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NICKEL INCORPORATION INTO Klebsiella aerogenes UREASE

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MANHYUNG LEE

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Robert P. Hausinger

Major professor
Dr. Robert P. Hausinger

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NICKEL INCORPORATION INTO *Klebsiella aerogenes* UREASE

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MAN HYUNG LEE

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ABSTRACT

NICKEL INCORPORATION INTO *Klebsiella aerogenes* UREASE

By

MAN HYUNG LEE

Although ureases play important roles in microbial nitrogen metabolism and in the pathogenesis of several human diseases, little is known of the mechanism of metallocenter biosynthesis in this Ni-containing enzyme.

Klebsiella aerogenes urease apo-protein was purified from cells grown in the absence of Ni. The purified apo-enzyme showed the same native molecular weight, charge, and subunit stoichiometry as the holo-enzyme. Chemical modification studies were consistent with histidinyl ligation of Ni. Apo-enzyme could not be activated by simple addition of Ni ions suggesting a requirement for a cellular factor.

Deletion analysis showed that four accessory genes (*ureD, ureE, ureF,* and *ureG*) are necessary for the functional incorporation of the urease metallocenter. Whereas the Δ*ureD*, Δ*ureF*, and Δ*ureG* mutants are inactive and their ureases lack Ni, the Δ*ureE* mutants retain partial activity and their ureases possess corresponding lower levels of Ni. UreE and UreG peptides were identified by SDS-polyacrylamide gel comparisons of mutant and wild type cells and by N-terminal sequencing. UreD and UreF peptides, which are synthesized at very low levels, were identified by using *in vitro* transcription/translation

methods. Cotransformation of *E. coli* cells with the complementing plasmids confirmed that *ureD* and *ureF* gene products act in *trans*.

UreE was purified and characterized. Immunogold electron microscopic studies were used to localize UreE to the cytoplasm. Equilibrium dialysis studies of purified UreE with $^{63}NiCl_2$ showed that it binds ~6 Ni in a specific manner with a K_d of 9.6 $\pm$ 1.3 μM. Results from spectroscopic studies demonstrated that Ni ions are ligated by 5 histidinyl residues and a sixth N or O atom, consistent with participation of the polyhistidine tail at the carboxyl termini of the dimeric UreE in Ni binding.

With these results and other known features of the urease-related gene products, a model for urease metallocenter biosynthesis is proposed in which UreE binds Ni and acts as a Ni donor to the urease apo-protein while UreG binds ATP and couples its hydrolysis to the Ni incorporation process.

To my parents,
to Hwa Bong, my wife,
and
to Jae Woo & Jae Sang, my sons,
with love.

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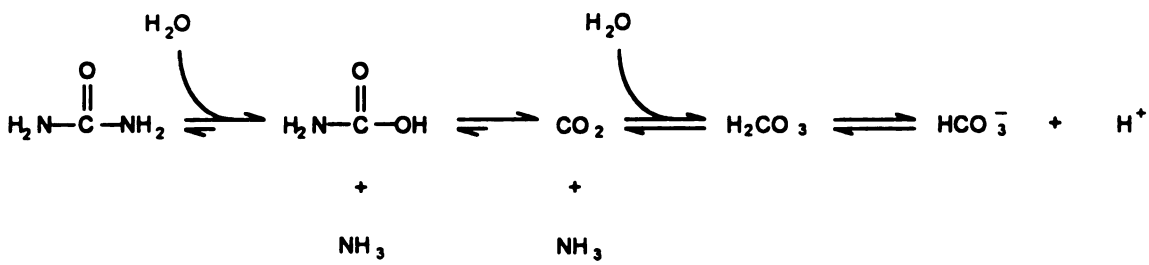
CHAPTER 1

INTRODUCTION

Urea is very stable in solution: its half-life for spontaneous degradation in aqueous solutions at 38°C is 3.6 years (8). In the environment, however, it is rapidly hydrolyzed to ammonia and carbamate through the action of the enzyme urease (urea amidohydrolase, EC 3. 5. 1. 5) (7). Carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid; carbonic acid then dissociates into a proton and bicarbonate. Two moles of ammonia and one mole of bicarbonate are the net products; thus urea hydrolysis results in a net increase in pH (scheme 1).



Scheme 1

The substrate in this reaction, urea, is constantly released into the environment through biological actions. For example, all mammals excrete urea in urine as a detoxification product (94). Urea is also formed by environmental catabolism of uric acid, the primary detoxification product excreted by birds, reptiles, and most terrestrial insects. Similarly, urea is a product of biodegradation of nitrogenous compounds including purines, arginine, agmantine, allantoin, and allantoic acid (95). The urea generated by these reactions is rapidly degraded by ureases found in a wide range of bacteria, several fungi, a few invertebrates, and a variety of plants. Urease activity is also found in soil, either associated with soil microbes, or as

extracellular urease, probably arising from the lysis of soil microbes or the decomposition of plant tissues (66). The objective of this chapter is to present a brief overview of the significance and enzymology of ureases, and to give an account of recent advances related to urease metallocenter biosynthesis.

Medical significance. Urease is an important virulence factor in several pathogenic states, especially those associated with urinary tract infections. Ureolytic microorganisms in this environment hydrolyze urea (at 0.4 M in human urine; 32) and cause a rise in urine pH which can lead to pyelonephritis (77). Elevated urine pH leads to supersaturation of the urine with respect to magnesium and calcium salts (32) which can precipitate forming stones; 15-20% of all kidney stones are associated with infections by ureolytic microorganisms (33). Precipitation of urine salts also affects patients with long term urinary catheterization; 86% of catheterized patients have ureolytic bacteria in their urine (61).

In addition to adverse effects caused by the elevation of pH, ureases release vast quantities of ammonia. Hyperammonemia (85), ammonia encephalopathy (81), and hepatic coma (78) are conditions caused by elevated levels of ammonia produced by ureolytic microbes in the intestines. Elevated ammonia can not be removed from the system by the liver, either because the liver is damaged or because the organism is producing an excessive amount of ammonia. Ammonia is known to have many toxic effects (21), and the additional ammonia released by ureolytic infections can contribute to the overall nitrogen burden of the organism.

Another type of infection in which urease contributes to the pathogenicity of a bacterium occurs in the mucosal lining of the mammalian

stomach (28). Colonization of this hostile environment by the acid-sensitive ureolytic bacterium *Helicobacter pylori* (formerly *Campylobacter pyloridis*) occurs at intercellular junctions (15). *H. pylori* is the etiologic agent of type B gastritis (28, 31) and has been implicated in the formation of peptic ulcers (37); eradication of the infection relieves the symptoms (28). *H. pylori* possesses an active urease which can rapidly hydrolyze serum urea, producing abnormal amounts of ammonia, initially thought to create a 'cloud' of neutral pH immediately surrounding the bacterium (30). Current hypotheses to account for *H. pylori* pathology include: bacterial secretion of mucin digesting enzymes (82), interference with the passage of H^+ ions from the gastric glands to the lumen (37), and/or toxicity due to elevated ammonia concentrations, as mentioned above (21, 83).

Agricultural significance. The lack of fixed nitrogen in the soil is a major limitation to plant growth; therefore, fertilizers are used to supplement soil nitrogen levels when growing commercially important crop plants. Urea is a good source of nitrogen because of its low cost, ease of use, and its high nitrogen content (4). Urea must be hydrolyzed by soil ureases before it can be assimilated into plant tissue; however, uncontrolled hydrolysis can lead to elevated soil pH, ammonia toxicity, and nitrogen loss as volatile NH_3 (up to 50% for submerged rice crops; 14). The simultaneous application of urease inhibitors and urea-based fertilizers has been shown to minimize crop damage and enhance the efficiency of nitrogen utilization (12, 14). Several studies have used urease inhibitors to reduce soil urea hydrolysis rates (11, 14, 52, 57, 61, 75).

Other roles for microbial ureases. Microbial ureases play an important role in the nitrogen metabolism of ruminants such as cattle, sheep, and other animals that contain a forestomach (42). Substantial amounts of animal-derived urea are recycled to the rumen, where ureolytic activity releases ammonia, the major source of nitrogen for most ruminal bacteria (13). The microbial biomass generated is then utilized as a nutrient by the ruminant. Exogenous urea can be used to supplement nitrogen-deficient feedstocks to enhance the quality of the ruminant diet. Urea hydrolysis also occurs in the intestinal tract of monogastric species such as humans, pigs, rats, cats, mice, and rabbits (94), but nitrogen cycling is quantitatively less important in these organisms compared with ruminants.

Plant ureases. Although many members of the Leguminosae are known to possess urease activity, jack bean (*Canavalia ensiformis*) and soybean (*Glycine max*) are the best studied plant sources. Jack bean urease was the first enzyme to be crystallized (86) and the first enzyme shown to contain nickel (25). The mechanism of this plant enzyme has been probed by kinetic and spectroscopic methods (2, 7, 26). The amino acid sequence of the jack bean enzyme was determined using classical biochemical methods (88). Soybean plants possess three immunologically and enzymologically distinguishable types of urease: the embryo-specific (seed), the ubiquitous (leaf) form, and the background urease (69, 70, 71). The roles for the plant enzymes remain unclear, but appear to be related to a large scale metabolic flux of nitrogen through urea, especially during germination.

Structural properties of purified ureases. In contrast to the homohexameric jack bean enzyme (subunit M_r = 90,770; 73), bacteria possess heteropolymeric ureases. The urease of *Klebsiella aerogenes* (90), *Proteus mirabilis* (10, 43), *Providencia stuartii* (64), *Selenomonas ruminantium* (35, 90), *Morganella morganii* (41), *Ureaplasma ureolyticum* (89), *Lactobacillus reuteri* (45), and *Lactobacillus fermentum* (46) consist of one large (α; M_r = 60,000 to 75,000) and two distinct small subunits (β and γ; M_r = 8,000 to 11,000). *Helicobacter pylori* is unusual in that it consists of one large (α; M_r = 66,000) and one medium-size subunit (β; M_r = 29,500) (40). Genetic studies have confirmed the presence of three structural genes in *Klebsiella aerogenes* (63), *Providencia stuartii* (64), *Proteus vulgaris* (62), *Proteus mirabilis* (43), and *Ureaplasma ureolyticum* (9) and two structural genes in *Helicobacter pylori* (49). These studies have also shown that these multiple structural genes are highly homologous to the single plant gene, which probably arose via gene fusion. Nickel has been found in all ureases examined (36). It was shown that the jack bean urease (24, 25) and the *K. aerogenes* enzyme (90, 91) have 2 mol nickel/mol catalytic unit. For other bacterial ureases, 0.8-2.1 mol nickel/mol large subunit has been calculated (reviewed by Mobley and Hausinger; 60) without determining the number of catalytic units per enzyme.

Urease nickel active site. Examination of the active site amino acids which participate in substrate binding, catalytic turnover, or metal binding is essential for elucidation of an enzyme mechanism. Despite the current absence of three-dimensional crystallographic structure, many aspects of the urease active site are being clarified by using kinetic inhibition studies, biophysical and

spectroscopic methods, chemical modification approaches, and site-directed mutagenesis studies.

Urease inhibitor analyses provided evidence for the presence of two nickel ions per catalytic unit (24, 25, 90, 91), demonstrated that nickel is at the active site rather than playing a structural role (8, 23, 91), and also provided some evidence for the presence of negatively charged residue, *e. g.* a carboxylate group (91).

Biophysical and spectroscopic analyses of the nickel center have only been performed with the jack bean and *K. aerogenes* enzymes. UV-visible spectroscopy of *K. aerogenes* urease in the presence of thiol inhibitors was reported by Todd and Hausinger (91), and compared to the jack bean enzyme results (8, 23). X-ray absorption spectroscopy (XAS) of jack bean (1, 18, 34) urease has demonstrated that the nickel is coordinated by a mixture of nitrogen and oxygen ligands. More recent work with the *K. aerogenes* enzyme (Scott *et al.*; unpublished) has provided compelling evidence for the presence of histidinyl ligands to the nickel.

One of the early chemical modification studies involved photooxidation. The investigators of jack bean urease (79) demonstrated that histidine residues were destroyed when the enzyme was subjected to intense light in the presence of methylene blue, whereas photooxidation was suppressed in the presence of tight-binding urease inhibitor, acetohydroxamic acid. Increased reactivity of *K. aerogenes* apo-urease towards a histidine-specific diethylpyrocarbonate (DEP) at pH 6.5 was observed (chapter 2; 50), consistent with histidine ligation of the nickel. A histidinyl residue may also serve as a general base in urease catalysis. The pH dependence of catalysis for both jack bean (27) and *K. aerogenes* (90) urease is consistent with the existence of a general base with a pK_a of 6.5, a value that is appropriate for a histidine residue. Furthermore, Park and

Hausinger (68) have shown that the native *K. aerogenes* enzyme is rapidly inactivated by diethylpyrocarbonate and that the inactivation rate is reduced in the presence of substrate or active site inhibitors. Inactivation was associated with a residue of pK_a 6.5 and spectroscopic changes were observed at 242 nm, consistent with histidine being the reactive residue. These results are compatible with the presence of an essential active site histidine residue that functions as a general base in catalysis. Several chemical modification studies have also implicated the presence of an essential cysteine residue in urease (67, 80, 87, 88, 92, 93), and the presence of a carboxyl group (27, 92).

Site-directed mutagenesis studies have been carried out to confirm the presence of the essential cysteine residue (56) and to study the roles of the conserved histidine residues (Park and Hausinger, unpublished) in the protein.

Reconstitution of urease apoenzyme. Urease apoenzyme is synthesized in both prokaryotic and eukaryotic cells when they are grown in the absence of nickel ion (65, 97). Thus, nickel ion is not involved in transcriptional regulation of urease.

K. aerogenes urease apoenzyme was purified (chapter 2; 50) from cells that were grown on nickel-depleted medium that contained the normal concentrations of other trace metal ions. The inactive protein possessed no significant levels of any metal ions as measured by plasma emission spectroscopy, but nevertheless it behaved identically to the holoprotein in terms of subunit stoichiometry and native size. Attempts to activate the protein by providing nickel ion in buffers containing no other additive or in buffer containing thiols, EDTA, guanidine hydrochloride, dimethyl sulfoxide, glycerol, KCl, and mixtures of these compounds were unsuccessful. These results clearly demonstrate the inability of nickel ion to form a functional active site by simple

association with the apoprotein. Furthermore, nickel addition to disrupted cells failed to generate active enzyme even upon addition of various concentrations of Mg-ATP, thiols, EDTA, glucose, or mixtures of these compounds. Similar negative results were reported for soybean apoenzyme that was treated with 1 mM nickel in the presence of KCN, dimethyl sulfoxide, or urea (97).

Urease apoenzyme can be activated under *in vivo* conditions. For example, preformed urease protein was activated by nickel addition to intact nickel-depleted *K. aerogenes* cells that were treated with an inhibitor of protein synthesis (50). Activation also appeared to be an energy-dependent process as shown by the inability to generate active enzyme in cells previously treated with a proton uncoupler, dinitrophenol, or an ATP synthase inhibitor, DCCD. *In vivo* reconstitution of urease apoenzyme also has been reported for another enteric bacterium, *Proteus mirabilis* (74), as well as for a purple sulfur bacterium (3), a cyanobacterium (55), several algae (76), and soybean (97). At this time, it is still unclear why disrupted cells are unable to activate the apoenzyme whereas urease activity is generated in intact cells.

Accessory genes for urease activation in eukaryotes. Several studies of fungal ureases are consistent with the presence of and requirement of urease genes that do not encode the urease subunits. For example, four complementation groups were identified and shown to be required for *Aspergillus nidulans* urease activity (54). Similarly, four loci (encoding unknown functions) are required for functional urease activity in *Neurospora crassa* (5) and in *Schizosaccharomyces pombe* (47). In *A. nidulans*, *ureA* is the structural gene for urea transport protein, *ureB* encodes the single subunit urease enzyme, *ureC* encodes a product needed for enzyme activity but of unknown function, and *ureD* is suspected to be necessary for synthesis or incorporation of the

nickel cofactor. A mutation in *ureD* can be overcome by growth in the presence of 0.1 mM nickel sulfate (53).

