JOURNAL OF BACTERIOLOGY, July 1997, p. 4081–4086 Vol. 179, No. 13
0021-9193/97/$04.00+0
Copyright © 1997, American Society for Microbiology

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

MARY BETH C. MONCRIEF† AND ROBERT P. HAUSINGER*

Departments of Biochemistry and Microbiology, Michigan State University, East Lansing, Michigan 28824

Received 19 November 1996/Accepted 21 April 1997

In vivo urease metallocenter assembly in *Klebsiella aerogenes* requires the presence of several accessory proteins (UreD, UreF, and UreG) and is further facilitated by UreE. In this study, UreG was isolated and shown to be a monomer with an M_r of 21,814 ± 20 based on gel filtration chromatography and mass spectrometric results. Although it contains a P-loop motif typically found in nucleotide-binding proteins, UreG did not bind or hydrolyze ATP or GTP, and it exhibited no affinity for ATP- and GTP-linked agarose resins. Site-directed mutagenesis of *ureG* allowed the substitution of Ala for Lys-20 or Thr-21 in the P-loop motif and resulted in the production of inactive urease in cells grown in the presence of nickel; hence, an intact P-loop may be essential for UreG to function in vivo. These mutant cells were unable to synthesize the UreD-UreF-UreG-urease apoprotein species that are thought to be the key urease activation complexes in the cell. An insoluble protein species containing UreD, UreF, and UreG (termed the DFG complex) was detected in cells carrying deletions in *ureE* and the urease structural genes. The DFG complex was solubilized in 0.5% Triton X-100 detergent, shown to bind to an ATP-linked agarose resin, and found to elute from the resin in the presence of Mg-ATP. In cells containing a UreG P-loop variant, the DFG complex was formed but did not bind to the nucleotide-linked resin. These results suggest that the UreG P-loop motif may be essential for nucleotide binding by the DFG complex and support the hypothesis that nucleotide hydrolysis is required for in vivo urease metallocenter assembly.

Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to yield ammonia and carbonic acid. The elevation of pH due to the ammonia generated by this enzyme has important ramifications for medicine. For example, urease is a virulence factor in pathogenic bacteria that cause gastric ulceration, urinary stone formation, pyelonephritis, and other human health-related problems (reviewed in reference 17). The best-characterized bacterial urease is that from *Klebsiella aerogenes*. Crystallographic analysis of this enzyme revealed that the three structural subunits (encoded by *ureA*, *ureB*, and *ureC*) associate as a trimer of trimers [(UreA-UreB-UreC)$_3$] containing one dinuclear nickel metallocenter per UreC (7). The two nickel ions are bridged by a carbamylated lysine and are separated by 3.5 Å. Ni-1 is tricoordinate, bound by two histidine ligands and the lysine carbamate. Ni-2 is pentacoordinate in which the carbamate, an aspartic acid, two histidines, and a solvent molecule serve as ligands.

In vivo activation of *K. aerogenes* urease involves the participation of four accessory proteins (UreD, UreE, UreF, and UreG) which are encoded by genes located in the same cluster (*ureDABCEFG*) as those encoding the enzyme subunits (13, 21). Although the precise function of these proteins in urease nickel metallocenter assembly has not been established, UreE has been shown to be a nickel-binding protein (14), and a series of UreD-urease (D-Apo) (24), UreD-UreF-urease (DF-Apo) (19), and UreD-UreF-UreG-urease (DFG-Apo) (25) apoprotein complexes has been identified. The auxiliary proteins within these complexes are thought to somehow alter the activation properties of the urease apoprotein (Apo) and, for the DFG-Apo complexes, allow the cell to obtain fully active enzyme. Apo, isolated from cells grown in the absence of nickel ion (12) or generated in accessory gene deletion mutants (13), has been partially activated in vitro and shown to require the presence of carbon dioxide (26). This finding can now be understood on the basis of structural studies demonstrating that the CO_2 is used to form the carbamylated lysine at the active site (7).

Of the four urease accessory proteins, UreG is the most highly conserved (18) and the only one of this group that exhibits clear sequence homology to other proteins. For example, UreG contains a P-loop motif (also called motif A [31] a less conserved or G-1 motif [1]; B motif or G-2, G-3, and G-4 motifs also are present as found in ATP- and especially GTP-binding proteins, where they function in nucleotide binding [30]). The presence of this putative nucleotide-binding site in UreG might be related to the in vivo energy requirement for urease activation (12). Besides the sequence similarity to various proteins based on the presence of the P-loop motif, the full-length UreG protein from *K. aerogenes* is approximately 25% identical to the sequence of the *Escherichia coli hypB* gene product (15). This gene is part of the hydrogenase pleiotropic operon required for activation of the three nickel-containing hydrogenases found in *E. coli*. HypB plays a role in nickel ion processing based on the demonstration that mutations in *hypB* can be complemented by addition of high levels of nickel ion to

* Corresponding author. Mailing address: 160 Giltner Hall, Department of Microbiology, Michigan State University, East Lansing, MI 48824-1101. Phone: (517) 353-9675. Fax: (517) 353-8957. E-mail: RHausing@msu.edu.

