, respectively. These data support the idea that biuret is the physiological substrate for the enzyme obtained here from *Rhizobium leguminosarum* bv. *viciae* strain 3841.

Biuret Hydrolase with Carboxybiuret. During the course of these studies, we became aware that cyanuric acid hydrolase does not produce biuret directly, but produces carboxybiuret (Richman and Cameron, Dodge and Cho, unpublished results),[23] which undergoes decarboxylation to yield biuret. The decarboxylation may be sufficiently rapid at neutral pH or below that the intermediacy of carboxybiuret was previously undetected.[21,22,24] In this context, we considered the possibility that biuret hydrolase might catalyze a physiologically relevant reaction with carboxybiuret. The biuret hydrolase might catalyze the hydrolysis of carboxybiuret to (A) biuret, (B) allophanic acid and carbamic acid, (C) urea and iminodicarboxylic acid, or (D) 1,3-dicarboxyurea (Scheme 2).

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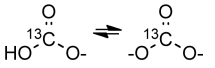
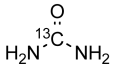
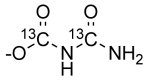
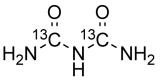
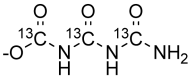
Table 2. Compounds Involved in ^{13}C Experiments

Compound	^{13}C Chemical Shift (ppm) (literature or standard)	^{13}C Chemical Shift (ppm) (observed)	Structure
Bicarbonate/ Carbonate	161.50[20] (pH 8.6)	160.35 (pH 7.7)	
Urea	162.82[a]	162.89	
Allophanate	159.36[a] 158.37	159.35 158.37	
Biuret	157.4[21]	157.49	
Carboxybiuret	—	157.13 156.80 155.00	

[a] Natural ^{13}C abundance used (this study); —, Not previously reported.

All of these reactions produce bicarbonate that shows a sharp signal via ^{13}C NMR. Scheme 2A generates bicarbonate directly.

Scheme 2B, C, and D produce unstable intermediates that yield bicarbonate in aqueous solution. In this context, we conducted a ^{13}C NMR experiment in which we monitored the disappearance of carboxybiuret and the concomitant appearance of bicarbonate in the absence or presence of biuret hydrolase. Uniformly labeled [^{13}C]-carboxybiuret was generated in situ from [U−^{13}C]-cyanuric acid using cyanuric acid hydrolase.

There was no difference in the time course of the disappearance of carboxybiuret without or with biuret hydrolase (Figure 4). Both followed first-order decomposition curves with R^2 values of 0.952 for Figure 4A and 0.950 for Figure 4B and had similar decay rates. The half-life was 12.7 min without biuret hydrolase and 12.5 min with biuret hydrolase. This suggested that carboxybiuret decarboxylation to biuret was likely nonenzymatic with a first order rate constant 18 min^{-1} under the conditions of the experiment. Thus, none of the hypothetical enzyme-catalyzed scenarios (Scheme 2A−D) occur. When the nonenzyme rate is compared to the first-order rate constant of biuret hydrolase with biuret, 240 min^{-1}, the enzyme rate is only 13-fold faster than the nonenzymatic decarboxylation reaction that feeds into it. The experiments with carboxybiuret were conducted here at pH 7.6, a pH close to the intracellular pH of many bacteria, which is typically near neutrality.[25] However, the pH may be lower inside bacteria. For example acid-tolerant *Rhizobium leguminosarum* strains were found to maintain intracellular pH values of 5.9−6.5[26] and under those conditions, the rate of nonenzymatic decarboxylation of carboxybiuret would be expected to be similar to the rate of enzymatic hydrolysis of biuret to allophanate. The decarboxylation of phenylallophanate, for example, was found to strongly increase with pH below pH 7.5.[27] As a positive control that the biuret hydrolase enzyme was reactive under the conditions used in these experiments, we observed the rapid formation of [U−^{13}C]-allophanate (data not shown).

