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Apoprotein isolation and activation, and vibrational structure of the *Helicobacter mustelae* iron urease

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Abstract

The micro-aerophilic pathogen *Helicobacter mustelae* synthesizes an oxygen-labile, iron-containing urease (UreA2B2) in addition to its standard nickel-containing enzyme (UreAB). An apoprotein form of the iron urease was prepared from *ureA2B2*-expressing recombinant *Escherichia coli* cells that were grown in minimal medium. Temperature-dependent circular dichroism measurements of holoprotein and apoprotein demonstrate an enhancement of thermal stability associated with the UreA2B2 metallocenter. In parallel to the situation reported for nickel activation of the standard urease apoprotein, incubation of UreA2B2 apoprotein with ferrous ions and bicarbonate generated urease activity in a portion of the nascent active sites. In addition, ferrous ions were shown to be capable of reductively activating the oxidized metallocenter. Resonance Raman spectra of the inactive, aerobically-purified UreA2B2 holoprotein exhibit vibrations at 495 cm⁻¹ and 784 cm⁻¹, consistent with ν_s and ν_{as} modes of an Fe(III)-O-Fe(III) center; these modes undergo downshifts upon binding of urea and were unaffected by changes in pH. The low-frequency mode also exhibits an isotopic shift from 497 to 476 cm⁻¹ upon ¹⁶O/¹⁸O bulk water isotope substitution. Expression of subunits of the conventional nickel-containing *Klebsiella aerogenes* urease in cells grown in rich medium without nickel resulted in iron incorporation into a portion of the protein. The inactive iron-loaded species exhibited a UV-visible spectrum similar to oxidized UreA2B2 and was capable of being reductively activated under anoxic conditions. Results from these studies more clearly define the formation and unique properties of the iron urease metallocenter.

Keywords

urease; nickel; metal-substituted; iron; dinuclear; oxygen sensitivity

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1. Introduction

A contribution on urease makes a fitting addition to this special issue of the *Journal of Inorganic Biochemistry* in honor of Professor Bert Vallee, for it was Dr. Vallee who carried out the initial metal ion analysis of jack bean urease in 1975 to establish it as the first nickel-containing enzyme (see acknowledgment in [1]). This notable achievement initiated an entirely new field of study: the characterization of nickel enzymes that now include such well-characterized examples as urease, [NiFe]-hydrogenase, carbon monoxide dehydrogenase, acetyl-coenzyme A synthase/decarbonylase, acireductone dioxygenase, methyl coenzyme M reductase, a unique subset of superoxide dismutases, and some forms of glyoxylase I [2, 3]. Although most well known for his work on zinc enzymes, Professor Vallee's paradigm-shifting analysis of urease illustrates his vastly broader impact to the field of metallobiochemistry.

Urease catalyzes a deceptively simple reaction; i.e., the hydrolysis of urea to form ammonia and carbamate which spontaneously decomposes into a second molecule of ammonia and carbonate. The enzyme is present in all plants, many fungi, some archaea, and a diversity of bacteria where it plays important roles in global nitrogen metabolism and virulence [4–9]. Jack bean urease was the first enzyme to be crystallized [10] as well as the premier nickel enzyme [1]. The structure of this plant urease [11] and bacterial representatives from *Klebsiella aerogenes* [12, 13], *Bacillus pasteurii* [14], and *Helicobacter pylori* [15] reveal identical dinuclear nickel metallocenters in various quaternary arrangements of the closely related subunit sequences [5, 6]. Each nickel is coordinated by two His side chains and terminal water molecules, one metal possesses an additional Asp ligand, and the two cations are bridged by a Lys-carbamate and a water molecule (Fig. 1). This dinuclear center is uniquely poised to hydrolyze urea, a highly stable molecule [4, 16]. The substrate carbonyl is thought to bind to and replace the terminal water on the five-coordinate metal, and one urea amide nitrogen may displace the terminal water from the six-coordinate site [14]. The bridging water attacks the urea carbon to form a tetrahedral intermediate that decomposes with release of ammonia. This chemistry is facilitated by several protein side chains, including a critical His residue on a loop that must open to allow substrate entry into the buried site and then close for catalysis with each turnover.

Metal content analyses of many ureases led to a consensus view that all ureases contain nickel; however, that notion came into question in 2008 from work involving *Helicobacter mustelae* [17]. This micro-aerophilic pathogen of ferrets synthesizes two distinct ureases: one involving the *ureABIEFGH* structural and accessory genes that are induced by nickel and a second encoded by the *ureA2B2* structural genes which are inversely regulated by this metal [17]. UreA2B2 subsequently was shown to be an oxygen-labile enzyme containing a dinuclear iron active site [18]. The 3.0 Å resolution structure of the inactive, oxidized form of UreA2B2 revealed a metallocenter that recapitulates all features of the metallocenter shown in Figure 1, except for possessing iron rather than nickel [18]. Such similarity is not entirely surprising given that UreA2 and UreB2 share 57.4% and 69.5% identity to their UreA and UreB counterparts in this microorganism. The oxidized UreA2B2 species exhibits an electronic spectrum resembling methemerythrin which contains μ -oxo bridged dinuclear ferric ions, and this spectrum was bleached as the protein was reactivated by reduction of the metallocenter under anaerobic conditions [18]. Whereas activation of nickel urease requires multiple accessory proteins, the iron urease is activated without any *ure*-encoded auxiliary proteins [17, 18]. Nevertheless, expression of *ureA2B2* in *Escherichia coli* yields enzyme with about half of its dinuclear active sites filled with iron, suggesting a partial deficiency in the activation process. The maximum specific activity observed for the UreA2B2 iron urease was less than 1% of that for typical nickel ureases; however, this activity is suggested to be sufficient for allowing *H. mustelae* to colonize the ferret stomach [17, 18].

