Characterization of the *Klebsiella aerogenes* Urease Accessory Protein UreD in Fusion with the Maltose Binding Protein[▽]

Eric L. Carter¹ and Robert P. Hausinger^{1,2*}

Department of Microbiology and Molecular Genetics,¹ and Department of Biochemistry and Molecular Biology,² Michigan State University, East Lansing, Michigan 48824

Received 30 October 2009/Accepted 21 February 2010

Assembly of the *Klebsiella aerogenes* urease metallocenter requires four accessory proteins, UreD, UreE, UreF, and UreG, to effectively deliver and incorporate two Ni²⁺ ions into the nascent active site of the urease apoprotein (UreABC). Each accessory protein has been purified and characterized with the exception of UreD due to its insolubility when it is overproduced in recombinant cells. In this study, a translational fusion was made between the maltose binding protein (MBP) and UreD, with the resulting MBP-UreD found to be soluble in *Escherichia coli* cell extracts and able to complement a Δ ureD-urease cluster in this host microorganism. MBP-UreD was purified as a large multimer (>670 kDa) that bound approximately 2.5 Ni²⁺ ions (K_d of ~50 μ M, where K_d is the dissociation constant) per UreD protomer according to equilibrium dialysis measurements. Zn²⁺ directly competes with 10-fold higher affinity (~4 Zn²⁺ ions per protomer; K_d of 5 μ M) for the Ni²⁺ binding sites. MBP pulldown experiments demonstrated that the UreD domain of MBP-UreD formed *in vivo* complexes with UreF, UreG, UreF plus UreG, or UreABC when these proteins were overproduced in the same *E. coli* cells. In addition, a UreABC-(MBP-UreD)-UreFG complex was observed in cells producing all urease components. Comparative *in vitro* binding experiments with purified proteins demonstrated an approximate 1:1 binding ratio between the UreD domain of MBP-UreD and the UreF domain of the UreEF fusion, only weak or transient interaction between MBP-UreD and UreG, and no binding with UreABC. These studies are the first to describe the properties of purified UreD, and they extend our understanding of its binding partners both *in vitro* and in the cell.

A requirement for accessory proteins in metalloenzyme maturation is a common theme in microbiology, with examples including the auxiliary proteins critical for synthesis of FeMo cofactor in bacterial nitrogenase, the creation of [NiFe] and [FeFe] metallocusters in microbial hydrogenases, and the delivery and incorporation of Ni²⁺ into the dinuclear active site of urease (15, 20, 26). Urease catalyzes the hydrolysis of urea to two molecules of ammonia and carbonic acid, a reaction dependent on the Ni²⁺-containing active site of the holoenzyme (3). The accessory proteins involved in activation of urease have been the focus of intensive research as this enzyme can act as a virulence factor in infectious microorganisms including *Helicobacter pylori* (25) and uropathogenic *Proteus mirabilis* (9). The best studied urease maturation system is that of *Klebsiella aerogenes*, for which the urease apoprotein and holoprotein structures are known (10, 11), and several accessory protein components and their complexes with urease have been extensively characterized (3).

K. aerogenes urease, encoded by the *ureDABCEFG* gene cluster, uses four accessory proteins, UreD, UreE, UreF, and UreG, to form the metallocenter in the UreABC apoenzyme (16). UreE is proposed to function as a metallochaperone that delivers Ni²⁺ to the nascent active site (17). This protein has been structurally characterized (28), shown by equilibrium dialysis methods to bind about six Ni²⁺ ions per homodimer with

a K_d (dissociation constant) of $9.6 \pm 1.3 \mu$ M (17), and further characterized by isothermal titration calorimetry, mutagenesis, and spectroscopic approaches (4–6, 8). UreD and UreF are insoluble when overproduced alone, leading to difficulty in characterizing these proteins. Recently, a UreE-UreF translational fusion was constructed, resulting in a functional and soluble form of UreF (12). Significant levels of urease activation take place in cells expressing the entire urease cluster with the *ureEF* fusion replacing the individual genes. Pulldown assays using the UreE domain of UreEF revealed the *in vivo* presence of a UreABC-UreD(EF)G complex in cells expressing all urease components. Moreover, the UreEF fusion protein associates with UreABC-UreD, as shown by a similar *in vitro* pulldown experiment (12). UreG contains a conserved P-loop motif compatible with its role in GTP binding and hydrolysis during urease activation (18, 30). The protein is present as the free monomer, but UreG also associates with UreABC-UreDF to form the UreABC-UreDFG complex which interacts with UreE and allows for urease activation at wild-type levels in a GTP-dependent manner (29).

UreD, the focus of the work described here, is the least characterized of the urease accessory proteins. Nevertheless, some clues to its role during urease apoenzyme maturation have been revealed. Extracts of *Escherichia coli* cells containing a Δ ureD-urease cluster contain negligible amounts of urease activity (<1 U mg⁻¹) compared to extracts from cells containing the wild-type gene cluster (~200 U mg⁻¹) (16). A UreD-urease apoprotein complex is known (22), and urease within UreABC-UreD can be partially activated *in vitro* in the presence of Ni²⁺ and bicarbonate (needed to form a carboxy-Lys residue that bridges the dinuclear center) to a greater level than

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, 6193 Biomedical Physical Sciences, Michigan State University, East Lansing, MI 48824-4320. Phone: (517) 884-5404. Fax: (517) 355-8957. E-mail: hausinge@msu.edu.

[▽] Published ahead of print on 5 March 2010.

TABLE 1. Description of plasmids used in this study

Plasmid	Description	Reference
pKK17	Wild-type <i>K. aerogenes</i> urease cluster (<i>ureDABCEFG</i>) inserted into pKK223-3	5
pET-EF	Translationally fused <i>ureEF</i> genes inserted into pET21 for production of the UreEF fusion protein	12
pIBA3+G	SacII-PstI <i>ureG</i> fragment cloned into similarly digested pASK-IBA3 (IBA, Göttingen, Germany) resulting in an in-frame C-terminal fusion of <i>ureG</i> with the plasmid-encoded <i>Strep</i> tag	24
pACT3	pACYC184-based <i>tac</i> promoter expression vector	7
pEC002	BamHI-HindIII <i>ureD</i> fragment cloned into similarly digested pMal-c2x resulting in a C-terminal fusion to MBP	This study
pEC004	KpnI-XbaI <i>ureABC</i> fragment cloned into similarly digested pACT3	This study
pEC005	KpnI-XbaI <i>ureFG</i> fragment with an engineered ribosomal binding site (5'-AGGAGGT-3') cloned into similarly digested pACT3	This study
pEC006	<i>ΔureD K. aerogenes</i> urease cluster (<i>ureABCEFG</i>) cloned into pACT3	This study
pEC007	KpnI-XbaI <i>ureE</i> fragment cloned into similarly digested pACT3	This study
pEC008	KpnI-XbaI <i>ureF</i> fragment cloned into similarly digested pACT3 containing the same engineered ribosomal binding site as the <i>ureFG</i> fragment in pEC005	This study
pEC009	KpnI-XbaI <i>ureG</i> fragment cloned into similarly digested pACT3	This study
pEC013	EcoRI-SbfI <i>malE-ureD</i> fragment cloned into similarly digested pKK17	This study
pMal-c2x	Commercially available expression vector for construction and production of MBP fusions; encodes MBP-LacZα.	New England Biolabs
pMBP2*	pMal-c2x with a stop codon inserted in the Factor-Xa cleavage site after the C-terminal Arg residue; used for overproduction of MBP	This study

UreABC alone (23). UreABC-UreDF and UreABC-UreDFG complexes also have been identified and found to have enhanced activation properties compared to the UreD-urease apoprotein complex (19, 30). In addition, UreD is found in an insoluble UreDFG complex that was hypothesized to function as a unit during urease activation (18).

Despite our knowledge of several UreD-containing urease complexes, the role of this protein during *in vivo* urease maturation remains unknown. The major bottleneck in carrying out a thorough biochemical analysis of the protein is the insolubility of UreD when *ureD* is overexpressed alone. To circumvent this problem, a translational fusion was constructed between UreD and the maltose binding protein (MBP). The linked species provides a functional and soluble form of UreD that provides us with the first analysis of this critical urease accessory protein.