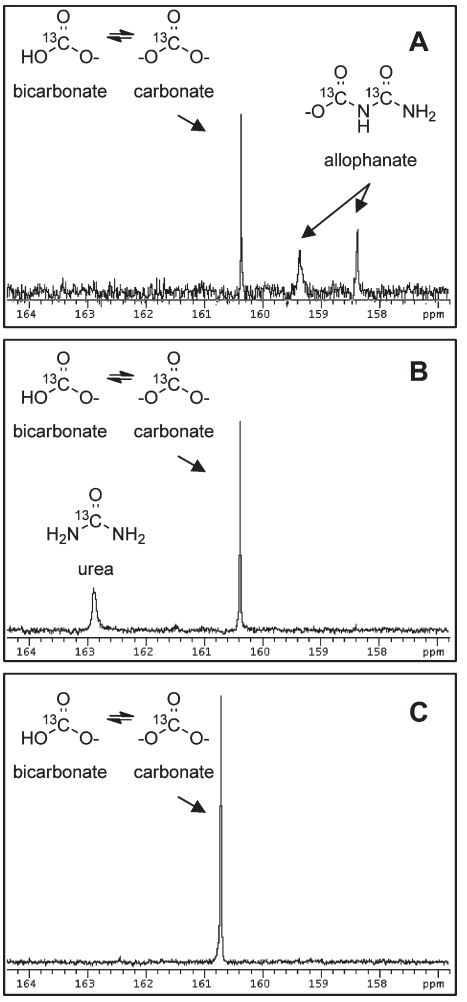
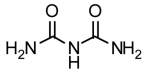
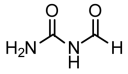
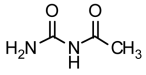
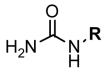
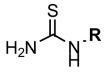
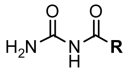
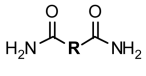
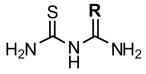
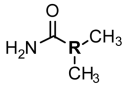
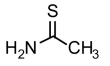
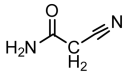
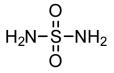
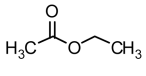


Figure 3. ^{13}C NMR of enzyme and nonenzyme formed products. (A) [U−^{13}C]-Biuret reacted with biuret hydrolase. Allophanate peaks were observed at 1.5 h. (B) Reaction mixture in (A) allowed to stand at 20 °C for 67.5 h. (C) Treatment of reaction mixture in (B) with urease. Urea peak undetectable after 1.25 h. NMR acquisition times vary.

In separate experiments that support the data above, 1-nitrobiuret, an isosteric and isoelectric analog of biuret carboxylate (the anion of carboxybiuret), was examined as a potential substrate or inhibitor. We found no evidence for release of ammonia or allophanate from 1-nitrobiuret in extended incubations. Moreover, we found no significant inhibition of biuret hydrolysis by 1-nitrobiuret at equimolar concentrations. These data are consistent with the idea that biuret hydrolase is not configured to bind and be reactive with carboxybiuret.

While the interposition of a nonenzymatic step into a metabolic pathway is not common, there are several precedents. A recent report describing a bacterial catabolic pathway for pyrimidines revealed two enzyme products that decay nonenzymatically to provide the ultimate substrate for the next enzyme in the pathway.[28] In the bacterial catabolic pathway for dihalomethanes, *S*-fluoromethyl glutathione was released as a reaction product by dichloromethane dehalogenase from methylotrophic bacteria when processing chlorofluoromethane.[29] The released product was observed to decay on the time scale of minutes to formaldehyde. Formaldehyde is a growth substrate for the bacteria. It is thought that dichloromethane reacts enzymatically to produce a

Table 3. Substrate Specificity of Biuret Hydrolase from *R. leguminosarum* bv. *viciae* 3841