The most detailed genetic analyses reported for any eukaryotic urease are those of soybean, a plant that is associated with three distinct urease isozymes: the embryo-specific, ubiquitous, and background enzymes (38, 39, 58, 59, 70, 71, 72, 84). The embryo-specific or seed urease is expressed at very high levels in the developing embryo and encoded by the *Eu1* locus (38, 58, 69). A portion of the embryo-specific urease gene has been cloned by Krueger *et al.* (48) who used synthetic oligonucleotides based on the jack bean and soybean urease sequences to screen a soybean genomic library. The isolated subclone possessed only a partial urease sequence, but it matched 108 of 130 amino acids determined for the jack bean enzyme. The ubiquitous isozyme is constitutively expressed at low levels in all soybean tissues from the *Eu4* locus (72). Finally, a background urease was identified in double mutants defective in both the embryo-specific and ubiquitous ureases (39). This background urease appears to be produced not by the plant, but rather by a phylloplane-associated bacterium, *Methylobacterium mesophilicum.* The isolated bacterium possesses urease with properties that are very similar to the urease activity in the plant double mutant. All three soybean-associated ureases appear to require nickel ion, as shown by the near absence of activity when plants are grown in nickel-depleted medium.

Accessory genes that facilitate nickel incorporation into urease appear to be required for the plant enzymes. Meyer-Bothling *et al.* (59) isolated pleiotropic mutants that were deficient in both plant-derived ureases. These mutations mapped to two distinct loci, *Eu2* and *Eu3*, neither of which was closely associated with the urease structural loci, *Eu1* and *Eu4*. They proposed that these loci may encode gene products that are required for a common urease

maturation function such as nickel ion emplacement. Curiously, mutants at either of the two plant accessory gene loci also eliminate urease activity in the phylloplane-associated bacterium (39). Bacteria isolated from these plant mutants were shown to possess transient urease- and hydrogenase-deficiencies that could be corrected by addition of nickel ion to the cultures.

Accessory genes for urease activation in bacteria. DNA sequence analysis of the *Klebsiella aerogenes* urease operon has revealed the presence of several additional genes that are part of the urease gene cluster. The *ureA, ureB,* and *ureC* genes encoding the urease subunits are immediately preceded by the *ureD* gene and followed by the *ureE, ureF,* and *ureG* genes (chapter 3; 51, 63). Sequence analysis of other bacterial urease genes clusters reveals that many of these accessory genes are conserved. *P. mirabilis* possesses highly homologous counterparts to at least the *ureD, ureE,* and *ureF* genes (44). *Helicobacter pylori* possesses analogues to each of the four *K. aerogenes* accessory genes (22), although the counterpart of *ureD* is located after *ureG* and was termed *ureH* by the authors. Portions of *ureD* and *ureE* genes can be observed in the sequences immediately surrounding the *P. vulgaris* urease subunit genes (62). Finally, all of these adjacent genes appear to be present in a ureolytic *E. coli* strain (20).

The non-subunit auxiliary genes recently have been shown to be required for urease activity. Transposon mutagenesis and other inactivation methods have demonstrated that *ureD*, *ureF*, and *ureG* (or unidentified upstream or downstream regions that are likely to correspond to these genes) are essential for obtaining a functional urease (19, 20, 29, 63, 64, 96). To begin assess the roles for the accessory genes, Lee *et al.* (chap 3; 51) examined the *K. aerogenes* urease properties in recombinant *E. coli* cells containing plasmids

with the intact urease gene cluster or deletion mutants in each of *ureD*, *ureE*, *ureF*, and *ureG* genes. In the deletions involving *ureD*, *ureF*, and *ureG*, the urease protein was synthesized in an inactive form and was shown to be devoid of nickel. Furthermore, mutants in *ureE* possessed a reduced urease activity and the nickel content of the purified urease enzyme was correspondingly reduced. Each of the four genes was shown to function via a trans-acting factor. Thus, all four accessory genes encode proteins that appear to be necessary for functional incorporation of nickel into urease.

Outline of this thesis

The following chapters describe my studies on *Klebsiella aerogenes* urease apoenzyme, accessory genes, and accessory proteins. In Chapter 2, I describe the purification of nickel-deficient apourease from *K. aerogenes* cells grown in nickel-free medium. I show that the apoenzyme possesses no significant levels of any metal ions, but nevertheless it behaves identically to holoprotein in terms of subunit stoichiometry and native size. These studies were combined with *in vivo* reconstitution analysis carried out by Scott Mulrooney and published in *J. Bacteriol.* **172**, 4427-4431 (1990) (50). In Chapter 3, I examine the *K. aerogenes* urease properties in recombinant *E. coli* cells containing plasmids with the intact urease gene cluster or deletion mutants in each of *ureD*, *ureE*, *ureF*, and *ureG* genes and show that all four accessory genes encode proteins that appear to be necessary for functional incorporation of nickel into urease. This work was combined with *ureD* sequence analysis and additional studies by others and published in *J. Bacteriol.* **174**, 4324-4330 1992) (51). In Chapter 4, I describe the purification of UreE protein and show that this protein is a dimer that specifically binds ~6 nickel ions with a K_d of 9.6 $\pm$ 1.3 μM. Finally, in Chapter 5, I summarize current concepts in urease metallocenter biosynthesis and present a model that should aid in stimulating future studies. As appendeces to this thesis, I provide a manuscript published in *J. Mol. Biol.* **227**,934-937 (1992) describing the preliminary crystallographic analysis of *K. aerogenes* urease that I supplied to the laboratory of Dr. Andy Karplus, and I provide immunogold electronmicroscopic results that localize UreG to the cytoplasm.

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CHAPTER 2

PURIFICATION AND CHARACTERIZATION OF UREASE APO-ENZYME FROM *Klebsiella aerogenes*

These studies were combined with *in vivo* reconstitution analyses by Scott B. Mulrooney and published in Journal of Bacteriology, 172:4427-4431. 1990.

ABSTRACT

Urease was purified from recombinant *Klebsiella aerogenes* cells which were grown in the absence of nickel. The protein was inactive and contained no transition metals, yet it possessed the same heteropolymeric structure as native enzyme, demonstrating that Ni is not required for inter-subunit association. Ni did, however, substantially increase the stability of the intact metalloprotein (T_m = 79°C) compared with apoenzyme (T_m = 62°C), as revealed by differential scanning calorimetric analysis. An increased number of histidine residues were accessible to diethyl pyrocarbonate in apourease compared with holoenzyme, consistent with possible Ni ligation by histidinyl residues. Addition of Ni to purified apo-urease or to cell-ruptured suspension did not yield active enzyme.

INTRODUCTION

Urease, a nickel-containing enzyme found in many microorganisms and plants, hydrolyzes urea to yield ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid (2, 17). Whereas the role of urease in plants is poorly understood (27), the microbial enzyme plays important roles in human and animal pathogenic states, in ruminant metabolism, and in environmental transformations of certain nitrogenous compounds (17). The best characterized plant urease is that isolated from jack bean; it was the first enzyme ever crystallized (22) and the first enzyme shown to possess nickel (5). This hexameric protein contains two Ni ions per subunit (M_r = 90,770), the sequence of which was recently reported (23). The best characterized bacterial urease is that from *Klebsiella aerogenes* (a non-nitrogen-fixing *K. pneumoniae*) which possesses three subunits [M_rs = 72,000 (α), 11,000 (β), and 9,000 (γ)] in an apparent $\alpha_2\beta_4\gamma_4$ stoichiometry (24). The native enzyme contains two catalytic sites, each of which is associated with 2 Ni ions (25). The genes for *K. aerogenes* urease were recently cloned and overexpressed such that urease accounted for over 10% of the cellular protein (19). Although the enzyme requires Ni for activity, no Ni-dependent regulation of gene expression was observed.

This report describes the isolation and characterization of apo-urease from *K. aerogenes*. Experiments were designed to ascertain (i) whether Ni is required for intersubunit association, (ii) if metal ions other than Ni are incorporated into urease during growth in Ni-free medium, (iii) how the presence of Ni affects the enzyme thermal stability and histidine reactivity, and (iv) whether Ni can be incorporated *in vitro* into the urease apo-enzyme.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. aerogenes* CG253 was transformed with plasmid pKAU19 (19), which possesses the urease genes from this microorganism. The recombinant bacterium was grown at 37°C in MOPS-glutamine medium (19) containing 30 μg/ml chloramphenicol. Ni-free cultures were grown without added Ni, whereas, for Ni-depleted growth all buffer components except metal salts were passed through a column of controlled pore glass-8-hydroxyquinoline (Pierce Chemical Co., Rockford, IL) to remove trace Ni contaminants as described by Eskew et al. (7). All glassware and plasticware were washed with 20 % nitric acid and rinsed with nickel-free water. Puratronic grade metal salts (Alpha Products, Danvers, MA) were then added.

Assays. Urease activity was determined by monitoring the rate of ammonia release from urea by formation of indophenol, which was measured at 625 nm (26). The assay buffer consisted of 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Sigma Chemical Co., St. Louis, MO), 50 mM urea, and 0.5 mM EDTA (pH 7.75). One unit of urease activity is defined as the amount of enzyme required to hydrolyze 1 μmole of urea per min at 37°C under the assay conditions described above. Protein was measured by the method of Lowry *et al.* (12) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. All denaturing gel electrophoresis was carried out by using the buffers of Laemmli (11) and a 10 to 15% polyacrylamide gradient resolving gel with a 4.5% stacking gel (bisacrylamide/acrylamide, 1:32). Samples were run after denaturing at 100°C for 5 min. The gels were stained with Coomassie brilliant blue (Sigma) and scanned by using a Gilford

Response spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) at 595 nm. The standards used were phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories, Richmond, CA). Non-denaturing gels (3 % stacking and 8 % running) were prepared in the same buffers except that SDS was omitted. Immunoblots were prepared by blotting gels onto nitrocellulose, probing with anti-*K. aerogenes* urease antibodies, and developing by using anti-rabbit IgG-alkaline phosphatase conjugates as previously described (19).

Purification of urease apo-enzyme. Cultures (3 L) of *K. aerogenes* CG253 (pKAU19) were grown to late exponential phase ($O.D._{600\ nm}$ = 2.7) in Ni-depleted medium and harvested by centrifugation. The cells were washed twice with PEB buffer (20 mM phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol [pH 7.5]), resuspended in an equal volume of PEB buffer containing 0.5 mM phenylmethylsulfonyl 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 x *g* for 90 min at 4^oC. The extracts were chromatographed on DEAE-Sepharose column (2.5 by 15 cm) in the same buffer and eluted with a 400-ml linear salt gradient to 1 M KCl. Highly purified apourease eluted from the column at 0.45 M KCl, identical to the elution position of holoenzyme (24). To remove most of the contaminants, the pooled sample was concentrated to 1 ml by using an Amicon pressure filtration stirred-cell 8050 (50 ml capacity; Amicon Corp., Dancers, Mass.) with an YM 30 (43 mm diameter) ultrafiltration membrane, and a Centricon 30, and subjected to Superose 12 chromatography (2.5 by 59 cm) in 50 mM HEPES-1 mM EDTA-1 mM 2-mercaptoethanol (pH 7.0) buffer supplemented with 0.1 M KCl on a Fast Protein Liquid Chromatography system (Pharmacia, Uppsala, Sweden) at room temperature. All resins and

columns were purchased from Pharmacia. The presence of urease protein in column fractions was assessed by denaturing gel electrophoresis as well as immunoblot analysis. For comparative analyses, urease holo-enzyme was also purified using the procedure described previously (25).

Characterization of urease apo-enzyme. The molecular weight for native *K. aerogenes* apo-urease was estimated by using Superose 12 chromatography in 50 mM HEPES, 1 mM EDTA, 1 mM 2-mercaptoethanol buffer [pH 7.0] containing 0.1 M KCl. The column (1.0 by 30 cm) was standardized by using thyroglobulin, gamma globulin, ovalbumin, myoglobin, and vitamin B-12 (M_rs = 670,000, 158,000, 44,000, 17,000, and 1,350; Bio-Rad Laboratories, Richmond, CA). Metal content was assessed at the Chemical Analysis Laboratory of the University of Georgia by using a Jarrell Ash 965 inductively coupled plasma emission spectrometer. The thermal stability of the apo-enzyme and holo-enzyme were analyzed by using a MicroCal MC2 differential scanning calorimeter (MicroCal, Inc., Northampton, MA). Samples (~1 mg/ml) in PEB buffer were scanned at a rate of ~80^{0}C/h, the baseline was selected by using the T(1)-T(4) option, and deconvolution was carried out by using the supplied fitting programs (DA2 version 2.1) assuming $\Delta H^0 = \Delta H^*$.

Urease apoprotein and holoenzyme (2μM) were compared for their reactivity toward diethyl pyrocarbonate (DEP, 0.5 mM) in 0.1 M phosphate-1 mM EDTA buffer (pH 6.5) by monitoring the time-dependent increases in absorbance at 242 nm due to the formation of carbethoxyhistidine (extinction coefficient = 3,200 M^{-1} cm^{-1}) (13). The DEP was freshly prepared in cold, anhydrous ethanol and the concentration was determined by reaction with a standard imidazole solution based on a carbethoxyimidazole extinction coefficient of 3,000 M^{-1} cm^{-1} at 230 nm. To confirm that protein histidine residues were the primary sites of

reaction, the wavelength maximum was determined for the modified samples (13). For the case of the native enzyme, aliquots were removed at the indicated time points and assayed to assess the effect of modification on urease activity. Native enzyme which had been inactivated by DEP was treated with 0.5 M hydroxylamine for 60 min and assayed to test for recovery of activity.

RESULTS

Characterization of urease apo-enzyme. Immunoblot analysis showed that *K. aerogenes* CG253(pKAU19) produced all three urease subunits even in the absence of nickel, in agreement with an earlier report (19). Using the stated growth conditions, approximately 10% of the protein in cell extracts was urease; however, no ureolytic activity was present. Purified urease apo-enzyme was over 95% homogeneous, as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis results (not shown). The three urease subunits [apparent M_r = 72,000, 11,000, 9,000] remained associated during apourease purification. The subunit stoichiometry was assessed by integrating the area under each peak in the scanned Coomassie blue stained gel and dividing by the apparent molecular weight, resulting in a 1.0:2.0:2.4 ratio. Such results can be affected by the dye binding affinity of each subunit, nevertheless they demonstrate that the apoenzyme has the same stoichiometry as the holo-protein which was estimated to possess a subunit ratio of 1.0:2.0:2.3. Furthermore, purified apoenzyme eluted from a Superose 12 gel filtration column at the same position as holoenzyme [M_r = 224,000 (24)]. This finding, when combined with the SDS gel results, indicates that the subunit stoichiometry of apourease is most likely

$\alpha_2\beta_4\gamma_4$, the same as the holoenzyme. Native gel electrophoresis established that the apoenzyme and holoenzyme migrated identically.

Plasma emission spectrometric analysis demonstrated that the apoenzyme was truly metal free and not substituted with other transition metal ions. No significant levels of Mg, Ca, Cr, Mn, Fe, Co, Cu, Zn, Mo or other multivalent biological metal ions were observed.

Addition of Ni to the apoprotein did not lead to enzyme activation, even in the presence of various concentrations of thiols (dithiothreitol or 2-mercaptoethanol), EDTA, guanidine-hydrochloride, dimethyl sulfoxide, glycerol, KCl and mixtures of these compounds. Addition of Ni to the disrupted cells grown in Ni-depleted medium in the presence of various concentrations of MgATP, thiols, EDTA, glucose, or mixtures of these compounds did not result in activation of apourease.

The presence of Ni stabilizes urease holoenzyme compared to apoprotein as shown by differential scanning calorimetry (Fig.1). Analysis of apoenzyme yielded a complex endotherm centered at 62°C which was deconvoluted into three transition temperatures (T_ms = 59.8, 62, and 64.5°C) possessing integrated heat capacities in a ratio of 31:53:16. By contrast, the holoenzyme exhibited an endotherm centered at 79°C which was fit to three transitions (T_ms = 73.6, 75.7, and 79.1°C) where the integrated heat capacities were in a ratio of 18:28:54. If the observed transitions reflect thermal denaturation of the same three domains in apo- and holo-protein, then the absence of Ni shifted the transition temperature of the largest domain by 15.9 °C, the second domain by 13.7°C, and the smallest by 10.4 °C.

