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MECHANISTIC STUDIES OF *Klebsiella aerogenes* UREASE AND ITS NICKEL METALLOCENTER ASSEMBLY PROCESS

By

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ABSTRACT

MECHANISTIC STUDIES OF *Klebsiella aerogenes* UREASE AND ITS NICKEL METALLOCENTER ASSEMBLY PROCESS

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Chemical modification and site-directed mutagenesis methods were used to examine the mechanism of *Klebsiella aerogenes* urease. The enzyme is inactivated by diethylpyrocarbonate (DEP) in a manner consistent with the presence of at least one essential histidine per catalytic unit. Each of ten conserved histidine residues (H96 in the γ subunit, H39 and H41 in β, and H134, H136, H219, H246, H312, H320 and H321 in the α subunit) was substituted with alanine. The γH96A, βH39A, βH41A, αH312A and αH321A mutant proteins possess activities and nickel contents similar to wild-type. The αH134A, αH136A and αH246A proteins exhibit no detectable activity and possess ≤50% of the nickel content of wild-type enzyme. The αH219A protein is active and has nickel (~1.9% and ~80%, respectively, when compared to wild-type protein), but exhibits a very high K_m value (1100±40 mM compared to 2.3±0.2 mM for the wild-type enzyme). Finally, the αH320A protein (K_m=8.3±0.2 mM) only displays ~0.003% of the wild-type enzyme activity, and has a normal nickel content. This mutant protein is not inactivated by DEP. These results are consistent with αH134, αH136 and αH246 functioning as nickel ligands, αH219 having some role in facilitating substrate binding, and αH320 being the DEP-reactive general base that facilitates catalysis.

In vivo assembly of the urease metallocenter is known to require four accessory proteins: UreD, UreE, UreF and UreG. *In vitro* activation of purified

K. aerogenes urease apoprotein has now been accomplished by simply providing CO_2 (half-maximal activation at ~0.2% CO_2) in addition to nickel ion. Activation requires that CO_2 be incorporated into urease in a pH-dependent reaction ($pK_a \geq 9$). I propose that CO_2 binding to urease apoprotein generates a ligand that facilitates productive nickel binding. Site-directed mutagenesis methods were used to overexpress *ureD* in the presence of the other urease genes, and UreD was found to co-purify with urease. The UreD-urease apoprotein complexes are activated in a CO_2-dependent manner. And, as for urease apoprotein, the activation is accompanied by non-productive nickel-binding. The presence of UreD, however, appears to reduce the rate of this inactivation step.

To my family,
with respect and love.

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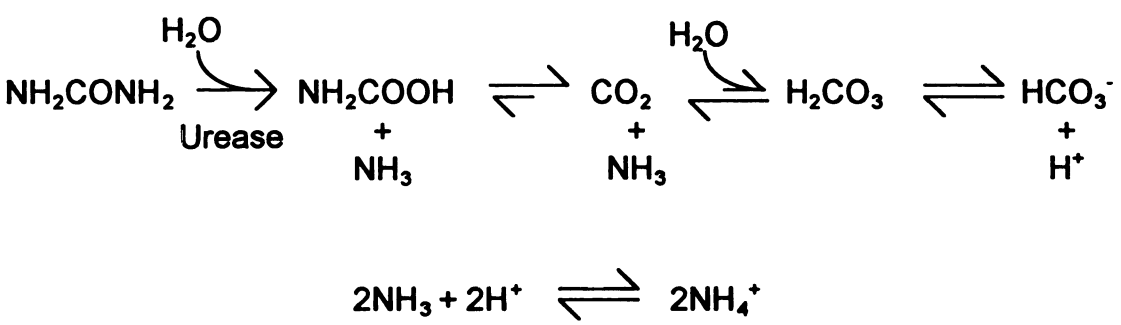
CHAPTER 1

INTRODUCTION

A large amount of urea is biologically produced. For example, urea is a product of biodegradation of nitrogenous compounds such as purines, arginine, agmatine, allantoin, and allantoic acid (1). Mammals excrete urea in urine as a detoxification product (2). Although urea is very stable in solution (its half-life is 3.6 years in aqueous solutions at 38°C (3)), it is rapidly hydrolyzed to ammonia and carbamate in the environment through the action of the enzyme urease (urea amidohydrolase, EC 3.5.1.5) (4). Carbamate spontaneously decomposes to form a second molecule of ammonia and carbon dioxide which hydrates to carbonic acid. At normal physiological pH, carbonic acid dissociates into a proton and bicarbonate and ammonia is protonated to yield ammonium ion. Since two moles of ammonium ion and one mole of bicarbonate are produced from the decomposition of one mole of urea, the hydrolysis results in a pH increase (Scheme 1).