Entry	Substrate		Specific Activity (μmol/min·mg)
1			6.08
2			0.17
3			0.04
4		R = H, CH_3, $CH{=}CH_2$, $CH_2C(O)CH_3$, CH_2CN, $C(O)NH_2$	<0.02
5		R = H, CH_3, CH_2CH_3, $CH_2CH{=}CH_2$, OH, NH_2	<0.02
6		R = H, CH_3, $C(O)CH_3$, NH_2	<0.02
7		R = O^-, OCH_2CH_3, $NH\text{-}NO_2$, NHOH	<0.02
8		R = CH_2, CH_2CH_2, $CH_2CH_2CH_2CH_2$, N=N	<0.02
9		R = S, NH	<0.02
10		R = N, CH	<0.02
11			<0.02
12			<0.02
13			<0.02
14			<0.02

similar *S*-halomethyl glutathione intermediate and the chlorinated intermediate decays much more rapidly than the *S*-fluoromethyl analog.[30]

The present study is the first characterization of a stable biuret hydrolase and it further identifies one of the protein superfamilies that contribute this enzymatic activity. Previous sequencing studies,[12] and the present work, indicate that biuret hydrolases derive from several distinct protein superfamilies. This contrasts to the cyanuric acid hydrolases for which all known examples derive from the same protein family.[24] In at least some cases, the cyanuric acid hydrolase and biuret hydrolase genes are contained on broad-host range plasmids[12] and thus there could be considerable mixing of genes via horizontal gene transfer events that contribute to the multiple origins of biuret hydrolase.

MATERIALS AND METHODS

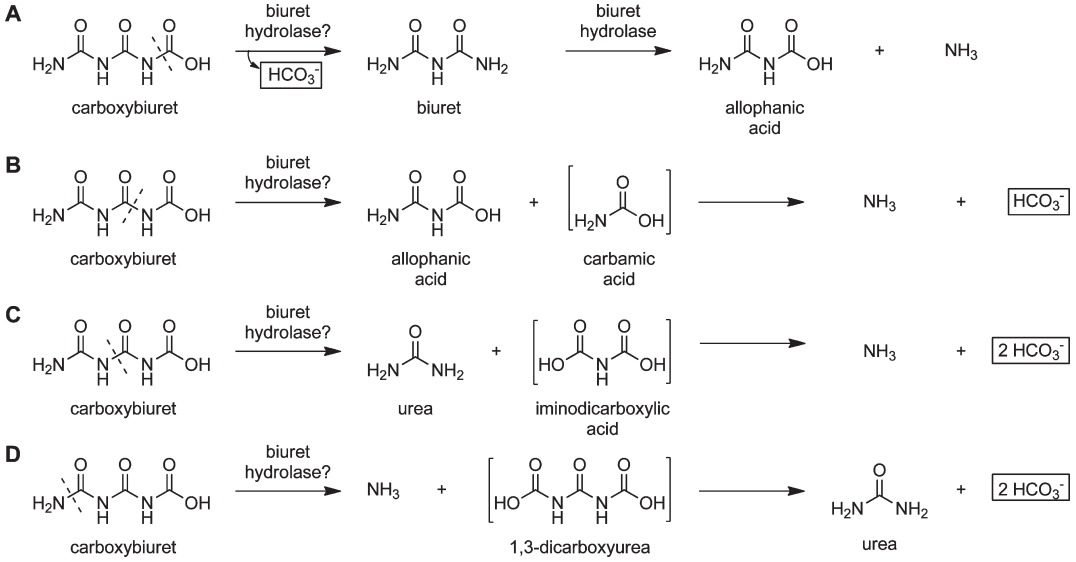
Chemicals and Reagents. The following chemicals and reagents were obtained from the sources indicated: Acros/Thermo Fisher Scientific (NJ) acetamide (99%), adipic dihydrazide (98%), 2-cyanoacetamide (99%), dicyandiamide (99.5%), 1,1-dimethylurea (98+%), ethyl allophanate (98%), *N*-formylurea (99%), isobutyramide (99%), malonamide (97%), oxamide (98%), semicarbazide hydrochloride (99+%), sulfamide (98%), thiosemicarbazide (99%); Alfa Aesar (Ward Hill, MA) acetylurea (98%), dithiobiuret (97%), thioacetamide (99+%); Cambridge Isotope Laboratories (Andover, MA) deuterium oxide (D99.9%); Fisher Scientific (Pittsburgh, PA) ethyl allophanate; Life Chemicals (Orange, CT) (2-chloroacetyl)urea (>90%); Mallinckrodt Baker, Inc. (Phillipsburg, NJ) sodium hydroxide, ferric chloride 6-hydrate; OmniSolv/EM Science (Gibbstown, NJ) formamide (99.8%); Sigma Aldrich Corp./Fluka (St. Louis, MO) acetoacetamide (97%), acetylthiourea (99%), acrylamide (99%), allophanic acid hydrazide, azodicarboxamide (97%), *N*-ethylurea (97%), hydroxyurea (98+%), 2-imino-4-thiobiuret (99%), jack bean urease (#94282), 1-methyl-2-thiourea (97%), methylurea (97%), 1-nitrobiuret, succinamide (98%), thiourea (99+%), 3-(trimethylsilyl)-1-propane-sulfonic acid sodium salt (97%), urea (99.5+%); Toronto Research Chemicals (North York, Ontario, Canada) [U−^{13}C]-cyanuric acid. Biuret (99%, Fluka) was recrystallized. Potassium allophanate was synthesized via hydrolysis of ethyl allophanate (Fisher Scientific, Pittsburgh, PA).[31]