Here, we present novel features of the iron urease. In particular, we describe the purification of an apoprotein form of UreA2B2, demonstrate the ability to activate this species with iron (but not nickel), examine the resonance Raman spectra of the unique metallocenter, and report the *in vivo* formation of a dinuclear iron site in *K. aerogenes* urease when only the structural genes are expressed in *E. coli* cells growing on rich medium.

2. Experimental procedures

2.1. Urease assays, protein analyses, and metal determination

Urease assays were carried out with the iron enzyme, UreA2B2, or the *K. aerogenes* protein, UreABC, in an anaerobic chamber with an atmosphere consisting of ~2.5% H₂ with N₂ balance (Coy Laboratory Products, Inc.) unless otherwise indicated. The standard urease assay buffer consisted of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.8, with 50 mM urea, except as noted. Aliquots were removed at selected time points after enzyme addition and activity was quantified by reacting the released ammonia with phenol to form indophenol which was monitored at 625 nm [19]. One unit of activity (U) is defined as the amount of enzyme necessary to degrade 1 μ mol of urea per min at 37 °C. Values shown are average $\pm$ standard deviation for three experiments.

Protein concentrations were determined by using the Bio-Rad Protein Assay with bovine serum albumin as the standard and the following molecular masses: *H. mustelae* UreA2B2, 86,949 Da, and *K. aerogenes* UreABC, 83,086 Da.

Metal analyses were carried out by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) at the University of Georgia Center for Applied Isotope Studies. An additional approach to measure iron content used the iron-specific chromophoric chelator 1,10-phenanthroline (Sigma). Samples (196 μ l, <100 μ M protein concentration, 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, containing 25 mM NaCl) were mixed with 2 μ l of 10% sodium dodecylsulfate (SDS) prepared in water and 2 μ l of 100 mM 1,10-phenanthroline dissolved in dimethyl sulfoxide. The mixtures were boiled for 5 min, sodium dithionite was added to 1 mM to reduce the oxidized iron, and the reaction incubated 5 min at ambient temperature before measuring the absorbance at 512 nm. The results were compared to a standard curve of ferrous sulfate, prepared by diluting a 10 mM ferrous sulfate stock solution containing 10 mM ascorbate. Results are presented as the average $\pm$ standard deviation for three replicates.

2.2. Purification of UreA2B2 apoprotein and UreA2B2 holoenzyme

The UreA2B2 apoprotein was generated in *E. coli* BL21-Gold (Stratagene) cells transformed with pEC015 [18] and grown in M9 minimal medium containing 100 μ g ml⁻¹ ampicillin and 0.4% mannitol. The culture was incubated at 37 °C with shaking to an optical density at 600 nm of 0.1–0.2, induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG, Calbiochem) and grown overnight. Cell pellets were resuspended in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA plus 1 mM β -mercaptoethanol and the slurry was sonicated (Branson Sonifier, 3–5 cycles of 2 min each, power level 1–4, 50% duty cycle with cooling in ice water/ethanol mixture). The disrupted cell material was centrifuged at 120,000 *g* for 1 h and the apoprotein was purified by DEAE-Sepharose, phenyl-Sepharose CL-4B, and Sephacryl S300-HR chromatographic methods in 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and 1 mM β -mercaptoethanol, as previously described for the holoenzyme [18]. Fractions were examined and pooled according to results from SDS-polyacrylamide gel electrophoresis (PAGE), using 12% running and 4% stacking gels [20], with the proteins visualized by Coomassie brilliant blue staining. Of note, the apoprotein was labile to freezing as judged by the loss of ability to activate the protein.

UreA2B2 holoprotein was purified from *E. coli* BL21-Gold [pEC015] cells grown in Lennox broth (LB, Fisher Scientific) containing 300 $\mu\text{g ml}^{-1}$ ampicillin, as reported earlier [18], except in some cases Superdex-200 (GE Healthcare) gel filtration matrix was used instead of Sephacryl S300-HR. The 320 and 380 nm extinction coefficients for the oxidized enzyme were obtained with a Shimadzu UV-2401PC spectrophotometer at room temperature by subtracting the spectrum of apoprotein from that of holoprotein, each at 100 μM heterodimer in HT buffer [50 mM HEPES buffer (pH 7.8) containing 1 mM tris(2-carboxyethyl)phosphine (TCEP)], and correcting for the amount of iron present (where holoprotein contained 1.0 equivalent and apoprotein contained about 0.2 equivalents). The effect of pH on the UreA2B2 spectrum was analyzed by comparing spectra of the protein (100 μM heterodimer) that was equilibrated in either 200 mM Tris-HCl, pH 7.4, or 200 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), pH 9.4. The influence of urea was monitored by comparing the spectrum of UreA2B2 (100 μM) in 28 mM Tris-HCl, pH 7.4, versus protein in the same buffer plus 20 mM urea.

2.3. Circular dichroism (CD) spectroscopy for measurement of thermal stability

CD spectra were collected on UreA2B2 holoprotein and apoprotein (10 μM heterodimer, 50 mM Na-phosphate, pH 7.4) by using a 1 mm Spectrosil quartz cuvette (Starna Cells) and a Chirascan CD Spectrometer (Applied Photophysics Limited; Leatherhead, U.K.) at 20 °C. Three scans were averaged by using Chirascan Pro-Data Viewer software. Thermal stability was examined by monitoring the changes in ellipticity at 220 nm as a function of temperature, which was controlled with a Melcor MTCA Series thermoelectric temperature controller (Laird Technologies) and a circulating water bath. Equipment parameters consisted of a 20.0–99.0 °C gradient with stepped ramping, 1 °C per step, with a 60 s equilibration time, and a 20 s read at each step subsequent to equilibration. Ellipticity profiles were fitted to a two-state model [21], using Igor Pro software (Wavemetrics).