Urease apoenzyme exhibited a greater reactivity toward DEP modification than did the holoenzyme (Fig. 2). Wavelength scans of the modified samples versus the unmodified protein established that carbethoxyhistidine (λ_{max} = 242

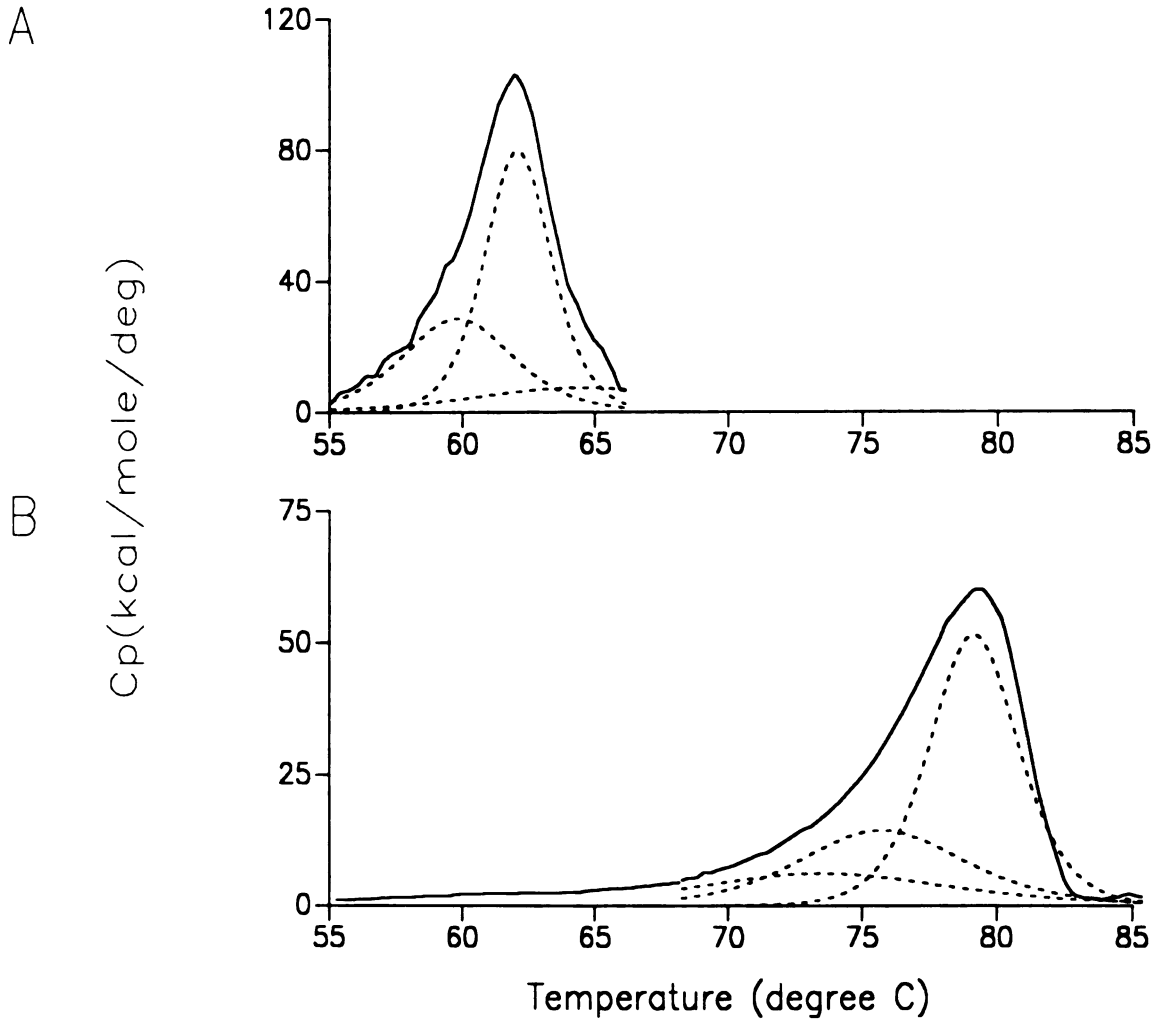

Figure 1. Effect of the Ni metallocenter on urease thermal stability. Holo- and apo-urease (1 mg/mL) were analyzed by differential scanning calorimetry. The baseline-subtracted heat capacity as a function of temperature is shown in solid lines for apo-protein (A) and native enzyme (B). Also shown by dashed lines are the deconvoluted transitions for each sample.

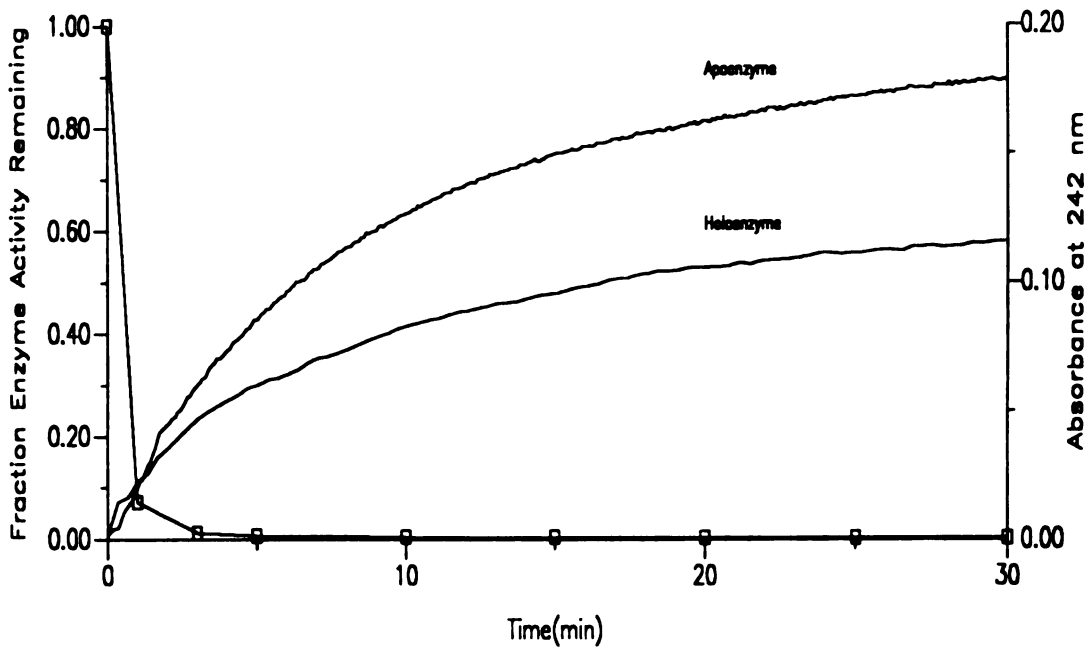

Figure 2. Comparison of DEP reactivity for apo- and holo-urease. 2 μM apo-protein (A) or native enzyme (B) were reacted with 50 mM DEP while monitoring the absorbance at 242 nm. The dashed line demonstrates loss of holo-enzyme activity during reaction with DEP.

nm) was the predominant reaction product. After 30 min incubation with DEP, the number of modified histidine residues in apo-urease was 28 versus only 19 in the holoenzyme. DEP modification of native urease is accompanied by a rapid loss of urease activity (Fig. 2), however, approximately 30% of the activity was restored by treatment with hydroxylamine for 60 min (not shown).

DISCUSSION

Ni has recently been shown to be an essential trace metal for several microorganisms and is specifically incorporated into four types of Ni-dependent enzymes (9). Although bacterial ureases contain tightly bound Ni at their active sites (17, 25), the mechanism by which Ni is incorporated into urease, the identity of the Ni ligands, the metallocenter structure, and the role for Ni are unclear.

Urease apo-enzyme purification and characterization. This work represents the first report of the purification and characterization of any urease apoenzyme. Despite the absence of Ni ions, the behavior of the apoprotein on anion exchange resins and during native gel electrophoresis is identical to that of the holoenzyme. This result is somewhat surprising when one considers that Ni is thought to be divalent in urease (2); however, several explanations can be offered. Although the apo-enzyme was free of many other biologically important multivalent transition metals, it is possible that Ni was replaced by K or Na ions whose concentrations were not accurately measured. Alternatively, Ni may bind to residues which become protonated in the absence of Ni. Finally, Ni may bind

to the protein in association with an anionic species such that the overall charge is neutral. Further studies must be carried out to resolve this question.

The demonstration that urease subunits associate in the proper stoichiometry in the absence of Ni demonstrates that Ni is not required for this interaction. The presence of Ni does, however, impart additional thermal stability to the protein. Ni does not appear to stabilize a single subunit or domain of the enzyme, as shown by the changes in each of the three deconvoluted temperature transitions for the two protein forms.

The finding that apourease has an increased number of accessible histidine residues over the holoenzyme is compatible with Ni ligation by these residues. Such interpretation must be made with caution, as the increased reactivity of apoenzyme may also be due to simple conformational changes. Nevertheless, a histidine-rich region of jack bean urease is known from sequence studies (23), and investigators have proposed that histidine residues may ligate the Ni in the plant enzyme (8, 21). For example, photooxidation studies of jack bean urease in the presence of methylene blue led to the destruction of histidine residues, and this photooxidation was partially suppressed by the presence of inhibitors (21). Furthermore, preliminary X-ray absorption spectroscopy studies of jack bean urease were interpreted as indicating histidinyl ligation of the active site Ni (8). However, more detailed X-ray absorption spectroscopic studies could only demonstrate that the ligands were a mixture of nitrogen and oxygen atoms (1).

Incorporation of the urease metallocenter. The inability to activate purified *K. aerogenes* urease apo-enzyme by simple addition of Ni ions indicates that a cellular factor may be required for incorporation of the metal center. Apourease synthesized in Ni-free *K. aerogenes* cultures could be activated by Ni

addition to whole cells even in the presence of protein synthesis inhibitors but not in dinitrophenol- or DCCD-treated cells (not shown; complementary studies carried out by Scott B. Mulrooney). Similar results have been previously reported for ureases from soybean (28), algae (20), a cyanobacterium (15), and purple sulfur bacteria (3). The lack of urease activation in the presence of an uncoupler or ATPase inhibitor could be explained by an energy dependence for Ni transport or for Ni incorporation into protein. We prefer the latter explanation because apourease could not be activated in disrupted cells where Ni transport is not a consideration. The energy dependence of reconstitution is not simply a requirement for ATP as shown by experiments where MgATP was added to purified apoprotein or to Ni-free cell extracts: Ni addition led to no observed generation of urease activity. Other studies have also shown the inability to generate urease activity by addition of Ni to disrupted, Ni-free cells of soybean (28), jackbean (6), and a cyanobacterium (15).

Preliminary studies in both plants and microorganisms are consistent with the requirement for urease-related accessory factors which activate apoenzyme. In soybean for example, Meyer-Bothling et al. (16) reported the isolation of pleiotropic mutants that were totally defective in urease activity yet expressed both urease isozymes. They concluded that the mutations, which mapped distant from the urease genes, led to a defect in a urease maturation factor. Similarly, a urease-deficient mutant of *Aspergillus nidulans* is thought to be defective in the production or incorporation of a nickel cofactor essential for urease activity (14). The addition of 0.1 mM Ni restored the ability of this mutant fungus to grow on urea and enhanced the urease activity to 5-8% of the wild-type levels, but Ni addition to cell extracts did not result in urease activity. Furthermore, the number of urease loci in *Neurospora crassa* (4) and *Schizosaccharomyces pombe* (10) exceeds that required for the urease

structural genes. The functions of the additional genes are unknown, but one could speculate that they may function in Ni incorporation or other maturation event. In this regard, DNA sequence analysis has established the presence of six genes in the *K. aerogenes* urease operon (18), which is three more than is required to encode the urease subunits. Further studies are necessary to identify whether these genes function in Ni incorporation.

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CHAPTER 3

CHARACTERIZATION OF ACCESSORY GENE DELETION MUTANTS

These studies were combined with sequence analysis and deletion mutation of *ureD* (by Scott B. Mulrooney and Yves Markowicz) and with a study on the effect of nickel concentration on urease activity of *K. aerogenes* cells grown in LB (by Michael J. Renner) and published in
Journal of Bacteriology, 174:4324-4431. 1992.

ABSTRACT

Deletions were generated in each of the genes (*ureE*, *ureF*, and *ureG*) located immediately downstream of the three structural genes of the *Klebsiella aerogenes* urease operon. These deletion derivatives, as well as two deletion derivatives of the upstream *ureD* gene*, were transformed into *Escherichia coli*. In all cases high levels of urease were produced, but the enzyme was inactive (deletions in *ureD, ureF* or *ureG*) or only partially active (deletions in *ureE*). Ureases were purified from the recombinant cells and shown to be identical to control enzyme when analyzed by gel filtration chromatography and SDS-polyacylamide gel electrophoresis; however, in every case the activity levels correlated to nickel contents as analyzed by atomic absorption analysis. UreD, UreE, UreF, and UreG peptides were tentatively identified by gel electrophoretic comparison of mutant and control cell extracts, by *in vivo* expression of separately cloned genes, or by in vitro transcription-translation analyses. The assignments were confirmed for UreE and UreG by amino terminal sequencing. The latter peptides (apparent M_r = 23,900 and 28,500) were present at high levels comparable to those of the urease subunits, whereas, the amounts of UreF (apparent M_r = 27,000) and UreD (apparent M_r = 29,300) were greatly reduced, perhaps because of the lack of good ribosome binding sites in the regions upstream of these open reading frames. These results demonstrate that these four accessory genes are necessary for the functional incorporation of the urease metallocenter.

* Two deletion mutants in *ureD* were generated by Yves Markowicz.

INTRODUCTION

Urease, a nickel-containing enzyme found in many microorganisms, hydrolyzes urea to yield ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid (12). In addition to being important in bacterial nitrogen metabolism, the enzyme has been implicated as a virulence factor in various human and animal diseases (reviewed in 12).

The most extensively characterized microbial urease is that from the Gram-negative enteric bacterium, *Klebsiella aerogenes* (a non-nitrogen-fixing *K. pneumoniae*). Combined genetic and biochemical analyses revealed that the enzyme activity is regulated by the global nitrogen regulatory system (10) acting through the *nac* (*n*itrogen *a*ssimilatory *c*ontrol) gene product (1, 9); thus, under conditions of low nitrogen availability urease activity is expressed. The urease enzyme possesses three different subunits [M_rs = 60,304 (α), 11,695 (β), and 11,086 (γ) (13)] in an apparent $\alpha_2\beta_4\gamma_4$ stoichiometry, and has two bi-nickel active sites (17, 18). When *K. aerogenes* cells are grown in the absence of nickel, urease apoenzyme is produced. Although the purified apoenzyme cannot be reactivated by the simple addition of nickel ions, an energy-dependent reconstitution was observed in intact cells (6). DNA sequencing of the *K. aerogenes* urease gene cluster revealed the presence of three genes (*ureE*, *ureF*, and *ureG*) located immediately downstream of the three urease structural genes (*ureA*, *ureB*, and *ureC*) (13). When a DNA fragment containing these three "accessory" genes was deleted from a plasmid containing the entire urease gene cluster, urease protein was still produced but in an inactive form which did not contain nickel. Cotransformation of these cells with a plasmid

containing the accessory genes under control of a *tac* promoter led to restoration of urease activity in the presence of isopropyl-β-D-thiogalactopyranoside. Thus, it was concluded that one or more of the downstream accessory genes encoded a protein which facilitated nickel incorporation into urease (13).

This report describes evidence that each of the three downstream accessory genes, and another upstream accessory gene — *ureD,* are involved in some aspect of urease metallocenter biosynthesis.

