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

Eric L. Carter and Robert P. Hausinger

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

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Assembly of the *Klebsiella aerogenes* urease metallocenter requires four accessory proteins, UreD, UreE, UreF, and UreG, to effectively deliver and incorporate two Ni\(^{2+}\) 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 *ΔureD*-urease cluster in this host microorganism. MBP-UreD was purified as a large multimer (>670 kDa) that bound approximately 2.5 Ni\(^{2+}\) ions (\(K_d\) of ~50 μM, where \(K_d\) is the dissociation constant) per UreD protomer according to equilibrium dialysis measurements. Zn\(^{2+}\) directly competes with 10-fold higher affinity (~4 Zn\(^{2+}\) ions per protomer; \(K_d\) of 5 μM) for the Ni\(^{2+}\) binding sites. MBP pulldown experiments demonstrated that the UreD domain of MBP-UreD formed *in vitro* complexes with UreF, UreE, 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] metalloclusters in microbial hydrogenases, and the delivery and incorporation of Ni\(^{2+}\) 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 Ni\(^{2+}\)-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 Ni\(^{2+}\) to the nascent active site (17). This protein has been structurally characterized (28), shown by equilibrium dialysis methods to bind about six Ni\(^{2+}\) ions per homodimer with a \(K_d\) (dissociation constant) of 9.6 ± 1.3 μM (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 UreF-UreE 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 *ΔureD*-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 Ni\(^{2+}\) and bicarbonate (needed to form a carboxy-Lys residue that bridges the dinuclear center) to a greater level than...
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′-TATAGGATCCGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′) with an engineered 5′ BamHI restriction site (shown in italics), and a reverse primer with a 5′ HindIII restriction site (5′-TATAGGATCCGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′). This fragment was digested and ligated into similarly digested pMal-c2x vector (New England Biolabs). The resulting pEC002 encoded an in-frame translational fusion of ureF with a stop codon inserted in the Factor-Xa cleavage site after the C-terminal Arg residue. This plasmid is denoted pMBP2*. For one-step purification of MBP, we used a C-terminal Strep tag fusion that was constructed by inserting the wild-type ureG into SacII-PstI-digested pASK-IBA3 (IBA, 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 ureABCΔEFG 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 and an engineered ribosomal binding site as the ureFG fragment in pEC005, and a reverse primer identical to the one used for construction of pEC002. Utilizing a unique BamHI restriction site within ureC, pKK17 was digested with BamHl 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 pEC006, 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′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′), and a reverse primer with a 5′ KpnI restriction site (5′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′). This fragment in pEC006 was PCR amplified by using pKK17 as a template, a forward primer with a 5′ BamHI restriction site (5′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′), and a reverse primer with a 5′ KpnI restriction site (5′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′). This fragment was ligated into similarly digested pACT3, resulting in pEC004. Utilizing a unique BamHI restriction site within ureC, pKK17 was digested with BamHl and HindIII, resulting in a fragment containing a 3′ end segment of ureC with full-length ureE, ureF, and ureG. This was PCR amplified by using pEC004 as a template, a forward primer with a 5′ EcoRI restriction site (5′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′), and a reverse primer with a 5′ KpnI restriction site (5′-GATCCGACACGAGGAAAGCTGGTCTCGACGCAGGAAGCAGCTGGTGTTTACCAACCTGAGCTG-3′). This fragment was ligated into similarly digested pACT3, resulting in pEC005. This plasmid was constructed by inserting the wild-type ureG into SacII-PstI-digested pASK-IBA3 (IBA, Göttingen, Germany) to produce pIBA3+G (24).

### TABLE 1. Description of plasmids used in this study

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

For one-step purification of UreG, we used a C-terminal Strep tag fusion that
Protein purification. MBP-UreD was purified from E. coli BL21-Gold(DE3) cells (Stratagene) transformed with pEC002 and grown with shaking in lysogenic broth (LB) supplemented with 300 μg ml⁻¹ ampicillin at 30°C until the cells reached an optical density of 0.60 (OD₆₀₀) 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 × g for 1 h at 4°C. Extracts were diluted at least 1:1 with TEB buffer containing 500 mM NaCl to be 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 A₂₈₀ 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 pull-down experiments or with 50 mM Tris-HCl, pH 7.5, containing 100 mM NaCl for equipping chromatography 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 procedure was the same as that used for the purification of the UreE H144U enzyme, cells were grown with shaking in LB supplemented with 300 μg ml⁻¹ ampicillin and 1 mM IPTG, incubated at 25°C for 12 to 16 h, and harvested by centrifugation. Cell pellets were resuspended in PEB buffer containing 800 μM NiCl₂ (or 500 μM ZnCl₂) and disrupted by sonication. Soluble cell extracts were obtained by centrifugation at 100,000 × g for 1 h at 4°C. Purification of UreEF fusion protein was initially purified from E. coli C41(DE3)(pET-Str)/H9251 (Str) was purified from E. coli BL21-Gold(DE3)/H9262 harboring pMBP2*. The MBP is identical in sequence regardless of which source was used for expression.