2.4. Activation of UreA2B2 apoprotein

The *H. mustelae* UreA2B2 apoprotein was subjected to activation conditions analogous to those previously developed for the *K. aerogenes* urease apoprotein [22, 23], except that an anaerobic atmosphere was maintained during activation and assays. To test the effect of sodium bicarbonate on the *in vitro* activation process, UreA2B2 apoprotein (233 μM in HT buffer) was diluted to 10 μM in 100 mM HEPES, pH 8.3, containing 150 mM NaCl and 0, 10, or 100 mM NaHCO_3 . The solutions were incubated at ambient temperature for 1 h before adding FeSO_4 to 100 μM , incubated an additional 1 h at 37 °C, and aliquots of the mixtures were assayed for urease activity. Alternatively, UreA2B2 (9.9–12.6 μM in 100 mM HEPES, pH 8.3, containing 150 mM NaCl) was incubated with 100 μM FeSO_4 with or without 100 mM NaHCO_3 for 1 h at 37 °C prior to activity assays. The effects of Ni^{2+} and Zn^{2+} ions on activation were tested by incubating 10 μM UreA2B2 apoprotein with 100 mM NaHCO_3 in 100 mM HEPES, pH 8.3, containing 150 mM NaCl for 4 h at ambient temperature, adding 100 μM NiCl_2 or ZnSO_4 (diluted from stocks prepared in 1 mM HCl), and incubating at 37 °C for 1 h prior to urease assays.

UreA2B2 activation by ferrous ions was examined as a function of time and pH by incubating UreA2B2 apoprotein (9.7–17 μM) in 100 mM buffer [N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid (TAPS), pH 8.6, HEPES, pH 7.6, or 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.6 and 5.6; each containing 150 mM NaCl] with 100 μM FeSO_4 at 37 °C. Aliquots of the mixtures were assayed for urease activity at various time points. The concentration dependence of UreA2B2 activation by ferrous ions was examined by mixing UreA2B2 apoprotein (71.4 μM heterodimer) in 100 mM HEPES buffer, pH 8.3, containing 150 mM NaCl with varying stoichiometric concentrations of FeSO_4 (diluted from a 1 mM stock prepared in 1 mM HCl). After

incubating at 37 °C for 1 h, aliquots were assayed for urease activity. The addition of FeSO₄ led to a dilution of the buffered protein solution by no more than 22%.

UreA2B2 apoprotein activation by sodium dithionite was investigated by incubating apoprotein (10 μM final concentration) with 1 mM sodium dithionite in 100 mM HEPES, pH 8.3, containing 150 mM NaCl at 37 °C for 1 hr prior to assaying the mixtures for urease activity. For all urease assays, the lower detection limit was ~0.05 U (mg protein)⁻¹.

2.5. Resonance Raman spectroscopy

UreA2B2 was subjected to resonance Raman analysis with excitation using the 363.8 nm line (100 mW) of an Ar⁺ ion laser (model I-300, Coherent Inc., CA) or the 413.1 nm line (5 mW) of a Kr⁺ ion laser (model I-90, Coherent Inc., CA). Several different samples were analyzed for Raman scattering by using 363.8-nm excitation. To test the effect of pH and urea on the UreA2B2 spectrum, protein (~0.6 mM) was equilibrated in either 28 mM Tris-HCl (pH 7.4), containing 20 mM urea, 28 mM TAPS (pH 8.4), or 28 mM CHES (pH 9.4). Oxygen isotope substitution was carried out by diluting 60 μl of UreA2B2 (2.7 mM in 200 mM Tris-HCl, pH 7.4) with 100 μl of either H₂¹⁶O or H₂¹⁸O followed by concentration to ~60 μl by 10,000 Da molecular weight cut-off Microcon centrifugal filter devices (Millipore; pre-washed with deionized water), second dilution with 100 μl of the respective water isotope leading to a final concentration of ~1 mM protein in 28 mM Tris-HCl, pH 7.4. Isotope enrichment of ¹⁸O sample was ~80%. These samples were incubated on ice for 4 d prior to Raman measurement.

To facilitate isotopic exchange of metal ligands, UreA2B2 was subjected to sequential reduction and reoxidation (where indicated). The protein samples were degassed with at least 20 vacuum/argon purge cycles on a Schlenk line apparatus, reduced by addition of a degassed sodium dithionite solution (100 mM prepared in 200 mM Tris-HCl, pH 7.4) to a final concentration of 5 mM, and incubated at ambient temperature for at least 100 min. For experiments involving oxidation of reduced UreA2B2 in the presence of isotopic water, samples (~1 mM protein in 28 mM Tris-HCl, pH 7.4, in either H₂¹⁶O or ~80% enriched H₂¹⁸O) were exposed to air for 1 h prior to analysis. For experiments involving oxidation of reduced UreA2B2 in the presence of ¹⁶O₂ or ¹⁸O₂, reduced anaerobic samples (100 μl of 0.5 mM UreA2B2 in 37 mM Tris-HCl, pH 7.4) in ~3 ml vials were oxidized by injecting 1 ml of pure gas into the vessel and incubating at room temperature for 10 min prior to Raman measurement. For the 413.1-nm excitation studies, the protein (1.9 mM in 200 mM Tris-HCl, pH 7.4; 100 μl final sample volume) was diluted 4-fold with H₂¹⁶O or H₂¹⁸O (H₂¹⁸O obtained from Isotec, 99% enrichment).