Bioinformatics. The cyanuric acid hydrolase protein sequence (NP_862537.1) from *Pseudomonas* sp. ADP was used to query the NCBI nonredundant protein sequences database by using blastp alogrithm 2.2.25+.[32] Genes adjacent to blast hits were examined for annotations as amidases using the Conserved Domain Database,[14] the UniProt Knowledgebase,[33] and the EMBL-EBI InterPro Database.[34,35] Three biuret hydrolase candidates were selected for gene synthesis and subsequent experimental investigation: NP_773922.1 (*Bradyrhizobium japonicum* USDA 110), YP_001237459.1 (*Bradyrhizobium* sp. BTAi1), and YP_770628.1 (*Rhizobium leguminosarum* bv. *viciae* strain 3841, plasmid 10) (see Supporting Information Figure S1). Theoretical pIs and molecular rates for the translated candidate amino acid sequences were calculated using the pI/MW Tool (http://ca.expasy.org/tools/pi_tool.html).[13] Multiple sequence alignments were made using ClustalW2.[36]

Plasmid Construction. Three synthetic genes were designed based on protein sequences available in the NCBI database (see Supporting Information Figure S1). The 5′-end of each gene was constructed to include nine nucleotides containing a *Hin*dIII restriction site, a 15 nucleotide spacer sequence and a His_6 tag. In addition, the construct based on YP_001237459.1 encoded an additional 96 nucleotides on the 3′ end to begin at what we consider to be the correct start codon. During gene synthesis, DNA 2.0 (Menlo Park, CA) optimized the codons for expression in *E. coli*.

Transformation and Expression: All 3 Candidates. Plasmids containing the synthesized gene candidates (pJexpress401::bhBJ110_optEc, pJexpress401::bhBBTAi1_optEc, pJexpress::bhpRL10_optEc) were each transformed into Subcloning Efficiency *E. coli* DH5α Chemically Competent Cells (Invitrogen,

Scheme 2. Hypothetical Modes of C−N Bond Cleavage by Biuret Hydrolase Demonstrating All Lead to Formation of HCO_3^-



Carlsbad, California) per directions. Transformants were cultured on LB medium (BD-Difco, Franklin Lakes, NJ) containing kanamycin (50 μg/mL) and incubated 1−2 days at 37 °C. Cells from each plate were stored −80 °C in 50% glycerol.

