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Scott B. Mulrooney

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Robert P. Hausinger
Major professor

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CLONING, SEQUENCE, AND CHARACTERIZATION OF THE KLEBSIELLA AEROGENES UREASE OPERON

By

Scott B. Mulrooney

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ABSTRACT

Microbial ureases play a significant role in agricultural nitrogen metabolism and the pathogenesis of several human diseases. The best-studied bacterial urease is from *Klebsiella aerogenes*: its regulation has been partially characterized and the heteropolymeric enzyme has been purified and shown to contain four nickel ions per native molecule. Cloning of the *Klebsiella aerogenes* urease genes was undertaken in order to obtain greater quantities of enzyme for study and to elucidate the regulation, genetic organization, and sequence of the urease genes.

Preliminary studies were carried out using the previously cloned urease genes from *Providencia stuartii*. Urease was purified and characterized from recombinant *Escherichia coli*, and urea-induced regulation of expression was demonstrated. Urease properties were identical to the parent organism when expressed in the heterologous host.

The *Klebsiella aerogenes* urease genes were cloned by selecting a urease-positive colony from a cosmid library and subsequently subcloned to a 5.7 kb fragment. When the recombinant plasmid was tested in several enteric hosts, urease was expressed during growth under nitrogen-limited conditions and repressed in nitrogen-rich media. *Klebsiella aerogenes* containing the recombinant plasmid expressed high levels of urease. These cells were used for immunogold electron microscopy to localize the enzyme to the cytoplasm.

In vivo incorporation of the nickel center into urease was examined in recombinant cells overexpressing urease in a nickel-free medium with several metabolic inhibitors. Addition of nickel restored activity when a protein synthesis inhibitor was used but not when energy-utilization

inhibitors were present or in sonicated cells. These results indicate that nickel ions are incorporated into pre-formed apo-urease in an energy dependent process.

Sequence analysis of the urease genes revealed an operon consisting of six open reading frames: three encoding the urease subunits (*ureA*, *ureB*, and *ureC*) and three with unspecified functions (*ureE*, *ureF*, and *ureG*). Deletion of the *ureE*, *ureF*, and *ureG* from the operon resulted in the synthesis of inactive apo-urease, however, these genes could act in trans to restore activity. This demonstrates that one or more of the UreE, UreF, and UreG gene products facilitate nickel incorporation into urease.

To my family and my parents

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CHAPTER 1

Literature Review

Urea (H_2N-CO-NH_2) is very stable in solution: its half-life for spontaneous degradation is 3.6 years at 38°C and non-catalyzed hydrolysis has never been observed (4). Most urea is therefore broken down enzymatically by urease (urea amidohydrolase, EC 3.5.1.5) which catalyzes the hydrolysis of urea to yield ammonia and carbamate. The carbamate spontaneously hydrolyzes to give additional ammonia and carbonic acid. In aqueous solution, the carbonic acid deprotonates and the ammonia becomes protonated resulting in a net increase of pH.

Urease occurs in organisms from many different taxonomic classifications, including over 200 species of bacteria, and several plants, yeast, algae, and invertebrates (73). Much of the early urease work was done on the enzyme purified from jack bean (*Canavalia ensiformis*) (1,109): indeed, in 1926 it was the first enzyme to be crystallized (104). It was not until nearly 50 years later that urease was demonstrated to be a nickel-containing enzyme (22). More recent studies have focused on the role of urease in particular human pathogenic conditions and in agricultural nitrogen economy. Molecular biological and genetic tools have been employed to give more information on enzyme structure, composition, nickel incorporation, and regulation. The objective of this chapter is to present a brief overview of the significance and enzymology of ureases, and give an account of recent advances in the regulation and genetics of this enzyme. The main focus will be on bacterial ureases, although examples from plant and other sources will be mentioned where appropriate.

Medical significance. Microbial urease activity has been shown to be a contributing factor in pathogenesis of several diseases. Hepatic encephalopathy (100), hyperammonemia (103), and hepatic coma (98) can occur from the effects of toxic nitrogenous compounds which have not been

metabolized by the liver. Ammonia released from urea hydrolysis contributes to these conditions (101). Furthermore, considerable recent research has focused on the role of urease in development of urinary stones and peptic ulcerations.

Human urine consists of 0.4 to 0.5 M urea (35) and presents a favorable environment for many microorganisms which can infect the urinary tract. One major consequence is infection-induced stone formation, which account for 20% to 40% of all urinary stones (34,36,94). Infection stones are formed when polyvalent struvite and apatite salts crystallize due to alkalinization from urea hydrolysis (68,69,73). Ureolytic bacteria have also been implicated in pyelonephritis, which occurs when urea hydrolysis and its associated increase in pH results in acute kidney inflammation and tubule necrosis (96). In addition, ureolytic microbes have been shown to be important in promoting incrustation and obstruction of urinary catheters (74,112). *Proteus mirabilis* is the major urease-producing uropathogen in humans (96). Experiments with animal models have shown urease to be a significant virulence factor in infections of the kidney epithilium (9,26,29,33,58). Treatment with urease inhibitors helps reduce these effects (2,59,82). Furthermore, recombinant DNA techniques have been used to construct urease-negative mutants of *Proteus mirabilis* (45) and *Staphylococcus saprophyticus* (30). In both cases, the mutants had significantly lower virulence.

Recently, the ureolytic bacterium *Helicobacter pylori* (formerly *Campylobacter pylori*) has been implicated in promoting peptic ulcerations (8,32,38,63,72,76). It only grows in a relatively neutral pH range and is very acid sensitive (32). A model has been proposed whereby the *Helicobacter pylori*, living in the stomach mucosa, survives by creating a

zone of favorable pH with the ammonia produced by urea hydrolysis (15). The *Helicobacter* urease, which has a relatively low K_m value of 0.2 to 0.8 mM (41,72), is able to utilize serum urea (1.7-3.4 mM) to modulate the pH of its local environment. Consequently, gastrointestinal inflamation and lesions develop, either by interfering with diffusion of acid from the gastric glands or by tissue damage directly from the localized high ammonia concentration (15,38).

Other roles for microbial ureases. Ureolytic microbes play a critical role in nitrogen cycling in ruminants: urea that has been generated from digestion and metabolism can be recycled through saliva and the bloodstream and returned to the rumen where it is subsequently hydrolyzed to give ammonia–the major source of nitrogen of the rumen microbial population (13,42,73). Urease activity is also found in soils, either in living organisms or as free enzyme (12,55,81). Indeed, application of urea fertilizers to areas with high soil urease activities can result in plant damage due to ammonia toxicity and elevated soil pH as well as nitrogen loss from volatilization of ammonia (81,99). Several studies have used urease inhibitors to reduce soil urea hydrolysis rates (11,14,54,64,89,90).

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 (104) the first demonstrated to contain nickel (22), and its mechanism has been probed by kinetic and spectroscopic methods (1,4,23). The amino acid sequence of the jack bean urease was determined by using classical protein methods (62,105). Soybean plants possess two immunologically and enzymologically distinguishable

types of urease: the embryo-specific (seed), and the ubiquitous (leaf) forms (84,85,86,87). Molecular biological strategies have been employed to examine the tissue-specific and temporal expression of the two isozymes (39). It was demonstrated that certain mutations would lead to the synthesis of inactive urease isozymes, indicating that some urease maturation factors may be required (71). A section of the soybean seed urease gene was cloned and sequenced (51).

Regulation of urease expression. Bacteria have three modes of regulating urease expression. Organisms such as *Helicobacter pylori* and *Morganella morganii* have ureases which are expressed constitutively and are not significantly affected by components in the growth medium (72,95). Another class, which possess urease genes that are inducible by urea, includes *Providencia stuartii* (Chapter 2; 75,79) and *Proteus mirabilis* (46). In the third group, urease expression is controlled by the global nitrogen (Ntr) system, and is best exemplified by *Klebsiella aerogenes* (Chapter 3; 28,60,80). Urease, like other Ntr-regulated genes, is expressed when the organism is exposed to nitrogen-limited conditions and repressed in the presence of nitrogen-rich constituents. Low nitrogen conditions initiate a complex regulatory cascade (60) which results in production the ntrA gene product (a specific sigma factor), which combines with core RNA polymerase and begins transcription of Ntr-regulated genes. There has been a recent proposal that transcription of a subset of *Klebsiella aerogenes* Ntr-regulated genes, including urease, is mediated by a newly discovered *nac* gene (*n*itrogen *a*ssimilation *c*ontrol; 3,61).

Cellular localization. Most reports of localization of urease in cells demonstrate that the enzyme is found mainly in the cytoplasm. Cell fractionation studies of various bacteria (43,44), *Providencia stuartii*

(75), *Proteus mirabilis*, (46), *Ureaplasma urealyticum*, (7,21,65, 92,97,110), and *Klebsiella aerogenes* (28), established that urease was in the cytoplasm. Immunogold electron microscopic localization studies of recombinant *Klebsiella aerogenes* urease confirmed earlier fractionation studies (Chapter 3; 80). Immunological methods were also used to localize jack bean urease in the cytoplasm (25). An alternate electron microscopic strategy was used for *Ureaplasma urealyticum* where electron-dense MnO_2 was precipitated by the alkaline pH from urea hydrolysis: urease was concluded to be in the cytoplasm (110).

Contrasting results were reported by McLean et al. (66,67), who used a cytochemical electron microscopic method with *Staphylococcus* species and *Proteus mirabilis*. In their strategy the bacteria were incubated with tetraphenylboron, which forms a precipitate with ammonia produced by urea hydrolysis. The tetraphenylboron-ammonia complex was then reacted to exchange the ammonium for silver ions which were then viewed by electron microscopy. For both *Staphylococcus* species urease was found to be membrane bound (66), and for *Proteus mirabilis* it was found in the periplasm and outer membrane (67). These anomalous results are probably due to the inability of tetraphenylboron to freely enter the cell; i.e., the reagent is reacting with the ammonia product diffusing out of the cell rather than the actual enzyme (73).

Urease purification. Sumner used a very simple procedure for obtaining crystalline jack bean urease (104) which involved extraction of jack bean meal with aqueous acetone and allowing crude crystals to form. This was eventually modified to include a DEAE-cellulose step (91). The first purification of a bacterial urease was from *Bacillus pasteurii* and included ammonium sulfate, calcium phosphate, and acetone fractionation

steps (52). Modern methods have relied on a series of ion exchange, gel filtration, and hydrophobic chromatographies to purify many ureases (73). Immuno-affinity chromatography has been used for *Ureaplasma urealyticum* (88,106). In addition, chromatography on immobilized substrate analogs have been used (20,70,83,113).

Structure and kinetic properties. Native ureases have molecular weights ranging from 125,000 to 590,000 and are composed of one or more subunit types. In contrast to the homohexameric jack bean enzyme (subunit M_r=90,770; 62), bacteria possess heteromeric ureases. The ureases of *Klebsiella aerogenes* (107), *Proteus mirabilis* (10,46), *Providencia stuartii* (Chapter 2; 79), *Selenomonas rumantium* (37,107), *Morganella morganii* (40), *Ureaplasma ureolyticum* (106), *Lactobacillus reutri* (49), and *Lactobacillus fermentum* (48) consist of one large (M_r=60,000 to 75,000) and two distinct small subunits (8,000 to 11,000). Many of the earlier accounts of purification of bacterial ureases reported that the enzyme consisted of multimers of a single large subunit (reviewed by Mobley and Hausinger, 73), however these studies did not look for subunits in the 8,000 to 11,000 range. Such subunits could easily be missed in SDS-polyacrylamide gel analysis unless proper precautions are taken (107). *Helicobacter pylori* urease is unusual in that it consists of one large (M_r=66,000) and one medium subunit (M_r=29,500) (41). Whereas most ureases exhibit maximum activity near neutral pH values, the recently purified enzymes from two *Lactobacillus* species were unusual in that they are most active at 65°C at pH 2 (48,49).

Jack bean urease possesses two nickel ions per subunit and most microbial ureases seem to contain 2 nickel ions per large subunit (22,37,48,79,107). Results reported for *Brevibacterium ammoniagenes* and

Bacillus pasteurii (16,83) are only half this amount. Active site titration studies on jack bean (22) and on *Klebsiella aerogenes* (108) ureases have shown that there are two nickel ions per active site.

K_m values for ureases can range from 0.1 to >100 mM (73). Ureases of ureopathogenic microbes are saturated with substrate, since urine is 0.4 to 0.5 M urea (35). Specific activity for jack bean urease is approximately 3,500 μmol urea min^{-1} mg^{-1} (1), while many bacterial enzymes range from 1,000 to 5,500 (73). Other ureases have lower reported specific activities, but this may be due to incomplete purification or inactivation (73).

Ureaplasma urealyticum produces an unusual urease in that specific activities have been reported as high as 180,000 (102). These high activities are consistent with a proposal that urease may be involved in energy transduction in *Ureaplasma*. Urea is an absolute requirement for growth of *Ureaplasma*, but very little of the ammonia or carbon is used by the cell (27). It is thought that the cytoplasmic increase in pH resulting from urea hydrolysis leads to formation of a proton gradient which drives ATP formation (65,93).

Cloning of urease genes. The screening method for all of the bacterial ureases that have been cloned, with the exception of *Ureaplasma urealyticum*, was to transform libraries of the urease-containing microbes into a urease-negative one; usually *E. coli*. Selections were made on agar plates which were only slightly buffered and contained urea and a pH indicator that gave a color change around urease-positive colonies. Christensen urea agar (17) has been used, but works best with constitutively expressed ureases. Other variations have been described which give an indicator color change upon overnight incubation

(19,24,46,50,79).

The urease genes of *Bacillus pasteurii* (50) and *Klebsiella pneumoniae* (31) were isolated by ligating size-fractionated chromosomal DNA into positive selection vectors. *Providencia stuartii* urease genes were cloned from its large conjugative plasmid by ligating fragments into vector pBR322 (75). The urease genes of a urease-positive *E. coli* (19), *Klebsiella aerogenes* (Chapter 3; 79), *Klebsiella pneumoniae* (31), *Morganella morganii* (40), and *Proteus mirabilis* (46,111) were cloned by partially digesting chromosomal DNA with *Sau*3A and ligating into various lambda and cosmid vectors. For *Proteus vulgarus* (77), the same strategy was used, except that plasmid pUC18 served as the vector. Furthermore, a similar approach was taken for *Staphylococcus saprophyticus* in which chromosomal fragments were ligated into a *Staphylococcus* vector and recombinant plasmids screened in urease-negative *Staphylococcus carnosus* (30).

In each of these cases, the cloned fragments contained the necessary regulatory regions that were recognized by the *E. coli* transcription and translation mechanisms. For *Providencia stuartii* and *Klebsiella aerogenes* clones, expression from the recombinant multi-copy plasmid allowed exceptionally high production of urease which was advantageous for purification of large amounts of enzyme (Chapter 2, 3; 79,80). To circumvent any possible problems of heterologous expression, *Helicobacter pylori* chromosomal fragments were cloned into a vector containing a *lac* promotor which controlled synthesis of the recombinant proteins (18).

The urease genes of *Ureaplasma urealyticum* were detected by hybridization to other cloned ureases rather than selecting for a urease-positive phenotype (6). Expression of urease activity was not

possible in heterologous hosts because *Ureaplasma* uses a UGA codon for tryptophan rather than a stop.