Aliquots of samples (80–100 μl) were subjected to Raman spectroscopy by using a spinning cell. Scattered light was collected at right angle geometry and analyzed with a single polychromator (model Triax 550, Jobin Yvon, NJ) equipped with a liquid N₂-cooled CCD detector (model Spectrum One, Jobin Yvon, NJ). Holographic notch filters (Kaiser Optical Systems, MI) were used to reject Rayleigh scattering. Spectra were acquired in 30 s intervals over 10–40 min and subsequently integrated in post-processing over the time period where the spectra demonstrated minimal changes due to laser illumination. A 2400 ln/mm grating (spectral slit width 5.9 cm⁻¹) and 3600 ln/mm grating (spectral slit width 7.1 cm⁻¹) were used with 413.1 nm and 363.8 nm excitation, respectively.

2.6. Purification of iron-containing *K. aerogenes* urease

BL21-Gold(DE3) cells harboring pEC004 [24], containing *K. aerogenes ureABC* with no urease accessory genes, were grown in LB containing chloramphenicol at 50 μg ml⁻¹ with shaking at 37 °C to OD₆₀₀ ~0.5, adjusted to 0.5 mM IPTG, and grown under the same

conditions overnight. Cells were harvested, suspended in 20 mM Na-phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM β -mercaptoethanol (PEB), sonicated, and the lysate cleared by ultracentrifugation as described above. Soluble cell-free extracts were loaded onto DEAE-Sepharose (2.5 cm $\times$ 13 cm) equilibrated in PEB, unbound species were removed by washing with PEB, and a linear gradient from 0–1 M KCl over ~6 column volumes was applied. Fractions containing urease apoprotein were identified by SDS-PAGE, pooled, and dialyzed against PEB containing 1.5 M KCl. The resultant pool was filtered (0.22 micron) and applied to phenyl-Sepharose CL-4B resin (2.5 cm $\times$ 13 cm) equilibrated in PEB with 1.5 M KCl. The column was washed with PEB plus 1.5 M KCl to eliminate unbound species, and urease apoprotein was eluted by using PEB with no salt. Fractions containing urease apoprotein were concentrated with a 10,000 molecular weight cut-off (MWCO) Amicon centrifugal filter device (Millipore) to 1 ml before being loaded onto Superdex-200 resin (1.5 cm $\times$ 68 cm) equilibrated in 50 mM HEPES, pH 7.8, containing 1 mM TCEP. Fractions containing pure urease apoprotein were verified with SDS-PAGE, the protein was concentrated with a 10,000 MWCO Amicon, and aliquots were snap-frozen in liquid N₂ prior to storage at –80 °C.

The isolated protein was reddish-yellow, unlike the colorless preparations of apoprotein purified from cells expressing the entire urease operon and grown in the absence of nickel. The electronic spectrum of the iron-containing *K. aerogenes* urease (131 μ M in 50 mM HEPES, pH 7.8, containing 1 mM TCEP) was used to determine the extinction coefficients at 320 and 380 nm after subtracting the spectrum of apoprotein (obtained from pKAU22 Δ ureD-1 [25] at the same concentration) and correcting for iron content (where holoprotein contained 1.0 equivalents and apoprotein contained 0.3 equivalents).

2.7 Urease activity of recombinant *E. coli* cells

To determine whether *E. coli* BL21-Gold(DE3) cells harboring pEC004 were ureolytic, cells were grown overnight in LB containing chloramphenicol at 50 μ g ml^{–1} with shaking at 37 °C. The culture was diluted 1:100 into 25 ml of fresh LB medium containing the same concentration of antibiotic and either 0.5 mM of FeCl₃ dicitrate (diluted from a 500 mM FeCl₃ stock prepared in 1 M citrate) or NiCl₂, grown with shaking at 37 °C until the cells reached an OD₆₀₀ of ~0.4, adjusted to 0.1 mM IPTG, and continued incubating overnight. Subsequently, the cells were harvested by centrifugation, the supernatant was discarded, and the cell pellets were suspended in 0.5 ml 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl and directly assayed for urease activity. Specific activities were calculated using the protein concentration in soluble cell-free extracts prepared by briefly sonicating cell slurries and clearing the lysate for 20 min at 16,000 *g*.

2.8 Reductive activation of iron-containing *K. aerogenes* urease

The reductive activation competence of the iron-containing *K. aerogenes* urease was ascertained by degassing a protein solution (263 μ M heterotrimer in 50 mM HEPES, pH 7.8, containing 1 mM TCEP) and transferring into an anaerobic chamber. The protein (25 μ M final concentration) was mixed with sodium dithionite (2.5 mM final concentration diluted from a 100 mM stock solution prepared in degassed 20 mM Tris-HCl, pH 7.4, containing 300 mM NaCl), and 20 mM Tris-HCl, pH 7.4, containing 300 mM NaCl in 50 μ l total volume. The reaction was incubated for 80 min at ambient temperature before assaying an aliquot for urease activity under anoxic conditions.

3. Results and discussion

3.1. The UreA2B2 apoprotein and holoprotein possess the same secondary structures, and the iron metallocenter confers thermostability

The apoprotein form of UreA2B2 cannot be generated by methods used with the conventional nickel urease. For example, *K. aerogenes* urease apoprotein is produced when *E. coli* cells containing the complete heterologous operon are grown in the absence of nickel [26] or when recombinant cells lacking *ureD* are grown in LB [23, 27]. These approaches do not work for UreA2B2 because iron is required for many essential cellular proteins and the iron urease is synthesized without the aid of accessory proteins. Due to these complications we could only approach a true apoprotein form of UreA2B2.