MATERIALS AND METHODS

Plasmid construction. Plasmid DNA was isolated by using the alkaline lysis method of Birnboim (2). Specific DNA fragments generated by restriction endonuclease digestion were isolated by using NA45-DEAE cellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.) after electrophoretic separation of DNA fragments on agarose gels. All restriction enzyme digestions, end fillings, and other common DNA manipulations were performed by standard procedures (15). The previously described plasmids pKAU17 (a pUC8 derivative) and pKAU19 (a derivative of pBR328) contain all genes required to express active *K. aerogenes* urease in *Escherichia coli* DH1(14). An *Eco*RI-*Hin*dIII fragment of pKAU17 was isolated by elution from a 1% agarose gel, treated with Klenow fragment of *E. coli* DNA polymerase to produce blunt ends, and inserted into the *Hin*cII site of pUC18 (22) to produce pKAU22 (Fig. 1). The *Nru*I-*Bcl*I, *Bsp*HI-*Rsr*II, and *Aat*II-*Hin*dIII fragments of pKAU17 were cloned separately into the *Eco*RV site (behind the tetracycline promoter) of plasmid pACYC184 (3) to generate plasmids pACYC-D, pACYC-F, and pACYC-G, respectively. In the case of pACYC-D construction, pKAU17 was isolated from

E. coli GM48 (a *dam* mutant strain), both the *Nru*I-*Bcl*I fragment and the restricted pACYC plasmid were treated with Klenow fragment to generate blunt ends, and the vector was dephosphorylated before ligation.

Deletion mutagenesis. Selective deletions within each of the *ureE*, *ureF*, and *ureG* genes were obtained by using a combination of restriction digestion and *Bal*31 digestion. For the *ureE* deletion, pKAU17 was partially digested with *Stu*I, a *Spe*I linker (U.S. Biochemical Corp., Cleveland, Ohio) was inserted into the cleavage sites, the appropriate subclone was identified on the basis of its restriction pattern, the unique *Spe*I site was restricted, the ends were partially digested with *Bal* 31, and the linear fragment was religated after making the ends flush with Klenow fragment. Two clones containing deletions of approximately 190 and 260-bp (designated Δ*ureE-1* and Δ*ureE-2*) were further analyzed as described below. The single *ureF* deletion was generated by removal of the 552-bp *Aat*II-*Avr*II fragment of pKAU17 followed by T4 DNA polymerase treatment to make blunt ends. Two *ureG* mutations containing deletions of approximately 310 and 360-bp (designated Δ*ureG-1* and Δ*ureG-2*) were generated by restriction with *Rsr*II, digestion with *Bal* 31, and religation. Deletion clones were verified by restriction analysis.

Assays. Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol, which was monitored at 625 nm (20). The assay buffer consisted of 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ehanesulfonic acid, 50 mM urea, and 0.5 mM EDTA (pH 7.75). The reactions were initiated by the addition of enzyme, the concentration of released ammonia was measured in timed aliquots, and rates were determined by linear regression analysis. One unit (U) of urease activity is defined as the amount of

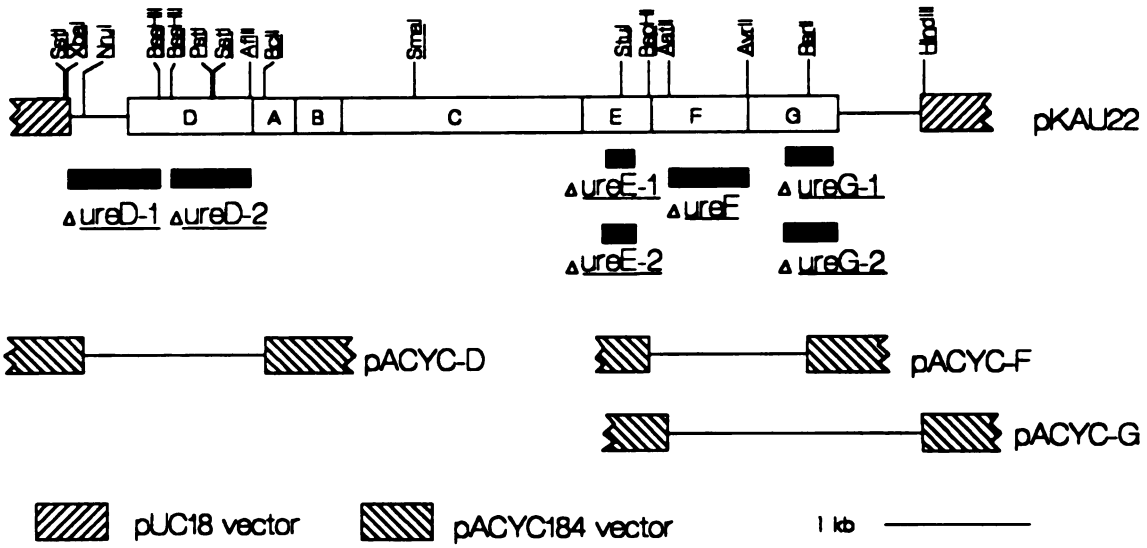

Figure 1. *K. aerogenes* urease gene cluster, selected subclones, and location of deletions in mutanat derivatives. The map of the *K. aerogenes* urease gene cluster in pKAU22 shows the location of the restriction sites used in sequence analysis, isolation of subclones, or generation of deletion derivatives. The sequence to the right of the *Sst*I site within the *ureD* gene has been published previously (13). Sites of deletions are indicated by solid bars; deletions in *ureD* were generated in pKAU22 whereas deletions in *ureE*, *ureF*, and *ureG* were obtained by using the precursor plasmid, pKAU17, as described in the text. Plasmids pACYC-D, pACYC-F, and pACYC-G are subclones of *ureD*, *ureF*, and *ureG* in the vector pACYC184.

enzyme required to hydrolyze 1 μmol of urea per min at 37°C under the assay conditions described above. When urease activity was determined in cultures, cells were disrupted by sonication as previously described (14). Protein content was determined by the method of Lowry et al. (7) with bovine serum albumin as the standard. Samples were assayed for nickel content after hydrolysis in 1 N HNO_3, drying, and dissolution in 50 mM HNO_3. A computer-interfaced Varian SpectraAA-400Z atomic absorption spectrometer equipped with an autosampler, a graphite furnace, and Zeeman background correction was used to measure the sample peak height.

Polyacryamide gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (5) and included either a 12 or 15% acrylamide 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 and scanned with a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 540 nm.

Amino terminal sequencing of accessory proteins. The peptides from partially purified protein fractions were resolved in a 0.75-mm denaturing gel, as described above, and transferred to a sheet of Pro-Blot membrane (Applied Biosystems, Foster City, Calif.) by standard procedures (11). The bands were visualized by Coomassie blue staining, cut from the membrane, and analyzed by using an Applied Biosystems 477A automated sequencer in the Michigan State University Macromolecular Structure Facility.

In vitro transcription-translation. Two μg of purified plasmid DNA (15) was transcribed and translated in the presence of [^{35}S]methionine (1,122 Ci/mmol) in 50 μl of an in vitro transcription-translation reaction mixture according to the manufacturer's instructions (Promega Corp., Madison, Wis.). Aliquots (5 μl) were analyzed by denaturing gel electrophoresis, as described above, with ^{14}C-labeled protein molecular weight markers (Amersham Corp., Arlington Heights, Ill.). Gels were fixed, dried, and used to expose X-ray film overnight.

Purification and characterization of urease from accessory gene mutants. Cell extracts were prepared from *E. coli* DH5 cells containing pKAU17 or plasmids deleted in the individual accessory genes and from control *K. aerogenes* cells containing pKAU19. Cells were disrupted in a French pressure cell and then membranes and cellular debris were sedimented for 90 min at 100,000 x *g*, as previously described (17). In some cases, the cell extracts were applied directly to a Mono-Q (HR 10/10) column equilibrated in 50 mM potassium phosphate – 1 mM EDTA – 1mM β-mercaptoethanol (pH 6.5) and eluted with a multistep gradient of increasing KCl in the same buffer. Alternatively, the cell extracts were subjected to chromatography on columns of DEAE-Sepharose, phenyl-Sepharose, and Mono-Q (HR 5/5), as previously described (17). In each case, urease accounted for greater than 90% of the protein in the final samples.

The purified ureases were compared by denaturing gel electrophoresis as described above. In addition, differences in native relative mass were assessed by gel filtration chromatography by using a Superose 12 (HR 10/30) column equilibrated in 0.2 M KC – 50 mM phosphate – 1 mM EDTA – 1 mM β-mercaptoethanol (pH 7.0).

RESULTS AND DISCUSSION

Effect of nickel concentration on urease activity. In order to characterize the effect of urease accessory gene deletions, it was necessary to use a medium that would allow rapid and reproducible growth of all cultures. Previously, MOPS medium was utilized for studies of urease regulation (14); optimal urease activity required the presence of at least 100 μM nickel concentrations, although no nickel-dependent regulation of protein expression was observed. We chose to grow all strains in Luria broth (LB) (15), which necessitated reexamination of the effect of nickel concentration on urease activity. *E. coli* DH5 (pKAU17) cells were observed to grow well in nickel concentrations up to 1.6 mM. Urease activity depended strongly on nickel levels and was not saturated until a nickel concentration of approximately 0.8 mM was present (Fig. 2). A similar pattern, although at much lower specific activity values because of the lower copy number of the plasmid, was noted for *K. aerogenes* (pKAU19); however, these cells could tolerate higher nickel levels and grew well up to a concentration of 2.5 mM nickel (not shown; complementary study carried out by Michael J. Renner). The effects of nickel concentration on urease activity were not due to nickel-dependent regulation of urease expression, as demonstrated by the identity of SDS-polyacrylamide gel staining patterns for samples of the various cultures (not shown); rather, LB simply appears to sequester nickel much more strongly than does MOPS medium. For the following experiments, *E. coli* cultures were grown in LB supplemented with 1 mM nickel.

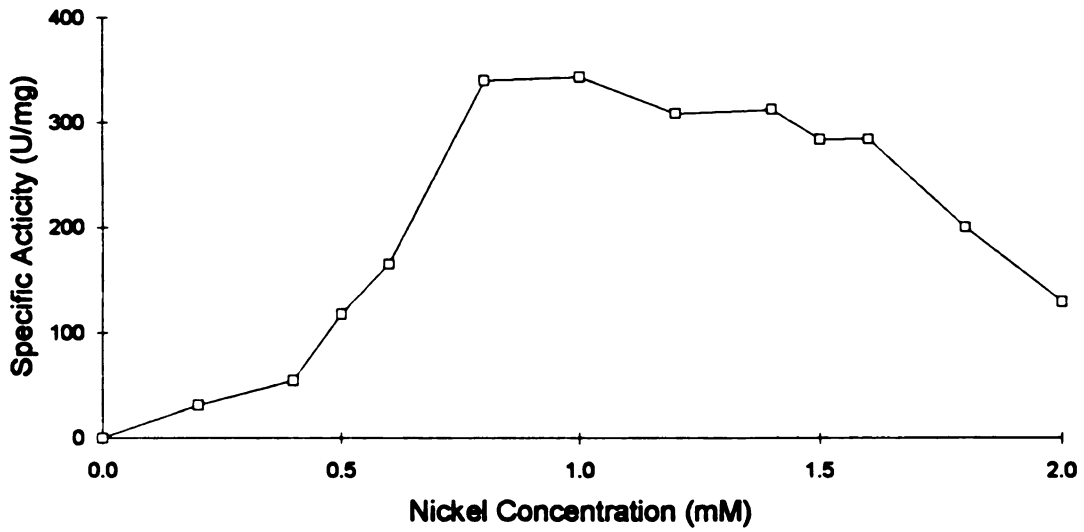

Figure 2. Effect of nickel chloride concentrations on urease activity for cells grown in LB medium. *E. coli* DH5 cells containing the intact urease gene cluster on plasmid pKAU17 were cultured overnight in LB medium in the presence of various nickel chloride concentrations. The cells were concentrated by centrifugation, disrupted by sonication, and assayed for specific activity (14).

Selective inactivation of *ureD*, *ureE*, *ureF*, and *ureG*. A map of the urease gene cluster indicating deletions within the *ureD*, *ureE*, *ureF*, and *ureG* genes is provided in Fig. 1. Also shown are restriction sites that were used for generating the deletion mutants, for subcloning, and for obtaining fragments for sequence analysis. Extracts of recombinant *E. coli* DH5 cells from a control culture and from each of the deletion mutants were examined for urease activity as shown in Table 1; the Δ*ureE* mutants retain partial activity, whereas the Δ*ureD*, Δ*ureF*, and Δ*ureG* mutants are inactive.

The depressed activity levels in the Δ*ureE* mutants may arise, at least in part, from polar effects on downstream genes which are required for urease activation. As discussed below, SDS-polyacrylamide gel analysis of these cell extracts is consistent with partial polarity. Similarly, the Δ*ureF* mutant could theoretically yield inactive urease because of polar effects on expression of the *ureG* gene; the 552-bp deletion in *ureF* leads to a frame shift at the end of an abbreviated UreF peptide, placing the new termination codon 22 bp downstream of the normal position and overlapping the start of *ureG* by 14 bp. To test whether the Δ*ureF* deletion mutant lacks activity because of polarity on *ureG*, cells were cotransformed with the Δ*ureF* plasmid and with a compatible plasmid, pACYC-G, containing an intact *ureG* gene. As shown by the data in Table 1, cotransformation of cells with Δ*ureF* and pACYC-G plasmids does not restore urease activity. In contrast, pACYC-G is capable of restoring activity in cells containing the Δ*ureG* plasmid, demonstrating the ability of the former plasmid to express an active UreG protein. Other cotransformation experiments included the demonstration that an intact *ureD* gene in pACYC-D could restore activity to cells containing Δ*ureD* plasmids, and pACYC-F, containing the *ureF* gene, could restore the Δ*ureF* mutant (Table 1). Thus, *ureD* and *ureF* encode trans-acting factors that are required for generating active urease.

Table 1. Urease specific activities in *E. coli* cell extracts from cells containing control plasmids and derivatives

Plasmid Derivative	Specific Activity[a] (U mg^{-1})
pKAU17	198.4
pKAU17Δ*ureE-1*	105.2
pKAU17Δ*ureE-2*	84.9
pKAU17Δ*ureF*	< 1
pKAU17Δ*ureF* + pACYC-F	66.5
pKAU17Δ*ureF* + pACYC-G	< 1
pKAU17Δ*ureG-1*	< 1
pKAU17Δ*ureG-1* + pACYC-G	129.7
pKAU17Δ*ureG-2*	< 1
pKAU17Δ*ureG-2* + pACYC-G	192.5
pKAU22	141.2
pKAU22Δ*ureD-1*	< 1
pKAU22Δ*ureD-1* + pACYC-D	48.6
pKAU22Δ*ureD-2*	< 1
pKAU22Δ*ureD-2* + pACYC-D	17.2

[a] Cells were grown overnight in LB containing 1 mM nickel chloride, concentrated by centrifugation, sonicated, and assayed for urease activity and protein content.

The extracts from the deletion mutants were subjected to SDS-polyacrylamide gel electrophoresis, as shown in Fig. 3. In each case, the urease subunits were present at high levels and comigrated with purified urease peptides; i.e., the mutations did not affect urease expression. The Δ*ureE* mutants lacked an intense band at an apparent M_r = 23,900 ± 1,000; additionally, in the case of Δ*ureE-2*, bands at M_rs of 27,000 and 28,500 were of diminished intensity. The Δ*ureF* mutant lacked a faint band at an M_r of 27,000 ± 1,000 and exhibited decreased intensity of a band at an M_r of 28,500, but was otherwise unaffected. The Δ*ureG* mutants were only deficient in an intense band at M_r = 28,500 ± 1,000. No changes from the control cultures were observed for Δ*ureD* mutants (data not shown). These results are consistent with the proposal that the *ureE*, *ureF*, and *ureG* genes encode proteins with M_rs of 23,900, 27,000, and 28,500, respectively, and that the Δ*ureE-2* and Δ*ureF* mutants exhibit partial polarity on downstream genes.

