응용약물학회지, The Journal of Applied Pharmacology 3, 311-315(1995)

Purification, Characterization and Cellular Localization of *Klebsiella aerogenes* UreG Protein

Mann Hyung LEE*

College of Pharmacy, Catholic University of Taegu-Hyosung, Kyongsan, Kyongbook, Korea

(Received November 22, 1995; accepted December 9, 1995)

Abstract—The *K. aerogenes* ureG gene product was previously shown to facilitate assembly of the urease metallocenter (Lee, M. H., Mulrooney, S. B., Renner, M. J., Markowicz, Y., and Hausinger, R. P. (1992) *J. Bacteriol.* 174, 4324-4330). UreG protein has now been purified and characterized. Although the protein is predicted to possess a putative NTP-binding P-loop motif, equilibrium dialysis studies showed negative results. Immunogold electron microscopic studies using polyclonal antibodies directed against UreG protein confirm that UreG is located in the cytoplasm as predicted in the DNA sequence.

Keywords □ urease, ureG, *K. aerogenes*

Urease (EC 3.5.1.5), a nickel-containing enzyme found in certain plants and many microorganisms, hydrolyzes urea to yield ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid (Andrews *et al.*, 1988; Mobley *et al.*, 1995). In addition to being important in nitrogen metabolism, the enzyme has been implicated as a bacterial virulence factor in various human and animal diseases (reviewed by Mobley *et al.*, 1995). The most extensively characterized microbial urease is that from the Gram-negative enteric bacterium, *Klebsiella aerogenes* (a non-nitrogen-fixing *K. pneumoniae*). The urease enzyme possesses three different subunits [M_rs=60,304 (α), 11,695 (β), and 11, 086 (γ) (Mulrooney and Hausinger, 1990)] where each catalytic unit is comprised of subunits in an $\alpha\beta\gamma$ stoichiometry and contains a bi-nickel active site (Todd and Hausinger, 1987, 1989, Jabri *et al.*, 1995).

DNA sequence analysis revealed the presence of several additional genes that are part of the *K. aerogenes* urease gene cluster. The three urease structural genes (*ureA, ureB,* and *ureC*) are immediately preceded by the *ureD* gene and followed by the *ureE, ureF,* and *ureG* genes (Mulrooney and Hausinger, 1990; Lee *et al.*, 1992). These nonsubunit auxiliary genes have been shown to be required for urease metallocenter assembly. Urease properties were examined in recombinant *Escherichia coli* cells containing plasmids with the intact *K. aerogenes* urease gene cluster or deletion mutants in each of the *ureD, ureE, ureF,* and *ureG* genes (Lee *et al.*, 1992). In the deletions involving *ureD, ureF,* and *ureG,* the urease protein is synthesized in an inactive form and was shown to be devoid of nickel, whereas mutants in *ureE* possess a reduced urease activity and the nickel content of the purified urease is correspondingly reduced. Each of the four genes appear to function via a *trans*-acting factor. Although specific functions have not been identified for the four accessory proteins that are required for nickel incorporation into urease, it was demonstrated that UreE is a nickelbinding protein and is believed to act as a nickel-donor during nickel metallocenter assembley (Lee *et al.*, 19 93). And recently Park *et al.* (1995) showed that soluble UreD can bind to urease apoprotein and this UreD-urease apoprotein complex is competent for activation upon addition of nickel. These authors speculated that UreD serves as a urease-specific chaperone protein that facilitates proper assembly of the metallocenter.

The roles of UreF and UreG peptides remain unknown. Predicted amino acid sequence of the UreG contains a P-loop motif (Saraste *et al.*, 1990) that is found in a variety of ATP and GTPbinding proteins. Furthermore, an energy dependence for *in vivo* nickel ion incorporation was observed (Lee *et al.*, 1990).

In this study, the UreG protein has been purified using combinationations of chromatographic resins, native molecular weight has been determined and cellu-

* To whom correspondence should be addressed.

lar location has been examined using immunogold-electron microscopic method.