† Present address: Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4965.

TABLE 1. Plasmids used in this study

Plasmid	Genotype or construction	Reference
pKAU17	*K. aerogenes* urease gene cluster	21
pKAUD2	pKAU17 with overexpressed *ureD*	24
pKAUG-1	Contains only pKAUD2 *ureG*	23
pKAU17K20A	pKAU17 with *ureG* mutation	This study
pKAU17T21A	pKAU17 with *ureG* mutation	This study
pKAUD2K20A	pKAUD2 with *ureG* mutation	This study
pKAUD2T21A	pKAUD2 with *ureG* mutation	This study
pKAUD2F+	pKAUD2 with overexpressed *ureF*	19
pKAUD2F+Δ*ureA*Δ*ureB*Δ*ureC*Δ*ureE*	Deletion in urease structural subunit genes and *ureE*	This study
pKAUD2F+T21AΔ*ureA*Δ*ureB*Δ*ureC*Δ*ureE*	*ureG* mutation and deletions in urease structural subunit genes and *ureE*	This study

the growth medium (32). *E. coli* HypB has been purified and found to bind and hydrolyze GTP (16). These findings raise the possibility that UreG binds nucleotides and catalyzes nucleotide hydrolysis as part of its role in facilitating urease metallocenter assembly.

This study describes purification and characterization of UreG and provides evidence from site-directed mutagenesis studies that is consistent with the UreG P-loop motif being needed for in vivo metallocenter assembly. Furthermore, we show that a wild-type P-loop sequence appears to be required for formation of the DFG-Apo complexes. Finally, we demonstrate the presence of a protein species containing UreD, UreF, and UreG and termed the DFG complex. Partially purified DFG complex is shown to bind to a nucleotide-linked resin, whereas DFG complex obtained from a UreG P-loop variant lacks the ability to bind to this resin. These studies suggest the possibility that UreG, when present in the DFG complex, couples nucleotide utilization to urease metallocenter assembly.

MATERIALS AND METHODS

Bacterial strains and plasmid construction. All molecular biology methods followed the general methods outlined in reference 28. All plasmids (Table 1) were transformed into *E. coli* DH5α. For routine purification of UreG, plasmid pKAUG-1, containing only *ureG* (23), was used. This plasmid was constructed by isolating the 4.1-kb *Ksp*I-*Eco*RI fragment from pKAUD2 (24), forming blunt ends with Klenow enzyme, and ligating the ends together.

To test the necessity for the UreG P-loop motif, we created two *ureG* mutants in which the encoded proteins were altered so that Lys-20 and Thr-21 were changed to Ala (K20A and T21A, respectively). A 1.5-kb *Sal*I-*Kpn*I fragment of pKAU17 (22) was subcloned into M13mp18 and mutagenized by the method of Kunkel et al. (9). Uracil-containing single-stranded template DNA was prepared from *E. coli* CJ236 [*dut-1 ung-1 thi-1 relA1*/pCJ105(Cam^r F′)]. By using oligonucleotide primer 5′-TCGGCTCCGGTGCAACCGCTCTGC-3′ or 5′-GCTCCG GTAAAGCCGCTCTGCTG-3′ (the underlined bases represent changes from the wild-type sequence), the phage DNA was mutagenized, and the mutant phage were isolated in *E. coli* MV1193 (Δ[*lac-proAB*] *rpsL thi endA spcB15 hsdR4* Δ[*srl-recA*]*306*::Tn*10*[Tet^r] F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15). Mutants were identified by sequence analysis using Sequenase 2.0 (United States Biochemical, Cleveland, Ohio) and the single-strand DNA sequence method of Sanger et al. (29). The 840-bp *Avr*II-*Kpn*I fragments were subcloned back into pKAU17 to generate pKAU17K20A and pKAU17T21A, and the sequences were confirmed by double-strand DNA sequencing methods. The 2.4-kb *Bam*HI-*Kpn*I fragments of pKAU17K20A and pKAU17T21A were also subcloned into the 5.7-kb *Bam*HI-*Kpn*I fragment of pKAUD2, containing a mutated *ureD* promoter region to allow overexpression of this gene (24), to generate pKAUD2K20A and pKAUD2T21A.

Plasmids were also constructed with *ureG* or mutated *ureG* for study of the DFG complex. The construct pKAUD2F+Δ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* (containing only *ureD*, *ureF*, and *ureG*) was created by isolating the 5.4-kb *Afl*II-*Bln*I fragment of pKAUD2F+ (19), filling in the ends with Klenow enzyme, and religating the ends. Plasmid pKAUD2F+T21AΔ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* was constructed by isolating the 8.2-kb *Eco*RI fragment of pKAUD2T21A and the 740-bp *Xba*I-*Bam*HI pETF fragment (19) containing the overexpressed *ureF* gene, filling in the ends of each fragment with Klenow enzyme, and ligating the fragments together. This plasmid, pKAUD2F+T21A, was digested with *Afl*II and *Bln*I, the 5.4-kb fragment ends were filled in with Klenow enzyme, and the ends were ligated together.