To verify the gene-peptide assignments for the two peptides which were expressed at a high level, their amino-terminal sequences were determined. The putative UreE and UreG peptides possessed sequences (Met-Leu-Tyr-Leu-Thr and Met-Asn-Ser-Tyr-Lys) that were identical to those predicted for these peptides on the basis of their DNA sequences (13). These peptides did not migrate in SDS-polyacrylamide gels according to their sizes (M_rs of 17,558 and 21,943) predicted by DNA sequencing. Gel scanning or visual inspection of Fig. 3 indicates that the UreE and UreG peptides are present at levels that are roughly comparable in intensity to the urease subunits. In contrast, the putative UreF peptide is present at barely detectable levels and UreD was not observed in *E. coli*(pKAU17) or *K. aerogenes*(pKAU19) cell extracts. To enhance the ability to detect plasmid-derived UreD peptide and to provide verification of the UreF peptide assignment, in vitro transcription-translation analysis was

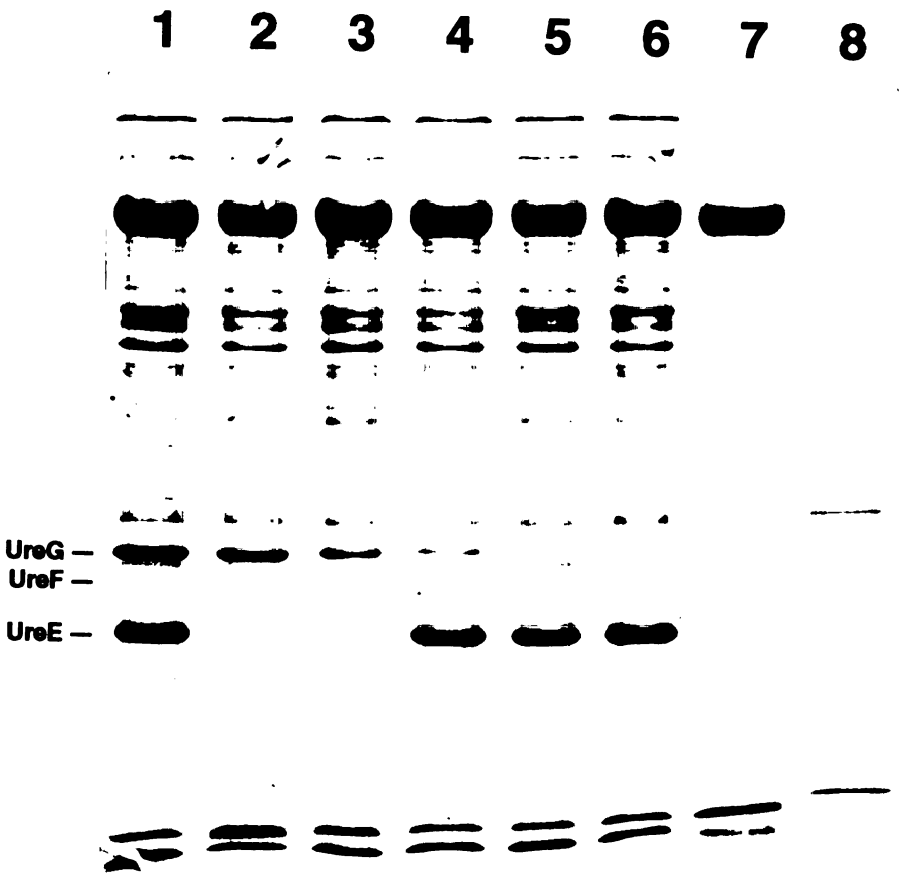

Figure 3. Analysis of deletion mutants by SDS-polyacrylamide gel electrophoresis. Cell extracts from a control *E. coli* DH5 culture containing pKAU17 (lane 1) and from cells containing pKAU17 with deletions in *ureE* (Δ*ureE-1* in lane 2 and Δ*ureE-2* in lane 3), *ureF* (lane 4), or *ureG* (Δ*ureG-1* in lane 5 and Δ*ureG-2* in lane 6) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10-15% gradient gel, followed by Coomassie blue staining. Standard urease (lane 7) and molecular weight markers (lane 8: phosphorylase b, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400.) were also examined. The proposed locations of the UreE, UreF, and UreG peptides are indicated.

performed (Fig. 4). As a control (Fig. 4A, lane 5), the pACYC-G plasmid yielded a unique band at an M_r of 27,000 ± 1,000, consistent with the results described above. The pACYC-D plasmid (Fig. 4A, lane 3) yielded a strong band (M_r = 34,900 ± 1,000) which coincided with a weak band that was also observed in the other pACYC184-derived plasmids (Fig. 4A, lanes 2,4, and 5) and in pKAU17 (Fig. 4A, lane 6). In addition, a faint band (M_r = 29,300 ± 1,000) was observed to be unique to the pACYC-D plasmid (Fig. 4A, lane 3). In a separate experiment comparing the products of pKAU22 and its Δ*ureD-1* derivative (Fig. 4B, lanes 2 and 3), a peptide band (M_r = 29,300 ± 1,000) was clearly missing in the latter sample, identifying this peptide as the *ureD* gene product. No unique band was observed for pACYC-F (Fig. 4A, lane 4), possibly because of overlap with the intense band of plasmid-derived chloramphenicol acetyltransferase at M_r = 25,700 ± 1,000. However, comparison of pKAU17 (Fig. 4A, lane 6) and its Δ*ureF* derivative (Fig. 4A, lane7) clearly shows the loss of a band (M_r = 25,800 ± 1,000) in the latter sample, as observed in cell extracts experiments. Also shown in the two pKAU17 samples are the three urease subunits, the UreE and UreG peptides, and the peptide associated with ampicillin resistance (M_r = 31,800 ± 1,000, also observed in lane 8 containing the pGEMβGAL control plasmid).

Effect of accessory gene mutations on urease. Urease was partially purified from the deletion mutants and from control cells. The specific activities and nickel contents of the mutant proteins are summarized in Table 2. Each of the four genes involved in urease activation affect the level of nickel incorporation into the enzyme. The specific activity of urease purified from *E. coli*(pKAU17) grown in LB containing 1 mM nickel was only 60% of that expected on the basis of the known specific activity of urease purified from *K. aerogenes*

(pKAU19) grown in MOPS medium containing 0.1 mM nickel. The low specific activity in the former cells may be related to the very high urease expression levels from this plasmid (urease accounts for well over 20% of the total protein, as illustrated in Fig. 3). Nevertheless, nickel incorporation into urease does not appear to be affected in these samples. The only mutants with significant activity, Δ*ureE-1* and Δ*ureE-2*, had nickel contents that correlated to their specific activities when compared with the pKAU17 control. The reduced nickel contents in urease from these mutants may relate, in part, to partial polar effects on downstream genes. Interestingly, cells containing deletions in *ureE* were more sensitive to growth inhibition at elevated nickel concentrations as if the UreE peptide protected cells from nickel toxicity. Deletions in *ureD*, *ureF*, and *ureG* resulted in the absence of activity and the near absence of nickel. No differences were discerned in the native relative mass of the mutant proteins, as judged by gel filtration chromatography, or in the subunit sizes, as assessed by SDS-polyacrylamide gel electrophoresis.

Figure 4. In vitro transcription-translation of urease-related plasmids. Purified DNA was transcribed and translated as described in the Materials and Methods section. After denaturing gel electrophoresis, the peptides obtained in each sample were visualized by exposure to X-ray film. **Panel A**: Radiolabeled molecular weight markers (lane 1) included myosin, M_r 200,000; phosphorylase b, M_r 92,500; bovine serum albumin, M_r 69,000; ovalbumin, M_r 46,000; carbonic anhydrase, M_r 30,000; and lysozyme, M_r 14,300. Lanes 2-5 contain samples from pACYC184 and its derivatives, pACYC-D, pACYC-F, and pACYC-G, respectively. Lane 6 is derived from pKAU17, whereas lane 7 is from pKAU17Δ*ureF*. Lane 8 contains the control plasmid pGEMβGAL, containing the β-lactamase gene. **Panel B**: Molecular weight markers (lane 1), pKAU22 (lane 2), and pKAU22Δ*ureD-1* (lane 3). The positions are shown for UreD, UreE, UreF, and UreG, for the urease subunits (X), for an uncharacterized peptide that is enhanced in pACYC-D (+), and for chloramphenical acetyltransferase (o) and β-lactamase (•).

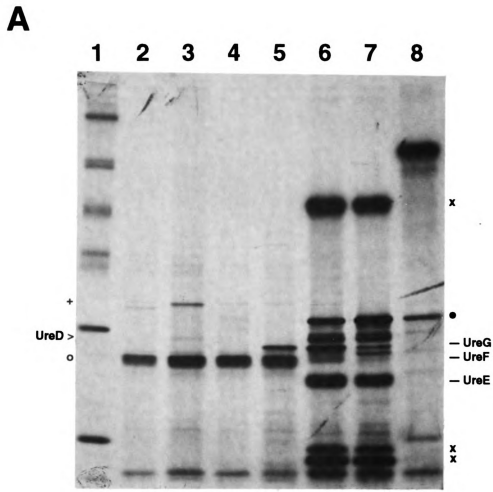
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Table 2. Properties of urease purified from *E. coli* containing intact urease plasmids and deletion mutants

Plasmid derivative	Location of deletion	Specific activity (μmol of urea $min^{-1}mg^{-1}$)	Number of Ni (per catalytic unit)
pKAU19	None	2,500	2
pKAU17	None	1,487 (100%)	2.3 (100%)
pKAU22	Δ *ureD-1*	< 1 (0%)	0.15 (7%)
pKAU22	Δ *ureD-2*	< 1 (0%)	0.2 (9%)
pKAU17	Δ *ureE-1*	966 (65%)	1.5 (65%)
pKAU17	Δ *ureE-2*	708 (48%)	1.15 (50%)
pKAU17	Δ *ureF*	< 1 (0%)	< 0.05 (2%)
pKAU17	Δ *ureG-1*	< 1 (0%)	< 0.05 (2%)
pKAU17	Δ *ureG-2*	< 1 (0%)	< 0.05 (2%)

Concluding remarks. These studies have demonstrated that all four urease accessory genes are necessary for the functional incorporation of the urease metallocenter. The role of these genes remains unknown; however, it is clear that the accessory proteins do not significantly alter the subunit or native size of the urease protein. Two of the accessory proteins (UreE and UreG) are expressed at levels comparable to those of the urease peptides, whereas UreD and UreF are expressed at undetectable to low levels. It is unclear whether these results reflect stoichiometric or catalytic roles for the proteins. Analysis of the UreD and UreF sequences has not revealed a model for their mode of action. In contrast, sequence analysis of *ureE* indicates that the predicted UreE protein possesses several potential metal-binding sites, consistent with a possible role of this protein as a metal transferase (13). Moreover, the predicted UreG sequence possesses a nucleotide binding motif (16), consistent with a hypothetical role in coupling ATP hydrolysis to metal incorporation; in vivo studies have provided evidence that energy is required for nickel activation (6). Further studies are in progress to address the function of these genes.

Although the structure and function of metal centers of metalloproteins have received a great deal of attention, very little is known with regard to how these centers are made. There are hints that metal processing genes exist in several other systems, but only a few clear examples have been reported (reviewed in 4). For example, genetic studies of *E. coli* have revealed the presence of several non-structural genes required for hydrogenase expression (*E. coli* hydrogenases are enzymes containing nickel and iron-sulfur centers); a mutation in one of these genes, *hypB*, can be complemented in part by the addition of very high levels of nickel (19). Interestingly, the predicted sequence for the HypB protein (8) is related (~25% identity) to that of the UreG peptide (21). Thus, the urease accessory genes and the hydrogenase accessory genes

may have diverged from a common ancestral nickel incorporation system. Analysis of the steps involved in incorporating nickel into urease may have a direct bearing on hydrogenase activation. In addition to systems requiring accessory genes for nickel incorporation, activation of non-nickel metalloproteins may also require accessory genes. For example, Zumft and coworkers (23) have reported that deletions in any of three genes immediately downstream of the structural genes for *Pseudomonas stutzeri* nitrous oxide reductase lead to the formation of copper-deficient enzyme. It is important to note that characterization of the genes involved in urease metallocenter biosynthesis may serve as a model system for the biosynthesis of other metalloproteins.

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CHAPTER 4

PURIFICATION AND CHARACTERIZATION OF UreE PROTEIN

ABSTRACT

The *K. aerogenes ureE* gene product was previously shown to facilitate assembly of the urease metallocenter (chapter 3). UreE protein has now been purified and characterized. Although the protein behaves as a soluble protein, it is predicted to possess an amphipathic β-strand and exhibits unusually tight binding to phenyl-Sepharose resin. Immunogold-electron microscopic studies confirm that UreE is cytoplasmic protein. Each dimeric UreE molecule (M_r = 35,000) binds ~6 nickel ions (K_d of 9.6 ± 1.3 μM) with high specificity according to equilibrium dialysis measurements. The nickel site in UreE was probed by extended X-ray absorption fine structure and variable temperature magnetic circular dichroism spectroscopies. The data are consistent with the presence of Ni(II) in pseudo-octahedral geometry where five of the ligands are imidazoles and the sixth ligand is a nitrogen or oxygen donor. We hypothesize that UreE binds intracellular nickel ion and functions as a nickel donor during metallocenter assembly into the urease apo-protein.

INTRODUCTION

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 (1, 24). 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 in 24). 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) (25)] where each catalytic unit is comprised of subunits in an apparent $\alpha_1\beta_2\gamma_2$ stoichiometry and contains a bi-nickel active site (29, 30). When *K. aerogenes* cells are grown in the absence of nickel, urease apoenzyme is produced. Although the purified apoenzyme cannot be reactivated by the simple addition of nickel ions, an energy-dependent reconstitution of the protein is observed in intact cells (17).

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 (18, 25). These non-subunit 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 (18). 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, UreE is a strong candidate for a nickel-binding protein since the translated *ureE* gene sequence reveals the presence of several potential metal binding sites including a carboxyl terminus where 10 of 15 residues are histidine (25).

Here we describe the purification and characterization of UreE protein. In particular, we demonstrate that UreE is indeed a nickel-binding protein and detail the properties of its novel nickel metallocenter.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. aerogenes* CG253 was transformed with plasmid pKAU19 (26) and *E. coli* DH5 was transformed with pKAU17 (26) or pKAU17ΔureE-1, a *ureE* deletion mutant (18). Recombinant *K. aerogenes* cells were grown at 37°C in MOPS-glutamine medium (26) containing 100 μM nickel chloride and chloramphenicol (30 μg/ml). Recombinant *E. coli* cells were grown at 37°C in LB medium containing 1mM nickel chloride and ampicillin (50 μg/ml) as previously described (18).

Purification of UreE 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 buffer (20 mM potassium phosphate, 0.5 mM EDTA, 1 mM 2-mercaptoethanol [pH 7.2]), resuspended in an equal volume of PEB buffer containing 0.5 mM phenylmethylsulfonyl 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 x *g* for 90 min at 4°C. The cell extracts were chromatographed on a DEAE-Sepharose column (2.5 by 15 cm) at 4°C, in the same buffer and eluted with a 400 ml-linear salt gradient to 1M KCl. UreE eluted from the column at approximately 0.3 M KCl. The pooled sample was desalted and concentrated by using an Amicon pressure filtration stirred-cell with a YM 10 ultrafiltration membrane in PEB buffer (pH 6.9), applied to a carboxymethyl-Sepharose column (2.5 by 17 cm), and eluted with a linear salt gradient to 0.5 M KCl. Nearly homogeneous UreE that was suitable for most experiments described below eluted approximately at 0.2 M KCl. For antibody preparation, however, this sample was further purified by using a Mono-S

column (0.5 by 5 cm) and a linear gradient to 1 M KCl at room temperature. All resins and columns were purchased from Pharmacia. The presence of UreE protein in column fractions was assessed by polyacrylamide gel electrophoresis.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (16) 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. Protein concentrations were routinely assessed by the spectrophotometric assay of Lowry *et al.* (21) using bovine serum albumin as a standard.

Peptide structural calculations. Prediction of the UreE structural properties made use of a set of sequence analysis programs described by Devereux *et al.* (8). In particular, the amphipathic moment was calculated by the MOMENT program, whereas, the hydrophobicity and the potentials for forming secondary structures were calculated by the PEPTIDESTRUCTURE program.