Scheme 1

Ureases have been found in a wide range of bacteria, several fungi, a few invertebrates, and a variety of plants (reviewed by Mobley and Hausinger, 5). Until recently, the best-studied urease was that from jack bean. This urease was the first enzyme to be crystallized (6) and also the first shown to contain nickel (7). More recently, urease studies have focused

on the bacterial enzymes because of the ease of their genetic manipulation and due to their medical importance. Microbial ureases are important in human health, rumen ecology, and soil nitrogen management. This chapter will present brief overviews of the significance, enzymology and metallocenter biosynthesis of ureases. I will focus this introduction on the ureases of bacteria, although examples from plant and other sources will be mentioned where appropriate.

Medical significance. Pathogenic ureolytic microorganisms have been isolated from humans and other animals where they can colonize the urinary tract, the colon, or the stomach.

Human urine consists of 0.4 to 0.5 M urea (8) and the urinary tract provides a favorable environment for many ureolytic microorganisms. The hydrolysis of urea by urease from these organisms causes a rise in urine pH leading to supersaturation and precipitation of magnesium and calcium salts (8) to form stones. Approximately 15-20% of all kidney stones are associated with infections by ureolytic microorganisms (9). *Proteus mirabilis* is the major urease-producing uropathogen in humans (10). Other urease-producing species associated with infection stones have been recognized, including *Pseudomonas, Klebsiella,* and *Staphylococcus* spp. (9). Urease has also been shown to be a virulence factor in urinary tract infections that result in pyelonephritis, an inflammation of the kidney (reviewed in 5).

Ureolytic microorganisms in the colon or the urinary tract can produce a large amount of ammonia, a toxic compound (11). Under normal conditions, the ammonia can be removed by the liver. However, when the amount of ammonia produced is too excessive or the liver is damaged, the elevated level can cause hyperammonemia (12), ammonia encephalopathy (13), and hepatic coma (14).

The urease-producing microbe *Helicobacter pylori* (formerly *Campylobacter pyloridis*) has been implicated in another type of infection. This bacterium has been reported to colonize at intercellular junctions of the mucosal lining of the mammalian stomach (15, 16). The urease of *H. pylori* rapidly hydrolyzes urea from serum to form large amounts of ammonia. The elevated level of ammonia causes a rise of pH in the immediate region of the stomach and allow the bacteria to grow in a relatively neutral pH range. This organism is associated with development of B gastritis (16, 17) and peptic ulcers (18).

Agricultural significance. A major limitation to plant growth is the lack of fixed nitrogen in the soil. Urea can be a good nitrogen source because of its ease of application and its high nitrogen content (19). Hydrolysis of urea by soil ureases is necessary before it can be used in the plant. However, uncontrolled hydrolysis can result in the rise of soil pH and ammonia toxicity. Urea hydrolysis may be able to be controlled by using urease inhibitors (20, 21). Although the urease inhibitor phenylphosphorodiamidate appears not to be effective for an increase in production of corn (22), this approach has the potential to solve the problems mentioned above .

Microbial ureases are also important in the nitrogen metabolism of ruminant livestocks that contain a forestomach (23). Urea produced from metabolism in liver or other tissues is recycled to the rumen through saliva or the bloodstream. Several ureolytic strains of bacteria including *Staphylococcus*, *Klebsiella, Selenomonas, Succinovibrio, Bifidobacterium, and Ruminococcus* spp. (24, 25) hydrolyze urea to produce ammonia, an important nitrogen source for the ruminal bacteria (26). The biomass of the bacteria can be then utilized as a nutrient by the ruminant (reviewed in 5).