Culture conditions. All cultures were grown at either 30 or 37°C to late stationary phase in 3 liters of Luria-Bertani (LB) broth supplemented with 100 μg of ampicillin per ml. *E. coli* DH5α cells containing plasmid pKAUG-1, pKAU17K20A, pKAU17T21A, pKAUD2K20A, or pKAUD2T21A were harvested by centrifugation and resuspended in 30 ml of PEB (20 mM phosphate [pH 7.4], 1 mM EDTA, 1 mM 2-mercaptoethanol) buffer. *E. coli* DH5α cells containing plasmid pKAUD2F+Δ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* or pKAUD2F+T21AΔ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* were harvested by centrifugation and resuspended in 30 ml of HMDG (25 mM HEPES [pH 7.4], 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10% glycerol) buffer.

Purification of UreG. *E. coli* DH5α cells carrying plasmid pKAUG-1 expressed *ureG* such that UreG was the most predominant cellular protein. Harvested cells were resuspended in PEB buffer, supplemented with 1 mM phenylmethylsulfonyl fluoride, and disrupted by passage through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in^2 (1 lb/in^2 = 6.89 kPa). Cell extracts, obtained after centrifugation at 100,000 × *g* for 45 min at 4°C, were applied to a DEAE-Sepharose column (2.5 by 19 cm; Pharmacia Biotech, Piscataway, N.J.) that had been equilibrated with PEB, and proteins were eluted by using a 400-ml linear salt gradient to 0.5 M KCl in the same buffer. Fractions containing UreG (eluting at approximately 0.1 to 0.2 M KCl) were pooled, dialyzed against PEB buffer containing 20% glycerol, and applied to a Mono Q HR 10/10 (Pharmacia Biotech) column equilibrated with the same buffer containing 20% glycerol. Flowthrough fractions containing UreG were pooled, dialyzed against PEB buffer to remove the glycerol, and applied to the reequilibrated Mono Q column in PEB buffer. A linear salt gradient to a concentration of 1 M KCl in PEB was used to elute UreG over a wide range at approximately 0.1 to 0.4 M KCl. Samples containing UreG were pooled, concentrated, dialyzed against 25 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 M KCl, and subjected to Superose 12 chromatography in this same eluent. For subunit molecular weight determination, matrix-assisted laser desorption ionization time-of-flight mass spectrometry was used (34). A 0.6-μg sample of purified UreG was loaded onto Zetabind nylon membrane, dried, washed with water, and mixed with 1 μl of sinapinic acid. The sample was run on a Vestec LaserTec Research time-of-flight mass spectrometer (Vestec, Houston, Tex.) equipped with a nitrogen laser (model VSL-337ND; Laser Science, Newton, Mass.).

Partial purification of DFG complex. *E. coli* DH5α cells carrying plasmid pKAUD2F+Δ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* or pKAUD2F+T21AΔ*ureA*Δ*ureB*Δ*ureC*Δ*ureE* were found to express *ureD*, *ureF*, and *ureG* (or mutated *ureG* in the latter case). Three-liter cultures were resuspended in HMDG buffer, supplemented with 1 mM phenylmethylsulfonyl fluoride, and disrupted by passage through a French pressure cell at 18,000 lb/in^2. The samples were centrifuged at 27,000 × *g* for 45 min at 4°C, and the supernatants were removed. The pellets were washed with 20 ml of HMDG buffer and recentrifuged as stated above, and the supernatants were discarded. The pellets were suspended in 20 ml of HMDG buffer containing 0.5% (wt/vol) Triton X-100 or Tween 20 and stored at 4°C for 17 h. After centrifugation at 27,000 × *g* for 45 min, the supernatant fractions containing the majority of the native and variant DFG complexes were collected. The solubilized protein samples were applied to DEAE-Sepharose columns (2.5 by 19 cm) that had been equilibrated with HMDG buffer containing 0.5% appropriate detergent, and the proteins were eluted by using a 400-ml linear salt gradient to 0.5 M KCl in the same buffer. Fractions containing the wild-type or altered DFG complexes were pooled, dialyzed against HMDG buffer with 0.5% detergent, and added to columns of Extracti-Gel D detergent-removing gel (1.7 by 7 cm; Pierce, Rockford, Ill.). The flowthrough fractions containing native or variant DFG complexes were pooled and immediately applied to ribose hydroxyl-linked GDP-, ribose hydroxyl-linked GTP-, or N-6-linked ATP-agarose resins (Sigma Chemical, St. Louis, Mo.) that had been equilibrated in detergent-free HMDG buffer. Columns were washed with HMDG buffer, and proteins were eluted with 10 mM ATP or 1 M KCl in HMDG buffer.