Sequence analysis. Recent DNA sequencing of the complete operons of *Proteus mirabilis* (47) revealed six open reading frames, three of which encode the urease subunits. The *Proteus mirabilis* urease subunit genes are preceded by a *ureD* gene, which may be involved in regulation (46). Next, are the γ, ß, and α urease structural genes, designated *ureA*, *ureB*, and *ureC*, respectively. Between *ureA* and *ureB* is a short region with homology to a eucaryotic splice junction, and the *ureB* and *ureC* genes overlap by four base pairs. Following *ureC* are *ureE*, and *ureF* which have unknown functions. These results, combined with earlier transposon mutagenesis studies, lead to the conclusion that all of the genes except *ureE* are necessary for urease activity. The *Klebsiella aerogenes* operon also consists of six open reading frames (Chapter 5; 78), however, there were two significant differences. An upstream *ureD* was lacking and an additional open reading frame, designated *ureG*, was found just downstream of *ureF*.

These findings, together with partial sequence information of the *Proteus vulgarus* (77), *Helicobacter pylori* (18), and *Ureaplasma urealyticum* (5) urease genes, reveal that the amino acid sequences of the multiple bacterial urease subunits are remarkably similar to the single subunit jack bean enzyme: there is about 50 to 60% identity between the sequences of the bacterial and the plant ureases. Indeed, 37% of the *Klebsiella aerogenes* amino acid residues are present in all four bacterial and the jack bean enzymes (Chapter 5).

Nickel incorporation into urease. Several studies have indicated that one or more gene products, in addition to the enzyme subunits, are

required to incorporate nickel ions into urease. For example, pleiotropic mutations have been found which result in the production of inactive ubiquitous and seed ureases in soybean (71). In *Aspergillis nidulans*, four genes are necessary for urease activity; mutation of two different loci caused production of inactive urease, and growth in high nickel could restore activity for one of these (56,57). In addition, transposon or deletion analysis of the *Providencia stuartii* (79), *Proteus mirabilis* (46), *Klebsiella pneumoniae* (31), *Klebsiella aerogenes* (Chapter 5; 78), and *Proteus vulgarus* (77) operons has resulted in mutants that express inactive urease subunits. Further evidence that a nickel insertion step is essential for urease activation is that *Klebsiella aerogenes* urease apoenzyme, which was purified from cells grown in the absence of nickel, could not be reactivated by nickel in vitro. Urease apoenzyme could be reactivated in whole cells even after treatment with protein synthesis inhibitors (Chapter 4; 51).

Aims of this thesis. At the time this thesis was started, bacterial ureases had been shown to consist of heterologous subunits and possess nickel. No urease genes had been cloned and it was not known if a heterologous host would express the urease protein, if the urease would be in an active or inactive form, or whether the nickel center could be correctly incorporated. This thesis reports on the first purification and characterization of a heterologously expressed urease from *Providencia stuartii* (Chapter 2), the cloning, regulation, overexpression, and cellular localization of *Klebsiella aerogenes* urease (Chapter 3), the in vivo reconstitution of urease apoenzyme (Chapter 4), and the sequence of the six open reading frames of the *Klebsiella aerogenes* urease operon and

indication that one or more genes are required for nickel incorporation (Chapter 5).

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CHAPTER 2

Purification and Characterization of Recombinant *Providencia stuartii* Urease Expressed by *Escherichia coli*

ABSTRACT

Recombinant urease from *Providencia stuartii* has been purified from *Escherichia coli*. Urease expression was induced by urea and repressed by nitrogen-rich components in the medium. The urease protein was purified 331-fold by DEAE-Sepharose, phenyl-Sepharose, Mono-Q, and phenyl-Superose chromatographies with a 7.3% yield. The enzyme possessed a K_m for urea of 9.3 mM and hydrolyzed urea at a V_{max} of 7,100 μmol/min per mg. *P. stuartii* urease is composed of three polypeptides (M_rs, 73,000, 10,000, and 9,000) denoted as α, ß, and τ. The native enzyme is best described as $(\alpha_1ß_2\tau_2)_2$, based on a native M_r of 230,000, obtained by gel filtration chromatography, and on the Coomassie blue staining intensities of the individual subunits. Atomic absorption analysis of the pure protein revealed 1.9 ± 0.1 nickel ions per $\alpha_1ß_2\tau_2$ unit.

INTRODUCTION

Urease, a nickel-containing enzyme that catalyzes the hydrolysis of urea to carbon dioxide and ammonia, is synthesized by a wide variety of bacteria, fungi, and plants (1,2,9,11). Bacterial urease may be a contributing factor in the development of pyelonephritis (4,16,24), hyperammonemia (25), and catheter encrustation (20), as well as kidney and bladder stone formation (7,23). In the last case, ammonia generated from urea hydrolysis alkalinizes urine, resulting in the precipitation of polyvalent anions and cations in the form of struvite and apatite salts (7). Although stones may develop from other causes, it has been estimated that 20-40% of all urinary stones are due to urease-positive bacteriuria (23).

In chronically catheterized patients, there is a high incidence of bacteriuria, with the organism *Providencia stuartii* a prevalent isolate (28,29). The urease genes of this microbe are located on a large conjugative plasmid in a number of isolates (6,18) and have been cloned and expressed in *Escherichia coli* (19). Preliminary minicell analysis of insertion and transposon mutants of the recombinant urease revealed that a region comprising 3.0 to 6.2 kilobase pairs (kb) of DNA was necessary for urease activity and encoded at least two polypeptides (M_rs, 73,000 and 25,500) (19). The kinetic parameters of the recombinant enzyme were similar to those of the native strain (19).

In addition to the urease genes of *P. stuartii*, those of *Bacillus pasteurii* (13), *Proteus mirabilis* (12), *Klebsiella aerogenes* (22; Chapter 3), *Klebsiella pneumoniae* (5), and *Morganella morganii* (10) have been

cloned in *E. coli* by selecting for a urease-positive phenotype. However, no detailed studies of urease expression, enzyme characterization or protein purification have been reported for any recombinant urease.

It was recently shown that the purified ureases of several bacterial species are composed of one large and two small polypeptides (27), which contrasts with the homomeric structure of the jack bean enzyme (1,2). The multimeric structure may be a general property of bacterial ureases.

This report extends the work of Mobley et al. (19) in which *P. stuartii* urease genes were cloned and some aspects of urease expression in *E. coli* were investigated. A purification scheme for recombinant urease is provided, factors affecting urease expression are examined, and the properties of the recombinant enzyme are described.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* HB101 (*recA pro leu rpsL hsdS*) containing pMID201 (*ure*$^+$ *tet*), which possesses urease genes cloned from a conjugative plasmid of *P. stuartii* BE2467 (19), were used for these experiments. The medium used (17) was either LB broth or ammonia-free M9 minimal medium supplemented with 5 or 10% (vol/vol) LB broth. When required, 1 ml of trace mineral solution (26) and 15 ml of filter-sterilized 1 M urea per liter were added. For growth of *E. coli* HB101(pMID201), all cultures contained 10 μg of tetracycline per ml.

Regulation of urease expression. For urease expression studies, 125-ml Erlenmeyer flasks containing 20 ml of medium were inoculated with 5 μl of a stationary LB culture and were grown overnight in a 37°C water bath with rapid shaking. Aliquots (5 ml) of the cultures were chilled on ice,

centrifuged, washed twice with an equal volume of ammonia-free M9 salts (M9 without glucose and ammonium chloride), and suspended in a final volume of 2.5 ml. The cells were sonicated four times for 30 s each time with a sonicator (Sonic Dismembranator; Ficher Scientific Co., Livonia, Mich.) by using a small probe (4-mm diameter tip) at 30% power. A 1-ml portion of the extract was centrifuged for 5 min at 4°C in a microcentrifuge (Eppendorf; Brinkmann Instruments, Inc., Westbury, N.Y.), and the supernatant was assayed for urease activity.

Assays. Urease activity was determined by monitoring the rate of ammonia released from urea by formation of indophenol, which was measured at 625 nm (30). 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 μmol of urea per min at 37°C under the assay conditions described above. Protein was measured by the method of Lowry et al. (15) by using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis. All electrophoresis was carried out by using the buffers of Laemmli (14), except that sodium dodecyl sulfate was omitted for native gels. Denaturing gels were run by using a 10 to 15% polyacrylamide gradient (bisacrylamide/acrylamide, 1:32) resolving gel with a 4.5% stacking gel. 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 Respense spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 562 nm. Nondenaturing gels were run by loading 1 U of urease activity on a 3% stacking gel and a 6% running gel, which was then stained for activity by using a phenol

red indicator, similar to the method of Blattler et al. (3).

Large-scale growth and urease purification. *E. coli* HB101(pMID201) was grown in ammonia-free M9 minimal medium supplemented with 10% (v\v) LB broth, 15 mM urea, 10 ug tetracycline per ml, and 1 ml trace mineral solution per liter. Cultures were grown at 37°C in either a 25-liter Microferm fermentor (New Brunswick Scientific Co., Edison, N.J.)(15 l cultures) with rapid mixing and aeration, or in a 20 l bottle (containing a 10-liter culture) immersed in a 37°C bath without shaking but with vigorous sparging. Cells were harvested by using a Pellicon concentrator (Millipore Corp., Bedford, Mass.), washed once with PEB buffer (20 mM phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.0), resuspended in an equal volume of PEB buffer and frozen at -20°C. The cells were thawed, disrupted by two passes through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 16,000 lb/in^2, and centrifuged at 100,000 x *g* for 60 min at 4°C. DEAE-Sepharose and phenyl-Sepharose chromatographies were performed on conventional columns at 4°C. All subsequent purification steps were carried out on a Fast Protein Liquid Chromatography system (Pharmacia, Uppsala, Sweden) at room temperature. All resins and columns were purchased from Pharmacia. PEB buffer with the stated additions was used in all phases of the purification.

Urease characterization. The reaction rates for purified urease were measured as the concentration of urea was varied from 1 to 100 mM, and the data were analyzed by the method of Wilkinson (31).

The molecular weight for native *P. stuartii* urease was estimated by using Superose 6 gel filtration chromatography in PEB buffer containing 0.1 M KCl. The column (1.0 X 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, Calif.).

The nickel content of the purified urease was determined by using a PE 5000 atomic absorption spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) equipped with an HGA 500 graphite furnace and an AS-1 autosampler. Samples were hydrolyzed in 1 M HNO_3, evaporated, and dissolved in 50 mM HNO_3. Nickel standards, prepared with and without bovine serum albumin (to mimic the enzyme matrix), were treated identically to the urease samples. Aliquots (20 μl) were dried at 120°C, charred at 1,200°C, atomized at 2,700°C, and quantitated for nickel by integrating the peak area while using the backround correction mode.

RESULTS

Optimization of cloned urease expression. The specific activities of crude cell extracts grown under various conditions are shown in Table 1. Urease expression in *E. coli* was greatest when urea was present, consistent with regulation by urea induction. In addition, urease expression was repressed in very rich medium (LB broth), but the enzyme was synthesized when the amount of nitrogen-rich constituents in the medium decreased to 5 or 10% the amount in LB broth. The presence or absence of trace minerals had little effect. When a growth curve was performed by using the medium described for large scale growth, the specific activity of the culture was highest during the early exponential phase, after which it gradually subsided (data not shown).

Purification of urease. The crude extract from 31 g (wet weight) of cells was applied to a DEAE-Sepharose column (2.5 x 15 cm) equilibrated

with PEB buffer. The urease was eluted with a 400 ml linear gradient of 0 to 1.0 M KCl in PEB buffer, resulting in a single peak of activity at 350 mM KCl. Peak fractions were adjusted to 1.0 M KCl and loaded onto a pre-equilibrated phenyl-Sepharose column (1.5 x 14 cm). After washing with 80 ml of 1.0 M KCl in PEB buffer, the urease was removed with a single step elution using 80 ml of PEB buffer. Washing the column with 20% dimethyl-sulfoxide in PEB buffer eluted only trace amounts of additional urease activity. Peak phenyl-Sepharose fractions were combined, diluted with an equal volume of PEB buffer and applied to a Mono-Q HR 5/5 Fast Protein Liquid Chromatography column. The activity eluted as a doublet at 350 mM KCl by using a linear KCl gradient in PEB buffer. The active fractions were pooled, adjusted to 2.0 M KCl, and loaded onto a phenyl-Superose HR 5/5 Fast Protein Liquid Chromatography column. The protein was eluted with a descending 2.0 to 0 M KCl gradient in PEB buffer yielding a major activity peak at 1.1 M KCl and a minor activity peak at 0.3 M KCl (Fig. 1). The latter peak was composed of both urease and contaminating protein, presumably as an aggregate. Fractions of the major peak were pooled and concentrated by ultrafiltration (Centricon 10; Amicon Corp., Lexington, Mass.). The purification procedure and results are summarized in Table 2.

Analysis of urease by gel electrophoresis. Denatured samples of purified urease were electrophoresed by using a sodium dodecyl sulfate-10 to 15% polyacrylamide gradient gel (Fig. 2). Three polypeptides were observed in the pure recombinant protein; they were similar to those found in several other bacterial ureases (27). Subunit ratios derived from scanning densitometry of the Coomassie blue-stained bands after normalization for molecular weights were 1:1.7:1.9 for the bands with M_rs of 73,000, 10,000, and 9,000, respectively. These peptides, in decreasing

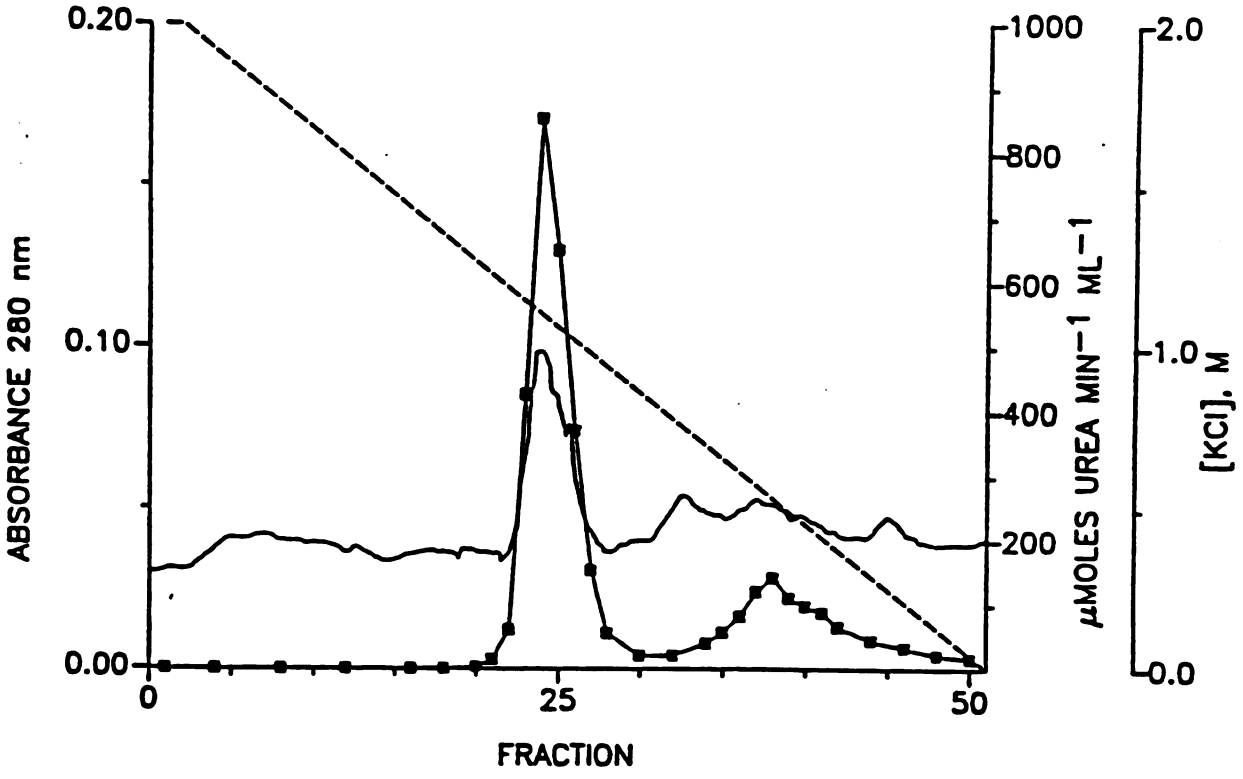

Fig. 1. Phenyl-Superose FPLC chromatography of recombinant *P. stuartii* urease expressed in *E. coli*(pMID201). Active fractions from Mono-Q chromatography were pooled, adjusted to 2M KCl, and chromatographed as described in the text by using a KCl gradient (---). Aliquots of the 1.0 ml fractions were assayed for urease activity (■), and absorbance was monitored (——).