MATERIALS AND METHODS

Plasmid construction. Molecular biology techniques were performed by using standard protocols (27), with primers obtained from Integrated DNA Technologies (Coralville, IA). All plasmids associated with this study are outlined in Table 1, and their correct construction was confirmed by sequence analysis. *E. coli* DH5α (Invitrogen) was used as the host for all cloning procedures described below.

For purification and pulldown studies of MBP-UreD, cells containing pEC002 were used for protein overproduction. This plasmid was constructed by PCR amplification of *ureD* using pKK17 (5) as a template, a forward primer (5'-TATAGGATCCGTGTTACCACCACTCAAAAAGGCTG-3') with an engineered 5' BamHI restriction site (shown in italics), and a reverse primer with a 5' HindIII restriction site (5'-TATAAGCTTTTAAGTCAGCCAGATTCTGGGGAAG-3'; restriction site is shown in italics). After amplification, the isolated BamHI-HindIII *ureD* fragment was digested and ligated into the similarly digested pMal-c2x vector (New England Biolabs). The resulting pEC002 encoded an in-frame translational fusion of *ureD* with *malE*, the open reading frame of MBP. A modified pMal-c2x vector also was used for overproduction of the UreD-free MBP by inserting a stop codon (using forward primer 5'-CGGGATCGAGGGAAGGTGATCAGAATTCTGGATC-3' and reverse primer 5'-GGATCCGAATTCTGATCACCTTCCCTCGATCCC-3'; mutations are in bold) after the bases encoding the C-terminal Arg residue. This plasmid is denoted pMBP2*.

For one-step purification of UreG, we used a C-terminal *Strep* tag fusion that

was constructed by inserting the wild-type *ureG* into SacII-PstI-digested pASK-IBA3plus (IBA GmbH, Göttingen, Germany) to produce pIBA3+G (24).

To test the ability of the *malE-ureD* fusion to complement a *ΔureD*-urease cluster, pEC006 and pEC013 were constructed. pEC006 consists of *ureABCEFG* inserted into the *tac* promoter-based expression vector pACT3 (7). Briefly, *ureABC* was PCR amplified by using pKK17 as a template, a forward primer with a 5' KpnI restriction site (5'-GATCGGGTACCTAAGAGAACGTTATGGAACTGACCC-3'), and a reverse primer with a 5' XbaI restriction site (5'-GATCGTCTAGATTAACAGAAAATATCGTTGCGC-3'; restriction sites are in italics). The isolated KpnI-XbaI *ureABC* fragment was digested and ligated into similarly digested pACT3, resulting in pEC004. Utilizing a unique BamHI restriction site within *ureC*, pKK17 was digested with BamHI and HindIII, resulting in a fragment containing the 3' end segment of *ureC* with full-length *ureE*, *ureF*, and *ureG*. This fragment was ligated into similarly digested pEC004, resulting in pEC006. pEC013 consists of pKK17 with *malE-ureD* replacing wild-type *ureD* within the urease cluster. *malE-ureD* was PCR amplified by using pEC002 as a template, a forward primer with a 5' EcoRI restriction site (5'-GATCGGAATTCATGAAAATCGAAGAAGGTAACTGGTAA-3'; restriction site is in italics), and a reverse primer identical to the one used for construction of pEC002. The isolated *malE-ureD* fragment was partially digested with EcoRI and SbfI (*ureD* contains a unique SbfI restriction site), and a resulting ~1.7-kb band was isolated via gel extraction and ligated to pKK17 digested with the same enzymes, resulting in pEC013.

Plasmids pEC005, pEC007, pEC008, and pEC009 were constructed to carry out coexpression studies involving *ureE*, *ureF*, and *ureG*. Plasmid pEC005 consists of the *ureFG* genes PCR amplified by using pKK17 as a template, a forward primer (5'-GATCGGGTACCAGGAGGTCTAGCATGTCGACAGCGGAACACG-3') with both a 5' KpnI restriction site and an engineered ribosomal binding site (underlined), and a reverse primer with a 5' XbaI restriction site (5'-GATCGTCTAGACTATTGCCAAGCATGCCTTTGTCT-3'; restriction sites are in italics). The isolated KpnI-XbaI *ureFG* fragment was digested and ligated into similarly digested pACT3. Plasmid pEC008 contains only *ureF* that was PCR amplified from the pKK17 template by using the same forward primer utilized for *ureFG* amplification and a reverse primer (5'-GATCGTCTAGACTAGGAACGGAATAATCGAGAGTA-3') specific for *ureF* with a 5' XbaI restriction site (in italics). This KpnI-XbaI *ureF* fragment was ligated into similarly digested pACT3. Finally, pEC007 and pEC009 containing *ureE* and *ureG*, respectively, were constructed in the same manner as pEC008; however, gene-specific primers for *ureE* and *ureG* amplification used forward primers (5'-GATCGGGTACCAGGAGAGCGGATGCTTTATTTAAAC-3' and 5'-GATCGGGTACCAGGAGAAGCCATGAACCTTATAA-3', respectively) with a 5' KpnI restriction site (in italics) and reverse primers (5'-GATCGTCTAGACTAGTGCTGTGAGCGTGGTGGTC-3' and the same as used for pEC005) with a 5' XbaI restriction site (in italics). The forward primers used in these reactions did not contain the engineered ribosomal binding site described for pEC008.

Protein purification. MBP-UreD was purified from *E. coli* BL21-Gold(DE3) cells (Stratagene) transformed with pEC002 and grown with shaking in lysogeny broth (LB) supplemented with 300 $\mu\text{g ml}^{-1}$ ampicillin at 30°C until the cells reached an optical density at 600 nm (OD_{600}) of ~ 0.5 . The cultures were induced with 0.5 mM isopropyl β -D-thiogalactopyranoside (IPTG; Calbiochem), incubated at 25°C for 12 to 16 h, and harvested by centrifugation. Cell pellets were resuspended in 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM β -mercaptoethanol (TEB) buffer with 500 mM NaCl and disrupted by sonication (Branson Sonifier; 5 cycles of 2 min each; power level 4; 50% duty cycle with cooling in an ice water-ethanol mixture). Cell extracts were obtained by centrifugation at $100,000 \times g$ for 1 h at 4°C. Extracts were diluted at least 1:1 with TEB buffer containing 500 mM NaCl before being loaded onto amylose resin (New England Biolabs) that had been preequilibrated with the same buffer. The column (30-ml bed volume) was washed with the same buffer until the A_{280} reached baseline, at which point the bound proteins were eluted with TEB buffer containing 500 mM NaCl plus 10 mM maltose. The eluted fractions were collected, pooled, and dialyzed against TEB buffer containing 15 mM NaCl overnight. Subsequent to dialysis, the MBP-UreD solution was loaded onto a Q-Sepharose column (2.5 cm by 14 cm; GE Healthcare) preequilibrated with TEB buffer containing 20 mM NaCl. The column was washed with at least 2.5 bed volumes of buffer before a linear gradient was applied from 20 mM NaCl to 1 M NaCl in TEB buffer. Fractions containing MBP-UreD were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before pooling. Depending on downstream applications, the purified MBP-UreD was concentrated by using a 10,000-molecular-weight-cutoff Amicon Ultra centrifugal filter device (Millipore) and dialyzed against either TEB buffer with 25 mM NaCl for pulldown experiments or with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl for equilibrium dialysis experiments.

Purified MBP (unlinked to UreD) was obtained by three methods: (i) direct purchase from New England Biolabs, (ii) factor Xa (New England Biolabs) proteolytic digest of MBP-UreD with a subsequent purification step using DEAE-Sepharose according to the manufacturer's instructions (GE Healthcare), and (iii) amylose resin purification of MBP overproduced in *E. coli* BL21-Gold(DE3) harboring pMBP2*. The MBP is identical in sequence regardless of which source was used.

The UreEF fusion protein was initially purified from *E. coli* C41(DE3)(pET-EF) by using Ni^{2+} -Sepharose High Performance medium (GE Healthcare) as previously described (12). Purified UreEF was dialyzed against TEB buffer containing 25 mM NaCl overnight and subjected to gel filtration chromatography by using a 1.5-cm by 69-cm Superdex 75 column (GE Healthcare) preequilibrated with TEB buffer plus 25 mM NaCl. The eluted fractions were analyzed by SDS-PAGE, and those containing pure UreEF were pooled.