Materials and Methods

Bacterial strains and growth conditions

K. aerogenes CG253 was transformed with plasmid pKAU19 (Mulrooney *et al.*, 1989) and *E. coli* DH5 was transformed with pKAU17 (Mulrooney *et al.*, 1989) or pKAU17ΔureG1, a *ureG* deletion mutant (Lee *et al.*, 1992). Recombinant *K. aerogenes* cells were grown at 37℃ in MOPS (3-(*N*-morpholino)propanesulfonic acid) glutamine medium (Mulrooney *et al.*, 1989) containing 100 mM nickel chloride and chloramphenicol (30 μg /ml). Recombinant *E. coli* cells were grown at 37℃ in LB medium containing 1 mM nickel chloride and ampicillin (50 mg/ml) as previously described (Lee *et al.*, 1992).

Purification of UreG protein

Cultures (3 L) of *K. aerogenes* CG253(pKAU19) or *E. coli* DH5 (pKAU17) were grown to late exponential phase (optical density at 600 nm=3.5) and harvested by centrifugation. The cells were washed twice with PEB (20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.2)) buffer, resuspended in an equal volume of PEB buffer containing 0.5 mM phenyl methylsulfonyl fluoride, disrupted by three passages through a French pressure cell (American Instrument Co., Silver Spring, MD) at 18,000 lb/in^2, and centrifuged at 100,000×g for 90 min at 4℃. The cell extracts were chromatographed on a DEAE-Sepharose column (2.5 by 15 cm) at 4℃, in the same buffer and eluted with a 400 mllinear salt gradient to 1 M KCl. UreG eluted from the column at approximately 0.35 M KCl. The pooled sample was applied to a phenyl-Sepharose column (2.5 by 17 cm), which had been equilibrated with 2 M KCl and eluted with 0 M KCl-PEB. Pooled fractions were desalted and concentrated by using an Amicon pressure filtration stirred cell with a YM-10 ultrafiltration membrane in PEB buffer (pH 6.9), then further purified on a FPLC by using a Superose-12 (1 by 30 cm) and Mono-Q (0.5 by 5 cm) columns. All resins and columns were purchased from Pharmacia. The presence of UreG protein in column fractions was assessed by polyacrylamide gel electrophoresis (Fig. 1).

Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (1970) and included either a 12 or 15% polyacrylamide running gel or a 10 to 15% polyacrylamide gradient running gel with a 4.5% polyacrylamide stacking gel. Gels were stained with Coomassie brilliant blue R-250.

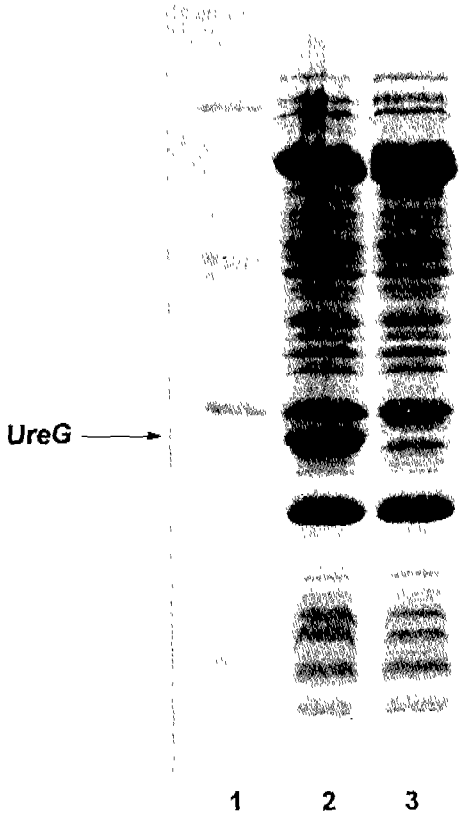

Fig. 1. SDS-polyacrylamide gel electrophoresis of UreG. Cell extracts from *E. coli* DH5 (pKAU17) (lane 2) and *E. coli* (pKAU17DureG1) (lane 3) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10~15% gradient gel, followed by Coomassie blue staining. Molecular weight markers(lane 1) were phosphorylase b, M_r=92,500; bovine serum albumin, M_r=66,200; ovalbumin, M_r=45,000; carbonic anhydrase, M_r=31,000; and soybean trypsin inhibitor, M_r=21,500.