TABLE 1. Optimization of recombinant *P. stuartii* urease expression by *E. coli*(pMID201)[a]

Medium[b]	Presence of		A_{600}[d]	Sp act[e]
	15mM urea	Trace minerals[c]		
LB	-	-	0.79	0.0
LB	+	-	0.69	0.8
LB	+	+	0.66	3.4
M9 + 10%LB	-	-	0.53	0.0
M9 + 10%LB	-	+	0.51	0.0
M9 + 10%LB	+	+	0.51	68.0
M9 + 10%LB	+	3+[f]	0.80	75.0
M9 + 5%LB	-	-	0.54	2.5
M9 + 5%LB	-	+	0.53	2.7
M9 + 5%LB	+	+	0.40	54.0

[a] *E. coli* HB101 is nonureolytic.
[b] See reference 17. All cultures contained tetracycline (10 μg/ml).
[c] See reference 26.
[d] A_{600} for the culture at time of harvest.
[e] Specific urease activity of extracts from disrupted cells expressed in μmol urea per min per mg.
[f] Three times the normal amount of trace minerals was added.

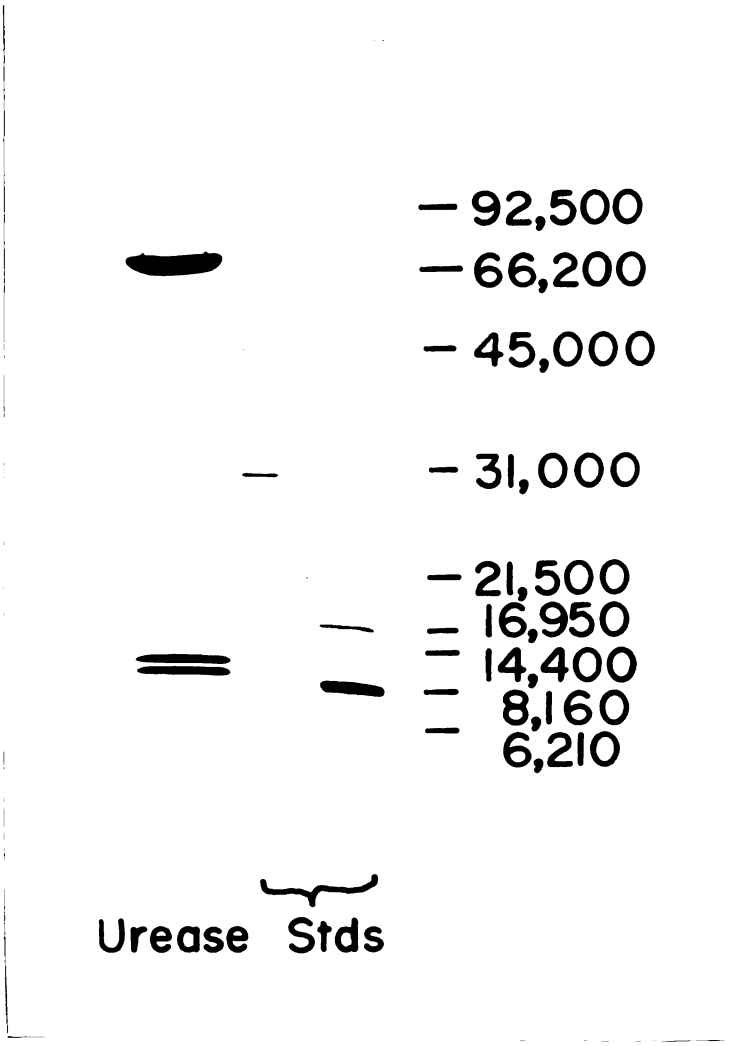

Fig. 2. SDS polyacrylamide gradient gel of purified recombinant P. stuartii urease expressed in E. coli. A sample of the purified enzyme was run as described in the text using 8ug of protein, and the gel was stained with Coomassie Brilliant Blue. The standards were: phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme (Bio-Rad, Richmond, CA), myoglobin, and myoglobin cyanogen bromide fragments I+II, I, and II (Sigma).

TABLE 2. Purification of recombinant *P. stuartii* urease from *E. coli*

Purification step	Specific Activity (μmol urea min^{-1} mg^{-1})	Purification (fold)	Total Activity (μmol min^{-1})	Total Protein (mg)	Recovery (%)
Crude extract	16.7	1	69,000	4,180	100
DEAE sepharose	190	11	45,300	239	67.5
Phenyl sepharose	881	53	33,500	38.0	48.6
Mono-Q	2,290	137	19,600	8.6	28.4
Phenyl-Superose	5,520	331	4,990	0.9	7.2

order of size are designated α, ß, and τ.

Samples of *P. stuartii* urease were run on nondenaturing gels and stained for activity. The final pure protein gave a single sharp band, whereas samples of the preparation taken at earlier stages in the purification showed several additional faint, slower-migrating bands (data not shown).

Kinetic parameters. A K_m of 9.3 ± 1.2 mM and a V_{max} of 7,100 ± 300 µmol of urea per min per mg were obtained for purified *P. stuartii* urease. This K_m value compares quite well with that reported for crude cell extracts from *P. stuartii* or from *E. coli* expressing recombinant urease (19).

Native molecular weight. The native molecular weight for the purified *P. stuartii* urease was estimated as 230,000 ± 20,000 by Superose 6 gel filtration chromatography. This value is significantly less than the value of 337,000 reported for crude cell extracts (19). This discrepancy may be due to association with other proteins in the more crude preparations.

Nickel analysis. Atomic absorption analysis showed that urease had 1.9 ± 0.1 mol of nickel per mol of $\alpha_1ß_2\tau_2$ structural unit when compared with a standard curve prepared as described in Materials and Methods.

DISCUSSION

Growth of *E. coli* containing the *P. stuartii* urease genes shows that urease expression is induced by urea, as reported for wild-type *P. stuartii* (18). This result differs from that of Mobley et al. (19), who reported that the expression of cloned urease gene sequences appeared to

be constitutive for recombinant cells grown in L broth. Nitrogen-rich conditions (e.g., LB medium) repressed urease expression in the experiments described above. Urease regulation by induction and repression indicates that the cloned segment must still possess regions necessary for regulating urease expression. In addition, *E. coli* regulatory components are probably necessary. The fact that omission of the trace mineral solution still resulted in high specific activities shows that there is enough trace nickel in the medium constituents to support active urease production. An ammonia-free M9 medium supplemented with 10% LB broth, trace minerals, and 15 mM urea was selected for large scale culturing; it was low enough in available nitrogen sources to promote high levels of urease, yet allowed the cells to grow rapidly for fast culturing of cells.

The early purification steps for cloned *P. stuartii* urease followed a protocol similar to that used for *K. aerogenes* (27), and *Selenomonas ruminantium* (8), indicating that the ureases may have similar physical properties. The presence of a single band, when stained either for protein or for activity, after native polyacrylamide gel electrophoresis demonstrated that a single isozyme of homogeneous urease was obtained. Sodium dodecyl sulfate-polyacrylamide gels showed that the recombinant *P. stuartii* urease has 3 polypeptides, which is consistent with the structure of several other bacterial ureases. The subunit ratio for *P. stuartii* urease is very close to the 1:2:2 ratio reported for the *K. aerogenes* enzyme (27). The native molecular mass of 230,000 kDa is consistent with a $(\alpha_1\beta_2\gamma_2)_2$ structure, as has been suggested for the *K. aerogenes* enzyme. Each $\alpha_1\beta_2\gamma_2$ unit was demonstrated to contain two nickel ions, a metal which has been found in a variety of plant, fungal, and bacterial ureases (9).

Recent transposon mutagenesis experiments using cloned *P. stuartii*

DNA gave data consistent with the above findings in which expression of at least three polypeptides are required for urease activity (21). These polypeptides may arise either from three separate genes, or a larger precursor protein which is subsequently processed.

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CHAPTER 3

Regulation of Gene Expression and Cellular Localization of Cloned *Klebsiella aerogenes (K. pneumoniae)* Urease

ABSTRACT

The genes for *Klebsiella aerogenes (K. pneumoniae)* urease were cloned and the protein was overexpressed (up to 18% of total protein consisted of this enzyme) in several hosts. The restriction map of the DNA encoding urease and the regulation of enzyme expression directed by the recombinant plasmid are distinct from other cloned ureases. Nickel concentration did not affect urease gene expression, as demonstrated by the high levels of apoenzyme measured in cells grown in nickel-free media. However, nickel was required for urease activity. The overproducing recombinant strain was used for immunogold electron microscopic localization studies to demonstrate that urease is a cytoplasmic enzyme.

INTRODUCTION

Nickel-containing ureases (EC 3.5.1.5), which hydrolyze urea to ammonia and carbon dioxide, play an important role in nitrogen metabolism of plants and microorganisms (11). One of the best-studied bacterial ureases is that from *Klebsiella aerogenes* [currently *Klebsiella pneumoniae* (30)]; its regulation has been characterized (8,20) and the three-subunit enzyme has been purified and shown to contain four nickel ions per native molecule (35). Our efforts are geared toward elucidating the structure and function of the *K. aerogenes* urease nickel center by using chemical, biophysical, and spectroscopic approaches which require large amounts of enzyme. Typical yields of urease are only 0.1 mg per liter of culture. Therefore, we sought to clone the *K. aerogenes* urease genes and to define conditions needed to optimize enzyme overexpression. In addition, the effect of nickel concentration on urease activity and expression was characterized and the cellular localization of the recombinant urease was defined.

MATERIALS AND METHODS

Gene cloning. K. aerogenes CG253 was obtained from Boris Magasanik and Alexander Ninfa (Massachusetts Institute of Technology). Chromosomal DNA of *K. aerogenes* was isolated (26) and partially digested with *Sau*3A to yield fragments approximately 40 kb in length. After phenol extraction and ethanol precipitation, the digestion mixture was ligated to *Bam*H1-cleaved, phosphatase-treated, cosmid vector pWH4 (12) and the resulting DNA was packaged into lambda phage by using an in vitro packaging system (Boehringer Mannheim) according to the manufacturer's instructions. The

phages were used to transfect *Escherichia coli* strain VCS257 (Strategene) and kanamycin-resistant (50 μg ml^{-1}) colonies were screened on urease indicator plates, which consisted of ammonia-free M9 minimal agar (21) adjusted to pH 6.8 and supplemented with 10% (v/v) LB medium, 20 mM urea, 20 μg phenol red ml^{-1} and 1 ml trace mineral solution l^{-1} (32). One of the 102 colonies tested positive, as shown by the development of a red halo after 24 h. The cosmid which encoded urease (designated pKAU1) was isolated (14) and transformed into *E. coli* DH1 (10); this plasmid conferred both kanamycin-resistance and urease-positive phenotypes. Purified cosmid pKAU1 was digested with *Bam*H1, and the fragments were ligated into *Bam*H1-cleaved vector pBR328 (33). One ampicillin-resistant, tetracycline-sensitive transformant was positive on urease indicator plates, and was found to contain a 10 kb insert in a plasmid derivative of pBR328 which was termed pKAU2687. Restriction fragments of the *K. aerogenes* insert in pKAU2687 were isolated (7), subcloned into vector pUC8 (36), transformed into *E. coli* JM101 (25) and screened on urease indicator plates.

Assays. Culture samples of cells grown in various media (0.5 ml) were centrifuged for 2 min in a microcentrifuge at 4°C, washed twice with 10 mM potassium phosphate, 1 mM EDTA, 1 mM 2-mercaptoethanol buffer (pH 7.5) and resuspended in the same buffer plus 0.5 mM phenylmethanesulphonyl fluoride. Cells were disrupted with a Fisher Sonic Dismembranator (micro probe) using three 20 s bursts at 30% power. Crude cell extracts were then centrifuged 15 min and the supernatant solutions were assayed for urease activity by converting released ammonia to indophenol, which was quantified spectrophotometrically (35). Protein was assayed by the Lowry method, with bovine serum albumin as the standard.

Nickel dependence studies. The effect of nickel concentration on the expression of recombinant urease protein and enzyme activity was determined by growing cultures to late exponential phase in ammonia-free MOPS minimal medium (29) containing defined nickel levels. Glutamine (10 mM) was used as the sole nitrogen source in order to derepress urease (see below); however, under these conditions urease activity was not required for microbial growth.

Immunological methods. Antibodies that recognized urease were generated in a New Zealand rabbit after injection with homogeneous enzyme, and the IgG fraction was purified from serum (22). For immunoblot analyses, samples were denatured, electrophoresed on an sodium dodecyl sulfate-10 to 15% polyacrylamide gradient gel (17), and blotted onto nitrocellulose. The blot was probed with anti-*K. aerogenes* urease antibodies and developed by using anti-rabbit IgG-alkaline phosphatase conjugates (4).

Immunogold electron microscopy. Wild-type *K. aerogenes* and *K. aerogenes*(pKAU19) (see below) were grown to stationary phase in ammonia-free MOPS medium supplemented with 10 mM arginine plus 100 μM $NiCl_2$. After centrifugation, the cells were washed once in 10 mM potassium phosphate, 1 mM EDTA (pH 7), 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) Nobel agar, dehydrated in ethanol, and embedded in Lowacryl K4M (1). Polymerization was carried out for 2 d at room or cold room temperatures 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% Tween 20) for 5 min and

transferred to 1% (w/v) bovine serum albumin in TBST for 15 min in order to block non-specific sites. The samples were transferred to the anti-urease IgG (35 $\mu g\ ml^{-1}$) in TBST for 1 h, washed three times for 5-15 min each in TBST, and floated on gold particles that were attached to anti-rabbit IgG (15 min, Jansen) for 1 h (3). 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.

RESULTS AND DISCUSSION

Gene cloning. The *K. aerogenes* urease genes were localized to a 10 kb DNA fragment which possesses the restriction map shown in Fig. 1. This region included two *Bam*H1 fragments of 7.0 and 3.0 kb, indicating that incomplete digestion had occurred in the subcloning of pKAU2687. When cloned individually, neither fragment conferred urease activity, indicating that sequences spanning the internal *Bam*H1 site are required. A *Pvu*II fragment (6.2 kb) overlapped this *Bam*HI site, yet no urease activity was detected in transformations containing this subcloned fragment (pKAU13). Partial *Sau*3A digestion of pKAU15 was used to generate a clone containing 5.7 kb (pKAU17) which conferred urease activity.