The C-terminal *Strep*-tagged UreG protein (UreG_{Str}) was purified from *E. coli* DH5 α transformed with pIBA3+G, grown with shaking in LB supplemented with 300 $\mu\text{g ml}^{-1}$ ampicillin at 37°C until the cells reached an OD_{600} of ~ 0.5 , induced with 200 ng ml^{-1} anhydrotetracycline, and incubated overnight before harvesting. Cell pellets were resuspended in 100 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA and then disrupted with sonication. Soluble cell extracts were obtained by centrifugation at $100,000 \times g$ for 1 h at 4°C. Purification of UreG_{Str} followed the manufacturer's instructions (IBA GmbH, Germany).

UreE was purified from *E. coli* BL21-Gold(DE3) cells transformed with pEC007, grown with shaking in LB supplemented with 50 $\mu\text{g ml}^{-1}$ chloramphenicol at 37°C until the cells reached an OD_{600} of ~ 0.5 , induced with 0.5 mM IPTG, and incubated overnight until the cells were harvested. The UreE purification procedure was the same as that used for the purification of the UreE H144* variant, as previously described (2).

Both holoprotein and apoprotein forms of urease were purified from *E. coli* BL21-Gold(DE3) cells transformed with pKK17. For purification of holoprotein, cells were grown with shaking in LB supplemented with 300 $\mu\text{g ml}^{-1}$ ampicillin and 1 mM NiCl_2 at 37°C until the cells reached an OD_{600} of ~ 0.5 , at which point the culture was induced with 0.1 mM IPTG and allowed to incubate overnight until the cells were harvested. Cell pellets were resuspended in PEB buffer (20 mM Na-phosphate buffer, pH 7.4, 1 mM EDTA, 1 mM β -mercaptoethanol) and disrupted by sonication. Soluble cell extracts were obtained by centrifugation at $100,000 \times g$ for 1 h at 4°C and applied to a 2.5-cm by 10-cm DEAE-Sepharose column preequilibrated with PEB buffer. A linear gradient from 0 M to 1 M KCl in PEB buffer was applied, and fractions containing urease were pooled, adjusted to 1.5 M KCl, and applied to a phenyl-Sepharose (GE Healthcare) column (2.5 cm by 9 cm) preequilibrated with PEB buffer containing 1.5 M KCl. The column was washed with equilibration buffer, and bound proteins were step-eluted with PEB buffer containing no KCl. Fractions containing pure urease were pooled and dialyzed against TEB buffer containing 25 mM NaCl. Urease apoprotein was purified in an identical manner as the holoprotein;

however, this protein was purified from cultures grown without the addition of NiCl_2 to the medium. Prior studies have demonstrated that urease activity is nearly undetectable under these conditions, presumably because the metal-binding components in the medium prevent uptake and incorporation of the metal (21).

Gel filtration analysis of MBP-UreD. To assess the native size of MBP-UreD, a sample was chromatographed on a column of Sephacryl S300-HR (1.5 by 65 cm; GE Healthcare) equilibrated in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl while the absorbance at 280 nm was monitored. Gel filtration standards (Bio-Rad) were used for calibration of the column. The effects of metal ions on the elution position of MBP-UreD were examined by repeating the analyses after equilibration of the column in 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl and a 100 μM concentration of either NiCl_2 or ZnCl_2 .

Analysis for metal content. The metal content of purified MBP-UreD was assessed by inductively coupled plasma atomic emission spectroscopy (Chemical Analysis Laboratory at the University of Georgia, Athens, GA). Control buffer samples were analyzed for comparison.

Equilibrium dialysis. One method for assessing the metal-binding properties of MBP-UreD made use of the metallochromogenic indicator 4-(2-pyridylazo)resorcinol (PAR; Sigma). Purified MBP-UreD (9.6 μM for Ni^{2+} experiments; 7.1 μM for Zn^{2+} experiments) and MBP (19.6 μM) were extensively dialyzed against 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl prior to equilibrium dialysis. Protein samples (300 μl) at the designated concentrations were dialyzed against the same buffer containing various concentrations of NiCl_2 or ZnCl_2 (500 μl) by using a Rapid Equilibrium Dialysis device (Pierce Biotechnology, Rockford, IL). The device was shaken at 200 or 300 rpm for 4 h at 37°C, and then 200- μl aliquots from each side of the membrane were assayed for $[\text{Ni}^{2+}]$ or $[\text{Zn}^{2+}]$ by mixing with 800 μl of PAR solution (100 μM in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl; prepared from 5 mM PAR aqueous stock solution titrated to pH 8.0 with 1 M KOH). The concentration of the PAR-metal complex was quantified by measuring the absorption at 500 nm for the aliquot from the protein-containing chamber, subtracting that for the protein-free aliquot, and comparing the result to standard curves that were developed by using Ni and Zn atomic absorption standards (Sigma). The data were analyzed directly or corrected for the metal ions bound to MBP and fit to the following equation for a single type of binding site (Sigma Plot, Systat Software, Inc.), where Y is the number of moles of metal ion bound per mole of protomer, B_{max} is the maximal number of moles of metal ion bound per mole of protomer, $[M]$ is the concentration of free metal ion, and K_d is the dissociation constant: $Y = (B_{\text{max}}[M]) / (K_d + [M])$.

A second approach for assessing Ni^{2+} binding to MBP-UreD and MBP as well as for examining the competition between Ni^{2+} and Zn^{2+} for the former protein made use of radiolabeled $^{63}\text{NiCl}_2$ (1,445 mCi/mmol; Du Pont NEN Research Products Inc., Wilmington, DE). MBP-UreD and MBP (approximately 10 μM and 30 μM , respectively) were dialyzed against various concentrations of $^{63}\text{NiCl}_2$ and unlabeled ZnCl_2 using the Rapid Equilibrium Dialysis device as described above. After shaking at 300 rpm for 4 h at 37°C, 200- μl samples were mixed with 10 ml of Safety-Solve scintillation fluid (Research Products International Corp.) and analyzed with a Beckman-Coulter LS6500 liquid scintillation counter. The data were fit as above (where $[M]$ is the concentration of free Ni^{2+}) or by using the competitive inhibition equation (where K_i is the inhibition constant of Zn^{2+}): $Y = (B_{\text{max}}[\text{Ni}]) / \{K_d(1 + [\text{Zn}]/K_i) + [\text{Ni}]\}$.

Polyacrylamide gel electrophoresis and Western blot analysis. SDS-PAGE was performed by using buffers previously described (14), 12% or 12.5% polyacrylamide running gels, and 4.5% or 5.0% stacking gels. Native PAGE was performed by using buffers without SDS, 6% polyacrylamide running gels, and 3% stacking gels. Protein bands were visualized with Coomassie brilliant blue stain, or the gels were electroblotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore) and blotted with anti-*K. aerogenes* urease antibodies (24). Reactive protein bands were visualized with anti-rabbit IgG alkaline phosphatase-conjugated antibodies and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium; Sigma) liquid substrate. Protein band intensities were measured by using the gel-scanning software AlphaEaseFC (Alpha Innotech Corp., San Leandro, California). The calculated molecular masses of UreA (11.1 kDa), UreB (11.7 kDa), UreE (17.6 kDa), UreG (21.9 kDa), UreG_{Str} (23.2 kDa), UreF (25.2 kDa), UreEF (42.8 kDa), UreD (29.8 kDa), MBP-LacZ α (50.8 kDa), UreC (60.3 kDa), and MBP-UreD (72.9 kDa) generally migrated during electrophoresis as expected, with the exception of UreG, which behaved as if it was larger than UreF.

Urease activity and protein assays. Urease activity was measured by quantifying the amount of ammonia released during urea hydrolysis by the formation of indophenol, which was monitored at 625 nm (32). One unit of activity is defined as the amount of enzyme necessary to hydrolyze 1 μmol of urea per min at 37°C. Standard assay buffer consisted of 50 mM HEPES, pH 7.8, and 50 mM

urea. Protein concentrations were determined by using a standard protein assay (Bio-Rad) with bovine serum albumin as the standard.