Cellular localization by immunogold electron microscopy

Antibodies directed against UreG protein were generated in a white, female, New Zealand rabbit by injecting 200 μl (2.5 mg/ml) of homogeneous protein in phosphate buffered saline emulsified with the same volume TiterMax adjuvant (CytRx corporation, Norcross, GA). The rabbit was boosted after 28 days, and after an additional 22 days, the IgG fraction was purified from the serum (McKinney and Parkinson, 1987). Antibodies were titrated by using standard dot blot (Cleveland *et al.*, 1981) and ELISA methods (Engvall and Perlmann, 1972). For immunogold detection, wild-type *K. aerogenes*, *K. aerogenes* (pKAU19), *E. coli* DH5 (pKAU17), and *E. coli* DH5 (pKAU17ΔureG1) were grown to stationary phase in LB medium supplemented with 1 mM nickel chloride. After centrifugation,

the cells were washed once in 10 mM potassium phosphate, 1 mM EDTA (pH 7.0), and fixed in 0.1 M potassium phosphate (pH 7.2) containing 1% (v/v) glutaraldehyde for 30~60 min at room temperature. The fixed cells were resuspended in 1% (w/v) Noble agar, dehydrated in ethanol, and embedded in Lowicryl K4M (Armbuster *et al.*, 1982). Polymerization was carried out for 2 days at 6℃ under UV irradiation. Thin sections were cut by using an LKB Ultratome III microtome and placed on Butvar B-98-coated nickel grids. Sections were floated first on a drop of TBST (Trisbuffered saline, pH 7.4, with 0.05% (v/v) Tween 20) for 5 min and transferred to 1 or 3% (w/v) bovine serum albumin in TBST for 15 min in order to block nonspecific binding. The samples were transferred to solutions containing anti-UreE IgG (200 μg/ml) in TBST for 1 hr, washed three times for 15 min each in TBST, and floated on gold particles that were attached to goat antirabbit IgG (15 nm, Jansen) for 1 hr (Bendayan, 1984). After washing in TBST and H_2O, the samples were stained with uranyl acetate and lead citrate. Sections were observed with a Philips CM-10 electron microscope.

Native size of UreG protein

The molecular weight for native *K. aerogenes* UreG protein was estimated by using a Superose 12 column (1.0 by 30 cm) in 20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol (pH 7.4) containing 0.2 M KCl. The column was standardized with thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B_{12} (Mrs=670,000, 158,000, 44,000, 17,000, and 1,350; Bio-Rad Laboratories, Richmond, CA).

Protein determination

Protein concentrations were routinely assessed by the spectrophotometric assay of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Equilibrium Dialysis

Equilibrium dialysis of UreG with ^{14}C-ATP (60 mCi/mmol; Amersham International Plc., Amersham, U.K.) diluted with various concentrations of unlabeled ATP was performed in an equilibrium microvolume dialyzer (Hoeffer Scientific Instruments, San Francisco, CA) with precut dialysis membranes (MWCO=12~14, 000). Purified UreG (2 μM) was analyzed for nickel binding in 50 mM sodium phosphate (pH 7.2), 50 mM HEPES (*N*(2-hydroxyethyl)piperazine-*N'*(2ethanesulfonic acid), pH 7.2), or 50 mM TrisHCl (pH 7.6) each containing 0.5% NaCl to reduce the Donnan effect. After a 3 hr equilibration period at room temperature, radioactivity was measured in aliquots from each compartment by using a Beckman LS7000 liquid scintillation system (Beckman Instruments, Inc., Fullerton, CA).