Expression and Purification of bhpRL10_optEc. *E. coli* DH5α (pJexpress401::bhpRL10_optEc) was added to 25 mL LB broth containing kanamycin (50 μg/mL) and cultured at 37 °C, 275 rpm for 17.25 h to a final optical density of 3.5 at 600 nm. This culture was used for a 1% inoculum of cultures for enzyme production (five 2 L Erlenmeyer flasks with 400 mL LB with 50 μg/mL kanamycin each). These cultures were induced with 1 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG) when A_{600} = 0.48−0.59. After 4 h, the average A_{600} = 3.8. Cells were harvested by centrifugation at 4420*g* for 15 min at 4 °C, resuspended in 10 mL 0.1 M potassium phosphate buffer, pH 7.0 and transferred to a chilled centrifuge tube containing a crushed EDTA-free Complete Mini protease inhibitor cocktail tablet (#11836170001, Roche Diagnostics, Mannheim, Germany). Cells were disrupted by two passes through a chilled French pressure cell at 19 000 p.s.i., and centrifuged for 90 min at 19 000*g*. The supernatant (containing the soluble proteins) was filtered through a 0.45 μm HT Tuffryn membrane (Acrodisc 25 mm syringe filter #4184, Pall Corp., Port Washington, NY). The filtrate was loaded onto an Amersham Biosciences LCC 501 FPLC with a 5 mL Ni (II)-loaded HisTrap HP affinity column (GE Healthcare, Waukesha, WI) equilibrated with 0.1 M potassium phosphate buffer, pH 7.0 at 4 °C. The expressed protein eluted with 0.5 M imidazole in 0.1 M potassium phosphate buffer, pH 7.0. SDS-PAGE (18% (wt/vol) separating gel, 8% (wt/vol) stacking gel) and Simply Blue Safestain (Invitrogen, Carlsbad, California) were used to analyze the fractions. Fractions 49−55 were pooled. At 4 °C, a 50 mL pressure concentrator (Amicon #8050) equipped with a 10,000 molecular weight cutoff ultrafiltration membrane (Ultracel regenerated cellulose, YM-10, Millipore, Billerica, MA) and nitrogen flow was used to remove the imidazole via buffer exchange with 0.1 M potassium phosphate buffer, pH 7.0 (3 times) and to concentrate the protein. A prior attempt with dialysis to remove the imidazole resulted in the protein quickly precipitating. Aliquots were flash frozen in liquid nitrogen and stored at −80 °C.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Purified biuret hydrolase (0.24 mg) from *R. leguminosarum* bv. *viciae* strain 3841 was hydrolyzed in 6.75 mL 0.2 M nitric acid and samples were incubated in a 95 °C water bath for 2.5 h with inversion mixing every 10−15 min. The sample was diluted to 0.1 M nitric acid, the internal standard, ^{45}scandium, was added, and transition metal content was determined by ICP-MS at the University of Minnesota Department of Geology & Geophysics (Minneapolis, MN). All chemical analyses were performed on a Thermo Scientific XSeries2 ICP-MS fitted with a hexapole collision/reaction cell using EPA method 200.8. Elements were analyzed at standard mass resolution using helium/hydrogen collision reaction mode (CCT) with kinetic energy discrimination (KED). Sample introduction was via an ESI PC3 FAST system with sample loops with a dwell time of 15 ms with 30 sweeps and 5 replicates. The calibration curve was generated using 4 multielement standards purchased from SPEX Industries. Detection limits ranged from 2 to 100 ppt.

***N*-Hydroxybiuret Synthesis.** *N*-hydroxybiuret was synthesized by the method of Larsen[37] with minor modifications as suggested by Exner.[38] In brief, ethyl allophanate was used in water instead of methyl allophanate in methanol. Also, the reaction was allowed to stand at 50 °C for 6−8 h instead of ambient temperature for 14 days. Samples run on TLC plates reacted with 5% iron(III) chloride in methanol to generate the expected blue spots. R_f = 0.4 using a solvent of methanol/benzene/ethyl acetate in a ratio of 2:4:1 containing 1% formic acid.

