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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 $^{16}O/^{18}O$ 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.

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

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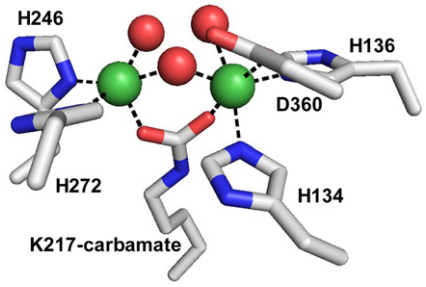

Fig. 1. Urease active site. The dinuclear nickel active site is depicted for *K. aerogenes* urease (PDB access code 1FWJ), but essentially identical metallocenters are present in the enzymes from jack bean (PDB 3LA4), *B. pasteurii* (PDB 2UBP), and *H. pylori* (PDB 1E9Z). UreA2B2 from *H. mustelae* contains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

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 Fig. 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% H_2 with N_2 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 μmol of urea per min at 37 °C. Values shown are average ± 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 μl, <100 μM protein concentration, 20 mM tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.4, containing 25 mM NaCl) were mixed with 2 μl of 10% sodium dodecylsulfate (SDS) prepared in water and 2 μl 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 ± 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 μg 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 μg 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.