Urease genes have been cloned from *Bacillus pasteurii* (16), a urease-positive *E. coli* (5), *Proteus mirabilis* (13,37), and *Providencia stuartii* (27,28). The restriction maps of these clones all differ significantly from that of the *K. aerogenes* urease gene fragment. *Klebsiella pneumoniae* urease genes also have been cloned by Gerlach et al. (9); however, the mechanism of gene regulation and properties of the recombinant enzyme were not studied. The restriction pattern, and the

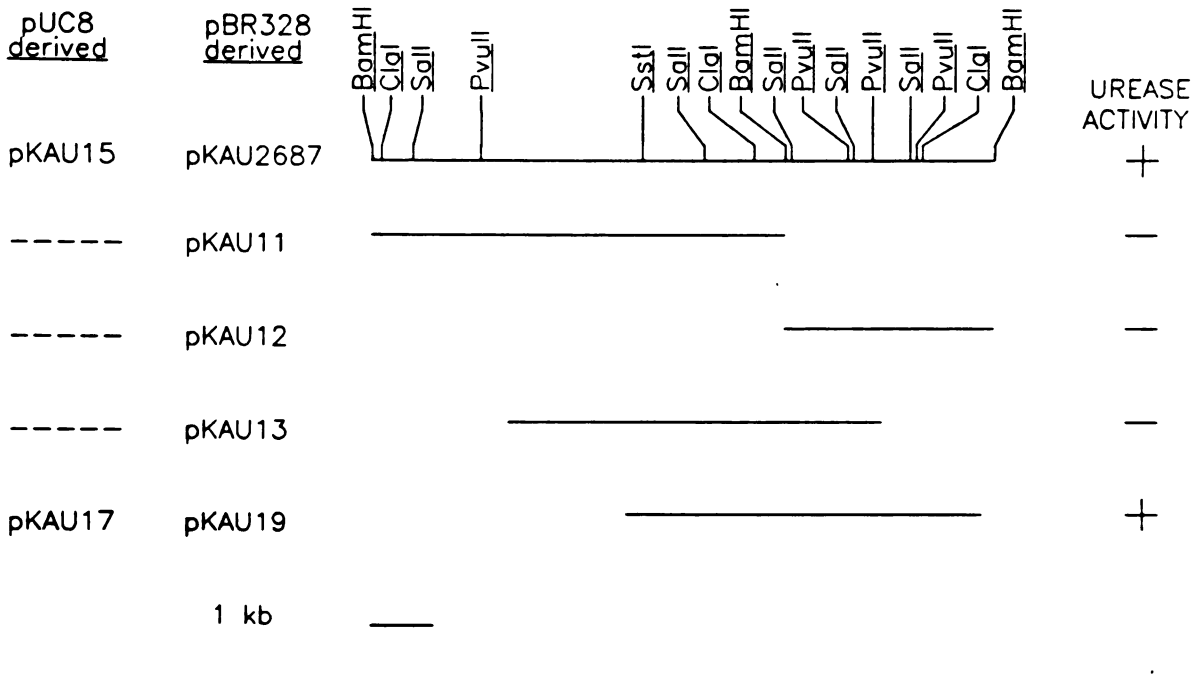

Fig. 1. Restriction map and summary of cloned *K. aerogenes* urease gene fragments. A restriction map is presented of a 10 kb DNA fragment containing the *K. aerogenes* urease genes. *Hin*dIII, *Eco*RI, and *Xba*I did not cleave this fragment. Subclones of the *K. aerogenes* DNA fragment were generated and tested for the presence (+) or absence (−) of urease activity based on results from indicator plates.

associated polypeptide sizes for the *K. pneumoniae* urease genes studied by Gerlach et al. (9) differ from that of the *K. aerogenes* DNA cloned in the present study.

Urease regulation. To increase their expression, the cloned urease genes were transformed into three hosts and the enzyme levels monitored under varied growth conditions. The 5.7 kb fragment was transferred from pUC8 to pBR328, which had more desirable antibiotic markers: pKAU17 was digested with *Eco*RI and *Hin*dIII, end-filled with polymerase I Klenow fragment, and ligated into *Sma*I-digested pBR328 to yield pKAU19. This plasmid was transformed into *E. coli* DH1, *K. aerogenes* CG253 (10), and into *Salmonella typhimurium* LT-2 (19). Since pKAU19 carried sequences homologous to the *K. aerogenes* host (which could lead to plasmid loss due to homologous recombination), colonies obtained directly from the transformation were used to inoculate starter cultures for overnight incubation. Specific activities were determined for cells of these strains and wild-type *K. aerogenes* grown to early exponential and stationary phase in different media that contained individual nitrogen sources; typical values are shown in Table 1. For comparison, pure *K. aerogenes* urease has a specific activity of 2200 μmol urea min^{-1} mg^{-1} (35).

Among the hosts tested, urease activity was regulated by nitrogen repression (20) as originally reported in the wild-type microbe (8). Low enzyme levels were observed during growth in nitrogen-rich medium (LB, or MOPS+CA+N, Table 1), whereas nitrogen limitation (MOPS+N, Gln, or Arg) led to derepression of urease activity. Enzyme levels exceeded 7% of the soluble protein in stationary-phase cultures of strains containing the cloned urease genes, and accounted for 18% in the case of *S. typhimurium* grown in MOPS+Arg calculated from the data in Table 1. However, the

Table I. Expression of Recombinant Klebsiella aerogenes Urease[a]

Culture	Exponential growth phase[b]					Stationary phase	
	LB[c]	MOPS[d] CA+N	MOPS N	MOPS Gln	MOPS Arg	MOPS Gln	MOPS Arg
K. aerogenes wt	0.0	0.1	0.3	0.4	3.3	1.1	1.7
K. aerogenes [pKAU19]	6.1	6.4	49.0	34.0	122	263	240
S. typhimurium [pKAU19]	2.5	11.2	111	48.3	29.5	219	403
E. coli DH1 [pKAU19]	1.4	9.1	107	109	165	119	176

[a] Units are expressed in umoles urea $min^{-1}mg^{-1}$.

[b] A_{600}=1 at time of harvest

[c] Abbreviations: LB, Luria-Bertani medium (7); N, 10mM NH_4Cl; Gln, 10mM glutamine; CA, 0.5% casamino acids; Arg, 10mM arginine.

[d] MOPS minimal medium (29) was used with the following modifications: NH_4Cl was omitted in most cases and replaced by various nitrogen sources at the indicated concentrations, and the trace mineral solution was replaced by that of Smith et al. (32). All cultures contained 100uM $NiSO_4$ and chloramphenicol.

highest activity per ml of culture was obtained for *K. aerogenes*(pKAU19). Regulation of other recombinant ureases has only been reported for urea-inducible, nitrogen-repressible *Providencia stuartii* (28) and urea-inducible *Proteus mirabilis* (13,37).

Characterization of the urease made by strains containing pKAU19. Crude cell extracts of *E. coli*DH1(pKAU19) and *S. typhimurium*(pKAU19) were examined by using immunoblot analysis (Fig. 2). Three urease subunits of the expected size were shown to be expressed in each case. Furthermore, enzyme purified from *K. aerogenes*(pKAU19) was demonstrated to be identical to wild-type enzyme in subunit composition, nickel content, specific activity, and inhibitor sensitivity (data not shown). Routine purification of urease is now carried our from stationary-phase cultures of *K. aerogenes*(pKAU19), resulting in 100- to 200-fold increased in yield over wild-type levels. These results demonstrate that urease-processing or nickel-insertion activities, if required, are encoded either on the 5.7 kb pKAU19 fragment or in the heterologous hosts.

Effects of nickel concentration on urease gene expression and urease activity. Derepressed, stationary-phase *K. aerogenes*(pKAU19) cultures grown in media of different nickel concentrations yielded identical intensities of anti-urease antibody cross-reactive material (Fig. 3). Hence, nickel does not affect expression of urease in strains with this plasmid. The urease activities, however, were greatly affected by nickel concentration, and 100 to 200 μM nickel was required for maximum activity (Fig. 3). The high requirement may reflect the ability of medium components to bind nickel. The production of inactive urease apoenzyme by this enteric bacterium is similar to the case of soybean, where apoenzyme was shown to be synthesized in the absence of nickel (38). Furthermore,

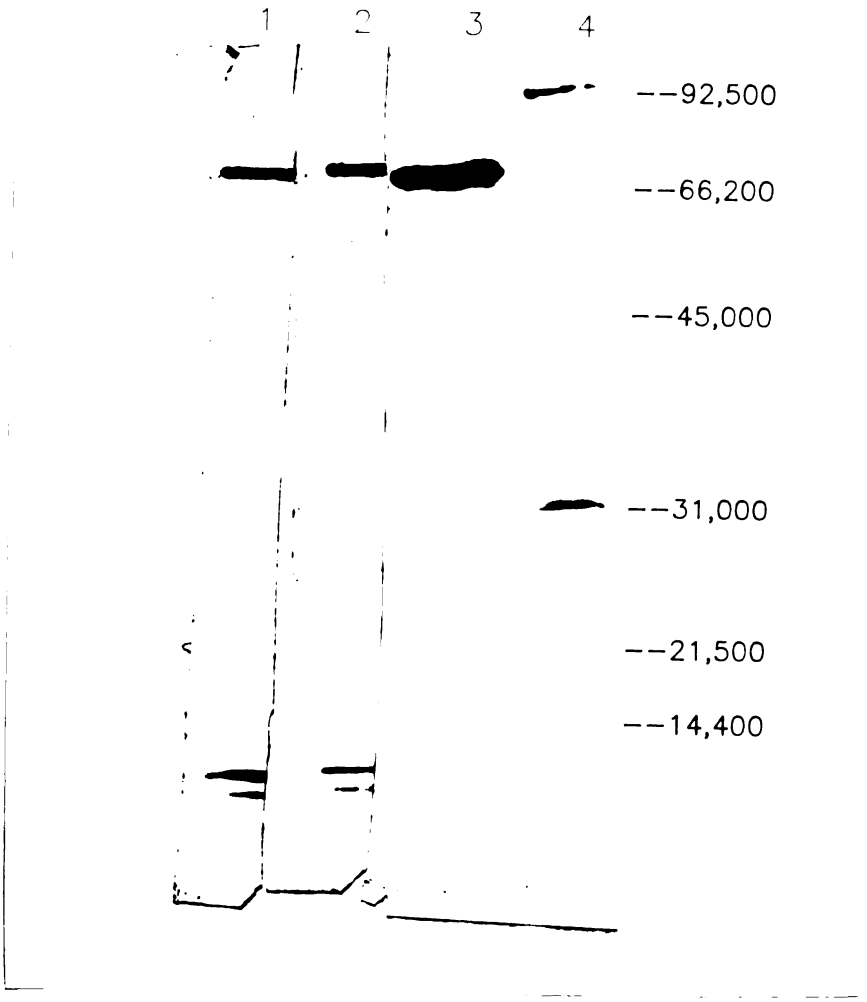

Fig. 2. Immunoblot analysis of recombinant urease expressed in *E. coli* DH1 and *S. typhimurium*. Samples of crude extracts of *E. coli* DH1(pKAU19) (lane 1) and *S. typhimurium*(pKAU19) (lane 2) containing 0.1 units (50 ng) of urease were analyzed. A standard of purified *K. aerogenes* urease (lane 3) and M_r standards (lane 4) were also run and stained for total protein with Amido black. The migration position of M_r markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme; Bio-Rad) are indicated to the right of the figure.

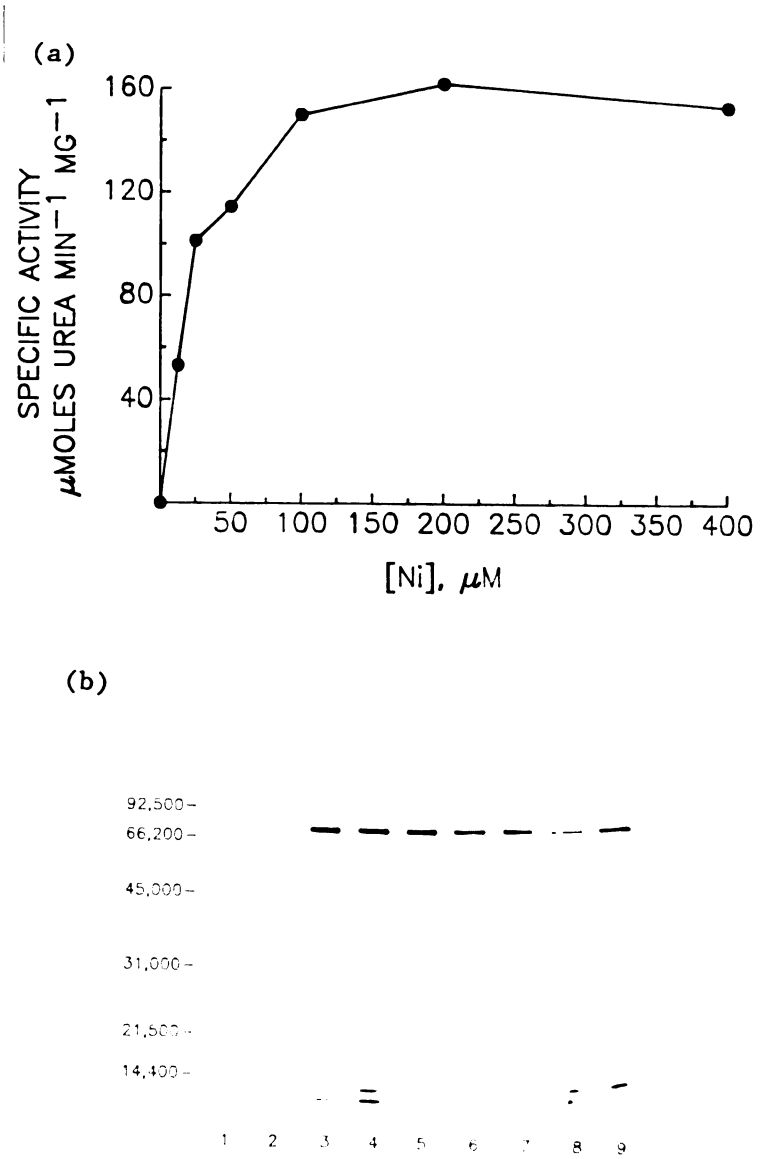

Fig. 3. Effect of nickel concentration on recombinant urease expression and activity. (a) Urease specific activity was determined for *K. aerogenes*(pKAU19) after growth in media containing the indicated nickel concentrations. (b) The level of urease protein expression was quantified for each sample by immunoblot staining. Lanes 1 and 2 are Amido black-stained M_r standards and purified urease, respectively. Lanes 3-9 represent the samples (2 μg total protein) obtained from cultures containing 0, 12.5, 25, 50, 100, 200, and 400 μM nickel respectively.

the algae *Phaeodactylum tricornutum* and *Tetraselmis subcordiformis* (31), the cyanobacterium *Anabaena cylindrica* (18), and the purple sulphur bacterium *Thiocapsa roseopersicina* (2) have also been suggested to synthesize urease apoenzyme in the absence of nickel. The microbial studies were based on reconstitution of urease activity for cells grown under nickel-free conditions when nickel was added, even in the presence of protein-synthesis inhibitors. In contrast to the lack of nickel-dependent regulation of ureases, nickel-containing hydrogenases from *Bradyrhizobium japonicum* and *Alcaligenes latus* have been shown to exhibit nickel-dependent expression (6,34).

Immunogold localization. The urease in cells of *K. aerogenes* containing pKAU19 is a cytoplasmic enzyme as shown by immunogold electron microscopy localization (Fig. 4). This result is consistent with the observed enzyme behavior during purification. Urease from wild-type *K. aerogenes* behaved identically to the recombinant enzyme during isolation, which is consistent with the reported cytoplasmic location from cell fractionation studies (8). Wild-type cells which contain 1-5 μM urease [calculated as in (15)], were insufficiently labeled by the immunogold technique to allow localization, as expected from the findings of Kellenberger et al. (15), who state that a cytoplasmic protein cannot be detected by this method at concentrations less than 10-100 μM.