In vivo interactions and complementation experiments. *E. coli* BL21-Gold(DE3) cells were transformed by following the manufacturer's instructions (Stratagene). Double transformants were generated by making the singly transformed cells competent using CaCl_2 , followed by a standard heat shock transformation protocol as previously described (27). Transformants were maintained on LB agar plates containing ampicillin ($100 \mu\text{g ml}^{-1}$) or chloramphenicol ($50 \mu\text{g ml}^{-1}$) for single transformants or with both antibiotics for double transformants. Precultures for *in vivo* pulldown experiments or complementation studies consisted of 5 ml of LB supplemented with $300 \mu\text{g ml}^{-1}$ ampicillin, $50 \mu\text{g ml}^{-1}$ chloramphenicol, or both antibiotics in the case of double transformants. These cultures were incubated overnight at 37°C in a shaking incubator and subcultured at 1:100 into 25 ml of LB supplemented with the same antibiotics plus 1 mM NiCl_2 in the case of complementation studies. The 25-ml cultures were incubated at 37°C with shaking until the cells reached an OD_{600} of ~ 0.5 , at which point they were induced with 0.5 mM IPTG for pulldown experiments or 0.1 mM IPTG for complementation studies. After induction, the cultures were incubated for an additional 12 to 16 h before being harvested. Cell pellets were resuspended in 1 ml of TEB buffer containing 25 mM NaCl or 1 ml of 50 mM HEPES, pH 7.8, for pulldown experiments or complementation studies, respectively. In both cases, the cell slurries were supplemented with 1 mM phenylmethylsulfonyl fluoride (Sigma) before disruption by sonication. Subsequently, the disrupted cultures were centrifuged at $16,000 \times g$ for 20 min in a tabletop microcentrifuge, the soluble cell extracts were kept, and the pellets were discarded. For complementation assays, soluble cell extracts obtained after sonication and centrifugation were directly assayed for urease activity. For some experiments, soluble cell extracts were mixed from two different types of recombinant cells that overproduced different individual proteins; in this case 800 μl of each preparation was mixed and incubated at room temperature for 1 h before the pulldown analysis.

For *in vivo* pulldown assays, the soluble cell extracts derived from the recombinant *E. coli* cells were mixed with a 200- μl bed volume of amylose resin that had been washed with water and equilibrated in TEB buffer containing 25 mM NaCl. The cell extract-amylose resin slurry was incubated at room temperature for 1 h with gentle rocking before the amylose resin was washed five times with 1 ml of TEB buffer containing 25 mM NaCl. After the final wash, the resin was pelleted and resuspended in 500 μl of TEB buffer plus 25 mM NaCl containing 10 mM maltose. This slurry was briefly vortexed, and the resin was pelleted for 1 min at $1,200 \times g$. The remaining supernatant was removed and analyzed by SDS-PAGE.

In vitro interaction experiments. Purified proteins were dialyzed against TEB buffer containing 25 mM NaCl before each experiment. Depending on the particular experiment, MBP-UreD or MBP (2 μM) was mixed with UreEF, UreE, UreG_{ure}, urease apoprotein, or holoenzyme (10 μM) in 250- μl , 500- μl , or 1-ml solutions by using TEB buffer plus 25 mM NaCl to adjust the volume. When necessary, the salt concentrations of the mixtures were adjusted to 1 M NaCl by using buffer containing 3 M NaCl. Additionally, in selected experiments soluble cell extracts of *E. coli* MG1655 (used as a standard wild-type cell) were prepared in TEB buffer with 25 mM NaCl as described for *E. coli* cells in the previous section and added at 1/6 the assay volume. After a mixing step, the protein solutions were incubated (at room temperature, 37°C , or 42°C) for 1 h, and amylose resin (1/5 the solution volume; previously washed and equilibrated in the buffer) was added to the mixture. The slurry was gently rocked for 1 h at room temperature and then microcentrifuged for 1 min to separate the amylose resin from the discarded supernatant. The resin was washed five times with one reaction volume of TEB buffer containing either 25 mM or 1 M NaCl and then resuspended in half the original reaction volume with TEB buffer containing 10 mM maltose. The maltose-containing slurry was briefly vortexed, and the resin was pelleted for 1 min at $1,200 \times g$. The remaining supernatant was removed and analyzed by SDS-PAGE.

RESULTS

Purification and properties of the MBP-UreD fusion protein. In order to generate a form of UreD that would be useful for biochemical analysis, an in-frame translational fusion between UreD and the MBP was created. The soluble MBP-UreD was purified to near homogeneity by sequential chromatography using amylose and Q-Sepharose resins and shown to exist as a large multimer (>670 kDa) according to gel filtration chromatography. Whereas MBP-UreD was highly soluble, the

addition of factor Xa to purified MBP-UreD at room temperature for 3 h liberated UreD, which was found to be unsuitable for further study. Chromatography of the factor Xa digest on DEAE-Sepharose led to irreversible interaction of UreD with the chromatographic resin. Furthermore, visible protein precipitation was observed after >6 h of digestion with factor Xa, attributed to UreD instability. Thus, further studies focused on the intact form of MBP-UreD.

To determine whether structural or tightly bound metal ions were present in MBP-UreD, the metal content of purified protein was determined by using inductively coupled plasma atomic emission spectroscopy. This analysis showed that no metals were associated with MBP-UreD that had been purified in the presence of 1 mM EDTA (a chelator commonly added to urease purification buffers and shown not to remove the enzyme-bound Ni^{2+}). Although the protein as purified was free of metal ions, it was of interest to assess the ability of the UreD domain of MBP-UreD to reversibly bind metals. In particular, we focused on Ni^{2+} because of its obvious relevance to urease activation and on Zn^{2+} because this metal is known to bind to UreE (2, 8) and UreG (34, 35) accessory proteins. Inclusion of 100 μM NiCl_2 or ZnCl_2 had no effect on the protein behavior as assessed by gel filtration chromatography.

In one approach to assess metal ion binding, MBP-UreD was subjected to equilibrium dialysis against various NiCl_2 or ZnCl_2 concentrations, and the concentrations of protein-bound metal versus free metal were determined by using the metallochromic indicator PAR. The MBP-UreD data (Fig. 1A) could be fit by assuming a single type of binding site with a maximum (B_{max}) of 2.1 ± 0.1 Ni^{2+} ions per protomer and a dissociation constant (K_d) of $33.8 \pm 4.1 \mu\text{M}$; however, significant binding of Ni^{2+} to MBP was observed over this metal ion concentration range. To correct for the nonspecific Ni^{2+} binding to MBP, a linear fit to the MBP data was subtracted from the MBP-UreD data, and the resulting points were analyzed to yield a B_{max} of 1.6 ± 0.1 Ni^{2+} ions per UreD protomer with a K_d of $24.3 \pm 3.1 \mu\text{M}$. Although the error ranges are quite small, we note that the greatest Ni^{2+} concentration tested was only 5-fold the apparent K_d , so the data do not approximate saturation. In contrast, the Zn^{2+} data (Fig. 1B) did approach saturation, and binding to MBP was less significant; thus, a B_{max} of 4.1 ± 0.2 Zn^{2+} ions per protomer with a K_d of $4.7 \pm 0.7 \mu\text{M}$ was confidently established.

As an alternative approach to measure Ni^{2+} interactions with MBP-UreD, various concentrations of radiolabeled $^{63}\text{NiCl}_2$ were used during equilibrium dialysis studies (Fig. 1C). Direct fitting of the data by assuming a single type of binding site yielded a B_{max} of 3.1 ± 0.1 Ni^{2+} ions per MBP-UreD protomer and a K_d of $62.0 \pm 5.8 \mu\text{M}$; however, correction of these data for the Ni^{2+} bound to MBP provided a B_{max} of 2.7 ± 0.1 Ni^{2+} ions per UreD protomer and K_d of $50.0 \pm 4.4 \mu\text{M}$. These data include Ni^{2+} concentrations that more closely approach saturation than were obtained in the PAR experiments and are considered to be more reliable.

The use of ^{63}Ni in equilibrium dialysis experiments also allowed for investigation of the competition between Ni^{2+} and Zn^{2+} . MBP-UreD was dialyzed against various concentrations of $^{63}\text{NiCl}_2$ with set ZnCl_2 concentrations of either 12.5 μM or 125 μM (Fig. 1C). The data from the experiment containing 12.5 μM Zn^{2+} were corrected for nonspecific Ni^{2+} binding to

FIG. 1. Equilibrium dialysis of MBP-UreD and MBP with NiCl_2 , $^{63}\text{NiCl}_2$, and ZnCl_2 . (A) MBP-UreD (closed circles) or MBP (open circles) were dialyzed against varied concentrations of NiCl_2 and the amounts of bound Ni^{2+} per protomer were determined by using the metallochromic indicator PAR. Data were fit by assuming saturation behavior at a single type of binding site for MBP-UreD or by using a linear equation for MBP. (B) Analogous results obtained for dialysis of MBP-UreD (closed circles) or MBP (open circles) against ZnCl_2 . The MBP-UreD data were fit as described above. (C) MBP-UreD was dialyzed against various concentrations of NiCl_2 containing radioactive ^{63}Ni along with 0 μM (closed circles), 12.5 μM (open circles), and 125 μM (closed triangles) ZnCl_2 . Concentrations of free and bound Ni^{2+} were determined by scintillation counting. Also shown are control data for MBP with varied concentrations of $^{63}\text{NiCl}_2$ (open triangles). Each data set was fit by assuming saturation behavior at a single type of binding site. (D) MBP-UreD was dialyzed against 100 μM $^{63}\text{NiCl}_2$ and the indicated concentrations of ZnCl_2 . Data were fit by using a competitive inhibition equation.