Cellular localization by immunogold electron microscopy. Antibodies directed against UreE protein were generated in a white, female, New Zealand rabbit by injecting 200 μl (2.5 mg/ml) of homogeneous protein in PBS (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 (23). Antibodies were titrated by using standard dot blot (6) and ELISA methods (9). For immunogold detection, wild-type *K. aerogenes, K. aerogenes* (pKAU19), *E.*

coli DH5 (pKAU17), and *E. coli* DH5 (pKAU17ΔureE-1) 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 (2). Polymerization was carried out for 2 days at 6°C 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 (Tris-buffered 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 non-specific 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 anti-rabbit IgG (15 nm, Jansen) for 1 hr (4). 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 and nickel content of UreE protein. The molecular weight for native *K. aerogenes* UreE 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} (M_rs = 670,000, 158,000, 44,000, 17,000, and 1,350; Bio-Rad Laboratories, Richmond, CA). Metal contents of ashed samples were determined by using a Varian Spectra AA-400Z atomic absorption spectrometer as previously described (18).

Equilibrium Dialysis. Equilibrium dialysis of UreE with $^{63}NiCl_2$ (1,455 mCi/mmol; Du Pont NEN Research products Inc., Boston, Mass.) diluted with various concentrations of unlabeled $NiCl_2$ was performed either in a Spectrum 5-cell equilibrium dialyzer (Spectrum medical industries Inc., Los Angeles, CA) or an equilibrium microvolume dialyzer (Hoeffer Scientific Instruments, San Francisco, CA) with precut dialysis membranes (MWCO= 12-14,000). Purified UreE (2 μM) was analyzed for nickel binding in 50 mM sodium phosphate (pH 7.2), 50 mM HEPES (pH 7.2), or 50 mM Tris-HCl (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). Calculation of the bound Ni was performed by standard procedures (3).

Spectroscopic characterization of UreE holoprotein. UreE (35 mg/ml) in the presence of 3.1 mM $NiCl_2$, 0.1 mM EDTA, 20 mM potassium phosphate buffer (pH 7.6), and 30 % glycerol was examined by extended X-ray absorption fine structure (EXAFS) by Shengke Wang and Robert A. Scott and examined by variable temperature magnetic circular dichroism (MCD) spectroscopy by Michael K. Johnson at the Center for Metalloenzyme Studies, Department of Chemistry, University of Georgia.

RESULTS

Purification of UreE. UreE was highly purified by using a combination of DEAE-Sepharose and carboxymethyl-Sepharose column chromatographies. Samples at this stage of purification were estimated to be over 95% homogeneous (Fig.1) and were deemed suitable for most of the experiments reported below. However, an additional step involving Mono S chromatography was included prior to generation of polyclonal monospecific antibodies. In spite of the presence of multiple histidine residues at its carboxyl terminus, UreE did not show any affinity to a chelating-Superose column that was charged with nickel. In contrast, UreE peptide does bind tightly to phenyl-Sepharose resin. Indeed, elution of protein from the hydrophobic resin requires the inclusion of 40% dimethylsulfoxide in the PEB buffer.

Cellular localization of UreE. The tight interaction observed between UreE and phenyl-Sepharose resin raises the possibility of a membrane localization for the peptide *in vivo*. Such an interaction is not predicted from calculations of UreE hydrophobicity that show a lack of significant hydrophobic regions. In contrast, however, an unusually strong feature (maximum of 0.85 with approximately 180^0 rotation per residue) can be found in the region from residue 18 to 34 in calculations of the amphipathic moment for UreE. The use of such predictive algorithms can lead to intriguing speculation (*vide infra*); however, it does not directly assess the *in vivo* interaction between the UreE peptide and the membrane. For this purpose, immunogold electron microscopic localization studies were carried out (Fig. 2). The gold particle labeling patterns clearly show that UreE 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

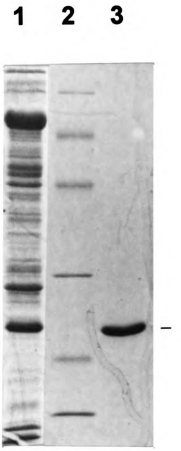


Figure 1. SDS-polyacrylamide gel electrophoresis of purified UreE. Cell extracts from *E. coli* DH5 (pKAU17) (lane 1) and purified UreE (lane 3) were subjected to SDS-polyacrylamide gel electrophoresis by using a 10-15% gradient gel, followed by Coomassie blue staining. Molecular weight markers(lane 2) 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; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400.

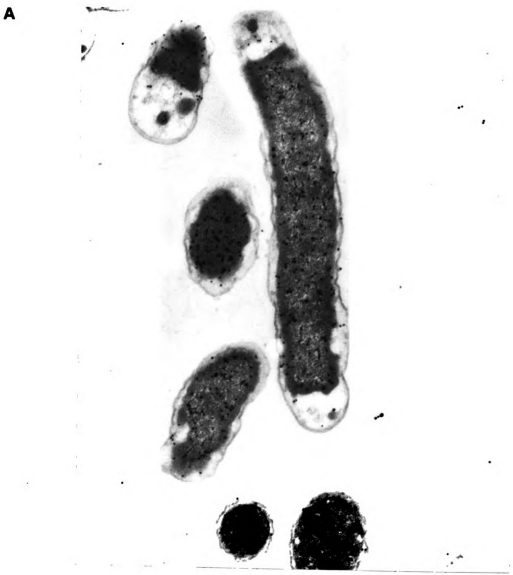
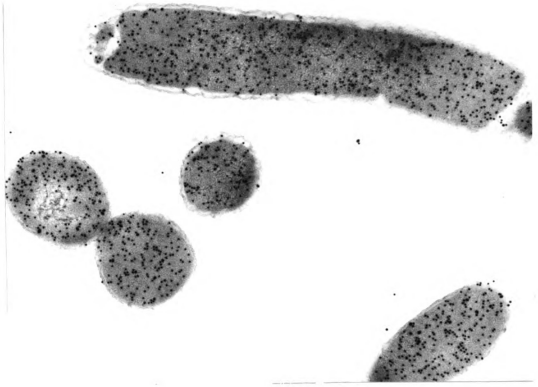


Figure 2. Immunogold localization of UreE in recombinant *K.aerogenes* and *E. coli* cells. Thin sections of (a) *K. aerogenes* (pKAU19), (b) *E. coli* (pKAU17), and (c) *E. coli* (pKAU17ΔureE-1) cells were reacted with anti-UreE antibodies and labeled with anti-rabbit IgG-gold particles. UreE was localized to the cytoplasmic portion of the cell.

B



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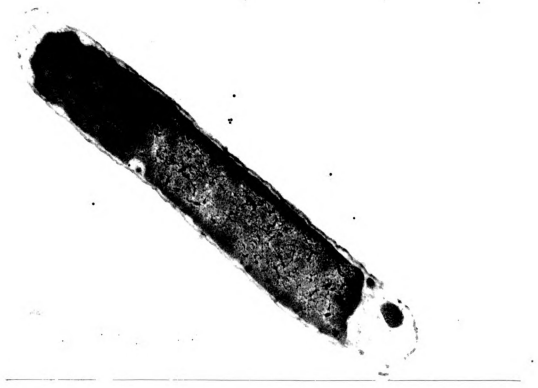


immunogold technique to allow localization (not shown), probably because of insufficient levels of ureE protein (15). *E. coli* (pKAU17ΔureE-1) that does not possess UreE did not bind significant levels of anti-UreE antibody (panel C).

Characterization of UreE: a nickel-binding protein. UreE protein was shown to exist as an apparent dimer (M_r = 35,000) when subjected to gel filtration analysis. When purified in the presence of buffer containing 0.5 mM EDTA, UreE was found to be free of metal ions; however, equilibrium dialysis experiments were used to demonstrate that dimeric UreE binds 6.05 ± 0.05 nickel ions with a K_d of 9.6 ± 1.3 μM (Fig. 3). This value is a combination of the individual values for each of the six nickel sites. Bovine serum albumin (BSA) and lysozyme were used as controls in this experiment. Lysozyme has no reported affinity for nickel ion, whereas, BSA is analogous to the human serum albumin which is known to bind 1 mole of nickel per mole of protein (10).

The specificity of nickel binding was assessed by examining the effects of various concentrations of other divalent cations on the binding of ^{63}Ni that was present at a concentration of 20 μM (Fig. 4, panel A). The presence of excess magnesium, manganese, and calcium ions had negligible effects on nickel binding even to concentrations of 400 μM. Cobalt ion appeared to compete reasonably well at approximately one of the four sites that bind nickel at this concentration and only weakly competed with the remaining nickel-binding sites. In a separate experiment, 200 μM cobalt was found to have little effect on the total number of nickel that UreE could bind and only slightly shifted the value for K_d (data not shown). Neglecting different affinities for cobalt among the six metal ion sites, the overall apparent K_i for cobalt ion was found to be ~ 640 μM. Copper ion also interferes differentially with the nickel binding sites, but in this case copper ion competed well at approximately three of the four binding sites

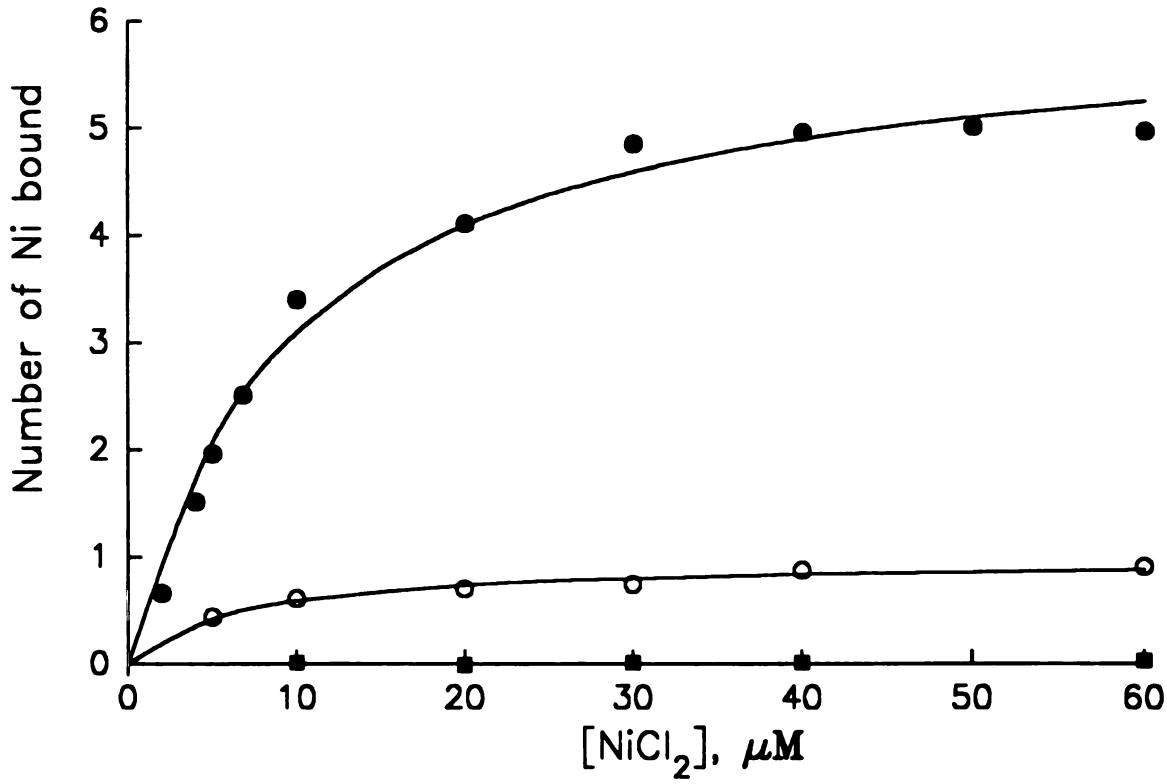
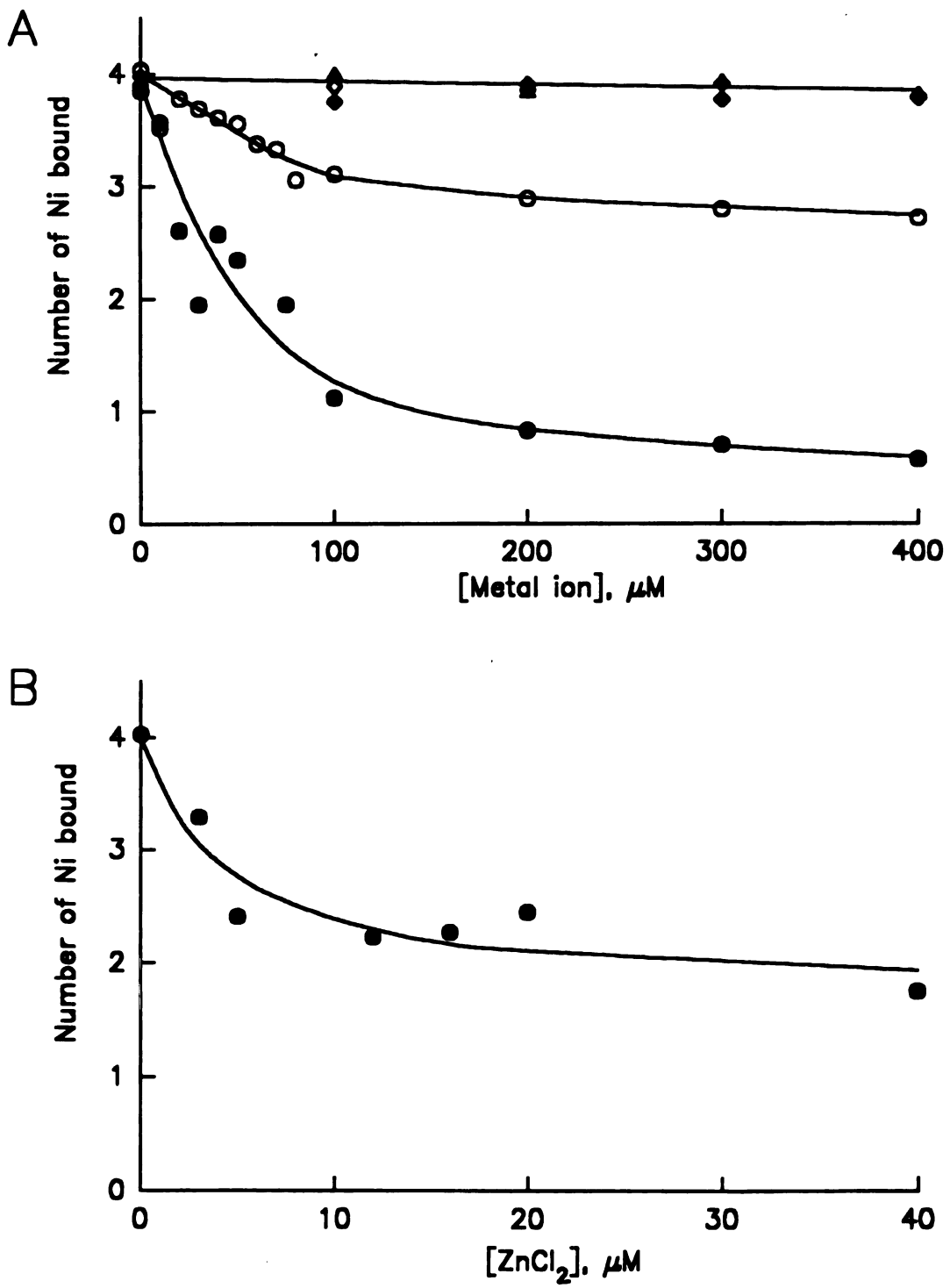


Figure 3. Equilibrium dialysis of UreE with $NiCl_2$. 2 μM UreE (●) in 50 mM sodium phosphate (pH 7.2) buffer containing 0.5% NaCl was equilibrated with the indicated concentrations of $NiCl_2$ solutions containing $^{63}NiCl_2$ (1.2 μCi/ml). Bovine serum albumin (O) and lysozyme (■) were used as controls.