The UreEF fusion protein was initially purified from E. coli C41(DE3)(pET-Str)/H9251 using Ni²⁺-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 (UreG₃) was purified from E. coli DH5α transformed with pBVA3+G, grown with shaking in LB supplemented with 300 μg ml⁻¹ ampicillin at 37°C until the cells reached an A₆₀₀ 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, dialyzed for 1 h at 4°C, then incubated overnight in 100 mM Tris-HCl, pH 7.5, containing 15 mM NaCl plus 10 mM maltose. The eluted fractions were collected, pooled, and dialyzed against TEB buffer containing 150,000 × g for 1 h at 4°C. Purification of UreG₃, 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 μg ml⁻¹ chloramphenicol at 37°C until the cells reached an A₆₀₀ 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 H144U variant, as previously described (2).

Both holoprotein and apoprotein forms of urease were purified from E. coli BL21-Gold(DE3) cells transformed with pKK7. For purification of holoenzyme, cells were grown with shaking in LB supplemented with 300 μg ml⁻¹ ampicillin and 1 mM NiCl₂ at 37°C until the cells reached an A₆₀₀ 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 × g for 1 h at 4°C and applied to a 2.5-cm by 10-cm DEAE-Sepharose column pre-equilibrated with PEB buffer. A linear gradient from 0 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) pre-equilibrated 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 holoenzyme; however, this protein was purified from cultures grown without the addition of NiCl₂ 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).

**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 used the metallochromicogenic indicator 4-[2-pyridylazo]resorcinol (PAR; Sigma). Purified MBP-UreD (9.6 μM for Ni²⁺ experiments; 7.1 μM for Zn²⁺ 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 NiCl₂ or ZnCl₂ (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 [Ni²⁺] or [Zn²⁺] by mixing with 800 μl of PAR solution 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 resultant 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 that were not bound to MBP by using the M₃₈₃ metal ion bound per mole of protomer, B₃₈₃, is the maximal number of moles of metal ion bound per mole of protomer, B₃₈₃, is the concentration of free nickel ion, and K₃₈₃ is the dissociation constant: Y = (B₃₈₃[M])/(K₃₈₃[M]).

A second approach for assessing Ni²⁺ binding to MBP-UreD and MBP as well as for examining the competition between Ni²⁺ and Zn²⁺ for the former protein made use of radiolabeled ⁶⁷NiCl₂ (1,445 Ci/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 ⁶⁷NiCl₂ and unlabeled ZnCl₂ 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 Safely-Solve scintillation fluid (Research Products International Corp.) and counted in a Lumi-1600 liquid scintillation spectrometer (Lumac, Amsterdam). The calculated mole masses of UreE (11.1 kDa), UreB (11.7 kDa), UreC (17.6 kDa), UreD (21.9 kDa), UreE₁₄₄U (23.2 kDa), UreF (25.2 kDa), UreEF (42.8 kDa), UreD (29.8 kDa), MBP-LacZα (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 UreE.

**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 NaCl.
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 CaCl2, followed by a standard heat shock transformation protocol as previously described (27). Transformants were maintained on LB agar plates containing ampicillin (100 μg ml−1) or chloramphenicol (50 μg 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 μg ml−1 ampicillin, 50 μg 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 NiCl2 in the case of complementation studies. The 25-ml cultures were incubated at 37°C with shaking until the cells reached an OD600 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 × 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 further used 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 amylase resin that had been washed with water and equilibrated in TEB buffer containing 25 mM NaCl. The cell extract-amylase resin slurry was incubated at room temperature for 1 h with gentle rocking before the amylase 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 × g. The remaining supernatant was removed and analyzed by SDS-PAGE.

In vitro interaction experiments. Purified proteins were diazylated 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, 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 amylase 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 amylase 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 × 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 Ni2+). 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 Ni2+ because of its obvious relevance to urease activation and on Zn2+ because this metal is known to bind to UreE (2, 8) and UreG (34, 35) accessory proteins. Inclusion of 100 μM NiCl2 or ZnCl2 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 NiCl2 or ZnCl2 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 (Bmax) of 2.1 ± 0.1 Ni2+ ions per protomer and a dissociation constant (Kd) of 33.8 ± 4.1 μM; however, significant binding of Ni2+ to MBP was observed over this metal ion concentration range. To correct for the nonspecific Ni2+ 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 Bmax of 1.6 ± 0.1 Ni2+ ions per UreD proteomer with a Kd of 24.3 ± 3.1 μM. Although the error ranges are quite small, we note that the greatest Ni2+ concentration tested was only 5-fold the apparent Kd, so the data do not approximate saturation. In contrast, the Zn2+ data (Fig. 1B) did approach saturation, and binding to MBP was less significant; thus, a Bmax of 4.1 ± 0.2 Zn2+ ions per proteomer with a Kd of 4.7 ± 0.7 μM was confidently established.