MBP in the same manner as done with the Zn^{2+} -free sample. These data were fit to a competitive inhibition equation using B_{max} and K_d values as described above, providing a Zn^{2+} K_i of 5.0 ± 0.3 μM , which is in close agreement to the K_d measured for Zn^{2+} by using PAR. At 125 μM Zn^{2+} nearly all of the Ni^{2+} binding sites were occupied by Zn^{2+} . A reciprocal experiment was performed in which MBP-UreD was dialyzed against various concentrations of ZnCl_2 with a set concentration of 100 μM $^{63}\text{NiCl}_2$ (Fig. 1D). The data were well fit by using the competitive inhibition equation, with B_{max} and K_d calculated above, yielding a Zn^{2+} K_i of 3.0 ± 0.2 μM . These data clearly demonstrate that the UreD domain of MBP-UreD reversibly binds both Ni^{2+} and Zn^{2+} and that Zn^{2+} exhibits high affinity for the Ni^{2+} binding sites.

***malE-ureD* replacement or complementation of a ΔureD -urease cluster.** To assess the ability of the UreD domain of MBP-UreD to function during *in vivo* urease maturation, it was necessary to show that *malE-ureD* could complement a ΔureD -urease gene cluster or functionally replace *ureD* within the urease cluster. *E. coli* BL21-Gold(DE3) cells were either cotransformed with pEC002, coding for the MBP-UreD fusion, and pEC006, a *tac*-based expression vector for overproduction of a ΔureD -urease cluster, or singly transformed with pEC013 encoding an intact urease cluster with *malE-ureD* replacing wild-type *ureD*. For comparison and control, the same cells were transformed with pKK17 (with the wild-type urease gene cluster from *K. aerogenes*) or with pMal-c2x plus pEC006 (where pMal-c2x overproduces an MBP-LacZ α fusion, with

TABLE 2. Urease-specific activity in soluble cell extracts of *E. coli* BL21-Gold(DE3) harboring the indicated plasmids

Plasmid derivative(s) (description)	Specific activity (U mg ⁻¹ of protein) ^a
pKK17 (wild-type urease cluster).....	171.2 ± 22.0
pEC002 (<i>malE-ureD</i>) + pEC006 (<i>ΔureD</i> -urease cluster).....	15.1 ± 2.4
pMal-c2x (<i>malE-lacZα</i>) + pEC006 (<i>ΔureD</i> -urease cluster).....	<0.1
pEC013 (urease cluster with <i>malE-ureD</i> replacing <i>ureD</i>).....	56.2 ± 4.2

^a Data are represented as the average ± standard deviation for triplicate biological samples.

the result that UreD is not present). Soluble cell extracts of cells harboring pEC002 and pEC006 possessed about 10% of the urease activity measured for extracts of cells containing the wild-type urease cluster (Table 2). Analogous extract preparations from cells containing pEC013 exhibited a specific activity of 56.2 ± 4.2 U mg⁻¹ or 33% of wild-type activity. The reduction in activity compared to cells with pKK17 is likely due to the requirement for dual plasmids or to steric hindrance introduced by the 42-kDa fusion protein. In contrast to these positive results, extracts of cells harboring pMal-c2x and pEC006 were inactive, demonstrating that the UreD domain of MBP-UreD was responsible for complementation and that MBP-UreD provides a functional form of UreD.

MBP-UreD interactions with other urease components *in vivo*. To map the cellular protein-protein interaction network of UreD with other urease components, *E. coli* BL21-Gold(DE3) cells were cotransformed with pEC002 (encoding MBP-UreD) and a series of other expression vectors encoding various urease-related proteins. The soluble extracts of these cells were examined directly by using SDS-PAGE or applied to amylose resin, and the subsequently bound proteins were eluted with buffer containing 10 mM maltose and analyzed by electrophoresis (Fig. 2). As controls, analogous experiments made use of cells cotransformed with pMal-c2x instead of pEC002. In all cases, the MBP-LacZα control experiments (where UreD was absent) demonstrated negligible copurification of urease components during amylose chromatography. This finding confirmed that the UreD domain of the MBP-UreD fusion, rather than MBP itself, was responsible for the observed protein-protein interactions.

The results depicted in Fig. 2 reveal the cellular interactions of MBP-UreD. The initial soluble extracts of all cells (odd-numbered lanes) contained a prominent band just below the position of UreF; this band most likely is attributed to chloramphenicol acetyltransferase and contributes little, if at all, to the intensity of the UreF band in samples bound to and released from the amylose resin (even-numbered lanes). The pulldown analysis of soluble extracts from cells cotransformed with pEC004 (encoding UreA, UreB, and UreC) confirm the copurification of UreABC with MBP-UreD (Fig. 2A, lane 2). The band corresponding to UreC was unclear on this gel due to its overlap with MBP-UreD; however, the presence of UreC was substantiated by immunoblot analysis using anti-urease antibodies (data not shown). Pulldown examination of soluble extracts from cells cotransformed with pEC005 (encoding

UreF and UreG) showed that both UreF and UreG copurify with MBP-UreD (Fig. 2A, lane 6). For pEC006 (encoding the *ΔureD*-urease cluster), UreABC, UreF, and UreG all copurified with MBP-UreD (Fig. 2A, lane 10), but UreE was not detected. A pulldown of soluble extracts from cells with pEC007 (encoding UreE) indicated that UreE does not copurify with MBP-UreD (Fig. 2B, lane 2). The same analysis for pEC008 (encoding UreF) confirmed that UreF copurified with MBP-UreD (Fig. 2B, lane 6). Lastly, pulldown studies involving pEC009 (encoding UreG) demonstrated copurification of UreG with MBP-UreD (Fig. 2B, lane 10). Densitometric analyses of the SDS-PAGE gels shown in Fig. 2 revealed a UreG/MBP-UreD ratio of only 0.07:1 for copurification of UreG with MBP-UreD, compared to a UreF/MBP-UreD ratio of 0.42:1 when UreF was overproduced alone with MBP-UreD or a UreF/UreG/MBP-UreD ratio of 0.16:0.19:1 when UreF and UreG were both overproduced with MBP-UreD. The ratios reflect the upper limit of the amount of bound UreF since we cannot completely rule out contamination of these samples with trace amounts of the chloramphenicol acetyltransferase.

In order to further investigate the interaction between UreD and urease apoprotein, soluble cell extracts of *E. coli*

FIG. 2. Interactions of MBP-UreD with other urease components *in vivo*. *E. coli* BL21-Gold(DE3) cells were cotransformed with either pEC002 (encoding MBP-UreD) or pMal-c2x (encoding MBP-LacZα) along with pEC004, pEC005, pEC006, pEC007, pEC008, or pEC009 (encoding UreABC, UreFG, UreABCEFG, UreE, UreF, or UreG, respectively). Soluble cell extracts were analyzed directly by SDS-PAGE (odd-numbered lanes) or subjected to amylose resin chromatography with the proteins eluted by maltose addition and analyzed by SDS-PAGE (even-numbered lanes). (A) MBP-UreD interactions with multiple urease components. (B) MBP-UreD interactions with single urease components. M, molecular mass markers.