Results

Purification of UreG

UreG was highly purified by using a combination

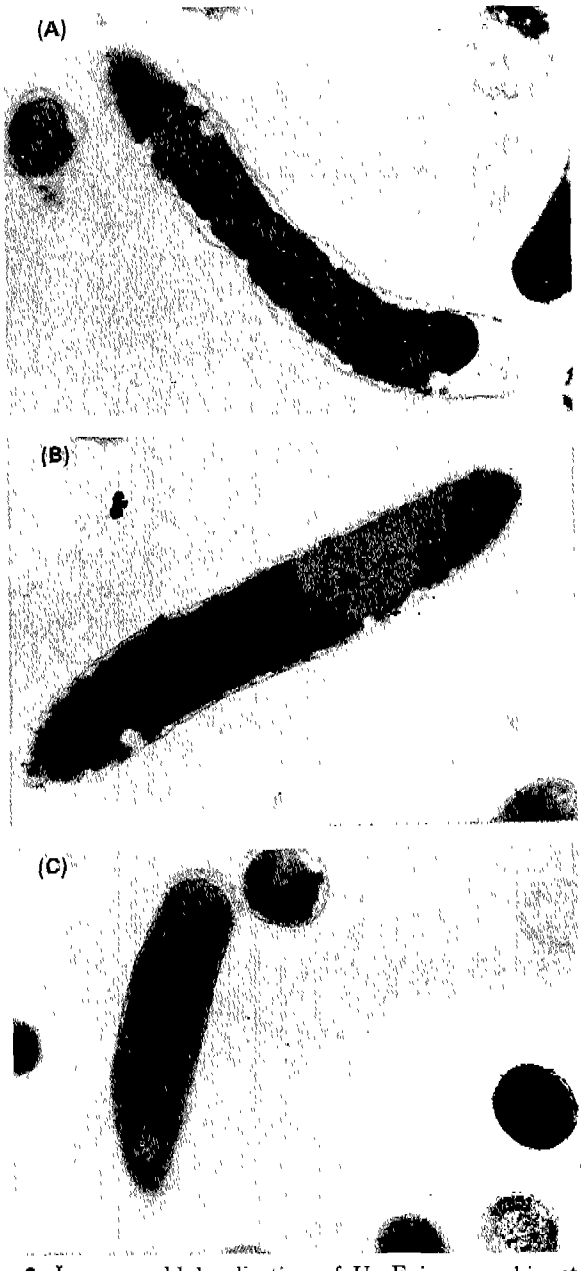

Fig. 2. Immunogold localization of UreE in recombinant *K. aerogenes* and *E. coli* cells. Thin sections of (A) *K. aerogenes* (pKAU19), (B) *E. coli* (pKAU17), and (C) *E. coli* (pKAU17DureG1) cells were reacted with anti-UreG antibodies and labeled with antirabbit IgG-gold particles. UreG was localized to the cytoplasmic portion of the cell.

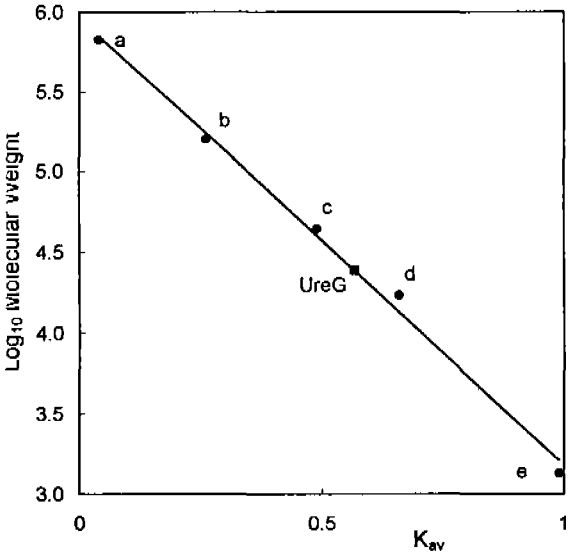

Fig. 3. Molecular weight determination of the UreG protein from *K. aerogenes*. (a) thyroglobulin, M_r=670,000; (b) gamma globulin, M_r=158,000; (c) ovalbumin, M_r=44,000; (d) myoglobin, M_r=17,000; (e) vitamin B_{12}, M_r=1,350; and (■) UreG M_r=24,000.