H. mustelae UreA2B2 was isolated from recombinant *E. coli* cells grown in minimal medium and shown to contain only 0.22–0.24 equivalents each of iron and 0.00–0.18 equivalents of zinc per UreA2B2 unit by ICP-AES. For simplicity, we refer to this sample as apoprotein. By contrast, protein isolated from *E. coli* cells grown in LB, where iron is more abundant, contained ~1.0 equivalent of iron and less than 0.1 equivalent of zinc [18]. Although only half of the dinuclear sites were filled in this sample, we term this holoprotein. The apoprotein sample behaved like the metal-loaded enzyme during purification, consistent with their possessing similar quaternary structures as expected for changes associated with a deeply buried metallocenter.

The CD spectra of UreA2B2 apoprotein and holoprotein are very similar (Fig. 2A), and deconvolution by the K2D method on the Dichroweb server [28] led to a secondary structure prediction of 31% α -helix, 11% β -sheet, and 58% random coil for the apoprotein and 32% α -helix, 12% β -sheet, and 56% random coil for the holoprotein. These estimated percentages of secondary structural elements closely match the assignments in the structure [18]. In both cases, the negative ellipticity at 220 nm was lost when the sample temperature was increased (Fig. 2B), leading to the irreversible denaturation of the protein. Fitting of the data to a two-state model yielded a melting temperature of 345.7 ± 0.1 K for the apoprotein, and 349.8 ± 0.1 K for the holoprotein. Whereas the fit to the holoprotein data agreed very well to a two-state model, the apoprotein data showed a slight non-coincidence to this model at the inflection points and at the highest temperature, consistent with some heterogeneity of the sample. Notably, the melting temperature of the holoprotein represents a lower limit as this species contains ~1 equivalent of iron per heterodimer; i.e., if all of the iron is in the dinuclear state the preparation would contain a mixture of 50% holoprotein and 50% apoprotein. Thus, a solution containing 100% holoprotein would likely have a higher melting temperature. These results demonstrate both that the apoprotein and holoprotein have essentially identical secondary structures and that the buried metallocenter contributes to the thermostability of the protein. This finding is similar to that reported for the enzyme from *K. aerogenes* where differential scanning calorimetry revealed increased stability of the nickel-loaded urease compared to the apoprotein species [26].

3.2. UreA2B2 apoprotein can be activated with ferrous ions, but not with nickel ions

Prior investigations had demonstrated the ability to partially convert the *K. aerogenes* urease apoprotein into active enzyme by incubation with bicarbonate and nickel ions [22, 23]. Bicarbonate (optimal concentration of 100 mM) is in equilibrium with carbon dioxide, the component identified as being needed to form a Lys-carbamate which serves as a bridging ligand for the two metal ions. Apoprotein activation was maximized when using 100 μ M nickel ions, a buffer at pH 8.3, and with NaCl added to 150 mM. Furthermore, this slow activation process required 60 min of incubation at 37 °C. By using radio-labeled bicarbonate to monitor Lys-carbamate formation and ICP-AES to measure nickel content in

sample incubated with these optimal conditions, it was found that about half of the nascent active sites bound carbon dioxide while all sites were fully loaded with nickel. Nevertheless, the activated apoprotein [$\sim 400 \text{ U (mg protein)}^{-1}$] exhibited only $\sim 16\%$ of the activity observed with as-purified holoenzyme [$2,500 \text{ U (mg protein)}^{-1}$]. This low extent of activation was attributed to subtle, and still undefined, structural perturbations at the metallocenter. Substitution of nickel by zinc or copper ions failed to yield any activity. By contrast, slight activity [7 and $9 \text{ U (mg protein)}^{-1}$] was observed with manganese and cobalt (for the C319A variant protein), respectively [27]. Iron substitution for the native nickel ion in *K. aerogenes* urease yielded an oxygen-sensitive and very labile form of the protein exhibiting a maximum activity of $\sim 9 \text{ U (mg protein)}^{-1}$ [18].

H. mustelae UreA2B2 apoprotein was incubated anaerobically using several conditions to examine its activation properties. Surprisingly, when the protein was subjected to activation conditions lacking added bicarbonate, $2\text{--}4 \text{ U (mg protein)}^{-1}$ of urease activity was observed, depending on the experiment/enzyme preparation. Inclusion of bicarbonate during these activations led to no further enhancement of activity. In contrast, mixing apoprotein with high levels of bicarbonate for 1 h prior to the addition of ferrous ions and incubation for an additional 1 h yielded $\sim 2\text{-fold}$ increased specific activity for samples with bicarbonate (Figure 3A). This enhanced activity is proposed to result from the slow incorporation of bicarbonate into the apoprotein to form new metallocenters. We propose that the background level of activity formed in the absence of bicarbonate is due to a distinct process. On the basis of results showing that sodium dithionite reductively activated apoprotein to near this basal level, we attribute the activity generated without added bicarbonate to reductive activation of the small amount of pre-existing metallocenter in the near apoprotein (0.24 Fe per heterodimer for the sample shown). Significantly, nearly full reductive activation is generated in the presence of only two equivalents of added iron (Fig. 3B).