Figure 4. Effect of divalent metal ions on nickel binding to UreE. Equilibrium dialysis of 2 μM of UreE with nickel ion at a concentration of 20 μM was performed in the presence of various concentrations of metal ions. Panel A: Experiments with $MgSO_4$ (◊), $MnCl_2$ (◆), and $CaCl_2$ (▲) were carried out in 50 mM sodium phosphate buffer (pH 7.2), $CoCl_2$ (O) in 50 mM HEPES buffer (pH 7.2), and $CuSO_4$ (●) in 50 mM Tris-HCl buffer (pH 7.6), each containing 0.5 % NaCl. Panel B: Experiments with $ZnCl_2$ were carried out in buffer containing 50 mM sodium phosphate (pH 7.2)-0.5% NaCl. Data at higher concentrations of zinc ion could not be obtained due to anomalous interference with the distribution of Ni ions across the dialysis membrane (See text).


A
Number of Ni bound
0
1
2
3
4
0
100
200
300
400
[Metal ion], μM
B
Number of Ni bound
0
1
2
3
4
0
10
20
30
40
[ZnCl2], μM

that bound nickel at 20 μM and competed weakly for the remaining site. Zinc ion exhibited the greatest ability to compete with nickel ion at the concentrations shown, but again this metal preferentially interfered with binding at a subset (approximately half) of the sites. At concentrations higher than 100 μM, zinc ion exhibited anomalous behavior as if it prevented equilibration of nickel ion across the dialysis membrane even in the control sample containing no protein.

Characterization of the UreE metallocenter. EXAFS data were of high quality and curve fitting required the presence of multiple imidazoles. The best fit to the data (Table 1) assumes a $Ni(imid)_5(N,O)$ coordination sphere. Variable temperature magnetic circular dichroism spectroscopic results were consistent with an octahedral geometry comprised of nitrogen ligands (data not shown).

Table 1. Curve-fitting results for Ni EXAFS of UreE protein[a]

Sample	Group	Shell	N_s	R_{as} (Å)	σ_{as}^2 (Å)	f[b]
UreE	imid	Ni-N_1	(5)[c]	2.08	0.0045	1.45
	imid	Ni-C_2		3.09	0.0070	
	imid	Ni-N_3		4.23	0.0090	
	imid	Ni-C_4		4.29	0.0095	
	imid	Ni-C_5		3.03	0.0075	
		Ni-N	(1)	2.11	0.0025	

[a] Data were fit by the EXCURVE fitting program. Group is the chemical unit defined for the multiple scattering calculation. N_s is the number of scatterers (or groups) per metal; R_{as} is the metal-scatterer distance; σ_{as}^2 is a mean square deviation in R_{as}.

[b] f' is a goodness-of-fit statistic normalized to the overall magnitude of the $k^3\chi(k)$ data:

$$f' = \frac{\left\{\sum\left[k^3(\chi_{obsd}(i) - \chi_{calc}(i))\right]^2 / N\right\}^{1/2}}{(k^3\chi)_{max} - (k^3\chi)_{min}}$$

[c] Numbers in parentheses were not varied during optimization.

DISCUSSION

Isolation of UreE. UreE, a cytoplasmic nickel-binding protein that assists in the functional incorporation of nickel ion into urease, has been purified and characterized. Mulrooney and Hausinger (25) speculated that UreE could function in binding nickel based on sequence analysis of the *ureE* gene. The protein sequence was shown to possess numerous potential metal ion binding sites including a carboxyl terminus that contained ten histidines among the last 15 residues. Other laboratories genetically attach polyhistidine tails to various proteins in order to facilitate their purification by metal ion affinity chromatography (12, 13, 19, 20, and 28). UreE thus represents a naturally occurring example of such a protein with a poly-His tail. In contrast to other poly-His proteins, however, we found that the UreE peptide does not bind to an iminodiacetic acid metal ion affinity resin. We speculate that UreE fails to bind to the resin because the metal ion is transferred from nickel-charged resin to the high affinity nickel-binding site on UreE. Although metal ion affinity chromatography was not useful for peptide isolation, UreE readily can be purified by a simple two-column procedure.

Structural features of UreE. Immunogold electron microscopic studies were used to localize UreE to the cytoplasm of the cell, consistent with the lack of hydrophobic regions in the peptide based on computer calculations. Yet, UreE tightly binds to phenyl-Sepharose resin. The observed interaction with this hydrophobic resin may be related to the high amphipathicity in the region from residue 18 to 34. The sequence of this region (Leu - Pro - Ile - $Asp^{(-)}$ - Val - $Arg^{(+)}$ - Val - $Lys^{(+)}$ - Ser - $Arg^{(+)}$ - Val - $Lys^{(+)}$ - Val -Thr - Leu) contains

alternating hydrophobic (underlined) and hydrophilic residues. I speculate that this region may form an amphipathic β-strand in which one face of the peptide is hydrophilic and the other face is hydrophobic. Such a structure could, perhaps, bind tightly to the phenyl-Sepharose resin. The hydrophobic face does not appear to bind to cellular membranes *in vivo*, but rather it may participate in protein-protein interactions. For example, this motif may account for the observed dimerization of the UreE peptide.

Nickel-binding properties of UreE. The dimeric UreE protein binds 6 nickel ions in a highly specific manner. The coordination sphere of UreE-bound nickel ions appears to be comprised of five histidine imidazole groups and a sixth nitrogen or oxygen donor. If one assumes that any one imidazole can ligate a single metal, then dimeric UreE would be able to bind 4 nickel ions at the carboxyl termini. The fact that additional nickel ions were bound demonstrates that other parts of the UreE structure are essential for ligating a portion of the nickel. The peptide possesses five additional histidine residues at positions 91, 96, 109, 110, and 112. The presence of distinct nickel-binding sites within UreE is consistent with the cobalt, copper, and zinc competition studies that indicate preferential competition with subsets of the total nickel. The *in vivo* effects of cobalt, copper, and zinc ions on urease metallocenter assembly are unknown; however, Lee *et al.* (17) demonstrated that these cations can not substitute for nickel during urease biosynthesis in the absence of nickel ion.

Possible role of UreE in urease metallocenter assembly. Although our results clearly demonstrate that the carboxyl terminus of *K. aerogenes* UreE protein can bind nickel ions, we have not established that this equilibrium process is important for incorporation of nickel into urease.

Indeed, the carboxyl termini of UreE peptides from *Proteus mirabilis* (14) and *Helicobacter pylori* (7) possess eight and only one histidine respectively. It is possible, however, that some other protein could functionally compensate for a UreE protein that was deficient in nickel-binding ability. Consistent with this view, deletion of most of the *ureE* gene from a plasmid containing the urease genes does not abolish all urease activity in recombinant *E. coli*, but only reduces the activity levels and correspondingly reduces the nickel content (18). Potential candidates for nickel-binding proteins that could substitute for UreE include auxiliary peptides that are essential for hydrogenase biosynthesis (hydrogenase is another nickel-containing enzyme (11)). For example, ORF5 in the *Azotobacter vinelandii hox* gene cluster possesses a 13-residue region containing 10 histidines (5) and ORF4 in the *Rhodobacter capsulatus hup* gene cluster possesses a region that contains 23 histidines in 62 residues (31). The roles of these hydrogenase-related genes have not been elucidated but they are related in sequence to *hypB*, an *E. coli* gene that is known to function in hydrogenase activation involving nickel incorporation (22).

UreE is only one of the four accessory proteins involved in urease metallocenter assembly. Nothing is known about the functions of the *ureD* and *ureF* gene products in this process. Similarly the role of UreG has not been demonstrated; however, sequence analysis reveals a P-loop motif (27) (...**GXXGXGKT**...) that is found in a variety of ATP- and GTP-binding proteins. Furthermore, an energy dependence for *in vivo* nickel ion incorporation was observed (17). These features, together with the above UreE-related results, lead us to hypothesize that UreE may bind nickel and act as a nickel donor to the urease apo-protein while UreG binds ATP and couples

its hydrolysis to the nickel incorporation process. Further experiments to test and expand this hypothesis are currently under investigation.

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CHAPTER 5

CONCLUSIONS AND FUTURE PROSPECTS

The main goal at the outset of the work described herein was to study how nickel ions are incorporated into *K. aerogenes* urease apo-enzyme. Although many questions about this process remain to be answered, I have made significant progress in understanding several aspects of this phenomenon.

As a preliminary step to performing nickel incorporation studies, I carried out experiments with the urease apo-protein from *K. aerogenes* (pKAU19) cells which were grown in the absence of nickel. This work represents the first purification and characterization of any apo-urease. I confirmed that the concentration of nickel ions in the growth medium does not affect the level of expression of the urease structural proteins. Apo-urease behaved analogously to holo-protein during purification and the purified apo-enzyme showed the same native molecular weight and the same subunit stoichiometry as the holo-enzyme. However, apo-enzyme was shown to be thermally less stable than holo-enzyme indicating that the presence of Ni somehow contributes to the integrity of the holo-enzyme. DEP modification studies of both holo- and apo-urease demonstrated that apo-protein has an increased number of histidine residues accessible to DEP, suggesting possible Ni ligation by these residues in holo-enzyme. The inability to activate purified *K. aerogenes* urease apo-enzyme by simple addition of Ni ions indicated that a cellular factor may be required for incorporation of the metal center.

To test whether the non-structural accessory genes are required for synthesis of active urease and to begin to characterize their functions, I generated selective deletions in these regions and assessed the resulting effects of these gene disruptions on the urease activity and the nickel

content of the enzyme. I found that all four accessory genes (*ureD, ureE, ureF,* and *ureG*) are necessary for the functional incorporation of the urease metallocenter. Whereas the Δ*ureD*, Δ*ureF*, and Δ*ureG* mutants are inactive and ureases in these mutants lack nickel, the Δ*ureE* mutants retain partial activity and their ureases possess corresponding lower levels of nickel. UreE and UreG peptides were identified by SDS-polyacrylamide gel comparisons of mutant and wild type cells and by N-terminal sequencing. UreD and UreF peptides, which are synthesized at levels that are too low to be convincingly identified on the SDS-polyacrylamide gel of the cell extracts, were identified by using *in vitro* transcription/translation methods. Cotransformation of *E. coli* cells with the complementing plasmids confirmed that *ureD* and *ureF* gene products indeed act in *trans*.

I purified and characterized the UreE protein. I used computerized peptide structure algorithms to identify a potential β-strand region that may be important in peptide dimerization. Furthermore, I used immunogold electron microscopic studies to localize UreE protein to the cytoplasm of the cell. To examine whether UreE binds nickel *in vitro*, I performed equilibrium dialysis studies of the purified UreE with $^{63}NiCl_2$. I found it binds ~6 Ni in a specific manner with a K_d of 9.6 ± 1.3 μM. Results from the EXAFS studies demonstrated that nickel ions are ligated by 5 histidinyl residues and a sixth N or O atom, consistent with the possible participation of the polyhistidine tail at the carboxyl termini of the dimeric UreE in nickel binding.

I provided samples of urease apo-protein and holo-enzyme to Prof. Andy Karplus for crystallographic analysis. A preliminary assessment of these crystals

has been published (Appendix I). Dr. Karplus' lab has collected X-ray diffraction data to better than 2 Å, obtained two heavy atom derivatives, and resolved the structure to 3 Å. A difference map of the holo-enzyme minus apo-protein clearly indicate that the native enzyme is a trimer of bi-nickel active sites. The nickel-nickel distance is estimated to be 3.3 to 3.4 Å. Another outcome of these crystallographic studies is the revelation of a trimeric structure for native urease indicating that urease subunits exist in either $\alpha_3\beta_3\gamma_3$ or $\alpha_3\beta_6\gamma_6$ stoichiometry. Whereas the latter stoichiometry is most consistent with gel scanning data which showed apparent $\alpha_1\beta_2\gamma_2$ subunit stoichiometry, the former stoichiometry is more consistent with the native molecular weight of urease. Because the determination of subunit stoichiometry by using gel scanning has an intrinsic uncertainty, since it is based on the assumption that the Coomassie dye has equal affinity to all peptides, and the sequence of the $\alpha\beta\gamma$ subunit of *Klebsiella aerogenes* align well with that of the single subunit of jack bean urease, the bacterial urease is most likely to possess an $\alpha_3\beta_3\gamma_3$ stoichiometry.

I localized UreG, another accessory gene product of *K. aerogenes* urease operon, to the cytoplasm by immunogold microscopy (Appendix II). The translated UreG possesses a P-loop motif which is found in a variety of ATP- and GTP-binding proteins, hence, this protein may bind ATP. This speculation is important in light of the energy dependence of *in vivo* activation of urease apo-enzyme that was shown by Scott B. Mulrooney.

Below, I provide a speculative model for urease metallocenter biosynthesis that incorporates my results with other known features of the urease-related gene products. UreE is proposed to bind nickel and act as a

nickel donor to the urease apo-protein while UreG binds ATP and couples its hydrolysis to the nickel incorporation process (figure 1).

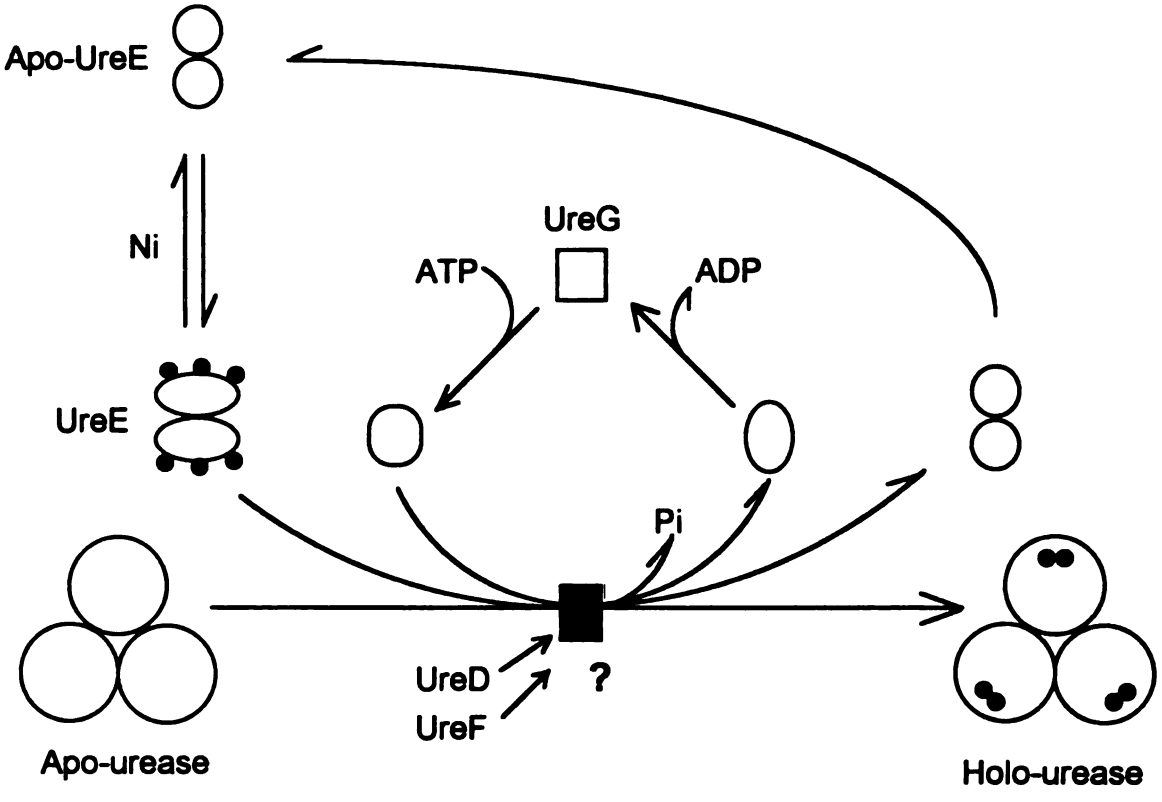

Figure 1. Model of possible roles of accessory gene products in Ni incorporation into *K. aerogenes* urease.

The roles of UreD and UreF are unknown (computer calculations indicate that these proteins are unlikely to be membrane associated) and the detailed mechanism of energy coupling remains obscured by the black box. This model serves as the basis for several testable hypotheses. For example, purified UreG could be examined for its ability to bind and/or hydrolyze ATP. Additionally, this model predicts the presence of a transient interaction between holo-UreE and

apo-urease. Since binding an effector molecule could affect protein conformation and association with other proteins, association of with UreG with other accessory gene products could be examined in certain conditions. Further understanding of this process will require the purification of UreD and UreF proteins, probably after genetic manipulations to enhance expression of this genes.