FIG. 3. Interactions of MBP-UreD with urease apoprotein. (A) *In vivo* complex formation. Soluble cell extracts of IPTG-induced *E. coli* BL21-Gold(DE3)/pEC002 (encoding MBP-UreD) and *E. coli* BL21-Gold(DE3)/pEC004 (encoding UreABC) were mixed, incubated at room temperature for 1 h, and chromatographed on amylose resin. Bound proteins were eluted in buffer containing 10 mM maltose and visualized by SDS-PAGE. Lanes: M, molecular mass marker; 1, pEC002 cell extracts; 2, pEC004 cell extracts; 3, coelution of UreABC with MBP-UreD from the amylose resin. (B) *In vitro* interactions. Purified MBP-UreD (2 μ M) was mixed with isolated urease apoprotein (10 μ M) in TEB buffer with 25 mM NaCl and, where indicated, cell extracts of *E. coli* MG1655. Reaction mixtures were incubated at room temperature for 1 h before the mix was subjected to amylose resin chromatography. Bound proteins were eluted with buffer containing 10 mM maltose. Lanes: M, molecular mass marker; 1, purified urease apoprotein; 2, purified MBP-UreD; 3, MBP-UreD plus urease apoprotein; 4, MBP-UreD plus urease apoprotein along with *E. coli* MG1655 cell extracts; 5, eluted fraction from lane 3; 6, eluted fraction from lane 4.

BL21-Gold(DE3)/pEC002 (containing MBP-UreD) and *E. coli* BL21-Gold(DE3)/pEC004 (containing UreABC) were mixed, incubated at room temperature for 1 h, and subjected to amylose resin pulldown analysis. In Figure 3A, lanes 1 and 2 depict soluble cell extracts of the two strains prior to mixing. Lane 3 shows the proteins eluted from amylose resin by maltose addition, clearly demonstrating that MBP-UreD and UreABC from separate cells can interact to form a complex by mixing the soluble extracts under these conditions.

MBP-UreD interactions with other urease components *in vitro*. The protein-protein interactions identified above by *in vivo* pulldown analyses were compared to the interactions observed when purified proteins were used (Fig. 3B and 4). Thus, MBP-UreD or MBP was mixed with various purified urease-

related proteins and subjected to amylose resin pulldown experiments, as already described. The quality of the samples is illustrated for urease apoprotein (Fig. 3B, lane 1), MBP (Fig. 4A, lane 1), MBP-UreD (Fig. 3B, lane 2, and 4A, lane 2), UreEF (Fig. 4A, lane 3), UreG_{Str} (Fig. 4A, lane 4), and UreE (Fig. 4B, lane 2).

Whereas a complex between MBP-UreD and urease apoprotein was formed when soluble cell extracts containing these separate components were mixed (Fig. 3A), purified MBP-UreD did not bind to isolated urease apoprotein (Fig. 3B). Samples examined prior to (lane 3) and after (lane 5) the pulldown analysis confirmed the absence of interaction. The same experiments were repeated in the presence of soluble extracts from wild-type *E. coli* MG1655 cells to assess whether

FIG. 4. Interactions of MBP-UreD with other urease components *in vitro*. Purified MBP-UreD and MBP (2 μ M) were tested for their abilities to form complexes with UreEF, UreE, or UreG_{Str} (10 μ M) in TEB buffer containing 25 mM NaCl (unless indicated otherwise). (A) SDS-PAGE analysis of purified components (lanes 1 to 4) and selected protein mixtures that were incubated at the indicated temperature and subjected to amylose resin chromatography with the fractions eluted by maltose analyzed (lanes 5 to 11). Lanes: M, molecular mass marker; 1, MBP; 2, MBP-UreD; 3, UreEF; 4, UreG_{Str}; 5, MBP-UreD and UreEF at 37°C; 6, MBP-UreD and UreEF at 42°C; 7, MBP-UreD and UreG_{Str} at 37°C; 8, MBP-UreD and UreG_{Str} at 42°C; 9, MBP and UreEF at 42°C; 10, MBP and UreG_{Str} at 42°C; 11, MBP-UreD and UreEF at 42°C in the presence of 1 M NaCl. (B) SDS-PAGE analysis of MBP-UreD interactions with UreE. Lanes: M, molecular mass marker; 1, MBP-UreD; 2, UreE; 3, maltose elution of chromatographed mixture of MBP-UreD and UreE incubated at 42°C. (C) Native-PAGE analysis of the interaction of MBP and MBP-UreD with UreEF. Lanes: 1, MBP; 2, UreEF; 3, MBP-UreD; 4, maltose elution of chromatographed mixture MBP-UreD and UreEF incubated at 42°C; 5, elution of MBP and UreEF incubated at 42°C.

any soluble intracellular constituents were responsible for the complex formation observed *in vivo*, but even after cells were incubated for 1 h, we observed no complex between urease apoprotein and MBP-UreD (Fig. 3B, lanes 4 and 6). Furthermore, incubation of MBP-UreD with urease apoprotein or holoenzyme at 37°C or 42°C failed to produce an interaction between the two species according to pulldown analysis (data not shown).

When MBP-UreD was incubated with the UreEF fusion protein at 37°C (Fig. 4A, lane 5), only a weak interaction (a UreEF/MBP-UreD ratio of 0.21:1 by densitometry) was detected. An identical experiment performed at 42°C resulted in a significant enhancement of interaction (UreEF/MBP-UreD ratio of 1.17:1), indicating that higher temperature increases the affinity of MBP-UreD for UreEF (Fig. 4A, lane 6). When the same sample at 42°C was examined in buffer containing 1 M NaCl, the UreEF/MBP-UreD ratio dropped (to 0.19:1), indicating that the interaction between these two species is sensitive to high ionic strength (Fig. 4A, lane 11). A similar set of experiments performed using UreG_{Str} indicated a weak interaction whether incubation was at 37°C (UreG/MBP-UreD ratio of 0.10:1) (Fig. 4A, lane 7) or at 42°C (UreG/MBP-UreD ratio of 0.18:1) (Fig. 4A, lane 8). The addition of 1 M NaCl to this protein mixture had no effect on complex formation (data not shown). To confirm that the UreD domain of MBP-UreD was responsible for the interaction with UreEF, an analogous pulldown experiment was carried out with purified MBP and UreEF. Because the UreEF and MBP bands overlap on the SDS-PAGE gel (Fig. 4A, lane 9), the interaction between these species was examined by utilizing native PAGE (Fig. 4C). This analysis confirmed that MBP and UreEF do not form a complex. To test whether the UreE domain of UreEF was responsible for the complex formation between UreEF and MBP-UreD, purified UreE (Fig. 4B, lane 2) was mixed with MBP-UreD and examined; no interaction between UreE and MBP-UreD was detected (Fig. 4B, lane 3). When MBP-UreD was mixed with both UreEF and UreG_{Str} at 42°C, no increase in the affinity of MBP-UreD for UreG_{Str} was noted; however, an approximately 1:1 complex between MBP-UreD and UreEF was present (data not shown). Therefore, a clear interaction between UreD and UreF was confirmed both *in vitro* and *in vivo*.

DISCUSSION

In this study we investigated the properties of a translational fusion between MBP and UreD, where the resulting MBP-UreD is the first soluble form of UreD to be described. The fusion protein is stable and readily purified via sequential chromatography on amylose and Q-Sepharose resins, whereas the free UreD liberated by factor Xa digestion was highly unstable and precipitated during chromatography or storage. In a similar way, digestion of an MBP-UreF fusion produced an unstable UreF (13). These results highlight the usefulness of the MBP domain for increasing the solubility of normally insoluble urease accessory proteins.

While MBP-UreD purified in the presence of EDTA was free of metal ions, it was shown that the UreD domain of this protein could bind reversibly either Ni²⁺ or Zn²⁺, similar to the case for UreE (2, 8) and UreG (34, 35) from various

microorganisms. All metal-binding data were reasonably well fit by assuming a single type of binding site although distinct sites with similar binding parameters are likely. For Ni²⁺, the PAR and radioactive ⁶³Ni methods provided different results, indicating 1.6 or 2.7 Ni²⁺ ions bound per UreD protomer, with a *K_d* of 24 or 50 μM, respectively. We suggest that the latter set of data is more reliable because of its greater range of Ni²⁺ concentrations and thus conclude that UreD binds approximately 2.5 Ni²⁺ ions per protomer. A noninteger number of bound Ni²⁺ ions can be explained by a binding site at the interface of two protomers in the quaternary structure. The PAR-derived data indicate the ability to bind four Zn²⁺ ions per protomer with a *K_d* of 5 μM, in excellent agreement with the Zn²⁺ *K_i* measured in two studies examining competition with ⁶³Ni²⁺ binding. Thus, we conclude that Zn²⁺ competes with 10-fold higher affinity for the Ni²⁺ binding sites on UreD and binds to at least one additional site. We emphasize, however, that the metal-binding properties of UreD may differ dramatically when it is in complex with urease. For example, increased Ni²⁺ specificity could potentially be present in the UreABC-UreD complex. Furthermore, activation of urease apoprotein within the UreABC-UreDFG complex by Ni²⁺, CO₂, GTP, and UreE takes place even in the presence of Ni²⁺-chelating agents; this linking of nucleotide hydrolysis to metalcenter assembly indicates that the process cannot simply be viewed from a thermodynamic perspective. Our finding that UreD binds metal ions suggests its involvement in Ni²⁺ translocation to the urease active site.