Preparation of polyclonal antibodies against UreG protein. Antibodies were generated in a New Zealand rabbit by injecting 200 μl (2.5 mg/ml) of purified UreG in phosphate-buffered saline that was emulsified with the same volume of

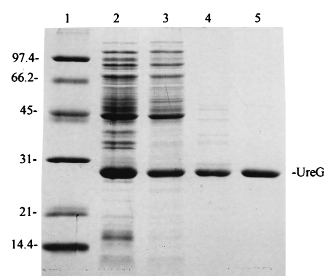

FIG. 1. Purification of UreG as monitored by SDS-polyacrylamide gel electrophoresis. Lanes: 1, molecular weight markers (phosphorylase *b*, M_r 97,400; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,000; and lysozyme, M_r 14,400); 2, *E. coli* DH5α(pKAUG-1) cell extracts; lane 3, DEAE-Sepharose pool; lane 4, flow-through pool from the first Mono Q pool chromatographic step using buffer containing glycerol; lane 5, second Mono Q pool using glycerol-free buffer.

TiterMax adjuvant (Vaxcel, Norcross, Ga.). The rabbit was boosted after 23 days, and after 97 days the immunoglobulin G fraction was purified from the serum by using an E-Z-Sep kit (Pharmacia Biotech). Antibodies were titrated by using standard dot blot (2) and immunoblotting (5) techniques.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (10) and included 4.5 and 12.5% polyacrylamide stacking and running gels. Nondenaturing gels used the same buffers without SDS and consisted of 3 and 6% polyacrylamide stacking and running gels. Denaturing gels were stained with Coomassie brilliant blue, while nondenaturing gels were electroblotted onto nitrocellulose, probed with either polyclonal anti-*K. aerogenes* urease antibodies (22) or anti-*K. aerogenes* UreG antibodies, and visualized by using goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugates (Sigma). When testing for the presence of biotin, we probed blots with a 1:5,000 dilution of ExtrAvidin-alkaline phosphatase (Sigma). Cytochrome *c*-biotin-labeled protein (Sigma) was used as a positive control. The presence of alkaline phosphatase was detected by using 3.3 mg of 4-bromo-4-chloro-3-indolyl phosphate and 0.165 mg of Nitro Blue Tetrazolium (Sigma) per ml. The band intensities of Coomassie blue-stained gels were measured with an AMBIS (San Diego, Calif.) gel scanner. For calculation of the ratios of UreD, UreF, and UreG, M_r values of 29,300, 27,000, and 21,800 were used.

Urease activity assays. Urease activity in cell extracts was measured by quantitating the rate of ammonia release from urea by formation of indolphenol, which was monitored at 625 nm (33). One unit of urease activity was defined as the amount of enzyme required to hydrolyze 1 μmol of urea per min at 37°C. The standard assay buffer consisted of 25 mM HEPES (pH 7.75), 0.5 mM EDTA, and 50 mM urea. Protein concentrations were determined by using a commercial assay (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard. Activation of urease apoprotein in cell extracts involved incubation of samples with 500 μM or 1 mM $NiCl_2$ in PEB or 25 mM HEPES (pH 7.75) buffer for up to 20 h at 37°C, followed by analysis using the standard urease assay.

Equilibrium dialysis determination of nucleotide binding. Nucleotide binding was determined by equilibrium dialysis measurements using (i) 50 mM sodium phosphate (pH 7.2) buffer containing 1 mM $MgCl_2$, 0.5% NaCl, and the desired nucleotide, (ii) 50 mM Tris (pH 7.5) buffer containing 100 mM NaCl, 5 mM $MgCl_2$, 0.09 mM dithiothreitol, 0.09 mM EDTA, and the desired nucleotide, or (iii) HMDG buffer containing 0.5% Triton X-100, 100 mM NaCl, and the desired nucleotide. [^{14}C]ATP (44 mCi/mmol; ICN Pharmaceuticals, Costa Mesa, Calif.) or [^{14}C]GTP (13.5 Ci/mmol; ICN) was diluted with various concentrations of unlabeled ATP or GTP, respectively. The protein concentration was 2 or 6 μM. Free ligand equilibrium was attained in an equilibrium dialyzer (Hoefer Scientific, San Francisco, Calif.), using Spectrapor 2 dialysis membranes at 4°C for approximately 14 h. Samples of 100 μl were removed from each compartment and measured for radioactivity with a Beckman LS7000 liquid scintillation system and 10 ml of Safety Solve scintillation fluid (Research Products International Corporation, Mount Prospect, Ill.).