Nuclear Magnetic Resonance (NMR) Standards and Product Confirmation. For the initial studies and the confirmation that allophanate was the product of biuret hydrolase with biuret (Figure 3), ^{13}C NMR spectra were acquired using a Varian Unity Inova 400 MHz NMR with VnmrJ 2.2D software and a Nalorac 4 Nuc probe in 0.1 M phosphate buffer, 10% deuterated water (D_2O), 1% dioxane, pH 7 to 7.7. Using 2.0 mg/mL cyanuric acid

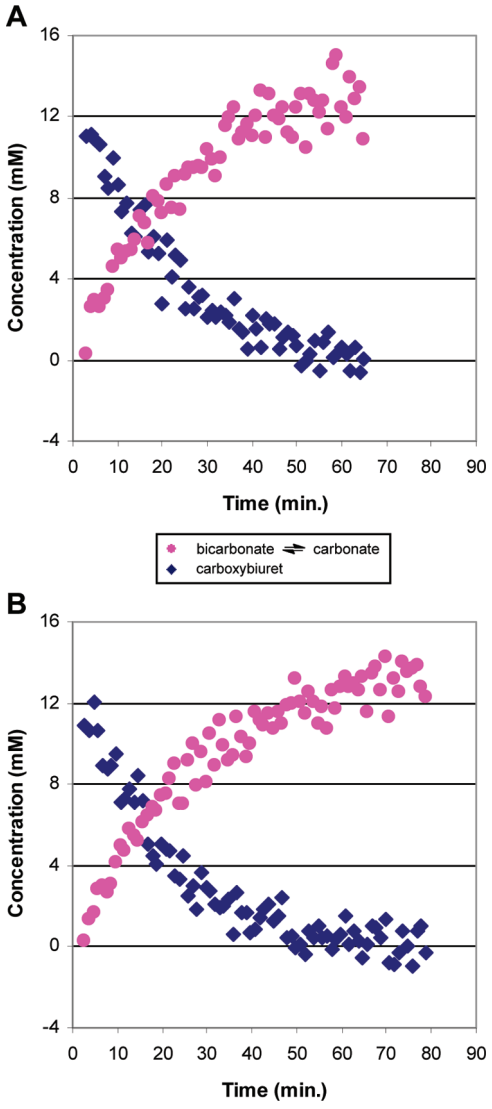


Figure 4. Disappearance of [U−^{13}C]-carboxybiuret and appearance of [U−^{13}C]-bicarbonate in the absence (A) or presence (B) of biuret hydrolase.

reduces the pH from 9.0 to 7.7. Tetramethylsilane (TMS) was used as an external standard (δ = 0) and dioxane as an internal standard (δ = 66.6). [U−^{13}C]-biuret was generated with 0.75 mL [U−^{13}C]-cyanuric acid (2.0 mg/mL) in 0.1 M potassium phosphate, 10% D_2O, 1% dioxane and 11 μg of cyanuric acid hydrolase.[24] Biuret hydrolase (3.1 μg) was added and allowed to react. After allophanate decomposed to urea, 125 μg urease was added to the reaction.

Enzymatic Assays, Substrate and Rate Determinations. One unit of biuret hydrolase activity was defined as the amount of enzyme converting 1 μmol biuret to 1 μmol ammonia in 1 min. One unit of allophanate hydrolase activity was defined as the amount of enzyme converting 1 μmol allophanate to 2 μmol ammonia in 1 min. Ammonia determination was performed via the Berthelot reaction as previously described.[39,40] Standard curves had a 1 μM to 1.2 mM linear range. Ammonia levels were also determined using an Ammonia Assay Kit (#AA0100, Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions.