In order to show that MBP-UreD could function during *in vivo* urease maturation, complementation studies were performed either by transforming *E. coli* cells with the plasmid encoding the fusion protein and a second vector carrying a *ΔureD*-urease cluster or by using a single plasmid encoding a urease cluster with *malE-ureD* replacing wild-type *ureD*. Cotransformed cells exhibited the capacity to activate urease apoprotein but only to ~10% of the activity found in cells carrying the wild-type urease gene cluster; this low percentage of complementation coincides with that in a previous study in which *E. coli* extracts from cotransformed cells (with plasmids producing the *K. aerogenes* wild-type *ureD* or a *K. aerogenes* urease cluster with a partial *ureD* deletion) yielded a specific activity of 17.2 U/mg, or 8.7% of that for extracts of cells containing the wild-type urease cluster (198.4 U/mg) (16). Thus, we conclude that MBP-UreD behaves similarly to wild-type UreD during analogous *in vivo* complementation assays. *E. coli* soluble extracts from cells transformed with a single plasmid encoding an intact urease cluster with the *ureD* fusion replacing wild-type *ureD* yielded a specific activity corresponding to 33% of wild-type activity. Although this value is higher than that obtained with the cotransformant system, it still does not meet wild-type levels, indicating that the MBP domain likely hinders UreD functionality. Nevertheless, these results provide strong support that MBP-UreD is at least a partially functional form of UreD.

Utilizing the MBP domain of MBP-UreD as a convenient affinity tag for amylose binding, a series of pulldown studies were performed to map the *in vivo* protein-protein interaction network of UreD with other urease components. When overproduced in the same cell, the UreD domain of MBP-UreD was shown to interact with the structural subunits UreABC in

the absence of other accessory proteins. This interaction was expected as a UreABC-UreD complex has been purified and characterized previously (22). When MBP-UreD was overproduced with a $\Delta ureD$ cluster, a pulldown study showed that UreABC-(MBP-UreD)-UreFG was formed within the cell. The wild-type version of this complex, UreABC-UreDFG, has been purified and shown to be highly competent for *in vitro* reconstitution with Ni^{2+} and bicarbonate (30). Also of interest was the demonstration that the UreD domain of MBP-UreD interacts with both UreF and UreG in the cell to form a soluble MBP-UreDFG complex. A previous study reported the purification of an insoluble UreDFG complex by using an ATP-linked column matrix (18), where the nucleotide binding was attributed to the UreG component of the heterotrimer (a UreDFG complex containing a mutation in the P-loop motif of UreG did not bind this resin). The UreDFG heterotrimer complex has been hypothesized to represent a physiologically relevant species that serves as a preformed molecular chaperone (18). Preparing this complex with soluble MBP-UreD, now shown to be feasible by the experiments described here, may allow further exploration of the function of this species during urease *in vivo* maturation.

In addition to its simultaneous interactions with multiple urease components in the cell, the UreD domain of MBP-UreD also formed individual complexes *in vivo* with UreF or UreG but not with UreE. These data are in agreement with

previous yeast two-hybrid studies in which a UreD-UreF interaction was seen in *P. mirabilis* (9) and a UreH (UreD orthologue)-UreF interaction was observed in *H. pylori* (31). Densitometry analysis demonstrated that MBP-UreD bound approximately six times more UreF than UreG in these individual complexes, whereas UreF and UreG were stoichiometric within the MBP-UreD-UreFG complex when these proteins were synthesized in the cell. The amount of UreG that coeluted with MBP-UreD increased 2.7-fold compared to the similar pulldown study without concomitant production of UreF. These data suggest that UreF within MBP-UreDF provides a cooperative binding surface that increases the affinity for UreG. Related to this result, a previous study focusing on the interactions of the UreEF fusion protein with other urease components demonstrated a weak or transient interaction between the UreF domain of that fusion protein and UreG. Together, these data indicate that it is more likely that UreD and UreF interact prior to UreG binding when forming the UreDFG complex than that UreG binds individually first with UreD or UreF.

To further investigate the interactions between the UreD domain of MBP-UreD and other urease proteins, purified components were mixed and monitored for their interactions by using a similar pulldown system. Surprisingly, isolated MBP-UreD did not form a complex with purified urease apoprotein even though a complex was formed when paired cell extracts

FIG. 5. Working model of urease activation. The urease apoprotein trimer of trimers (A) sequentially binds UreD, UreF, and UreG to form UreABC-UreD (B), UreABC-UreDF (C), and UreABC-UreDFG (D). Alternatively, UreDFG may exist as a preformed molecular chaperone that directly binds the urease apoprotein, as indicated by the asterisk. Urease apoprotein binds to UreD and, less well, to UreF; UreD and UreF bind each other; and UreG binds to these proteins with no direct interactions to urease. The UreE metallochaperone delivers Ni^{2+} via an interaction with UreG (E). *In vivo*, urease activation requires CO_2 to carboxylate a nascent active site lysine along with GTP, which is bound and hydrolyzed by UreG in the activation complex. Coupled to this process, we propose the directed transfer of Ni^{2+} from UreE to UreG to UreD and finally into the urease apoprotein to generate active enzyme (F).

containing the two proteins were mixed. Furthermore, the addition of cell extracts to the mixture of purified proteins failed to stimulate complex formation. These results demonstrate that concomitant folding is not a prerequisite for these two species to interact; however, they suggest that the purification process somehow renders MBP-UreD or urease apoprotein incompetent to bind each another. For MBP-UreD and UreEF, a direct ~1:1 stoichiometric complex was observed when the two species were incubated at 42°C prior to the pulldown experiment. The interaction was shown to exist between the UreD domain of MBP-UreD and the UreF domain of UreEF since purified MBP did not interact with UreEF and since UreE did not interact with MBP-UreD under identical conditions. Additionally, the inclusion of 1 M NaCl to the pulldown assay resulted in a substoichiometric complex, indicating that UreD and UreF may bind one another via electrostatic interactions. In contrast, only a weak or transient interaction was seen between the UreD domain of MBP-UreD and UreG; furthermore, this interaction was not enhanced by the addition of UreEF. The distinction between these results and the *in vivo* pulldown investigations, where UreF increased the interaction between UreG and MBP-UreD, may be due to steric interference by the UreE domain of UreEF, to the need for an unidentified cellular component that was not present in the *in vitro* assay, or to an unidentified change in one of the proteins during purification.

In conclusion, our studies with MBP-UreD have uncovered the previously unknown metal-binding capability of UreD and revealed new aspects of its interactions with other urease components. Consistent with previous studies that characterized urease apoprotein alone and in complex with UreD, UreDF, and UreDFG (19, 22, 30), we show here that MBP-UreD can form such complexes *in vivo* and, in a subset of these cases, *in vitro* as well. Each of the different urease apoprotein complexes has distinct activation properties. Of particular significance, the importance of UreD is shown by the finding that ~30% of the nascent active sites in purified UreABC-UreD can form active enzyme, whereas only ~15% of UreABC alone is activated in solutions containing Ni²⁺ and bicarbonate ions (23). These data suggest that UreD plays an important role in apoprotein activation, but that role remains enigmatic. The metal-binding capacity of MBP-UreD leads us to hypothesize that these binding sites contribute to UreD's function during the activation process. Two other urease accessory proteins, UreG and UreE, are known to bind various metal ions. For example, UreG from *Bacillus pasteurii* binds 2 Zn²⁺ or 4 Ni²⁺ ions per dimer, and that from *H. pylori* binds 0.5 Zn²⁺ or 2 Ni²⁺ ions per monomer (34, 35). Similarly, UreE proteins from *K. aerogenes*, *B. pasteurii*, and *H. pylori* are known to bind both Ni²⁺ and Zn²⁺ (1, 2, 33). Taking together our equilibrium dialysis of MBP-UreD and the protein-protein interaction network we have clarified, we speculate that the accessory proteins work together to carry out sequential transfers of Ni²⁺ from UreE to UreG to UreD to the urease active site (Fig. 5). Regardless of the physiological relevance of this model for sequential metal binding, the present results provide clear support for the hypothesis that UreD directly participates in Ni²⁺ transfer to urease apoprotein during *in vivo* maturation.