Our results contrast with two previous electron microscopic localization studies involving urease from a *Staphylococcus* species (23) and from *Proteus mirabilis* (24). The earlier workers utilized tetraphenylboron, a compound which reacts with ammonia to form a precipitate; ammonia was exchanged for electron-dense silver ions to allow visualization by electron microscopy after thin sectioning. The

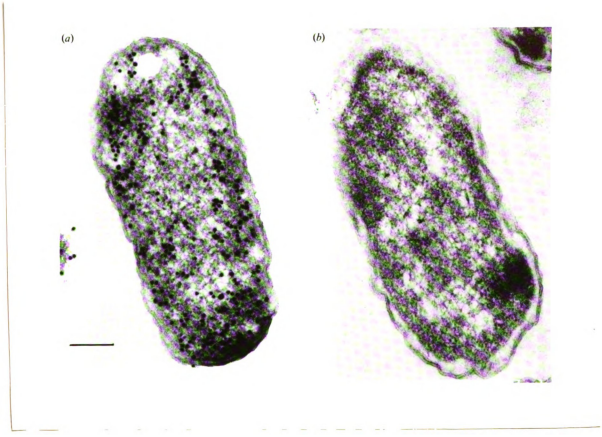

Fig. 4. Immunogold localization of recombinant *K. aerogenes* urease. (a) Thin sections of *K. aerogenes*(pKAU19) expressing recombinant urease (274 μmol urea hydrolyzed min^{-1} mg^{-1}) were reacted with anti-urease antibodies and labeled with anti-rabbit IgG-gold particles. Urease was localized to the cytoplasmic portion of the cell. (b) Similar experiments were carried out with wild-type *K. aerogenes* (2.0 μmol urea hydrolyzed min^{-1} mg^{-1}). Bar, 0.2 μm.

precipitated metal was observed on the membrane of the Gram-positive *Staphylococcus* sp. (23) or in the periplasmic space and on the outer membrane of the Gram-negative *P. mirabilis* (24). The discrepancy between these earlier studies and our findings may be due to the inability of tetraphenylboron to cross the cytoplasmic membrane, thus it could only react with external ammonia.

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CHAPTER 4

In Vivo Reconstitution of

Klebsiella aerogenes urease apo-enzyme

Abstract

Recombinant *Klebsiella aerogenes* cells overexpressing urease protein were grown in the absence of nickel and reactivation of urease activity upon restoration of nickel to the growth medium was examined in control cells and cells treated with protein synthesis and energy uncoupling inhibitors. Control cultures gradually gained activity after nickel addition primarily due to new urease synthesis, and this increase continued for several hours after culture growth had ceased. In the presence of a protein synthesis inhibitor, nickel addition also led to an increase in urease activity, although at reduced rates when compared to untreated cultures. This activity arose from activation of pre-formed apo-protein. Energy dependence of nickel incorporation into apo-urease was demonstrated by the failure to generate urease activity in dicyclohexyl-carbodiimide and dinitrophenol-treated cells. Sonicated cells also lost the ability to activate urease apo-enzyme. These results indicate that nickel is incorporated into pre-formed apo-enzyme in an energy-dependent process which is destroyed by cell disruption.

INTRODUCTION

Urease, a nickel-containing enzyme found in many plants and microorganisms, hydrolyzes urea to yield carbonic acid and ammonia (1,14). Whereas the role of urease in plants is poorly understood (23), the microbial enzyme plays important roles in human and animal pathogenic states, in ruminant metabolism, and in environmental transformations of certain nitrogenous compounds (14). The best characterized plant urease is that isolated from jack bean; it was the first enzyme ever crystallized (18) and the first enzyme shown to possess nickel (4). This hexameric protein contains two Ni ions per subunit (M_r = 90,770), the sequence of which was recently reported (19). The most studied bacterial urease is that from *Klebsiella aerogenes* (currently *K. pneumoniae*) which possesses three subunits [M_rs = 72,000 (α), 11,000 (β), and 9,000 (γ)] in an $\alpha_2\beta_4\gamma_4$ stoichiometry (20). The native enzyme contains two catalytic sites, each of which is associated with 2 Ni ions (21). The genes for *K. aerogenes* urease were recently cloned and overexpressed such that urease accounted for over 10% of the cellular protein (Chapter 3; 16). Although the enzyme requires Ni for activity, no Ni-dependent regulation of gene expression was observed.

This chapter describes the in vivo reconstitution of apo-urease in *K. aerogenes* cells. Experiments were designed to ascertain whether Ni ions are incorporated into the enzyme during synthesis, or if the Ni is incorporated into pre-formed apo-enzyme. Possible energy requirements of Ni-incorporation are also explored.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *K. aerogenes* CG253 was transformed with plasmid pKAU19 (16), which possesses the urease genes from this microorganism. The recombinant bacterium was grown at 37°C in MOPS-glutamine medium (16) containing 30 μg/ml chloramphenicol. Ni-free cultures were grown without added Ni, and 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 (22). 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. (10) with bovine serum albumin as the standard.

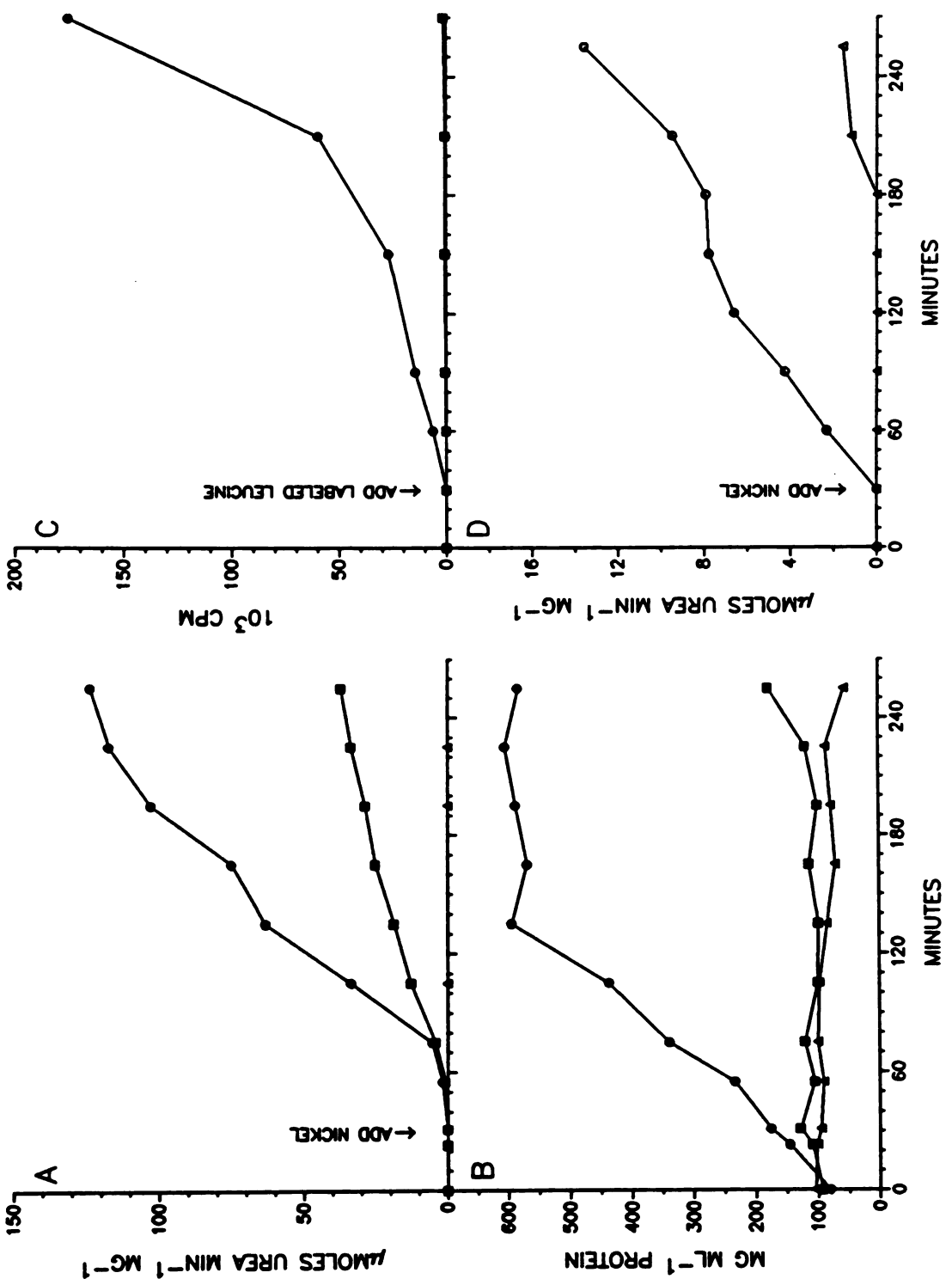
In vivo activation of apo-urease. Early exponential phase Ni-free cultures of *K. aerogenes* CG253(pKAU19) were treated with an inhibitor of protein synthesis (50 μg/ml spectinomycin), an uncoupler of electron transport phosphorylation (2 mM dinitrophenol), or an ATPase inhibitor [5 mM (DCCD)]. In the latter case, 10 mM ammonium chloride was used as the nitrogen source and 20 mM malate was used as the sole carbon source to preclude the possibility of substrate level phosphorylation (6). After 30 minutes, $NiSO_4$ was added (50 μM final concentration) to these treated cultures and to an untreated Ni-free control. Portions of the cultures were taken at several time points, washed, disrupted by sonication, and

assayed for protein and urease activity as previously described (15). To investigate whether protein synthesis was completely abolished in the presence of spectinomycin, 1 mM leucine (supplemented with 1 μCi/ml of 1-[3,4,5-^{3}H(N)]-leucine at 153 Ci/mmole; New England Nuclear, Boston, MA) was added to control and inhibited cultures, and aliquots were removed at selected timepoints. The aliquots were mixed with an equal volume of 30% trichloroacetic acid, placed on ice for 30-60 min, heated to 80°C for 30 min, and filtered on Whatman GF/C filters. After washing and drying, the filters were counted in a toluene based scintilation solution.

RESULTS

Addition of Ni to Ni-free *K. aerogenes* CG253(pKAU19) cultures led to an increase in urease specific activity which may represent both newly synthesized enzyme and activation of previously formed apo-protein (Fig. 1). Following Ni addition there was a delay of 30 min, then urease activity continuously increased until two hours after protein synthesis ceased. This result is consistent with a slow incorporation of Ni into previously synthesized apo-enzyme. Furthermore, activation of pre-formed apo-urease was observed in cultures treated with spectinomycin, an inhibitor of protein synthesis. [^{3}H]-leucine uptake studies were carried out (Fig. 1C) to confirm that protein synthesis was fully inhibited in these cultures, e.g., that small amounts of protein turnover did not occur. Similar to the control culture, in vivo activation of urease apo-protein was a very slow process and exhibited a 30 min lag. After 4 hr, the urease specific activity for spectinomycin-treated cultures was approximately 1/3 that of the control culture, perhaps due to non-specific effects of long-term exposure to the inhibitor. Similar results were

Fig. 1. In vivo reconstitution of urease apo-protein. Exponentially growing nickel-free cultures of *K. aerogenes*(pKAU19) in MOPS-10 mM glutamine were treated with no inhibitor (●), 50 μM spectinomycin (■), or 2 mM dinitrophenol (▲) followed after 30 min by addition of Ni (50 μM) as indicated by the arrow. Aliquots were assayed for urease specific activity (panel A), total protein (panel B), and level of protein synthesis (panel C). In the latter case, [^{3}H]-leucine was added to the cultures where indicated and the incorporation of labeled leucine into trichloroacetic acid-precipitable protein was assessed. In addition, cells were grown in MOPS-10 mM NH_4Cl with 20 mM malate as the sole carbon source (Panel D). The cultures were treated with no inhibitor (●), or with 5 mM DCCD (▲). The low specific activities in the malate control culture are due to the non-optimal medium conditions which were required in order to insure a minimum of substrate level phosphorylation.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observed for wild-type *K. aerogenes* (not shown). In contrast, cells treated with dinitrophenol or DCCD gave no urease activity upon addition of nickel (Fig. 1). Furthermore, if the cells were disrupted prior to adding Ni no urease activity was generated.

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 (7). Although bacterial ureases contain tightly bound Ni at their active sites (14, 21), 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.

Purified *K. aerogenes* urease apo-enzyme could not be activated by simple addition of Ni ions, hence, a cellular factor may be required for incorporation of the metal center. Apo-urease synthesized in Ni-free *K. aerogenes* cultures could be activated by Ni addition to whole cells even in the presence of protein synthesis inhibitors; similar results have been previously reported for ureases from soybean (24), algae (17), a cyanobacterium (12), and purple sulfur bacteria (2). In contrast, apo-enzyme was not activated in dinitrophenol—or DCCD—treated cells. This result could be explained by an energy dependence for Ni transport or for Ni incorporation into protein. We prefer the latter explanation because apo-urease 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 apo-protein or to Ni-free cell extracts: Ni addition did not yield urease activity (9). Other studies have also shown the inability to

generate urease activity by addition of Ni to disrupted, Ni-free cells of soybean (24), jack bean (5), and a cyanobacterium (12).

Preliminary studies in both plants and microorganisms are consistent with the requirement for urease-related accessory factors which activate apo-enzyme. In soybean for example, Meyer-Bothling et al. (13) 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 (11). 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* (3) and *Schizosaccharomyces pombe* (8) exceeds that required for the urease structural genes. The function of the additional genes is 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 (Chapter 4), which is three more than is required to encode the urease subunits.

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CHAPTER 5

Sequence of the *Klebsiella aerogenes* Urease Genes and Evidence for Accessory Proteins Facilitating Nickel Incorporation

ABSTRACT

A 4.8-kilobase-pair region of cloned DNA encoding the genes of the *Klebsiella aerogenes* urease operon has been sequenced. A total of six closely-spaced open reading frames were found: *ureA* (encoding a peptide of 11.1 kilodaltons, kDa), *ureB* (11.7-kDa), *ureC* (60.3-kDa), *ureE* (17.6-kDa), *ureF* (25.2-kDa), and *ureG* (21.9-kDa). Immediately following the *ureG* gene is a putative rho-dependent transcription terminator. The three subunits of the nickel-containing enzyme are encoded by *ureA*, *ureB*, and *ureC* based on protein structural studies and sequence homology to jack bean urease. Potential roles for *ureE*, *ureF*, and *ureG* were explored by deleting these accessory genes from the operon. The deletion mutant produced inactive urease which was partially purified and found to have the same subunit stoichiometry and native size as the active enzyme, but contained no significant levels of nickel. The three accessory genes were able to activate apo-urease in vivo when they were cloned into a compatible expression vector and co-transformed into cells carrying the plasmid containing *ureA*, *ureB*, and *ureC*. Thus, one or more of the *ureE*, *ureF*, or *ureG* gene products are involved in nickel incorporation into urease.