of DEAE-Sepharose, phenyl-Sepharose column chromatographies and FPLC using prefilled Superose-12 and Mono-Q columns. Samples at this stage of purification were estimated to be over 95% homogeneous and were deemed suitable for most of the experiments reported below. In spite of the presence of nucleotide-binding P-loop motif in the amino acid sequence, UreG did not show any affinity to either Blue-dye column or Green-dye columns (Amicon Inc., Beverly, MA) which are supposed to show significant binding capacity to any nucleotide-containg proteins.

Cellular localization of UreG

Immunogold electron microscopic localization studies were carried out as described above (Fig. 2). The gold particle labeling patterns clearly show that UreG in *K. aerogenes* (pKAU19) (panel A) and *E. coli* (pKAU17) (panel B) is a cytoplasmic protein. Wild type *K. aerogenes* cells were insufficiently labeled by immunogold technique to allow localization (not shown), probably because of insufficient levels of ureG protein (Kellenberger *et al.*, 1987). *E. coli* (pKAU17ΔureG1) that does not possess UreG did not bind significant levels of anti-UreG antibody (panel C).

Characterization of UreG

UreG protein was shown to exist as an apparent monomer (M_r=24,000) when subjected to gel filtration analysis (Fig. 3). The K_{av}-values given in Fig. 3 were calculated from $(V_e \sim V_o)/(V_t \sim V_o)$ where V_e is the elution volume, V_o the void volume, and V_t the total volume of the gel matrix (Andrews, 1964). Equilibrium dialysis experiments failed to demonstrate that monomeric UreG binds ATP.

Discussion

UreG, a monomeric protein that assists in the functional incorporation of nickel ion into urease, has been purified and characterized. Immunogold electron microscopic studies were used to localize UreG to the cytoplasm of the cell, consistent with the lack of hydrophobic regions in the peptide based on computer calculations. Sequence analysis reveals a P-loop motif (Saraste *et al.*, 1990) that is found in many ATP and GTPbinding proteins. However, equilibrium dialysis analyses with ^{14}C-ATP showed negative results. Furthermore, during trial purification steps, UreG peptide did not bind to dye-ligand column resins that are known to have binding property to nucleotide containing proteins.

We cannot, however, exclude the possibility that UreG with a different conformation in a multipeptide-complex form can still act as an energy donor *in vivo* since Park *et al.* (1995) recently provided an evidence for the presence of urease apoprotein complexes containing UreD, UreF and UreG in recombinant *E. coli* cells that are competent for *in vivo* enzyme activation. Also an energy dependence for *in vivo* nickel ion incorporation was observed (Lee *et al.* 1990). *K. aerogenes* UreG sequence is approximately 25% identical to the *E. coli hypB* gene product. Purified HypB was shown to bind and hydrolyse GTP and proposed to provide energy for nickel-metallocenter assembly of hydrogenase.

The role of UreF in nickel metallocenter assembly is still unknown and its characterization is hindered by the minute level of peptide translation despite that the *ureF* gene is under the influence of the same promoter as other accessory genes. Overexpression of the *ureF* gene by changing the ribosome-binding site upstream of that open reading frame is currently under progress.

Acknowledgement

This work was supported in part by the Korean Science and Engineering Foundation grant 951-0705-051-2, and in part by the the general research grant from Catholic University of Taegu-HyoSung.

References

Andrews, P. (1964). Estimation of the molecular weight of proteins by Sephadex gel filtraion. *Biochem. J.* **91**, 222-233.