ACKNOWLEDGMENTS

We thank Scott Mulrooney, Rachel Morr, and Scott Taber for assistance with some experiments.

This work was supported by NIH grant DK04586.

REFERENCES

- Benoit, S., and R. J. Maier. 2003. Dependence of *Helicobacter pylori* urease activity on the nickel-sequestering ability of the UreE accessory protein. *J. Bacteriol.* **185**:4787–4795.
- Brayman, T. G., and R. P. Hausinger. 1996. Purification, characterization, and functional analysis of a truncated *Klebsiella aerogenes* UreE urease accessory protein lacking the histidine-rich carboxyl terminus. *J. Bacteriol.* **178**:5410–5416.
- Carter, E. L., T. G. Flugga, J. L. Boer, S. B. Mulrooney, and R. P. Hausinger. 2009. Interplay of metal ions and urease. *Metallomics* **1**:207–221.
- Colpas, G. J., T. G. Brayman, J. McCracken, M. A. Pressler, G. T. Babcock, L.-J. Ming, C. M. Colangelo, R. A. Scott, and R. P. Hausinger. 1998. Spectroscopic characterization of metal binding by *Klebsiella aerogenes* UreE urease accessory protein. *J. Biol. Inorg. Chem.* **3**:150–160.
- Colpas, G. J., T. G. Brayman, L.-J. Ming, and R. P. Hausinger. 1999. Identification of metal-binding residues in the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. *Biochemistry* **38**:4078–4088.
- Colpas, G. J., and R. P. Hausinger. 2000. *In vivo* and *in vitro* kinetics of metal transfer by the *Klebsiella aerogenes* urease nickel metallochaperone, UreE. *J. Biol. Chem.* **275**:10731–10737.
- Dykxhoorn, D. M., R. St. Pierre, and T. Linn. 1996. A set of compatible *tac* promoter expression vectors. *Gene* **177**:133–136.
- Grossoehe, N. E., S. B. Mulrooney, R. P. Hausinger, and D. E. Wilcox. 2007. Thermodynamics of Ni²⁺, Cu²⁺, and Zn²⁺ binding to urease metallochaperone UreE. *Biochemistry* **46**:10506–10516.
- Heimer, S. R., and H. L. Mobley. 2001. Interaction of *Proteus mirabilis* urease apoprotein and accessory proteins identified with yeast two-hybrid technology. *J. Bacteriol.* **183**:1423–1433.
- Jabri, E., M. B. Carr, R. P. Hausinger, and P. A. Karplus. 1995. The crystal structure of urease from *Klebsiella aerogenes*. *Science* **268**:998–1004.
- Jabri, E., and P. A. Karplus. 1996. Structures of the *Klebsiella aerogenes* urease apoprotein and two active-site mutants. *Biochemistry* **35**:10616–10626.
- Kim, J. K., and R. P. Hausinger. 2006. The UreF fusion protein provides a soluble and functional form of the UreF urease accessory protein. *J. Bacteriol.* **188**:8413–8420.
- Kim, K. Y., C. H. Yang, and M. H. Lee. 1999. Expression of the recombinant *Klebsiella aerogenes* UreF protein as a MalE fusion. *Arch. Pharm. Res.* **22**:274–278.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Leach, M. R., and D. B. Zamble. 2007. Metallocenter assembly of the hydrogenase enzymes. *Curr. Opin. Chem. Biol.* **11**:159–165.
- Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. P. Hausinger. 1992. *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. *J. Bacteriol.* **174**:4324–4330.
- Lee, M. H., H. S. Pankratz, S. Wang, R. A. Scott, M. G. Finnegan, M. K. Johnson, J. A. Ippolito, D. W. Christianson, and R. P. Hausinger. 1993. Purification and characterization of *Klebsiella aerogenes* UreE protein: a nickel-binding protein that functions in urease metallocenter assembly. *Protein Sci.* **2**:1042–1052.
- Moncrief, M. B. C., and R. P. Hausinger. 1997. Characterization of UreG, identification of a UreD-UreF-UreG complex, and evidence suggesting that a nucleotide-binding site in UreG is required for *in vivo* metallocenter assembly of *Klebsiella aerogenes* urease. *J. Bacteriol.* **179**:4081–4086.
- Moncrief, M. B. C., and R. P. Hausinger. 1996. Purification and activation properties of UreD-UreF-urease apoprotein complexes. *J. Bacteriol.* **178**:5417–5421.
- Mulrooney, S. B., and R. P. Hausinger. 2003. Nickel uptake and utilization by microorganisms. *FEMS Microbiol. Rev.* **27**:239–261.
- Mulrooney, S. B., H. S. Pankratz, and R. P. Hausinger. 1989. Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes* (*K. pneumoniae*) urease. *J. Gen. Microbiol.* **135**:1769–1776.
- Park, I.-S., M. B. Carr, and R. P. Hausinger. 1994. *In vitro* activation of urease apoprotein and role of UreD as a chaperone required for nickel metallocenter assembly. *Proc. Natl. Acad. Sci. U. S. A.* **91**:3233–3237.
- Park, I.-S., and R. P. Hausinger. 1995. Requirement of carbon dioxide for *in vitro* assembly of the urease nickel metallocenter. *Science* **267**:1156–1158.
- Quiroz-Valenzuela, S. 2008. New insights in the urease activation process obtained by characterization of apourease complexes and the UreG accessory protein of *Klebsiella aerogenes*. Ph.D. thesis. Michigan State University, East Lansing, MI.
- Rain, J.-C., L. Selig, H. de Reuse, V. Battaglia, C. Reverdy, S. Simon, G. Lenzen, F. Petel, J. Wojcik, V. Schächter, Y. Chemama, A. Labigne, and P. Legrain. 2001. The protein-protein interaction map of *Helicobacter pylori*. *Nature* **409**:211–215.

26. **Rubio, L. M., and P. W. Ludden.** 2005. Maturation of nitrogenase: a biochemical puzzle. *J. Bacteriol.* **187**:405–414.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
28. **Song, H. K., S. B. Mulrooney, R. Huber, and R. P. Hausinger.** 2001. Crystal structure of *Klebsiella aerogenes* UreE, a nickel-binding metallochaperone for urease activation. *J. Biol. Chem.* **276**:49359–49364.
29. **Soriano, A., G. J. Colpas, and R. P. Hausinger.** 2000. UreE stimulation of GTP-dependent urease activation in the UreD-UreF-UreG-urease apoprotein complex. *Biochemistry* **39**:12435–12440.
30. **Soriano, A., and R. P. Hausinger.** 1999. GTP-dependent activation of urease apoprotein in complex with the UreD, UreF, and UreG accessory proteins. *Proc. Natl. Acad. Sci. U. S. A.* **96**:11140–11144.
31. **Voland, P., D. L. Weeks, E. A. Marcus, C. Prinz, G. Sachs, and D. Scott.** 2003. Interactions among the seven *Helicobacter pylori* proteins encoded by the urease gene cluster. *Am. J. Physiol. Gastrointest. Liver Physiol.* **284**:G96–G106.
32. **Weatherburn, M. W.** 1967. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **39**:971–974.
33. **Won, H.-S., Y.-H. Lee, J.-H. Kim, I. S. Shin, M. H. Lee, and B.-J. Lee.** 2004. Structural characterization of the nickel-binding properties of *Bacillus pasteurii* UreE in solution. *J. Biol. Chem.* **279**:17466–17472.
34. **Zambelli, B., M. Stola, F. Musiani, K. De Vriendt, B. Samyn, B. Devreese, J. Van Beeumen, A. Dikiy, D. A. Bryant, and S. Ciurli.** 2005. UreG, a chaperone in the urease assembly process, is an intrinsically unstructured GTPase that specifically binds Zn^{2+} . *J. Biol. Chem.* **280**:4684–4695.
35. **Zambelli, B., P. Turano, F. Musiani, P. Neyroz, and S. Ciurli.** 2009. Zn^{2+} -linked dimerization of UreG from *Helicobacter pylori*, a chaperone involved in nickel trafficking and urease activation. *Proteins* **74**:222–239.