INTRODUCTION

Urease (EC 3.5.1.5) was the first enzyme to be crystallized (37) as well as the first shown to contain nickel (6). Early studies involving urease made use of enzyme purified from jack bean; this homohexameric plant enzyme has continued to be extensively studied, as reviewed by Andrews et al. (1). Recently, interest has focused on ureases from bacterial sources, which differ from the plant enzyme in subunit composition and native molecular weight (30). The urease of *Klebsiella aerogenes* (currently *K. pneumoniae*) is similar to those of several other bacteria; it contains 4 tightly-bound nickel ions distributed among two active sites per native molecule and consists of three subunits in an $\alpha_2\beta_4\gamma_4$ stoichiometry (39, 40). The genes for *K. aerogenes* urease were recently cloned, and the enzyme was overexpressed in *E. coli* (Chapter 3; 33). Inactive urease apo-enzyme was synthesized when recombinant cells were grown in the absence of nickel ion (33), ruling out nickel-dependent transcriptional regulation. Subsequent addition of nickel to whole cells led to apo-enzyme activation, even after treatment with protein synthesis inhibitors (Chapter 4; 23). In contrast, purified apo-urease could not be activated in vitro by addition of nickel indicating that some cellular component may be required for nickel incorporation into the enzyme (23).

This report presents the DNA sequence of the six open reading frames in the *K. aerogenes* urease operon. Three of these genes are shown to encode the urease subunits. The precise role of the remaining three genes is unknown; however, evidence is presented that these accessory genes function in activating urease apo-enzyme by incorporating nickel.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains JM109 (43) or XL-1-B (2), grown at 37°C in 2xYT medium (28), served as hosts for M13 clones used for sequencing. *E. coli* DH1 (14), used as the recipient for all studies involving urease expression, was grown at 37°C in MOPS-glutamine medium supplemented with 100 μM $NiSO_4$ and appropriate antibiotics as previously described (33).

DNA sequencing. All restriction enzyme digestions, end-fillings, and other common DNA manipulations, unless otherwise stated, were performed according to standard procedures (27, 34). Sequencing was carried out on a portion of the urease operon from the upstream *Sst*I site to the downstream *Hin*dIII site of plasmid pKAU17 (33), illustrated in Fig. 1. This fragment contains all of the urease operon genes but lacks urease activity due to partial deletion of the upstream promotor region. Portions of the *Sst*I-*Hin*dIII fragment were cloned into phage vector M13mp19 (43) and a series of unidirectional deletions were constructed for each strand by using exonuclease III according to the method of Henikoff (15). Phage DNA from selected deletion clones was purified (28) and sequenced with Sequenase enzyme (U.S. Biochemicals, Cleveland, OH) by using [^{35}S] dATP according to the manufacturer's instructions. Duplicate reaction sets were made for each clone: one with dGTP and the other with dITP. Sequencing gels consisted of 6% acrylamide (19:1 acrylamide:bisacrylamide) and were either wedged shaped (0.4-1.2 mm) or straight (0.4 mm), in which case they were run by making the bottom buffer reservoir 1 M sodium acetate before electrophoresis (35). Sequence analysis was carried out for both strands. Alignments of overlapping sequence data were made by using the GENEPRO program (Riverside Scientific, Seattle, WA). Database searches and open

reading frame assignments were performed with the Wisconsin Genetic Computer Group software package version 6.1 (5). The Profilesearch program was used to look for homologous proteins in the National Biomedical Research Foundation (NBRF) protein sequence database. The sequences determined here have been deposited in Genbank under the accession number (M36068).

Construction of plasmids pKAU601 and pKAU506. Plasmid pKAU17 was partially digested with *Aat*I, the DNA fragments were electrophoresed in 1% agarose, and the band corresponding to a 1.5-kilobase-pair (kb) deletion was isolated by interception on DEAE paper (Whatman DE81). The DNA was eluted, ethanol precipitated, recircularized by T4 DNA ligase, and transformed into *E. coli* DH1. The resulting plasmid, pKAU520, was digested by several restriction enzymes to verify the proper deletion. In order to take advantage of more appropriate antibiotic markers, the pKAU520 fragment was cleaved from the pUC8 vector by digestion with *Eco*RI and *Hin*dIII. After treatment with Klenow fragment of *E. coli* DNA polymerase to produce blunt ends, the fragment was ligated into *Pst*I cut and Klenow-treated pBR328 (36) to yield pKAU601 (Fig. 1).

A second subclone was made by digesting pKAU2687 (33), a precursor to pKAU17, with *Bam*HI. The resulting 2.9-kb fragment, which contains the *ureE*, *ureF*, and *ureG* region, was isolated as described above and ligated into *Bam*HI-cleaved pMMB66HE (10, obtained from Michael Bagdasarian, Michigan Biotechnology Institute). The desired recombinant plasmid was verified by restriction analysis and designated pKAU506 (Fig. 1).

Assays. Urease activity was measured by quantitating the rate of ammonia released from urea by formation of indophenol which was monitored at 625 nm (42). The assay buffer consisted of 25 mM HEPES (N-2-

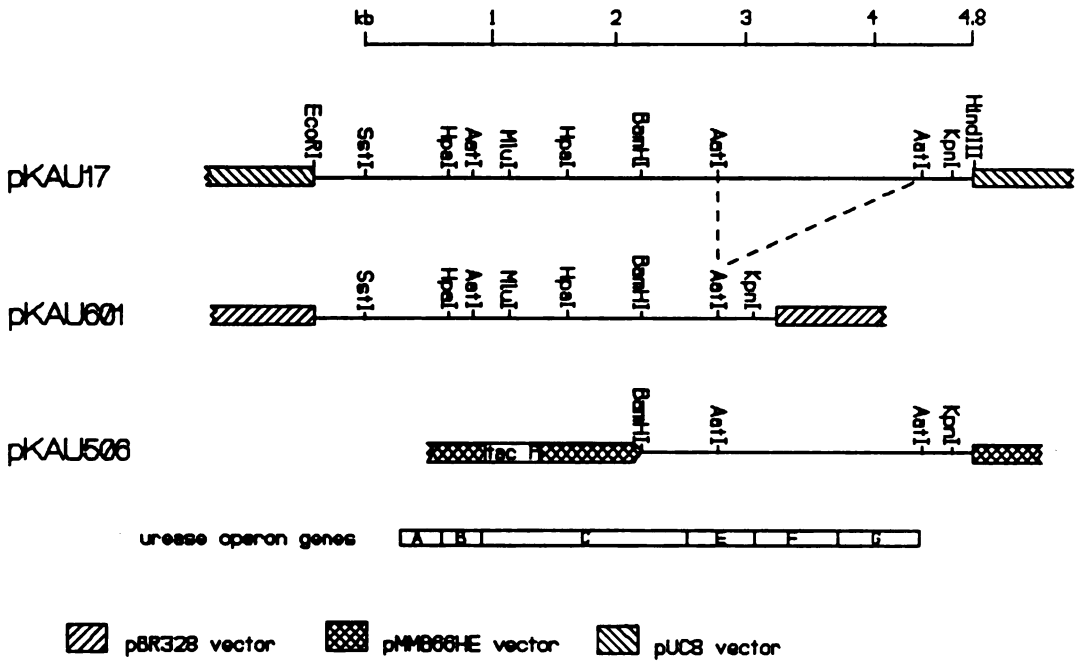

Fig. 1. Structure of the urease operon and two subclones. The restrictionmap for pKAU17, containing the cloned urease operon, is shown along with two subclones derived from this plasmid. As described in the text and indicated at the bottom of the figure, the complete operon is comprised of six genes. The first three genes encode the urease structural subunits, whereas the function of the last three accessory genes may be related to nickel incorporation into urease. The structural genes and urease promoter region were subcloned into the ampicillin resistance gene of vector pBR328, yielding pKAU601. Similarly, the accessory genes were cloned under control of the *tac* promoter of expression vector pMMB66HE to give pKAU506.

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. When urease activity was determined in cultures, cells were disrupted as previously described (33). Protein content was determined by the method of Lowry et al. (24) by using bovine serum albumin as the standard.

SDS-Polyacrylamide gel electrophoresis. All gels for protein analysis were prepared by using the buffers of Laemmli (21) and consisted of either a 15% polyacrylamide (acrylamide:bisacrylamide, 32:1) or a 10-15% polyacrylamide gradient resolving gel with a 4.5% stacking gel. Gels were stained with Coomassie brilliant blue (Sigma) and scanned by using a Gilford Response spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) at 540 nm.

Purification and characterization of pKAU601-derived urease. A 2-liter stationary phase culture of *E. coli* DH1 containing plasmid pKAU601 was grown as described above. The cells were collected by centrifugation, resuspended in 80 mL PEB buffer (20 mM phosphate, 1 mM EDTA, 1 mM β-mercaptoethanol, pH 7.0) containing 0.5 mM phenylmethylsulfonyl fluoride, and sonicated 3 min at 50% duty and 30% power using a Branson Sonifier (Danbury, CT) equipped with a 0.5 in diameter tip. Cell debris was removed by sedimentation at 100,000 x *g* for 60 min at 4°C. Further purification of the pKAU601-derived urease from the cell extracts made use of room temperature chromatography on DEAE-Sepharose, Superose 6, and Mono-Q resins obtained from Pharmacia (Uppsala, Sweden) by using methods which were previously described for urease isolated from cells grown in the absence of nickel ion (23). Because the urease from this strain was

enzymatically inactive, its presence was assayed by SDS-polyacrylamide gel electrophoresis and by comparison to authentic urease enzyme. The nickel content of the urease preparation was assessed by using atomic absorption spectrometry as previously described (32).

RESULTS AND DISCUSSION

Sequence analysis of the urease operon. The sequence of a 4.8-kb region encoding the urease operon is shown in Fig. 2. Analysis of the sequence revealed six open reading frames that are designated *ureA*, *ureB*, *ureC*, *ureE*, *ureF*, and *ureG*. These genes are all transcribed in the same direction and are predicted to encode peptides of 101, 106, 567, 158, 224, and 205 amino acids with M_r= 11,086, 11,695, 60,304, 17,558, 25,221, and 21,943, respectively. The very close spacing of the genes results in 3 cases where the ribosome binding site for one gene overlaps the coding region of the previous gene. Furthermore, the end of *ureB* overlaps the beginning of *ureC* by 8 nucleotides. *K. aerogenes* urease is expressed under nitrogen limited conditions by the global Ntr system (9,26). Detailed characterization of the urease upstream regulatory region will be described separately (Markowicz, Mulrooney, and Hausinger, in preparation), and the sequence is provided as an appendix to this thesis. *P. mirabilis* contains an additional urease gene (*ureD*) located immediately upstream of *ureA* (20); the *ureD* gene is thought to function in urea induction, a mode of regulation which does not occur in *K. aerogenes*. A potential rho-dependent transcriptional termination site has been identified immediately after the *ureG* gene. In addition, the first 75 amino acids of another open reading frame was detected starting at base

Fig. 2. Nucleotide sequence of the urease genes. The deduced amino acid sequences for the six open reading frames are shown for *ureA* (bp 264-566), *ureB* (bp 576-896), *ureC* (bp 889-2592), *ureE* (2602-3078), *ureF* (bp 3080-3754), and *ureG* (bp 3763-4380). Putative Shine-Dalgarno sites are underlined and a possible rho-dependent transcription termination sequence is indicated by arrows.

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                .         .         .         .         .         .

  1 CTCTCGCCGAACGTCCCTGGGTCGGCACTTTGCTGTGCTATCCGGCTACCGATGCCCTGC

                .         .         .         .         .         .

 61 TCGACGGGGTGCGCGACGCGCTGGCGCCGCTCGGTCTCTACGCCGGCGCCAGCCTGACCG

                .         .         .         .         .         .

121 ACCGCCTGCTGACGGTGCGTTTCCTCAGTGACGATAATCTGATTTGCCAGCGGGTGATGC

                .         .         .         .         .         .

181 GCGACGTATGGCAGTTTCTGCGCCCTCATCTCACCGGTAAATCTCCCGTACTTCCCCGAA

                .         .         .         .         .         .

241 TCTGGCTGACTTAAGAGAACGTTATGGAACTGACCCCCCGAGAAAAAGACAAGCTGTTGC
                          M  E  L  T  P  R  E  K  D  K  L  L  L

                .         .         .         .         .         .

301 TGTTTACCGCCGCGCTGGTGGCGGAGCGTCGCCTGGCCCGCGGCCTGAAGCTCAACTATC
      F  T  A  A  L  V  A  E  R  R  L  A  R  G  L  K  L  N  Y  P

                .         .         .         .         .         .

361 CGGAGTCCGTGGCCCTGATCAGCGCCTTTATTATGGAAGGCGCTCGGGACGGCAAAAGCG
      E  S  V  A  L  I  S  A  F  I  M  E  G  A  R  D  G  K  S  V

                .         .         .         .         .         .

421 TGGCCTCGCTGATGGAGGAAGGCCGTCACGTCCTGACCCGCGAGCAGGTGATGGAGGGCG
      A  S  L  M  E  E  G  R  H  V  L  T  R  E  Q  V  M  E  G  V

                .         .         .         .         .         .

481 TCCCGGAAATGATCCCGGATATCCAGGTCGAAGCCACCTTCCCGGACGGCTCGAAGCTGG
      P  E  M  I  P  D  I  Q  V  E  A  T  F  P  D  G  S  K  L  V

                .         .         .         .         .         .

541 TCACCGTTCACAACCCGATTATCTGAGGTAGCGCCATGATCCCCGGTGAATATCACGTTA
      T  V  H  N  P  I  I  *              M  I  P  G  E  Y  H  V  K

                .         .         .         .         .         .

601 AGCCCGGTCAGATAGCCCTGAATACCGGCCGGGCAACCTGTCGCGTGGTCGTTGAGAACC
      P  G  Q  I  A  L  N  T  G  R  A  T  C  R  V  V  V  E  N  H

                .         .         .         .         .         .

661 ACGGCGATCGGCCGATTCAGGTCGGTTCGCACTACCATTTCGCCGAGGTTAACCCGGCGC
      G  D  R  P  I  Q  V  G  S  H  Y  H  F  A  E  V  N  P  A  L

                .         .         .         .         .         .

721 TGAAGTTCGACCGTCAGCAGGCCGCCGGCTATCGCCTGAATATCCCGGCGGGCACGGCGG
      K  F  D  R  Q  Q  A  A  G  Y  R  L  N  I  P  A  G  T  A  V

                .         .         .         .         .         .

781 TACGCTTTGAACCCGGCCAGAAACGCGAGGTCGAGCTGGTGGCCTTCGCCGGTCACCGCG
      R  F  E  P  G  Q  K  R  E  V  E  L  V  A  F  A  G  H  R  A

                .         .         .         .         .         .

841 CCGTCTTCGGCTTCCGCGGCGAGGTCATGGGCCCTCTGGAGGTAAACGATGAGTAATATT
      V  F  G  F  R  G  E  V  M  G  P  L  E  V  N  D  E  *
                                                      M  S  N  I

                .         .         .         .         .         .

901 TCACGCCAGGCCTATGCCGATATGTTCGGCCCCACCGTCGGCGACAAGGTGCGCCTGGCA
    S  R  Q  A  Y  A  D  M  F  G  P  T  V  G  D  K  V  R  L  A

                .         .         .         .         .         .

961 GATACCGAGCTGTGGATCGAGGTGGAGGACGATTTGACCACCTACGGGGAAGAGGTCAAA
    D  T  E  L  W  I  E  V  E  D  D  L  T  T  Y  G  E  E  V  K

                .         .         .         .         .         .