Chromatographic measurement of nucleotide hydrolysis. For quantitating the rates of hydrolysis of ATP, 0.033 μM UreG, 0.6 mM ATP, and 10 mM $MgCl_2$ in 0.1 M potassium phosphate buffer (pH 7.3) were incubated at room temperature for 1 to 13 h. Reactions were quenched with 2 volumes of column equilibration buffer, and 1/10 of the sample was chromatographed by using a C_{18} reverse-phase HR 5/5 column (Pharmacia Biotech) equilibrated with 19% acetonitrile, 30 mM potassium phosphate, and 10 mM tetrabutylammonium phosphate (pH 2.65) (3). GTP hydrolysis was measured by diluting [^{3}H]GTP with unlabeled GTP to a final concentration of 5 μM and incubating it at room temperature with 1.2 μM UreG, 10 mM $MgCl_2$, and 50 mM Tris (pH 8.0). At various time intervals, samples were removed and chromatographed on a Mono Q HR 10/10 column equilibrated with 20 mM phosphate (pH 7.3). Radioactivity in the resulting fractions was detected by using a Beckman LS7000 liquid scintillation system.

RESULTS

UreG characterization. UreG was synthesized as the most abundant protein in cell extracts of *E. coli* DH5α(pKAUG-1). The soluble protein was purified by using a combination of DEAE-Sepharose, Mono Q, and Superose 12 chromatographies (Fig. 1). From a 1-liter culture, about 10 to 15 mg of UreG was recovered. Native UreG was well behaved during gel filtration chromatography, and the size was estimated to be approximately 19.1 kDa. A more precise size estimate of the UreG subunit was determined by using matrix-assisted laser desorption ionization time-of-flight electrospray-mass spectrometry. UreG was shown to possess an M_r of 21,814 ± 20, which agrees well with the expected value (21,812) for protein lacking the N-terminal methionine based on the translated DNA sequence (21).

To begin to characterize the function of UreG, its ability to bind or hydrolyze nucleotides and its biotin content were examined. The presence of a P-loop motif in the UreG sequence suggested the possibility of nucleotide binding by this protein; however, no nucleotide was associated with isolated protein (4), neither ATP nor GTP binding was detected for purified UreG by using equilibrium dialysis methods, no interaction with ATP- or GTP-linked resins was observed, and no nucleotide hydrolysis was found by using chromatographic procedures. Similarly, the requirement for CO_2 in urease activation implicated the presence of biotin or other carboxyl group carrier in one of the accessory proteins; however, UreG possessed no covalently associated cofactor (shown by mass spectrometry studies) and did not contain biotin when further analyzed by Western blot (immunoblot) analysis using avidin-linked alkaline phosphatase (data not shown).

Site-directed mutagenesis of UreG. To assess the importance of the conserved (18) P-loop motif in UreG, site-directed mutagenesis methods were used to prepare constructs that formed altered UreG species. Since the Lys and Thr residues in the motif are known to be essential for nucleotide binding in a variety of other proteins (30), mutated genes encoding K20A and T21A forms of UreG were created and subcloned back into pKAU17 and pKAUD2. The UreG P-loop variants did not affect formation of D-Apo or DF-Apo complexes, but they prevented formation of DFG-Apo complexes when *E. coli* cells

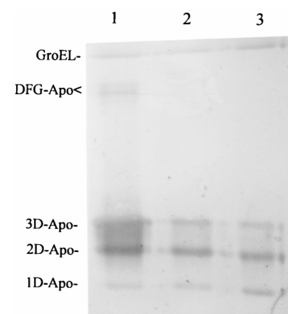

FIG. 2. Effects of alterations to the UreG P-loop sequence on formation of urease apoprotein complexes. *E. coli* DH5α cells carrying pKAUD2 (encoding wild-type UreG) or mutants of this plasmid (encoding UreG P-loop variants) were grown in the absence of added nickel ions, extracts were analyzed on a nondenaturing polyacrylamide gel, and the gel was probed with anti-*K. aerogenes* urease antibodies. Lanes: 1, 40 μg of cell extracts from *E. coli* DH5α(pKAUD2); 2, 40 μg of cell extracts from *E. coli* DH5α(pKAUD2K20A); 3, 40 μg of cell extracts from *E. coli* DH5α(pKAUD2T21A). The positions of the comigrating D-Apo and DF-Apo complexes (containing one, two, or three UreD or UreD plus UreF molecules per urease apoprotein trimer [19, 24]) and the DFG-Apo complexes (25) are indicated. The anti-urease antibodies also cross-react with GroEL that is present in these extracts (19).