Urease structural properties. Bacterial ureases are heteropolymeric proteins. For example, the ureases of *Klebsiella aerogenes* (27), *Proteus mirabilis* (28, 29), *Providencia stuartii* (30), *Selenomonas ruminantium* (27, 31), *Morganella morganii* (32), *Ureaplasma ureolyticum* (33), *Lactobacillus reuteri* (34), *Lactobacillus fermentum* (35), and *Bacillus* sp. strain TB-90 (36) consist of one large (α; M_r = 60,000 to 75,000) and two distinct small subunits (β and γ; M_r = 8,000 to 11,000). In contrast, the bacterial strain, *H. pylori* possesses a urease of two subunits; one large subunit (M_r = 66,000) is homologous to the α subunit of the other bacterial ureases, whereas the other subunit (β; M_r = 29,500) looks like a fusion of the β and γ subunits of others (37). The stoichiometry of the subunits for bacterial ureases may not be consistent in the different sources. *K. aerogenes* urease was reported to possess a $(\alpha\beta_2\gamma_2)_2$ structure as deduced by native size and denaturing gel scanning methods; however, a $(\alpha\beta\gamma)_3$ structure has been established in preliminary X-ray crystallographic analysis. Stoichiometries reported for those of *L. reuteri* (34) and *L. fermentum* (35) were $\alpha\beta_2\gamma$ and $(\alpha\beta_2\gamma_2)_2$ for *P. stuartii* (30), $\alpha_6\gamma_6$ for *H. pylori* (37), $\alpha_2\beta_4\gamma_2$ for *Streptococcus mitior* (38); however, these enzymes are also likely to be trimers of dimers or trimers.

The urease structural genes have been cloned and sequenced from *K. aerogenes* (39), *Proteus vulgaris* (40), *P. mirabilis* (29), *U. ureolyticum* (41), *Bacillus* sp. strain TB-90 (36), *H. pylori* (42), *Yersinia enterocolitica* (43), *Rhizobium meliloti* (44), *Helicobacter felis* (45), *Bacillus pasteurii* (46), *Staphylococcus xylosus* (47), *Lactobacillus fermentum* (48) and jack bean (49). In contrast to the bacterial enzyme, jack bean urease is homohexameric (subunit M_r = 90,770; 73). Comparison of the amino acids and DNA sequences of jack bean (49, 50) and bacterial ureases indicates the genes are highly conserved through evolution. It is likely that the single plant gene

of the plant urease was formed via the gene fusion of the multiple structural genes from bacteria.

Urease active site. Urease is the first enzyme shown to have nickel (7). All ureases examined possess nickel as a cofactor (5, 51). *K. aerogenes* urease apoprotein has been prepared from a nickel-deficient culture. In spite of lacking enzyme activity, the apoprotein showed characteristics similar to holoprotein in terms of the composition of urease subunits (52). Furthermore, preliminary X-ray crystallographic analysis showed no difference between the structures of apoprotein and holoprotein except the absence of nickel in the former protein. These results are consistent with the nickel cofactor playing an important role in enzyme catalysis rather than participating in a structural role. Bacterial and plant ureases have been shown to possess 0.8-2.1 mol nickel/mol large subunit (reviewed in 5). In combination with the analysis of slow-binding inhibitors, the *K. aerogenes* (27, 53) and jack bean (7, 54) ureases have 2 mol nickel/mol catalytic unit. In spectroscopic studies, the interaction of the plant urease with β-mercaptoethanol was shown to produce an increase in absorbance at ~320, ~380, and 425 nm due to a thiolated anion→Ni(II) charge transfer interaction. The change is associated with a K_d of 0.95 mM which is compatible with the K_i value of 0.72 mM determined for the competitive inhibitor (55). *K. aerogenes* urease also interacts with β-mercaptoethanol to result in increased absorbance at 322, 374, and 432 nm with a K_d of 0.38 mM (53). This value is again consistent with the K_i of 0.55 mM determined kinetically. The close relationship of spectroscopically measured thiol binding to nickel of urease and the inhibitory effect of the thiol on urea hydrolysis supports the assumption that urea binds to nickel during the catalysis. These results again suggest that nickel ions are located proximal to the active site and play a catalytic role (3, 55).

X-ray absorption spectroscopy (XAS) of jack bean urease (56, 57, 58) has demonstrated that the nickel is coordinated by a mixture of nitrogen and oxygen ligands, implying the involvement of amino acids containing those atoms in their side chains. In a study involving photooxidation of jack bean urease, it was demonstrated that histidine residues are destroyed when the enzyme is illuminated by intense light in the presence of methylene blue. The photooxidation was found to be suppressed in the presence of the tight-binding urease inhibitor, acetohydroxamic acid (59). This early chemical modification study suggests the presence of one or more histidine residue located proximal to the active site. *K. aerogenes* urease apoprotein showed reactivity with diethylpyrocarbonate (DEP), a histidine-selective modifier (52). In the following site-directed mutagenesis studies (Chapter 3, 60), H134, H136, and H246 residues of the α subunit of *K. aerogenes* urease were substituted with alanine. The αH134A, αH136A and αH246A mutant proteins have no detectable activities and possess one or fewer nickel ion bound per active site (60). These results strongly suggest the ligation of nickel by these histidine residues.