As an alternative approach to measure Ni2+ interactions with MBP-UreD, various concentrations of radiolabeled 65NiCl2 were used during equilibrium dialysis studies (Fig. 1C). Direct fitting of the data by assuming a single type of binding site with a maximum (Bmax) of 3.1 ± 0.1 Ni2+ ions per MBP-UreD proteomer and a Kd of 62.0 ± 5.8 μM; however, correction of these data for the Ni2+ bound to MBP provided a Bmax of 2.7 ± 0.1 Ni2+ ions per UreD proteomer and Kd of 50.0 ± 4.4 μM. These data include Ni2+ concentrations that more closely approach saturation than were obtained in the PAR experiments and are considered to be more reliable.

The use of 65Ni in equilibrium dialysis experiments also allowed for investigation of the competition between Ni2+ and Zn2+. MBP-UreD was dialyzed against various concentrations of 65NiCl2 with set ZnCl2 concentrations of either 12.5 μM or 125 μM (Fig. 1C). The data from the experiment containing 12.5 μM Zn2+ were corrected for nonspecific Ni2+ binding to...
MBP in the same manner as done with the Zn$^{2+}$-free sample. These data were fit to a competitive inhibition equation using $B_{\text{max}}$ and $K_d$ values as described above, providing a Zn$^{2+}$ $K_i$ of $5.0 \pm 0.3$ μM, which is in close agreement to the $K_i$ 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 $^{63}$NiCl$_2$ (Fig. 1D). 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 $^{63}$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 $^{63}$NiCl$_2$ and the indicated concentrations of ZnCl$_2$. Data were fit by using a competitive inhibition equation.

**Fig. 1.** Equilibrium dialysis of MBP-UreD and MBP with NiCl$_2$, $^{63}$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 $^{63}$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 $^{63}$NiCl$_2$ and the indicated concentrations of ZnCl$_2$. Data were fit by using a competitive inhibition equation.

**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-LacZα fusion, with
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). Analogue extract prepara-
tions from cells containing pEC013 exhibited a specific activity
of 56.2 ± 4.2 U mg⁻¹ of
protein, revealing a specific activity
for extracts of cells containing the
soluble cell extracts of
E. coli
BL21-Gold(DE3)
harboring the indicated plasmids

<table>
<thead>
<tr>
<th>Plasmid derivative(s) (description)</th>
<th>Specific activity (U mg⁻¹ of protein)</th>
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<tr>
<td>pKK17 (wild-type urease cluster)</td>
<td>171.2 ± 22.0</td>
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<tr>
<td>pEC0002 (malE-ureD) + pEC006</td>
<td>15.1 ± 2.4</td>
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<tr>
<td>(ΔureD-urease cluster)</td>
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<tr>
<td>pMal-c2x (malE-lacZu) + pEC006</td>
<td>&lt;0.1</td>
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<tr>
<td>(ΔureD-urease cluster)</td>
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<tr>
<td>pEC013 (urease cluster replacing ureD)</td>
<td>56.2 ± 4.2</td>
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Data are represented as the average ± standard deviation for triplicate biological samples.

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 of 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-LacZu 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 amylose resin (even-numbered lanes). The pulldown analysis of soluble extracts from cells cotransformed with pEC004 (encoding UreA, UreB, and UreC) confirmed 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 UreF/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-LacZu) 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.](http://jb.asm.org/Downloadedfrom)
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<sub>Str</sub> (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
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$_{so}$ 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$_{so}$ at 42°C, no increase in the affinity of MBP-UreD for UreG$_{so}$ 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 resin, 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 Ni$^{2+}$ or Zn$^{2+}$, 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 Ni$^{2+}$, the PAR and radioactive $^{65}$Ni methods provided different results, indicating 1.6 or 2.7 Ni$^{2+}$ 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 Ni$^{2+}$ concentrations and thus conclude that UreD binds approximately 2.5 Ni$^{2+}$ ions per protomer. A noninteger number of bound Ni$^{2+}$ ions can be explained by a binding site at the interface of two proteomes in the quaternary structure. The PAR-derived data indicate the ability to bind four Zn$^{2+}$ ions per protomer with a K$_{d}$ of 5 µM, in excellent agreement with the Zn$^{2+}$ K$_{d}$ measured in two studies examining competition with $^{65}$Ni$^{2+}$ binding. Thus, we conclude that Zn$^{2+}$ competes with 10-fold higher affinity for the Ni$^{2+}$ 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 Ni$^{2+}$ specificity could potentially be present in the UreABC-UreD complex. Furthermore, activation of urease apoprotein within the UreABC-UreDFG complex by Ni$^{2+}$, CO$_{2}$, GTP, and UreE takes place even in the presence of Ni$^{2+}$-chelating agents; this linking of nucleotide hydrolysis to metallocenter assembly indicates that the process cannot simply be viewed from a thermodynamic perspective. Our finding that UreD binds metal ions suggests its involvement in Ni$^{2+}$ 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 $\Delta$ureD-urease cluster or by using a single plasmid encoding a urease cluster with malE/ureD replacing wild-type ureD. Co-transformed 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 over-produced 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 ΔureD 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 Ni²⁺ 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 Ni²⁺ via an interaction with UreG (E). In vivo, urease activation requires CO₂ 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 Ni²⁺ 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 other. 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 vitro and, in a subset of these cases, in vivo 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.

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