Andrews, R. K., Blakeley, R. L. and Zerner, B. (1988). Urease-a Ni(II) metalloenzyme, in *The bioinorganic chemistry of nickel* (J. R. Lancaster, Jr. ed.) pp. 141-165, VCH Pub., Inc., New York, NY.

Armbruster, B. L., Carlemalm, E., Chiovetti, R., Garavito, R. M, Hobot, J. A., Kellenberger and Villiger, W. (1982). Specimen preparation for electron microscopy using low temperature embedding resins. *J. Microscopie* **126**, 77-85.

Bendayan, M. (1984). Protein A gold electron microscopic immunochemistry: methods, applications, and limitations. *J. Electron Microscopy Tech.* **1**, 243-270.

Cleveland, P. H., Wickham, M. G., Goldbaum, M. H., Ryan, A. F. & Worthen, D. M. (1981). Rapid and efficient immobilization of soluble and small particulate antigens for solid phase radioimmuoassay. *J. Immunoassay* **2**, 117-136.

Engvall, E. and Perlmann, P. J. (1972). Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzymelabeled antiimmunoglobulin in antigencoated tubes. *J. Immunol.* **109**, 129-135.

Jabri, E., Carr, M. B., Hausinger, R. P. and Karplus P. A. (1995). The crystal structure of urease from *K. aerogenes*. *Science* **268**, 998-1004.

Kellenberger, E., Durrenberger, M., Villiger, W., Carlemalm, E. and Wurtz, M. (1987). The efficiency of immunolabel on Lowicryl sections compared to theoretical predictions. *J. Histochem. Cytochem.* **35**, 959-969.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* (London) **227**, 680-685.

Lee, M. H., Mulrooney, S. B. and Hausinger, R. P. (1990). Purification, characterization, and *in vivo* reconstitution of *Klebsiella aerogenes* urease apoenzyme. J. Bacteriol. **171**, 4427-4431.

Lee, M. H., Mulrooney, S. B., Renner, M. J., Markowicz, Y. and Hausinger, R. P. (1992). *Klebsiella aerogenes* urease gene cluster: sequence of *ureD* and demonstration that four accessory genes (*ureD, ureE, ureF* and *ureG*) are involved in nickel metallocenter biosynthesis. *J. Bacteriol.* **174**, 4324-4330.

Lowry, O. H., Rosebrough N. J., Farr, A. J. and Randall, R. J. (1951). Protein measurement with the folin pheno reagent. *J. Biol. Chem.* **193**, 265-275.

McKinney, M. M. and Parkinson, A. (1987). A simple, nonchromatographic procedure to purify immunoglobulins from serum and ascites fluid. *J. Immun. Meth.* **96**, 271-278.

Mobley, H. L. T., Island M. D. and Hausinger, R. P. (1995). Molecular biology of microbial ureases. *Microbiol. Rev.* **59**, 451-480.

Mulrooney, S. B. and Hausinger, R. P. (1990). Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J. Bacteriol.* **172**, 5837-5843.

Mulrooney, S. B., Pankratz, H. S. and Hausinger, R. P. (1989). Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes (K. pneumoniae)* urease. *J. Gen. Microbiol.* **135**, 1769-1776.

Park, I. S., Carr, M. B. and Hausinger, R. P. (1995). *In vitro* activation of urease apoprotein and role of UreD as a chaperone required for nickel metallocenter assembly. *Proc. Natl. Acd. Sci. USA* **91**, 3233-3237.

Saraste, M., Sibbald, P. T. and Wittinghofer, A. (1990). The P-loop: a common motif in ATP and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430-434.

Todd, M. J. and Hausinger, R. P. (1987). Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*. *J. Biol. Chem.* **262**, 5963-5967.

Todd, M. J. and Hausinger, R. P. (1989). Competitive inhibitors of *Klebsiella aerogenes* urease: mechanisms of interaction with the nickel active site. *J. Biol. Chem.* **264**, 15835-15842.