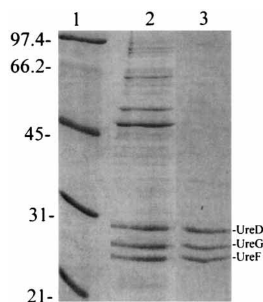

FIG. 3. Analysis of DFG complex binding to an ATP-linked agarose resin as analyzed by SDS-polyacrylamide gel electrophoresis. Lanes: 1, molecular weight markers as in Fig. 1; 2, Extracti-Gel DFG pool; 3, sample eluted from ATP-linked agarose resin by 1 M KCl in HMDG buffer.

containing pKAUD2K20A or pKAUD2T21A were grown in the absence of nickel (Fig. 2). Moreover, the P-loop-specific alterations to pKAU17 and pKAUD2 resulted in the absence of in vivo urease activity when cells were grown in LB containing nickel. By contrast, the various urease apoprotein species in extracts of these cells were competent for in vitro activation. For example, when cell extracts of *E. coli* DH5α(pKAU17K20A or pKAU17T21A) were incubated with 1 mM $NiCl_2$ for 20 h, the urease specific activities reached 38 and 44 U/mg, approaching the value of 49 U/mg observed for extracts of *E. coli* DH5α (pKAU17) grown in the absence of nickel ions and treated similarly. Activation of urease apoprotein in extracts of *E. coli* DH5α(pKAUD2K20A or pKAUD2T21A) was also observed, but the measured level of activity (reaching approximately 10 U/mg) was less than that obtained for extracts of *E. coli* DH5α (pKAUD2) grown in the absence of nickel ions and activated similarly (approximately 50 U/mg). This difference could arise from an inability to form DFG-Apo complex in the cells with mutated *ureG*.

Identification, partial purification, and characterization of the DFG complex. When subcloned independently from other urease genes, *ureD* and *ureF* were expressed as insoluble proteins in the cell (unpublished observations), whereas *ureG* expression led to soluble protein (described above). Using *E. coli* DH5α(pKAUD2F+Δ*ureA*Δ*ureB*Δ*ureC*Δ*ureE*), *ureD*, *ureF*, and *ureG* were cooverexpressed in the absence of the urease structural genes. The UreD and UreF peptides were found to be located in the insoluble cell fraction of disrupted cells. In addition, nearly all of the UreG protein was present as an insoluble species, as shown by the near absence of UreG in cell extracts that were examined by Coomassie blue staining of SDS-polyacrylamide gels (data not shown). This result was confirmed by Western blot analysis of cell extracts using anti-UreG antibodies. A variable portion of UreG was solubilized by low concentrations of nonionic detergents, and it appeared to cosolubilize as a complex with UreD and UreF. This heterotrimeric accessory protein complex (DFG complex) was partially purified by using DEAE-Sepharose chromatography. Most of the detergent was removed by passage of the sample through Extracti-Gel D resin; however, the detergent-free complex was unstable and UreD precipitated out of solution in less than 1 week. Based on gel scanning analysis of the freshly prepared pool, the DFG complex consisted of 0.85 UreD and 0.82 UreF per UreG. The detergent-free DFG pool was added to various nucleotide-linked agarose resins to test for nucleotide-binding capabilities. The DFG complex did not bind to GDP- or GTP-linked agarose resins, but binding was observed to an ATP-linked agarose column. The bound complex eluted upon addition of 10 mM ATP or 1 M KCl (Fig. 3) to the column in HMDG buffer. The DFG complex did not bind to the resin if the DEAE-Sepharose column step was omitted, consistent with the removal of a competing ligand during ion-exchange chromatography. In addition, binding to the nucleotide-linked resin was prevented by the presence of detergent. Finally, no interaction with the ATP-agarose column was observed for the altered DFG complex that had been purified from *E. coli* DH5α(pKAUD2F+21AΔ*ureA*Δ*ureB*Δ*ureC*Δ*ureE*). These cells, containing a UreG P-loop variant, were able to form the DFG complex (data not shown), and the complex chromatographed in a typical manner on DEAE-Sepharose resin; however, the UreG P-loop variant complex exhibited aberrant interactions with the ATP-linked agarose resin.

The DFG complex was further studied for its ability to bind ATP and for its biotin content. Using equilibrium dialysis methods, we detected no nucleotide binding for a DEAE-Sepharose pool of the DFG complex in the presence or absence of Triton X-100. Lack of binding for the detergent-containing sample was not unexpected since nonionic detergents also interfered with binding to the ATP-linked agarose resin. The lack of binding to the detergent-free sample may be related to the instability of DFG complex under these conditions, although equilibrium dialysis studies were performed immediately after detergent removal. As in the case of purified UreG, the DFG complex did not contain biotin as determined by Western blot (immunoblot) analysis using avidin-linked alkaline phosphatase (data not shown).

DISCUSSION

Our results include the purification and characterization of UreG, evidence that a likely nucleotide-binding region in UreG is critical to urease metallocenter assembly, and identification of a UreD-UreF-UreG accessory protein complex that may be important for urease maturation. The findings are discussed in more detail below.