The ferrous ion-dependent activation of the UreA2B2 apoprotein in the absence of added bicarbonate was a slow process that required 60 min to achieve maximal activity (Fig. 3C) in TAPS buffer at pH 8.6. The loss in activity after this time might relate to the exquisite oxygen sensitivity of this enzyme. The rate of activation was greatly reduced when using HEPES buffer at pH 7.6, and activation was not detectable when using buffers at pH 6.6 or pH 5.6. In sum, these results are consistent with the UreA2B2 metallocenter having a reduction potential significantly greater than that of ferrous ions in these activation conditions. Whereas the aqueous ferrous/ferric reduction potential is 0.771 V , this value can shift dramatically for a cation that is complexed to buffer components or to protein (e.g., three His coordination environments for iron in designed metalloproteins resulted in reduction potentials ranging from 0.43 V to 0.01 V [29]) or for different ligand protonation states. While the reduction potential of the UreA2B2 metallocenter is unknown, the met/semi-met and semi-met/deoxy states of *Thermotoga zostericola* hemerythrin were measured as 0.11 V and 0.31 V , respectively [30]. The activity formed in the bicarbonate-containing incubation mixtures at pH 8.3 under standard activation conditions represents both partial reductive activation and true apoprotein activation. The maximal level of true apoprotein activation was estimated to be $\sim 2.8 \text{ U (mg protein)}^{-1}$ or 20% of the activity for enzyme directly isolated from the native host when using anaerobic conditions [18]. The incomplete activation of UreA2B2 closely coincides with the partial activation of *K. aerogenes* urease apoprotein [22, 23]. Significantly, no activity was formed when UreA2B2 was incubated with zinc or nickel ions. Given the close structural similarity between the *K. aerogenes* and *H. mustelae* active sites, the reason for the lack of activation by nickel is unclear.

3.3. Raman spectroscopy reveals oxygen vibrations in the dinuclear center

The UV-visible absorbance spectrum of UreA2B2 [18] resembles that of methemerythrin or metmyohemerythrin (Table 1), which contain a $\mu\text{-oxo}$ bridge between two Fe(III) atoms

[31–33]. The UreA2B2 absorbance spectrum did not change when shifting the pH from 7.4 to 9.4, or when 20 mM urea was present. A number of anionic ligands are known to perturb the absorption spectrum of methemerythrin [34], raising the question of whether similar perturbations would occur in UreA2B2. The addition of 50 mM azide or thiocyanate had no effect on the spectrum of oxidized UreA2B2 (100 μ M) (data not shown), indicating distinct metallocenter differences from methemerythrin. To further probe the unique iron urease metallocenter, the protein was examined by resonance Raman spectroscopy.

The resonance Raman spectrum of oxidized UreA2B2 holoprotein (pH 7.4) obtained with 363.8 nm excitation revealed several protein vibrations with the strongest band appearing at ~ 495 cm^{-1} and several prominent vibrations appearing at higher frequencies (Fig. 4). Raman spectra remained invariant between pH 7.4 and pH 9.4. Excitation at 413.1 nm revealed noticeably different spectra strongly dominated by a single vibration at 500 cm^{-1} (~ 4 – 5 cm^{-1} upshifted from the frequency observed with 383.8 nm excitation) and no clear vibrations observed at higher frequencies. The addition of urea to UreA2B2 upon excitation at 363.8 nm led to a downshift of the 495 cm^{-1} band to 486 cm^{-1} along with spectra changes around 784 cm^{-1} . The difference between the Raman spectra of UreA2B2 with and without urea revealed a downshift of two vibrations due to urea binding at 497/484 cm^{-1} (visible in the absolute spectra) and 790/763 cm^{-1} . The latter, weaker band is not seen in the absolute spectra due to overlap with modes at 784 cm^{-1} and 754 cm^{-1} , neither of which shows sensitivity to urea. Significantly urea-sensitive bands coincide well with the symmetric and asymmetric vibrational modes of Fe(III)-O-Fe(III) sites. For example, methemerythrin from *Phascolopsis gouldii* exhibits a vibration at 511 cm^{-1} [35], metmyohemerythrin from the same organism shows a vibration at 505 cm^{-1} , and a variant of the protein reveals a feature at 492 cm^{-1} [33]. Extensive comparisons of ν_s and ν_{as} versus Fe-O-Fe angles for structurally characterized proteins and model complexes [36, 37] suggest that the Fe-O-Fe angle in oxidized UreA2B2 is $\sim 130^\circ$. The low resolution of the UreA2B2 crystal precludes accurate estimation of the Fe-O-Fe angle, but for comparison the Ni-OH-Ni angle is 119° for *B. pasteurii* urease or 121° for *K. aerogenes* urease [14].

The 500 cm^{-1} vibration detected when exciting at 413.1 nm showed no evidence of rapid exchange with bulk water as indicated by the lack of sensitivity to $\text{H}_2^{16}\text{O}/\text{H}_2^{18}\text{O}$ isotope substitution (not shown). Using 363.8 cm^{-1} excitation, prolonged incubation of UreA2B2 in isotopically labeled water revealed the presence of an oxygen isotope-sensitive vibration at 497/476 cm^{-1} for $^{16}\text{O}/^{18}\text{O}$, respectively. An isotopic shift of 21 cm^{-1} is consistent with either the $\nu_{\text{Fe-O}}$ of terminal solvent bound to iron or the symmetrical stretching mode ν_s of a μ -oxo bridge. Another weak feature in the $\text{H}_2^{16}\text{O} - \text{H}_2^{18}\text{O}$ difference spectrum was detected at 528 cm^{-1} for ^{16}O and an apparent trough at 512 cm^{-1} , although the trough position may be significantly affected by overlap with the ^{16}O mode at 497 cm^{-1} . No isotopic shifts were detected for the second urea sensitive mode at 790 cm^{-1} , possibly due to its significant lower intensity. It was noticed that isotopic exchange did not cause a quantitative shift of the 495 cm^{-1} band in the absolute spectrum suggesting that either the yield of exchange is small or, more likely considering vibrational differences between 363.8-nm and 413.1-nm spectra and band shape of the 495 cm^{-1} mode, that the 495 cm^{-1} band consists of several individual vibrations with varying oxygen sensitivity or exchangeability. The UreA2B2 spectra can be compared to the high pH spectra of *P. gouldii* and *Thermotoga dyscritum* methemerythrins which contain an extra terminal hydroxide on one iron atom, providing hydroxomethemerythrin [35]. The hydroxide ligand causes a shift of the 511 cm^{-1} vibration to 506 cm^{-1} , while introducing a new vibration at 490 cm^{-1} attributed to coupled Fe(III)-OH stretching and O-H bending. This precedent points to a possible explanation for the multiple vibrations near 500 cm^{-1} in UreA2B2 as arising from vibrations of the μ -oxo and Fe(III)-OH components of its metallocenter. Another possibility that we cannot exclude is that some of the observed vibrations are associated with the Lys-carbamate bridging ligand. $\text{D}_2^{16}\text{O}/$