1021 TTCGGCGGCGGCAAAGTGATCCGCGACGGCATGGGCCAGGGACAGATGCTGGCCGCCGAC
     F  G  G  G  K  V  I  R  D  G  M  G  Q  G  Q  M  L  A  A  D
```

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1081 TGTGTCGACCTGGTGCTCACCAACGCGTTGATCGTCGATCACTGGGGGATCGTTAAGGCC
     C  V  D  L  V  L  T  N  A  L  I  V  D  H  W  G  I  V  K  A

1141 GATATCGGCGTGAAGGACGGCCGGATCTTCGCCATCGGCAAAGCCGGCAACCCCGACATC
     D  I  G  V  K  D  G  R  I  F  A  I  G  K  A  G  N  P  D  I

1201 CAGCCCAACGTCACCATCCCCATCGGCGCTGCGACGGAAGTGATCGCCGCCGAAGGAAAA
     Q  P  N  V  T  I  P  I  G  A  A  T  E  V  I  A  A  E  G  K

1261 ATTGTCACCGCCGGCGGGATCGATACCCATATTCACTGGATCTGTCCGCAGCAGGCGGAA
     I  V  T  A  G  G  I  D  T  H  I  H  W  I  C  P  Q  Q  A  E

1321 GAGGCGCTGGTCTCTGGCGTGACCACCATGGTCGGCGGCGGCACCGGCCCGGCCGCGGGC
     E  A  L  V  S  G  V  T  T  M  V  G  G  G  T  G  P  A  A  G

1381 ACCCATGCCACCACCTGCACCCCGGGCCCGTGGTATATCTCACGCATGCTGCAGGCGGCC
     T  H  A  T  T  C  T  P  G  P  W  Y  I  S  R  M  L  Q  A  A

1441 GACAGCCTGCCGGTCAATATCGGCCTGCTGGGCAAGGGAAACGTTTCTCAGCCGGATGCC
     D  S  L  P  V  N  I  G  L  L  G  K  G  N  V  S  Q  P  D  A

1501 CTGCGCGAGCAGGTGGCGGCAGGCGTTATTGGCCTGAAGATCCATGAGGACTGGGGCGCC
     L  R  E  Q  V  A  A  G  V  I  G  L  K  I  H  E  D  W  G  A

1561 ACCCCGGCGGCGATCGACTGTGCGTTAACCGTCGCCGATGAAATGGACATCCAGGTCGCC
     T  P  A  A  I  D  C  A  L  T  V  A  D  E  M  D  I  Q  V  A

1621 CTGCACAGCGACACCCTGAATGAATCCGGTTTTGTGGAAGACACCCTCGCCGCCATCGGC
     L  H  S  D  T  L  N  E  S  G  F  V  E  D  T  L  A  A  I  G

1681 GGGCGCACCATCCACACCTTCCATACCGAAGGGGCCGGCGGCGGCCATGCGCCGGACATC
     G  R  T  I  H  T  F  H  T  E  G  A  G  G  G  H  A  P  D  I

1741 ATCACCGCCTGCGCCCACCCGAACATTTTGCCGTCGTCCACCAACCCAACGCTGCCCTAC
     I  T  A  C  A  H  P  N  I  L  P  S  S  T  N  P  T  L  P  Y

1801 ACCCTCAACACCATCGATGAACATCTCGATATGCTGATGGTCTGCCACCATCTGGACCCG
     T  L  N  T  I  D  E  H  L  D  M  L  M  V  C  H  H  L  D  P

1861 GACATCGCCGAGGACGTGGCCTTTGCCGAGTCGCGCATTCGCCGGGAAACCATCGCTGCG
     D  I  A  E  D  V  A  F  A  E  S  R  I  R  R  E  T  I  A  A

1921 GAAGACGTGCTGCACGATCTCGGCGCCTTCTCGCTCACCTCCTCCGATTCGCAGGCCATG
     E  D  V  L  H  D  L  G  A  F  S  L  T  S  S  D  S  Q  A  M

1981 GGCCGCGTCGGGGAAGTGATTCTCCGCACCTGGCAGGTGGCGCATCGCATGAAGGTGCAG
     G  R  V  G  E  V  I  L  R  T  W  Q  V  A  H  R  M  K  V  Q

2041 CGCGGAGCGCTGGCGGAGGAGACCGGGGATAACGACAACTTCCGCGTGAAGCGCTACATC
     R  G  A  L  A  E  E  T  G  D  N  D  N  F  R  V  K  R  Y  I

2101 GCCAAATACACCATCAACCCGGCGCTGACCCACGGCATCGCACACGAAGTCGGATCCATT
     A  K  Y  T  I  N  P  A  L  T  H  G  I  A  H  E  V  G  S  I
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2161 GAGGTGGGTAAGCTGGCTGACCTCGTGGTCTGGTCACCAGCCTTCTTCGGCGTGAAACCG
     E  V  G  K  L  A  D  L  V  V  W  S  P  A  F  F  G  V  K  P

2221 GCCACCGTGATCAAAGGCGGCATGATCGCCATCGCGCCGATGGGCGATATCAATGCCTCT
     A  T  V  I  K  G  G  M  I  A  I  A  P  M  G  D  I  N  A  S

2281 ATTCCGACCCCGCAGCCGGTGCACTACCGCCCGATGTTTGGCGCGCTGGGCAGCGCCCGC
     I  P  T  P  Q  P  V  H  Y  R  P  M  F  G  A  L  G  S  A  R

2341 CATCACTGCCGCCTCACCTTCCTGTCGCAGGCGGCGGCAGCCAATGGCGTTGCCGAGCGG
     H  H  C  R  L  T  F  L  S  Q  A  A  A  A  N  G  V  A  E  R

2401 CTGAACCTGCGCAGCGCGATCGCCGTGGTGAAAGGCTGCCGTACGGTGCAGAAAGCCGAC
     L  N  L  R  S  A  I  A  V  V  K  G  C  R  T  V  Q  K  A  D

2461 ATGGTGCACAACAGTCTGCAGCCTAACATCACCGTCGACGCCCAGACCTATGAGGTGCGG
     M  V  H  N  S  L  Q  P  N  I  T  V  D  A  Q  T  Y  E  V  R

2521 GTGGATGGCGAACTTATCACCAGCGAGCCGGCAGACGTTCTGCCGATGGCGCAACGATAT
     V  D  G  E  L  I  T  S  E  P  A  D  V  L  P  M  A  Q  R  Y

2581 TTTCTGTTTTAAGGAGAGCGGATGCTTTATTTAACTCAACGTCTGGAGATCCCCGCCGCC
     F  L  F  *           M  L  Y  L  T  Q  R  L  E  I  P  A  A

2641 GCGACCGCCAGCGTTACGCTGCCGATTGATGTTCGCGTCAAAAGCCGGGTTAAGGTCACC
     A  T  A  S  V  T  L  P  I  D  V  R  V  K  S  R  V  K  V  T

2701 CTCAACGATGGCCGGGATGCCGGCCTGCTGCTGCCCCGCGGCCTGCTACTACGCGGCGGC
     L  N  D  G  R  D  A  G  L  L  L  P  R  G  L  L  L  R  G  G

2761 GATGTGCTCAGCAACGAAGAAGGCACCGAGTTTGTGCAGGTGATTGCCGCTGATGAAGAG
     D  V  L  S  N  E  E  G  T  E  F  V  Q  V  I  A  A  D  E  E

2821 GTGTCGGTAGTGCGCTGCGACGATCCGTTTATGCTGGCGAAGGCCTGCTACCACCTCGGC
     V  S  V  V  R  C  D  D  P  F  M  L  A  K  A  C  Y  H  L  G

2881 AACCGTCACGTGCCGCTGCAGATCATGCCGGGCGAGCTGCGCTACCATCACGATCACGTG
     N  R  H  V  P  L  Q  I  M  P  G  E  L  R  Y  H  H  D  H  V

2941 CTGGACGATATGCTGCGCCAGTTCGGCCTGACGGTGACCTTTGGCCAGCTGCCGTTCGAG
     L  D  D  M  L  R  Q  F  G  L  T  V  T  F  G  Q  L  P  F  E

3001 CCGGAAGCCGGCGCTTACGCCAGCGAGAGCCACGGTCATCATCATGCTCATCATGACCAC
     P  E  A  G  A  Y  A  S  E  S  H  G  H  H  H  A  H  H  D  H

3061 CACGCTCACAGCCACTAGCATGTCGACAGCGGAACAACGCCTGCGGCTGATGCAGCTGGC
     H  A  H  S  H  *  M  S  T  A  E  Q  R  L  R  L  M  Q  L  A

3121 CAGCAGCAACCTGCCGGTAGGGGGTTACAGCTGGTCCCAGGGGCTGGAGTGGGCTGTGGA
      S  S  N  L  P  V  G  G  Y  S  W  S  Q  G  L  E  W  A  V  E

3181 AGCCGGCTGGGTGCTGGACGTCGCGGCCTTCGAGCGCTGGCAGCGACGCCAGATGACGGA
      A  G  W  V  L  D  V  A  A  F  E  R  W  Q  R  R  Q  M  T  E
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3241 AGGCTTTTTTACCGTTGACCTGCCGCTGTTCGCCCGCCTGTACCGCGCCTGCGAACAAGG
      G  F  F  T  V  D  L  P  L  F  A  R  L  Y  R  A  C  E  Q  G

3301 CGATATCGCTGCGGCCCAGCGCTGGACCGCCTATCTGCTGGCCTGCCGGGAAACTCGTGA
      D  I  A  A  A  Q  R  W  T  A  Y  L  L  A  C  R  E  T  R  E

3361 ACTGCGGGAGGAAGAGCGCAACCGCGGCGCGGCGTTTGCCCGTCTGCTGAGCGACTGGCA
      L  R  E  E  E  R  N  R  G  A  A  F  A  R  L  L  S  D  W  Q

3421 GCCGGACTGTCCGCCGCCGTGGCGCTCCCTGTGCCAGCAAAGCCAGCTCGCCGGGATGGC
      P  D  C  P  P  P  W  R  S  L  C  Q  Q  S  Q  L  A  G  M  A

3481 CTGGCTCGGCGTGCGCTGGCGTATCGCCCTGCCCGAGATGGCCCTCAGCCTGGGCTATAG
      W  L  G  V  R  W  R  I  A  L  P  E  M  A  L  S  L  G  Y  S

3541 CTGGATTGAGAGCGCCGTGATGGCCGGCGTCAAGCTGGTCCCCTTCGGCCAGCAGGCCGC
      W  I  E  S  A  V  M  A  G  V  K  L  V  P  F  G  Q  Q  A  A

3601 CCAGCAGCTGATTTTACGTCTTTGTGACCACTACGCGGCCGAGATGCCCCGCGCGCTGGC
      Q  Q  L  I  L  R  L  C  D  H  Y  A  A  E  M  P  R  A  L  A

3661 CGCGCCGGACGGCGATATCGGATCGGCCACCCCGCTCGCCGCCATCGCCTCTGCCCGGCA
      A  P  D  G  D  I  G  S  A  T  P  L  A  A  I  A  S  A  R  H

3721 TGAAACCCAATACTCTCGATTATTCCGTTCCTAGGAGAAGCCATGAACTCTTATAAACAC
      E  T  Q  Y  S  R  L  F  R  S  *           M  N  S  Y  K  H

3781 CCGCTGCGCGTCGGCGTCGGCGGCCCGGTCGGCTCCGGTAAAACCGCTCTGCTGGAAGCG
      P  L  R  V  G  V  G  G  P  V  G  S  G  K  T  A  L  L  E  A

3841 CTGTGTAAAGCGATGCGCGATACCTGGCAGCTGGCGGTGGTCACTAACGACATCTATACC
      L  C  K  A  M  R  D  T  W  Q  L  A  V  V  T  N  D  I  Y  T

3901 AAAGAAGATCAGCGCATCCTCACCGAAGCGGGCGCGCTGGCGCCTGAACGCATCGTCGGT
      K  E  D  Q  R  I  L  T  E  A  G  A  L  A  P  E  R  I  V  G

3961 GTGGAAACCGGCGGCTGCCCGCATACGGCGATCCGCGAAGATGCCTCAATGAACCTCGCC
      V  E  T  G  G  C  P  H  T  A  I  R  E  D  A  S  M  N  L  A

4021 GCCGTGGAAGCGCTGAGTGAAAAGTTCGGTAACCTCGACCTTATCTTCGTGGAAAGCGGC
      A  V  E  A  L  S  E  K  F  G  N  L  D  L  I  F  V  E  S  G

4081 GGCGATAACCTGAGCGCCACCTTCAGCCCGGAGCTGGCGGATCTGACCATCTACGTCATC
      G  D  N  L  S  A  T  F  S  P  E  L  A  D  L  T  I  Y  V  I

4141 GATGTGGCCGAAGGGGAGAAGATCCCGCGCAAAGGCGGACCGGGGATCACCAAATCCGAT
      D  V  A  E  G  E  K  I  P  R  K  G  G  P  G  I  T  K  S  D

4201 TTCCTGGTGATCAATAAAACCGACCTTGCCCCCTATGTGGGCGCGTCGCTGGAGGTGATG
      F  L  V  I  N  K  T  D  L  A  P  Y  V  G  A  S  L  E  V  M

4261 GCGAGCGATACCCAGCGTATGCGCGGCGATCGCCCATGGACCTTCACCAATCTGAAGCAG
      A  S  D  T  Q  R  M  R  G  D  R  P  W  T  F  T  N  L  K  Q
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4321 GGCGACGGCCTGAGCACCATTATCGCCTTCCTCGAAGACAAAGGCATGCTTGGCAAATAG
     G  D  G  L  S  T  I  I  A  F  L  E  D  K  G  M  L  G  K  *

4381 GCCTGTTGCACCAGCCGGGCGCAAGCGCGTCCGGATGGTGCAGCCTCTGCTCTCTCTTTA

4441 TATCATCCTGCCTCCACCTCCGCGCCACGCCTGCCCTGCAATATGGCATAAGGTTTGCTA

4501 ATTCAAGTCATGCCTAACCATTAAGGAATGACTATGTCATCACTGGATCTTAACCCTGAA

4561 TTACCCGCGACAACGCGGACTTCCGGTACCCGGGAAACCTTAGAAGATTACACCTTACGT

4621 TACGCCCCGCTGAGCTTCCGCCGCTGGGGTCCGGGCGTCGTCGCGGTCACCGCGCTGGGC

4681 GGCATCGCCTATCTGGCCGACTTTTCCATCGGCGCCAGCATCGGTATGGCCTGGGGCACC

4741 AGCAACGCCATCTATTCGATC  4761
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4534. It is preceded by two potential NtrC binding sites (4388-4402, 4407-4421) and an NtrA binding site (4483-4499) consistent with nitrogen-dependent regulation. The partial open reading frame was not homologous to any sequence in the DNA or protein database and is not part of the urease operon.

Homology comparisons. The predicted sequences for the first three genes of the *K. aerogenes* urease operon display significant homology to the reported amino acid sequence of jack bean urease (38): UreA is 59% identical to residues 1-101, UreB is 52% identical to residues 132-237, and UreC is 60% identical to residues 271-840 of the plant protein. The amino-terminal protein sequence of the large subunit of *K. aerogenes* urease (30) confirmed the assignment of the *ureC* gene to this polypeptide. Furthermore, the gene sequences encoding the *K. aerogenes* urease subunits are 72%, 71%, and 58% identical to recently reported sequences encoding urease subunits from *Proteus mirabilis* (20), *Proteus vulgaris* (31), and *Helicobacter pylori* (3). Indeed, 42% of the *K. aerogenes* amino acid residues are present in all three bacterial ureases and the jack bean enzyme. No significant homology was detected between the urease structural genes and any non-urease sequences in the NBRF data bank. Gene fusion or gene disruption may have occurred during evolution to explain the single subunit plant protein, the two subunit *H. pylori* sequence, and the three subunit ureases found in other bacteria.

The predicted sequences for UreE, UreF, and UreG displayed no homology to the amino acid sequence of jack bean urease. Furthermore, little homology was observed when these sequences were compared to other sequences in the NBRF data bank. By contrast, sequences determined for the *ureE* and *ureF* genes of the *P. mirabilis* urease operon (20) were 53% and

38% identical to the *K. aerogenes* genes. Moreover, the reported *P. mirabilis* sequence included the start of an open reading frame (located 60 nucleotides beyond the termination of *ureF*) which was homologous to *ureG* from *K. aerogenes*. In addition, the limited DNA sequence data reported for the urease operons from *P. vulgaris* (31) and *H. pylori* (3) included regions corresponding to the start of *ureE*.

Complementation analysis of the urease operon genes. When a region extending from the middle of *ureE* to the end of *ureG* was deleted from the urease operon (plasmid pKAU601, Fig. 1), no activity could be detected in transformed cells (Table 1). SDS-polyacrylamide gel analysis showed that the *ureA*, *ureB*, and *ureC* gene products were being expressed, although at lower levels than are seen with the intact operon (results not shown). A fragment containing the missing *ureE*, *ureF*, and *ureG* genes was subcloned behind the *tac* promoter of the compatible expression vector pMMB66HE to yield pKAU506 (Fig. 1). Expression of the genes was verified by SDS-polyacrylamide gel analysis; polypeptides corresponding to the predicted sizes for UreE (17-kDa), UreF (25-kDa), and UreG (22-kDa) were clearly seen upon induction with 1 mM IPTG (Fig. 3). Furthermore, centrifugation of the extracts resulted in a partial loss of the *ureE* gene product and complete disappearance of the *ureG* gene product. Extraction of the washed pellet with SDS showed the presence of the UreG protein (Fig. 3).

When plasmids pKAU506 and pKAU601 were cotransformed into the *E. coli* host, the cotransformant was ureolytic and IPTG enhanced urease activity 2.4 fold (Table 1). The high levels of urease activity in the uninduced controls may indicate that small amounts of the accessory gene products are able to activate large amounts of inactive urease. Thus, *ureE*, *ureF*, and *ureG* gene products could act in *trans* with *ureA*, *ureB* and

TABLE 1. Urease specific activities of recombinant *E. coli* cultures.

Culture	Specific activity* (μmole urea min^{-1} mg^{-1}) -IPTG	+IPTG
E. coli DH1(pKAU506)	< 0.2	< 0.2
E. coli DH1(pKAU601)	< 0.06	< 0.06
E. coli DH1(pKAU506 + pKAU601)	12.9	31.2

* For comparison, *E. coli* DH1(pKAU17) typically has a specific activity of 120 U mg^{-1}

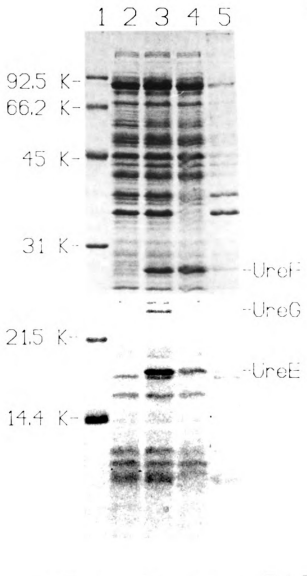

Fig. 3. Polyacrylamide gel analysis of UreE, UreF, and UreG. Sonicated cell extracts of *E. coli* containing pKAU506 were compared for uninduced (lane 2) and IPTG-induced (lane 3) cells by SDS-polyacrylamide (15%) gel electrophoresis. Extracts from the induced culture were centrifuged at 12,000 x *g* for 45 min and analyzed (lane 4). The pellet was washed, resuspended in SDS sample buffer and run (lane 5). Standards (Bio Rad low MW, Richmond, CA) are shown for comparison (lane 1). All samples were boiled 5 min before running.

ureC to give active urease and at least one of these accessory genes is required. In contrast, no activity was observed when sonicated cells containing pKAU601 were mixed with equal amounts of sonicated IPTG-induced cells containing pKAU506. Inclusion of 1 mM ATP in the 20 mM phosphate, 1 mM β-mercaptoetanol, pH 7.0 reaction buffer had no effect. Lack of urease activation under these conditions may indicate a requirement for intact membranes, a need for an energy source other than ATP, or a temporal requirement for the accessory proteins during urease folding.

A requirement for accessory genes has also been demonstrated for ureases from soybean (29), the fungus *Aspergillus nidulans* (25), *Providencia stuartii* (32), *P. mirabilis* (19, 41), a urease positive *E. coli* (4), *K. pneumoniae* (13), *P. vulgaris* (31), and *Staphylococcus saprophyticus* (11). Genetic studies of soybean demonstrated the presence of two loci which are distinct from the embryo-specific or ubiquitous urease isozyme structural genes (29). Mutations in these loci result in the production of inactive urease protein, consistent with a possible role in maturation. *A. nidulans* has been shown to have four loci involved in urea utilization: *ureA* encoding a urea permease, *ureB* encoding urease, *ureC* of unknown function, and *ureD* suggested to participate in the synthesis or incorporation of a nickel cofactor (25). In the bacterial cases, transposon insertion mutants or deletion mutants downstream of the urease structural genes produced all three urease subunits but possessed little or no urease activity (4, 11, 13, 19, 31, 32, 41). Several of these mutants were defective in non-urease subunit peptides which may correspond to one or more of the accessory genes described here. Furthermore, the *P. mirabilis* urease operon included sequences that are analogous to *ureE*, *ureF*, and the start of *ureG* (20). A deletion of *ureG* and the very end of

ureF led to substantial losses of activity in the *P. mirabilis* clone.

Purification and characterization of pKAU601-derived urease. Although plasmid pKAU601 contained the normal promoter region and the three urease subunit genes, cells containing this plasmid had no urease activity. In order to assess the role of the missing genes in this construct, inactive urease protein was purified by procedures which were nearly identical to that used for the native enzyme. Because no activity was present, the presence of urease protein in column fractions was assessed by SDS-polyacrylamide gel electrophoresis. The intensity of bands on the gels demonstrated that cell extracts contained greatly decreased levels of urease protein compared to that found in cells containing pKAU17. Moreover, the amount of urease protein purified from this clone (approximately 1.5 mg) was less than 5% of that typically observed in cells containing pKAU17.

The final preparation of pKAU601-derived urease was only 51% homogeneous as estimated by densitometric analysis of Coomassie blue stained SDS-polyacrylamide gels, nevertheless, several properties of the protein were characterized. Although differing somewhat from the predicted sizes, the three urease subunits were identical in size to that found in native enzyme (apparent M_r = 9, 11, and 72 kDa). Moreover, they clearly remained associated throughout the purification, which included ion exchange and gel filtration chromatography. Gel scanning demonstrated that the ratio of 60.3-kDa : 11.7-kDa : 11.1-kDa subunits was 1.1 : 1.8 : 1.7, nearly identical to that observed in the native enzyme (39). Furthermore, the chromatographic properties of the pKAU601-derived urease matched that of the native enzyme indicating a similar size [$(60\text{-kDa})_2(12\text{-kDa})_4(11\text{-kDa})_4$] and charge. The only observed distinction from the native urease was

in the nickel content. Whereas the native enzyme was shown to possess 4 moles of nickel per mole of enzyme (39), the pKAU601-derived protein had less than 0.25 moles of nickel per mole of enzyme. Thus, one or more of the accessory gene products is involved in facilitating assimilation of nickel into apo-urease.

One possible role for the accessory genes involves nickel transport into the cell. However, we feel that this can not be the only role for the accessory genes because nickel can enter *E. coli* cells via the magnesium transport system (17). Since our cells were grown in a medium containing 0.1 mM nickel, it seems probable that some nickel would enter the cell, yet we could not detect any measurable activity.

A second possible role for the accessory genes involves nickel incorporation into apo-urease. We had previously reported that *K. aerogenes* cells containing the recombinant urease plasmid pKAU19 grown in nickel-free medium synthesized urease apo-enzyme, and that the apo-enzyme was activated upon addition of nickel even after treating the cells with protein synthesis inhibitors (Chapter 4; 23). The purified apo-urease could not be reactivated by addition of nickel, indicating that nickel incorporation does not occur by passive binding. It was proposed that some additional component was necessary to facilitate insertion of nickel into the apoenzyme. The accessory genes may participate in this process. In this regard, the carboxyl terminal sequence predicted for UreE is particularly interesting: ten of the last 15 amino acids are histidine residues. Histidine-rich regions are involved in metal binding sites for other proteins [e.g., the zinc-binding protein from albacore tuna plasma (7) and the copper-containing hemocyanin (12)]; thus, such a sequence may bind nickel ions which are subsequently transferred to apo-urease. The

only other known function for a histidine-rich region involves a 16 amino acid peptide containing 7 adjacent histidinyl residues; this sequence participates in regulation of the *Salmonella* histidine operon (18).

In summary, the formation of active urease requires activation by accessory proteins which function in nickel incorporation. Urease is not the only metalloenzyme which requires accessory proteins for metal incorporation. For example, multiple gene products are required for proper incorporation of molybdenum into several enzymes, as reviewed by Hinton and Dean (16). Moreover, evidence has been presented for a copper-insertase required for N_2O reductase biosynthesis (22). There is also evidence that in vivo nickel incorporation into *Rhodospirillum rubrum* carbon monoxide dehydrogenase may require nickel processing (8). Further characterization of the accessory proteins involved in activating urease may aid in elucidating some of the general incorporation mechanisms for metalloproteins. Future efforts will be directed at characterizing how the *ureE*, *ureF*, and *ureG* gene products are involved in this process.

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CHAPTER 6

Conclusions and Future Prospects

There were three main goals established at the onset of the work described herein: to clone the *Klebsiella aerogenes* urease genes; to ascertain the best combination of plasmid vector, host strain, and growth conditions to yield maximum production of urease; and to determine the sequence of the urease genes.

As a prelude to the *Klebsiella aerogenes* investigations, experiments were carried out with recombinant *Providencia stuartii* urease. This work represents the first purification and analysis of a urease expressed in a heterologous host, demonstrating that *E. coli* could synthesize urease that was identical in every respect to the enzyme purified from the original strain. Furthermore, the regulation of enzyme expression observed in *E. coli* was identical to that of the parent *Providencia stuartii*. These results supported our expectation that the *Klebsiella aerogenes* urease genes could be successfully cloned and overexpressed.

The *Klebsiella aerogenes* urease genes were successfully cloned by screening a cosmid library on modified urease indicator plates. Several subcloning steps reduced the size of the insert DNA to 5.7 kb. This fragment was cloned into vector pBR328 (pKAU19) and urease expression was tested in several host strains. The optimal growth conditions were found to be a MOPS buffered minimal medium containing low nitrogen and high nickel concentrations. Greatest expression of urease was obtained by transforming pKAU19 back into *Klebsiella aerogenes*; yields were 100 to 200-fold higher than in the wild-type organism carrying a single copy of the urease genes. Expression of these high levels greatly simplified purification providing a large supply of enzyme for biophysical studies. Furthermore, it allowed the use of immunogold localization to confirm that urease was a cytoplasmic enzyme.

Sequencing of the urease genes revealed an operon much larger and more complex than anticipated. Six open reading frames were present, three of which were the urease subunit genes: these genes show a high degree of homology to the ureases of jack bean and other bacteria. The three additional genes have unspecified functions, but their deletion from the urease operon results in the synthesis of inactive urease apoenzyme. In addition, these three genes can act in trans to produce active urease and thus may play a role in nickel incorporation. Additional information on the mechanism of nickel incorporation was obtained by growing cells in a nickel-free medium in the presence of various metabolic inhibitors: addition of nickel restored urease activity in cells inhibited in protein synthesis but not in cells inhibited in energy production or in sonicated cells. These results led to the proposal that urease is initially synthesized as an apoenzyme and is subsequently activated in an energy-dependent nickel incorporation step.

Future directions. The results presented here lay a foundation for many new experimental paths. Several possibilities are summarized below:

a) Characterization of UreE, UreF, and UreG proteins. These three accessory proteins are highly expressed from plasmid pKAU506, facilitating their purification and analysis. Metal chelation chromatography might be a good choice as a UreE purification step because of its unusual histidine-rich carboxy terminus. Antisera can be produced against all three peptides and immunogold electron microscopy used for localization using the same techniques as for *Klebsiella aerogenes* urease. Functional characterization of these peptides would include metal analysis.

b) Complementation among urease genes of different species. Since it was

demonstrated that the *Klebsiella aerogenes ureE*, *ureF*, and *ureG* can complement, in trans, *ureA*, *ureB*, and *ureC*, it seems reasonable to try this with other cloned ureases. For example, there are mutants of *Proteus mirabilis*, *Providencia stuartii*, *Klebsiella pneumoniae*, and *Proteus vulgarus* which express inactive urease subunits. Furthermore, the bacterial accessory genes could be used to attempt complementation of the soybean urease structural gene. The ability of plasmid pKAU506 to restore activity in these mutants would indicate a common mechanism of nickel incorporation in these other ureases and would indicate conserved regions essential for maintaining the structure and function of the accessory proteins.

c) The high production of urease described in chapter 2 should allow purification of large enough quantities for biophysical and crystallization studies with the long term goals of determining the metallocenter function and the three dimensional structure of the enzyme.

d) Site directed mutagenesis. Once the active site is located by labeling and peptide isolation, specific amino acids could be changed to test the effects on kinetic parameters. One obvious target would be the one cysteine that is conserved among all of the sequenced ureases: a cysteine is implicated in the active site of *Klebsiella aerogenes* and jack bean enzymes. Mutagenesis could also be performed on several of the highly conserved histidines, which could serve as nickel ligands.

Other targets for mutagenesis would be the accessory proteins. For example, urease activity could be measured in UreE mutants containing amino acid substitutions or truncation of the histidine-rich carboxy terminus.

APPENDIX

Sequence of the urease upstream region from a *Sau*3A site to the *Sst*I site (base 751 here is base 1 of Fig. 4, Chapter 5)