APPENDIX I

J. Mol. Biol. (1992) 227, 934–937

CRYSTALLIZATION NOTES

Preliminary Crystallographic Studies of Urease from Jack Bean and from *Klebsiella aerogenes*

Evelyn Jabri[1], Mann H. Lee[2], Robert P. Hausinger[2]† and P. Andrew Karplus[1]†

[1]*Section of Biochemistry, Molecular and Cell Biology Cornell University, Ithaca, NY 14853, U.S.A.*

[2]*Departments of Microbiology and Biochemistry Michigan State University, East Lansing, MI 48824-1101, U.S.A.*

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Ureases from both jack bean (*Canavalia ensiformis*) seeds and *Klebsiella aerogenes* have been crystallized by the hanging drop method. The plant-derived urease crystals are regular octahedra analogous to those obtained by Sumner. Preliminary X-ray diffraction studies show that the crystals belong to the cubic space group $F4_132$, with $a = 364$ Å, and appear to contain one or two subunits in the asymmetric unit. Using a synchrotron source, the crystals diffract to near 3·5 Å resolution. Crystals of urease from *K. aerogenes* belong to the cubic space group $I23$ or $I2_13$, with $a = 170{\cdot}8$ Å and appear to contain a single catalytic unit per asymmetric unit. The crystals diffract to better than 2·0 Å resolution and are well suited for structural analysis.

Keywords: urease; nickel; protein crystallography

Urease (urea amidohydrolase; EC 3.5.1.5) is a nickel-dependent enzyme (Dixon *et al.*, 1975) that catalyzes the hydrolysis of urea to form ammonia and carbamate. The carbamate then spontaneously decomposes to form carbonic acid and a second molecule of ammonia. For unknown reasons, some seeds are particularly rich sources of urease, and the best characterized example of this enzyme comes from seeds of the jack bean (*Canavalia ensiformis*). It was originally isolated as a pure, crystalline enzyme by Sumner (1926), and these crystals, the first obtained for any enzyme, played a decisive role in proving the proteinacious nature of enzymes. Ureases have since been isolated from a wide variety of organisms including other higher plants, fungi and bacteria (for a review, see Mobley & Hausinger, 1989). Although the crystals of jack bean urease have long been available, a structure determination has not been carried out. A passing reference to some X-ray diffraction studies of jack bean urease crystals appears in a recent review (Zerner, 1991).

The quaternary structures of a number of ureases have been characterized. The jack bean enzyme, as a representative of higher plant ureases, exists as a hexamer of identical 91 kDa subunits (Takishima *et al.*, 1988) with two Ni/subunit, the catalytic unit (Dixon *et al.*, 1980*a*). The *Klebsiella aerogenes* enzyme is representative of most bacterial ureases; it possesses three subunits of 60·3 kDa (α), 11·7 kDa (β) and 11·1 kDa (γ) (Mulrooney & Hausinger, 1990) in apparent $\alpha_2\beta_4\gamma_4$ stoichiometry and has two binickel active sites (Todd & Hausinger, 1989). The amino acid sequences (Takishima *et al.*, 1988; Riddles *et al.*, 1991; Mulrooney & Hausinger, 1990) show that despite their different quarternary structures, the jack bean and *K. aerogenes* enzymes are indeed homologous: the γ, β and α chains of *K. aerogenes* urease correspond to jack bean urease residues 1 to 101, 132 to 237 and 271 to 840, respectively. This clear correspondence of amino acid sequences suggests that the catalytic unit of *K. aerogenes* urease may be $\alpha\beta\gamma$ rather than $\alpha\beta_2\gamma_2$ as was suggested by the staining intensity of electrophoretically separated subunits (Todd & Hausinger, 1987) and by chemical modification experiments (Todd & Hausinger, 1991). Crystallographic analysis of the bacterial enzyme would resolve this ambiguity in subunit stoichiometry.

All known ureases are nickel-dependent, despite the fact that the chemistry of urea hydrolysis would not seem to require metal involvement (Zerner, 1991). Spectrophotometric characterization of both the jack bean and bacterial urease nickel centers has shown that at least one of the two bound nickel ions per catalytic unit has an essential role in catalysis, as it is directly involved in binding substrates and inhibitors (Dixon *et al.*, 1980*b*; Todd & Hausinger,

† Authors to whom all correspondence should be addressed.

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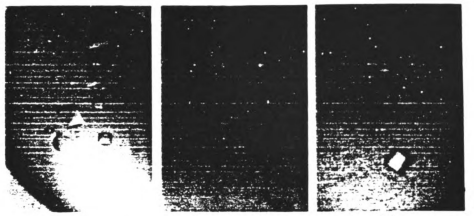

(a) (b) (c)

Figure 1. Crystals of (a) jack bean urease (scale = 120 μm/cm) grown at 4°C from hanging drops containing 3 μl of a 2·25 mg/ml solution of urease in buffer A (pH 7·0) mixed with an equal volume of 10% polyethylene glycol 1000 or 6% polyethylene glycol 8000 in 50 mM-sodium citrate, 1 mM-β-mercaptoethanol (pH 7·0) and equilibrated against the same. (b) *K. aerogenes* urease (scale = 90 μm/cm) grown at 4°C from hanging drops containing 3 μl of a 10·2 mg/ml solution of protein in buffer A mixed with an equal volume of 14% ammonium sulfate in 0·2 M-sodium citrate/phosphate buffer (pH 8·5), 0·02% (w/v) NaN_3. (c) *K. aerogenes* urease (scale = 130 μm/cm) grown at 25°C from hanging drops containing 10 μl of a 19 mg/ml solution of urease in 20 mM-Tris buffer (pH 7·0), 1 mM-EDTA, 1 mM-β-mercaptoethanol mixed with an equal volume of 1·5 to 1·7 M-Li_2SO_4 in 100 mM-Hepes buffer (pH 7·25 to 7·75) and equilibrated against the same.

1989). In the current model for urease catalysis, the two nickel ions have unique roles: one polarizes the carbonyl oxygen of urea and the second increases the nucleophilicity of a water molecule that attacks this carbonyl carbon (Dixon *et al.*, 1980*c*). The two nickel ions in jack bean urease are apparently coordinated by oxygen and nitrogen ligands (Clark *et al.*, 1990) and have been shown to be in close proximity to each other by variable temperature magnetic circular dichroism spectroscopy (Finnegan *et al.*, 1991). Analogous biophysical studies carried out with the bacterial enzyme have revealed near identities to the jack bean urease bi-nickel active site (unpublished results). Chemical modification and other studies are consistent with the presence in both the plant and bacterial enzymes of a carbonyl group, a sulfhydryl group, and an unidentified base located at or near the active site (for reviews, see Mobley & Hausinger, 1989; Zerner, 1991). The single cysteine residue identified by chemical modification is conserved in both enzymes (Takishima *et al.*, 1988; Todd & Hausinger, 1991).

Structural studies of urease are needed to provide further insight into the mechanism of catalysis. The plant enzyme is an obvious choice for structural analysis because of its historical significance and because methods for purification and crystallization have been reported. Characterization of bacterial urease is also important because ureolytic microorganisms contribute to the development of urinary stones, pyelonephritis, ammonia encephalopathy, urinary catheter encrustation and peptic ulceration, and, in addition, soil microbial ureases decrease the efficiency of fertilizers (for a review, see Mobley & Hausinger, 1989). Structural characterization of a bacterial urease will facilitate the design of inhibitors of medical and agronomic significance. For these reasons, our laboratories have undertaken X-ray crystallographic studies of urease from both jack bean and *K. aerogenes*.

The jack bean enzyme has been purified from jack bean meal based on the method of Blakeley *et al.* (1969). Briefly, jack bean meal (British Drug Houses) was extracted with buffer A (20 mM-sodium phosphate, 1 mM-EDTA, 1 mM-β-mercaptoethanol (pH 7)) for one hour at 4°C. The meal was removed through centrifugation, and 28% acetone was added to the supernatant. The suspension was incubated at 0°C overnight and the precipitated proteins were removed by centrifugation. The concentration of acetone in the supernatant was then increased to 31·6% and, after stirring at room temperature for ten minutes, the precipitate was removed by centrifugation. The resulting supernatant was directly applied to a 1 cm × 27 cm Q-Sepharose (Sigma) anion exchange column, which was subsequently washed with buffer B (20 mM-sodium phosphate, 1 mM-EDTA, 5 mM-β-mercaptoethanol (pH 7·5)). The column was washed with 100 ml of 0·1 M-NaCl in buffer B prior to elution of the urease by a 200 ml linear gradient of 0·1 M to 0·3 M-NaCl in buffer B. Urease eluted at approximately 0·17 M-NaCl. Active fractions were pooled and concentrated by using an Amicon YM10 filter and Centricon-30. Urease was then applied to a 1 cm × 117 cm Ultragel ACA34 gel filtration

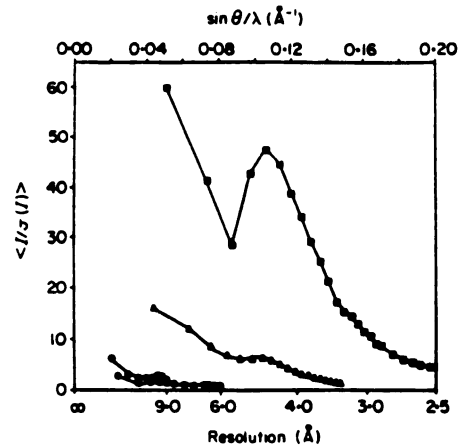

Figure 2. Diffraction strength as a function of resolution for urease crystals. Diffraction strength is given by $\langle I/\sigma(I)\rangle$ over all reflections in a given resolution range, where I is the integrated intensity and $\sigma(I)$ is the standard error of the intensity measurement. The curves shown are based on laboratory diffraction data for *K. aerogenes* urease crystals (■), and laboratory (○, ●) and synchrotron (▲) diffraction data for jack bean urease crystals. Approximate volumes of the 3 jack bean urease crystals used were $5{\cdot}4 \times 10^6$ (○), $3{\cdot}4 \times 10^6$ (●) and $1{\cdot}6 \times 10^6$ (▲) μm^3; and that for the *K. aerogenes* crystal was 70×10^6 μm^3. The laboratory diffraction data were collected with a single San Diego Multiwire Systems detector on a Rigaku RU-200 rotating anode X-ray generator (CuKα radiation; 0·5 mm × 5 mm focal spot; running at 50 kV, 150 mA, graphite monochrometer). Parameters for the *K. aerogenes* data collection: crystal-to-detector distance = 770 mm through a helium path; frame width = 0·08° in ω; rate = 30 s/frame; 0·5 mm collimator. Equivalent parameters for the jack bean data: 1040 mm through a helium path; 0·1° in ω: 120 s/frame (○) or 150 s/frame (●); 0·3 mm collimator. The synchrotron data were collected at beam line F1 at the Cornell High Energy Synchrotron Source using λ = 0·91 Å radiation and a 0·2 mm collimator. Four contiguous 10 s, 1° oscillation images were recorded from a single crystal on 8 inch × 10 inch Kodak storage phosphors using a crystal-to-film distance of 287 mm through air. Even though a smaller crystal was used for the synchrotron data set, the increased signal-to-noise relative to the laboratory data is dramatic.

column equilibrated in buffer A. The fractions containing urease were pooled and concentrated for use in crystallization trials. Throughout the preparation, urease activity at 37 °C and pH 7·0 was monitored using the Bertholet ammonia assay (Weatherburn, 1967) and protein concentration was determined by using the BCA reagent assay (Pierce). Ten grams of jack bean meal yielded 1 mg of urease with a specific activity of 2300 units/mg protein (1 unit = 1 μmol urea hydrolyzed/min).

Crystals of jack bean urease were obtained by using the hanging drop method with three different coprecipitants. Both 20% (v/v) acetone and 15% (v/v) 2-methyl-2,4-pentanediol as coprecipitants yield regular octahedral crystals of approximate dimensions 40 μm × 20 μm × 20 μm. Larger octahedral crystals, 200 μm × 160 μm × 160 μm, were obtained using 10% (w/v) polyethylene glycol 1000 or 6% (w/v) polyethylene glycol 8000 (Fig. 1(a)). For space group characterization, simulated precession photographs were collected with a San Diego Multiwire Systems detector (see the legend to Fig. 2).

Precession photographs of the *hk*0, *hhl* and *h*0*l* planes showed that these crystals belong to the cubic space group $F4_132$ with $a = 364$ Å (1 Å = 0·1 nm). As each urease subunit occupies about 23% of the volume of the asymmetric unit, it is conceivable that anywhere from one to three subunits could fit in the asymmetric unit leaving space for 77% to 31% solvent (Matthews, 1968). Given the weak diffraction of the native crystals, it seems most likely that one or two subunits occupy the asymmetric unit and the crystals are greater than 50% solvent. Using our laboratory source, significant diffraction can be seen only out to near 6 Å resolution (Fig. 2). However, crystals exposed using a synchrotron source consistently showed diffraction out to near 3·5 Å (Fig. 2), suggesting that it is the small crystal size (which can be compensated for by a more intense X-ray source), rather than intrinsic disorder which limits the laboratory diffraction to 6 Å resolution. The large unit cell and relatively weak diffraction of these crystals complicate crystallographic investigation; nevertheless, by using synchrotron radiation, a structure determination should be possible.

X-ray diffraction studies of crystals of urease from *K. aerogenes* are much more promising. Purfication of this urease from *K. aerogenes* containing plasmid pKAU19 is outlined by Todd & Hausinger (1989). The enzyme possessed a specific activity of ≥2500 units/mg under our standard assay conditions. Initially, trapezoidal plates of dimensions 600 μm × 400 μm × 40 μm could be obtained from 44 to 46% $(NH_4)_2SO_4$. The larger crystals often showed mica-like layering (Fig. 1(b)) and, although they diffracted to 3 Å resolution, they were disordered in one plane. Using the quick screen method devised by Jancarik & Kim (1991), a condition (no. 16) was found which gave single crystals. After optimization of conditions, cubic crystals of dimension 400 μm^3 were obtained. After setting up the drop, a light precipitate forms within a day and the crystals grow in the presence of precipitant over two to three weeks (Fig. 1(c)). Precession photos of the *h*0*l* and *hk*0 zones were identical and showed mm symmetry and centering. Subsequent analysis of a small data set showed the crystals had I rather than F-centering, so that the crystals belong to the space group $I23$ or $I2_13$ with $a = 170{\cdot}8$ Å. These space groups cannot be distinguished based on the diffraction pattern alone. Assuming one or two catalytic units per asymmetric unit, the crystals would contain 45% or 12% solvent, respectively. Since

12% solvent is outside of the range normally observed for protein crystals (Matthews, 1968), we conclude that there is a single catalytic unit per asymmetric unit. Native diffraction data have been collected at 2·4 Å resolution (Fig. 2), and the search for heavy-atom derivatives is underway. Since the crystals diffract to better than 2 Å resolution (data not shown), the structure solution will yield details suitable for studying the mechanism and carrying out inhibitor design.

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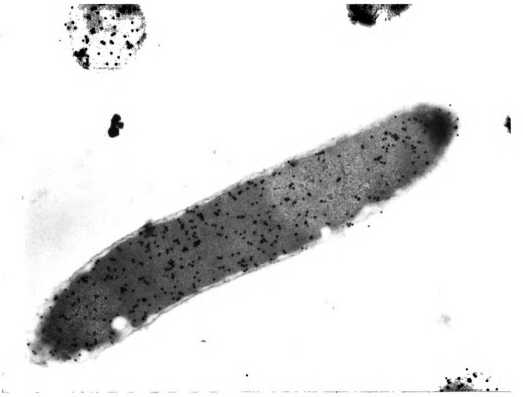
APPENDIX II

Figure 1. Immunogold localization of UreG in recombinant *K.aerogenes* and *E. coli* cells. Thin sections of (a) *K. aerogenes* (pKAU19), (b) *E. coli* (pKAU17), and (c) *E. coli* (pKAU17ΔureG-2) cells were reacted with anti-UreG antibodies and labeled with anti-rabbit IgG-gold particles. UreG was localized to the cytoplasmic portion of the cell.

B



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