H₂¹⁶O substitution suggests that the 495 cm⁻¹ band is hydrogen isotope sensitive and that such sensitivity may be significantly higher than the corresponding ¹⁶O/¹⁸O sensitivity, although further studies are necessary for unambiguous resolution and assignment of the 495 cm⁻¹ band. Reduction and oxidation of UreA2B2 is expected to facilitate exchange of metal ligands with bulk solvent; however, such treatment of the sample with ¹⁶O₂ in H₂¹⁶O or H₂¹⁸O immediately prior to Raman measurement did not reveal any additional oxygen isotope-sensitive modes or significant enhancement of the mode(s) observed with long exchange without reduction. In contrast, the feature at 528 cm⁻¹ appears to have lost intensity. The difference spectrum between the oxidation products obtained with ¹⁶O₂ and ¹⁸O₂ (both in H₂¹⁶O, Fig. 4) indicated no oxygen isotope-sensitive bands, suggesting that either ligand exchange is fast (min) or neither of the vibrations observed with this excitation wavelength originate from the products of O₂ reduction.

3.4. A dinuclear iron metallocenter can form *in vivo* in *K. aerogenes* urease

E. coli whole cells expressing *K. aerogenes* ureABC (but lacking the accessory genes) and grown in LB supplemented with excess iron were non-ureolytic while cells grown in the presence of nickel demonstrated low levels of urease activity, ~0.1 U (mg protein)⁻¹. Urease purified from the cells grown in non-supplemented LB was reddish-yellow and its spectrum closely resembled that of oxidized UreA2B2 [18] (Table 1), in contrast to the colorless apoprotein formed when *E. coli* cells containing the entire *K. aerogenes* urease gene cluster are grown in minimal medium lacking nickel [26] or when *E. coli* cells containing *K. aerogenes* ureABCEFG (i.e., missing *ureD*) are grown in LB [22, 27]. The spectrum closely resembled that of oxidized UreA2B2 [18], and both of these spectra are similar to that of methemerythrin [32]; however, methemerythrin has distinct peaks at 320 and 380 nm, whereas the urease features were less defined. On the basis of the resonance Raman results, this distinction may arise from terminal solvent-Fe(III) electronic transitions superimposed on the Fe(III)-O-Fe(III) spectrum. The *K. aerogenes* urease possessed 1.025 ± 0.005 equivalents of iron per UreABC unit as measured by using 1,10-phenanthroline. The protein was inactive as purified, however anaerobic reduction with dithionite led to a specific activity of 8.1 ± 0.3 U (mg protein)⁻¹. For comparison, the same sample was subjected to the standard *in vitro* activation conditions and resulted in 356 ± 9 U (mg protein)⁻¹ of oxygen-stable nickel-dependent activity when assayed in standard buffer containing 0.5 mM EDTA [or ~16% of the fully active enzyme exhibiting 2,500 U (mg protein)⁻¹].

5. Conclusions

We have demonstrated that UreA2B2 apoprotein exhibits the same properties during its purification and possesses the same secondary structure as the holoprotein, except that the metal-containing sample has greater thermal stability. UreA2B2 apoprotein was slowly and partially activated by ferrous ions and bicarbonate, but not with nickel or zinc ions. Surprisingly, activation also was observed without added bicarbonate, consistent with reductive activation of the small amount of oxidized dinuclear iron site found in this protein by ferrous ions. The level of activity catalyzed by UreA2B2 is quite low, so an alternative reaction cannot be excluded; however, ureolytic activity by this enzyme is sufficient to allow *H. mustelae* to survive acid shock if urea is provided [17]. The oxidized metallocenter exhibited a resonance Raman spectrum consistent with ν_s and ν_{as} vibrations expected for a Fe(III)-O-Fe(III) metallocenter, and these modes were downshifted by added urea. Considering the inability of oxidized UreA2B2 to turn over substrate, this system provides a unique probe for monitoring the structural nature of urea binding at the urease metallocenter. The spectrum contained additional vibrations around 500 cm⁻¹ with variable susceptibility to exchange with bulk solvent, likely corresponding to several oxygenic metal ligands. Finally, we demonstrated the *in vivo* formation of a dinuclear iron center in *K. aerogenes* urease when recombinant cells containing the structural subunit genes, but lacking the set of

urease accessory genes, were grown in LB. This result suggests that iron can serve as the default metal in urease when the maturation proteins are not present, consistent with one of the roles for these auxiliary components being to prevent mis-incorporation of metal in the nickel enzyme.

In *H. mustelae*, the lack of UreA2B2-associated accessory proteins may be used as a mechanism to impart the desired metal specificity for this protein. The UreA2B2 proteins of gastric pathogens possess a cohort of conserved residues that are unique when compared to the sequences of their nickel-containing UreAB counterparts [18]. Many of these unique residues are on the outer surface of the dodecameric assembly, possibly rendering UreA2B2 incapable of interacting with the endogenous maturation proteins encoded by the UreAB urease cluster. Under these conditions, UreA2B2 would spontaneously acquire ferrous ions as the default metal, similar to what we observed for *K. aerogenes* urease when it is produced without accessory proteins. Whereas cells producing UreA2B2 are ureolytic [18], the *E. coli* cells producing Fe-containing *K. aerogenes* urease lacked urease activity. This exclusive aspect of UreA2B2 is consistent with the *H. mustelae* protein possessing the unique ability to utilize iron rather than nickel as the catalytic cofactor despite containing a highly conserved active site structure that is nearly identical to that of nickel ureases.