```
1   GATCAGGATATTGACGGCAAAGATTATCCGGGTGAATAATCCGGAGCGGTTATGCCGCTT
60  TCCCCTCCGCGCCCGTCGCGACGCTCCGGGTTACGGATGACATAAGCGTTTCGTATGACC
120 GGGATAAACTCCCGCCGATCAATACTCATTGCTGCTGTTTTATCTTGATTTTGCAGGGGC
180 GCAACATTGCACGGCACCGTGTTACCACCACTCAAAAAAGGCTGGCAGGCCACGCTGGAT
240 CTCCGCTTTCACCAGGCCGGCGGCAAGACCGTTCTCGCCAGCGCCCAACACGTCGGCCCG
300 CTGACCGTCCAGCGCCCGTTTTACCCGGAAGAAGAGACCTGTCACCTCTATCTGCTTCAC
360 CCGCCCGGCGGCATCGTCGGCGGTGATGAGCTGACAATTAGCGCGCACCTTGCCCCCGGC
420 TGCCATACGCTGATAACCATGCCTGGCGCCAGCAAGTTTTACCGCAGCAGCGGCGCGCAA
480 GCGCTAGTTCGCCAGCAGTTGACCCTTGCCCCGCAGGCGACCCTGGAGTGGCTCCCGCAG
540 GATGCCATCTTCTTTCCCGGGGCCAATGCCCGGCTGTTCACCACCTTTCATCTTTGCGCC
600 TCCAGCAGGCTGCTGGCCTGGGATCTGCTCTGCCTTGGCCGCCCGGTGATTGGCGAAACC
660 TTCAGCCACGGCACCCTCAGCAACCGGCTGGAGGTATGGGTGGACAATGAGCCGCTGCTG
720 GTCGAGCGCCTGCACCTGCAGGAGGGAGAGC 751
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