UreG purification and partial characterization. *ureG* is expressed at high levels in *E. coli* cells carrying a plasmid with the wild-type *K. aerogenes* urease gene cluster. Since this protein is required for functional nickel insertion into the urease active site and contains a sequence suggestive of function (i.e., a P-loop motif), its purification and characterization was a logical step in examining metallocenter assembly. Prior studies (11) had used immunogold electron microscopic methods to provide evidence for a cytoplasmic location of UreG. The purification described here is consistent with this localization and yields a soluble, monomeric 21.8-kDa protein. Despite possessing a P-loop motif, isolated UreG did not possess bound nucleotide, it failed to bind or hydrolyze added ATP or GTP, and it did not bind to ATP- or GTP-linked agarose resins. Further characterization of purified UreG focused on its possible involvement in providing CO_2 for carbamylation of Lys-217, which then serves as a nickel ligand. Specifically, the presence of biotin, a carrier of CO_2 in many carboxylases (reviewed in reference 8), was shown to be absent in purified UreG from *E. coli* cells containing pKAUG-1. This result does not rule out a role for UreG in CO_2 delivery, as other biotin-free carboxylases are known including ribulosebisphosphate carboxylase/oxygenase (6) and the pyrimidine biosynthesis enzyme PurK (20).

The requirement of the P-loop motif for UreG function. Although it contains a P-loop motif that is commonly found in nucleotide-binding proteins (30), nucleotide binding and hydrolysis by isolated UreG were not detected. This result contrasts with the situation found in the *E. coli* HypB protein, to

which UreG exhibits 25% identity. *E. coli* HypB binds and hydrolyzes GTP (albeit very slowly [$k_{cat} = 0.17\ min^{-1}$]), likely related to its suggested role in nickel ion processing for hydrogenase activation (16). Significantly, the HypB homolog from *Rhizobium leguminosarum* does not bind or hydrolyze GTP or ATP (27) even though its proposed nucleotide-binding domain is conserved. For *R. leguminosarum* HypB and *K. aerogenes* UreG, it is possible that additional accessory proteins must be present for nucleotide binding and hydrolysis to be observed. For example, many GTPases exhibit their activities only when present in heterotrimeric complexes, not when present as the isolated proteins (1). An appealing model is that the UreG P-loop motif is involved in the previously reported (12) in vivo energy requirement for urease activation only when UreG is present in an appropriate complex.

To further assess the importance of the UreG P-loop motif, site-directed mutagenesis was performed to replace with Ala either of two conserved residues (Lys-20 and Thr-21) that could be expected to bind the Mg-nucleotide complex. *E. coli* cells containing the intact *K. aerogenes* urease gene cluster except for these mutations were grown in the presence of nickel and shown to lack urease activity. The cell extracts were able to be activated in vitro, presumably from Apo, D-Apo, or DF-Apo complexes which are unaffected in the UreG P-loop variants. By immunoblot analysis, DFG-Apo complexes were undetectable in soluble extracts of the P-loop variants. In summary, these data indicate that the P-loop motif in UreG is likely to be essential for in vivo incorporation of nickel into the urease active site, and this sequence appears to be important for DFG-Apo formation or stability.

The DFG complex. A complex containing UreD, UreF, and UreG was detected in *E. coli* cells carrying the urease gene cluster with deletions in *ureE* and the urease subunit genes. This complex was localized to the insoluble fraction after cell lysis and centrifugation but was solubilized upon addition of low concentrations of nonionic detergents. We propose that trace levels of this complex may be present in soluble form in cells containing the wild-type gene cluster where *ureD* and *ureF* are not highly expressed. Following DEAE-Sepharose and Extracti-Gel D chromatographies, DFG was shown to bind to an ATP-linked agarose resin. A P-loop variant in UreG did not affect formation of the DFG complex, indicating that the wild-type sequence is not required for UreD, UreF, and UreG association. In contrast to the DFG complex comprised of peptides with wild-type sequences, the complex containing an altered UreG P-loop sequence did not bind to the ATP-linked agarose resin. These data support the idea that the UreG P-loop sequence is needed for binding of nucleotides to the DFG complex but is not essential for complex formation. We note, however, that binding of radiolabeled ATP to DFG complex was not observed by equilibrium dialysis methods. As with purified UreG, the DFG complex did not contain biotin.

Although the DFG complex was observed under highly artificial conditions (i.e., *ureD* and *ureF* were overexpressed compared to their normal expression levels, and the three peptides were synthesized in the absence of the urease subunits), these results could provide significant clues related to the in vivo urease metallocenter assembly mechanism. For example, DFG-Apo, the urease apoprotein complex that has been proposed to be the key species needed for in vivo activation (25), perhaps could be formed by association of Apo with trace levels of DFG complex, rather than by a stepwise assembly of Apo to D-Apo (24) to DF-Apo (19) to DFG-Apo (25) species.

ACKNOWLEDGMENT

This work was supported by the National Institutes of Diabetes and Digestive Kidney Diseases (grant DK45686).