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Abbreviations

CD	Circular dichroism
CHES	2-(cyclohexylamino)ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ICP-AES	Inductively-coupled plasma-atomic emission spectroscopy
IPTG	Isopropyl β -D-1-thiogalactopyranoside
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MWCO	Molecular weight cut-off
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
TAPS	N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid
TCEP	Tris(2-carboxyethyl)phosphine
Tris	Tris(hydroxymethyl)aminomethane

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Fig. 1.

Urease active site. The dinuclear nickel active site is depicted for *K. aerogenes* urease (PDB access code 1FWJ), but essentially identical metallocenters are present in the enzymes from jack bean (PDB 3LA4), *B. pasteurii* (PDB 2UBP), and *H. pylori* (PDB 1E9Z). UreA2B2 from *H. mustelae* contains a dinuclear iron metallocenter with the same configuration of ligands (PDB 3QGA). The metal ions are shown as green spheres, water molecules as red spheres, metal ligand carbon atoms as grey, oxygen atoms as red, and nitrogen atoms as blue.

Fig. 2.

CD comparison of UreA2B2 apoprotein and holoprotein. (A) Raw CD spectra of UreA2B2 (10 μ M) apoprotein (dashed line) and holoprotein (solid line) at 20 °C. Data are normalized as ellipticity per residue and are representative of three scans with baseline subtracted. (B) Changes in ellipticity at 220 nm are shown for holoprotein (filled circles, right y-axis) and apoprotein (open circles, left y-axis) as a function of temperature. Solid lines represent the nonlinear fits of experimental data to a two-state model.

Fig. 3.

In vitro activation of UreA2B2 apoprotein. All incubations and assays were carried out under anaerobic conditions. (A) Bicarbonate dependence of UreA2B2 apoprotein activation. Apoprotein (10 μ M heterodimer) was incubated with 0, 10, or 100 mM bicarbonate for 1 h at ambient temperature, ferrous ions were added to 100 μ M, and the mixtures were incubated at 37 $^{\circ}$ C for an additional 1 h prior to urease assays. (B) Ferrous ion concentration dependence of activation. Varying concentrations of UreA2B2 were incubated for 1 h at 37 $^{\circ}$ C in 100 mM HEPES buffer, pH 8.3, containing 150 mM NaCl and the indicated number of equivalents of FeSO₄ prior to activity assays. (C) Time and pH dependence of urease activation. UreA2B2 apoprotein was incubated at 37 $^{\circ}$ C in 100 mM HEPES, pH 7.6 (9.7 μ M heterodimer; open circles), or 100 mM TAPS, pH 8.6 (17.0 μ M heterodimer; closed circles),

with both solutions also containing 100 μM FeSO_4 and 150 mM NaCl. Aliquots were removed at the indicated times and assayed for urease activity using standard conditions.

Fig. 4.

Resonance Raman spectra of oxidized UreA2B2. Top: Absolute and oxygen isotope difference spectra of UreA2B2 at pH 7.4. UreA2B2 was sequentially reduced and reoxidized with $^{16}\text{O}_2$ or $^{18}\text{O}_2$ in H_2^{16}O or H_2^{18}O , as indicated. $\text{H}_2^{16}\text{O} - \text{H}_2^{18}\text{O}$ isotope difference spectrum obtained without reoxidation for samples in the corresponding medium for four d at 4 °C also is shown. Isotope difference spectra ($\text{H}_2^{16}\text{O} - \text{H}_2^{18}\text{O}$ and $^{16}\text{O}_2 - ^{18}\text{O}_2$) were calculated by subtracting the corresponding absolute spectra, which cancels all vibrations that are not sensitive to the oxygen mass. Middle: Absolute spectra of UreA2B2 samples at pH 8.4, pH 9.4, and pH 7.4 (with added urea). The urea-induced difference spectrum of UreA2B2 is shown in comparison with the absolute spectrum of urea in buffer. Middle and top spectra were obtained with 363.8 nm excitation. Bottom: Absolute spectrum of oxidized UreA2B2 upon excitation at 413.1 nm. Asterisks indicate the positions of major plasma lines.

Table 1Absorption spectral properties of diferric μ -oxo core-containing proteins

Protein	Wavelength (nm)	Extinction coefficient ($M^{-1} cm^{-1}$)	Wavelength (nm)	Extinction coefficient ($M^{-1} cm^{-1}$)
<i>H. mustelae</i> UreA2B2	320	4129 ^a (8258) ^b	380	1994 ^a (3988) ^b
<i>K. aerogenes</i> UreABC	320	2856 ^a (5712) ^b	380	1589 ^a (3178) ^b
<i>Phascolopsis gouldii</i> myohemerythrin ^c	324	6600	366	6400
<i>Desulfovibrio vulgaris</i> (Hildenborough) DcrH hemerythrin domain ^d	322	6500	380	4700
<i>Methylococcus capsulatus</i> (Bath) hemerythrin ^e	327	4431	376	3715

^aCalculated from experimental data on the basis of protein concentration; extinction coefficients represent protein containing 1.0 equivalent of iron heterodimer⁻¹.

^bCalculated assuming full diferric center occupancy; i.e., 2.0 iron heterodimer⁻¹.

^cFrom [38].

^dFrom [39].

^eFrom [32].