Assay, Substrate Analogs, and Steady-State Kinetics. Assays contained 3 mM biuret in 0.1 M buffer; 1.92 μg/mL of biuret hydrolase was allowed to react with the biuret for 3 min at ambient temperature and then subjected to the Berthelot assay. The following buffers were tested: (0.1 M each, within 1 pH unit of a pK_a): succinate, 3-(*N*-morpholino)propanesulfonic acid, *N*-[tris-(hydroxymethyl)methyl]glycine, 2-(cyclohexylamino)ethanesulfonic acid, sodium carbonate, 2-(*N*-morpholino)ethanesulfonic acid, potassium phosphate, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid, borate, and 3-(cyclohexylamino)-1-propanesulfonic acid. Substrate analog assays were conducted in 0.1 M potassium phosphate buffer, pH 8.5, at ambient temperature. For substrate tests, 500 μM substrate was incubated with purified biuret hydrolase (1.92 μg/mL). Ammonia release was measured using the Berthelot assay (30 min incubation, reading at 630 nm). Control samples without enzyme were analyzed in parallel and no significant nonenzymatic ammonia release was observed. To preclude that any substrate generated false negatives from the Berthelot assay, 800 μM ammonium chloride was separately added to 500 μM substrate solutions, then assayed. No significant interference was detected. Kinetic parameters for biuret were calculated from initial hydrolysis rates at eleven different biuret concentrations ranging from 10 μM−300 μM. Triplicates for each biuret concentration with the same enzyme preparation were performed. Apparent K_m and v_{max} values were estimated by nonlinear regression according to the Michaelis−Menten equation, $v_0 = (V_{max} \times [S])/(K_m+[S])$, using GraphPad Prism software (version 5).

Inhibition by Sulfhydryl Modifying Reagents. Biuret hydrolase (14.8 μM) was incubated with either *N*-ethylmaleimide (NEM, 500 μM) or iodoacetamide (50 μM) in 0.1 M potassium phosphate buffer, pH 7.1 in the absence of biuret at ambient temperature. Ten microliter aliquots were taken between 0 and 5 min and immediately added to 990 μL of 3 mM biuret in 0.1 M potassium phosphate, pH 7.1 to initiate activity assays. Enzyme activity was detected using the Berthelot assay as previously described.

NMR-Determined Decarboxylation Kinetics. To follow the kinetics of carboxybiuret disappearance and bicarbonate appearance, the ^{1}H-decoupled ^{13}C NMR spectra for the reactions in Figure 4 were acquired with a Varian Inova 600 MHz NMR spectrometer using a 5 mm Auto-X Dual Broadband probe at 25 °C. In each NMR tube, the reaction mixture contained 14.4 mM [U−^{13}C]-cyanuric acid (prepared in 0.1 M potassium phosphate, 10% D_2O, 1% dioxane, pH 9.07) and 0.08 mg/mL cyanuric acid hydrolase[24] (purified in 0.1 mM potassium phosphate, pH 7). One tube also contained 0.024 mg/mL biuret hydrolase that was mixed with cyanuric acid hydrolase prior to the start of the reaction. The pH of each reaction mixture at time zero was pH 7.7. Following the initiation of each reaction by adding cyanuric acid hydrolase, array spectra were acquired each minute for approximately 70 min. Compound concentrations were determined by following the peak integrals at 155.0 ppm for carboxybiuret and 160.4 for bicarbonate/carbonate. Using the Varian VnmrJ 2.2D software, data were transformed and analyzed in the following manner: integrals were selected and the internal standard, dioxane, was set to 100 on the first spectra (66.6 ppm) of each array. Integrals were imported into Microsoft Office Excel 2002 SP3 and normalized to the dioxane value in the first spectra. Acquisition time was converted to enzyme reaction time. Data were imported into GraphPad Prism 5 software, plotted and analyzed using the nonlinear regression package.

■ ASSOCIATED CONTENT

Supporting Information. Data related to the biuret hydrolase gene candidates (nucleotide sequences for gene contructs, source strains and protein ID numbers, theoretical pI and molecular weight values); SDS-PAGE image of the purified biuret hydrolase from *Rhizobium leguminosarum* bv. *viciae* strain 3841. This material is available free of charge via the Internet at http://pubs.acs.org.

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