REFERENCES

1. **Bourne, H. R., D. A. Sanders, and F. McCormick.** 1991. The GTPase superfamily: conserved structure and molecular mechanism. Nature **349:**117–127.
2. **Cleveland, P. H., M. G. Wickham, M. H. Goldbaum, A. F. Ryan, and D. M. Worthen.** 1981. Rapid and efficient immobilization of soluble and small particulate antigens for solid phase radioimmunoassay. J. Immunoassay **2:** 117–136.
3. **Coleman, J. P., W. B. White, B. Egestad, J. Sjovall, and P. B. Hylemon.** 1987. Biosynthesis of a novel bile acid nucleotide and mechanism of 7α-dehydroxylation by an intestinal *Eubacterium* species. J. Biol. Chem. **262:**4701–4707.
4. **Colpas, G. J., and R. P. Hausinger.** Unpublished data.
5. **Engvall, E., and P. J. Perlmann.** 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J. Immunol. **109:**129–135.
6. **Hartman, F. C., and M. R. Harpel.** 1994. Structure, function, regulation, and assembly of D-ribulose-1,5-bisphosphate carboxylase/oxygenase. Annu. Rev. Biochem. **63:**197–234.
7. **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.
8. **Knowles, J. R.** 1989. The mechanism of biotin-dependent enzymes. Annu. Rev. Biochem. **58:**195–221.
9. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
11. **Lee, M. H.** 1992. Thesis. Michigan State University, East Lansing, Mich.
12. **Lee, M. H., S. B. Mulrooney, and R. P. Hausinger.** 1990. Purification, characterization, and in vivo reconstitution of *Klebsiella aerogenes* urease apoenzyme. J. Bacteriol. **172:**4427–4431.
13. **Lee, M. H., S. B. Mulrooney, M. J. Renner, Y. Markowicz, and R. Hausinger.** 1992. 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.
14. **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.
15. **Lutz, A., A. Jacobi, V. Schlensog, R. Böhm, G. Sawers, and A. Böck.** 1991. Molecular characterization of an operon (*hyp*) necessary for the activity of the three hydrogenase isoenzymes in *Escherichia coli*. Mol. Microbiol. **5:**123–135.
16. **Maier, T., A. Jacobi, M. Sauter, and A. Böck.** 1993. The product of the *hypB* gene, which is required for nickel incorporation into hydrogenases, is a novel guanine nucleotide-binding protein. J. Bacteriol. **175:**630–635.
17. **Mobley, H. L. T., M. D. Island, and R. P. Hausinger.** 1995. Molecular biology of microbial ureases. Microbiol. Rev. **59:**451–480.
18. **Moncrief, M. B. C., and R. P. Hausinger.** 1996. Nickel incorporation into urease, p. 151–171. *In* R. P. Hausinger, G. L. Eichhorn, and L. G. Marzilli (ed.), Mechanisms of metallocenter assembly. VCH Publishers, New York, N.Y.
19. **Moncrief, M. B. C., and R. P. Hausinger.** 1996. Purification and activation properties of UreD-UreF-urease apoprotein complexes. J. Bacteriol. **178:** 5417–5421.
20. **Mueller, E. J., E. Meyer, J. Rudolph, V. J. Davisson, and J. Stubbe.** 1994. N^5-carboxyaminoimidazole ribonucleotide: evidence for a new intermediate and two new enzymatic activities in the *do novo* purine biosynthetic pathway of *Escherichia coli*. Biochemistry **33:**2269–2278.
21. **Mulrooney, S. B., and R. P. Hausinger.** 1990. Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. J. Bacteriol. **172:**5837–5843.
22. **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.
23. **Park, I.-S.** 1994. Thesis. Michigan State University, East Lansing, Mich.
24. **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. USA **91:**3233–3237.
25. **Park, I.-S., and R. P. Hausinger.** 1995. Evidence for the presence of urease apoprotein complexes containing UreD, UreF, and UreG in cells that are competent for in vivo enzyme activation. J. Bacteriol. **177:**1947–1951.
26. **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.
27. **Rey, L., J. Imperial, J.-M. Palacios, and T. Ruiz-Argueso.** 1994. Purification of *Rhizobium leguminosarum* HypB, a nickel-binding protein required for

hydrogenase synthesis. J. Bacteriol. **176:**6066–6073.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Saraste, M., P. R. Sibbald, and A. Wittinghofer.** 1990. The P-loop—a common motif in ATP- and GTP-binding proteins. Trends Biochem. Sci. **15:**430–434.
31. **Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay.** 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide fold. EMBO J. **1:**945–951.
32. **Waugh, R., and D. H. Boxer.** 1986. Pleiotropic hydrogenase mutants of *Escherichia coli* K-12: growth in the presence of nickel can restore hydrogenase activity. Biochimie **68:**157–166.
33. **Weatherburn, M. W.** 1967. Phenol-hypochlorite reaction for determination of ammonia. Anal. Chem. **39:**971–974.
34. **Zaluzec, E. J., D. A. Gage, and J. J. Watson.** 1994. Quantitative assessment of cysteine and cystine in peptides and proteins following organomercurial derivatization and analysis by matrix-assisted laser desorption ionization mass spectrometry. J. Am. Soc. Mass Spectrom. **5:**359–366.