The presence of two ionizable groups essential for urease activity was proposed from the pH dependence studies with the *K. aerogenes* urease enzyme (29). One group (pK_a=6.5) must be deprotonated and a second group (pK_a=8.9) must be protonated for catalysis. Although the identity of these residues was unknown at the time, the former residue was proposed to act as a general base and the latter one as a general acid. In succeeding experiments with the same urease it was found that the histidine-selective reagent DEP rapidly inactivates the enzyme (Chapter 2, 61) in a modification reaction that is associated with a pK_a of 6.5, consistent with modification and inactivation of the residue which acts as the general base. Substitution of αH320 with alanine by site-directed mutagenesis resulted in a mutant protein

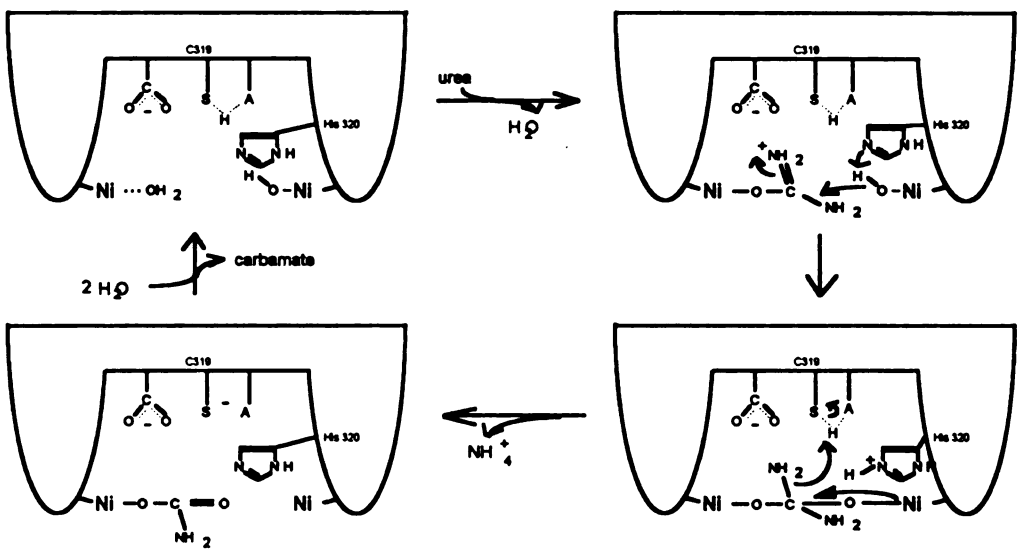
with very low activity (~0.003% of wild-type protein) that was apparently resistant to DEP (Chapter 3, 60), consistent with this being the residue acting as a general base in urea hydrolysis.

K. aerogenes urease is also susceptible to disulfide and alkylating agents. The pH dependence of the inactivation kinetics is compatible with the presence of a single essential thiol interacting with a second ionizable residue together yielding macroscopic pK_a values of <5 and 12 (62). Active site labeling studies have identified the cysteine as residue 319 in the α subunit of the urease protein (63). Three site-directed mutant proteins (C319A, C319S, and C319D) showed a shift in pH optimum compared to wild-type enzyme. Based on all these results, the authors proposed that the cysteine residue ionically interacts with a second residue, together acting as a proton donor (general acid) during catalysis (64).

Inhibitor studies and inactivator with the same enzyme indicate the presence of a negatively charged residue at the active site. Uncharged or positively charged thiolates were found to have lower K_i values than inhibitors possessing a negatively charged carboxyl group (53). Similarly, negatively-charged chemical reagents inactivate the enzyme more slowly than uncharged or positively charged compounds (62). It is suggested that a negative charge in the active site may repel the carboxylated inhibitors and inactivators.

Urea hydrolysis reaction mechanism. A mechanism for the hydrolysis of urea has been proposed for the jack bean urease (65) and the mechanism is retained here (Scheme 2) with some elaboration. One nickel ion in the active site is supposed to bind to the carbonyl oxygen of urease. A general base (His-320) is thought to activate a hydroxide bound to the other nickel ion and it nucleophilically attacks the urea carbon. A molecule of

ammonia is released with the action of a general acid (comprising Cys-319 and another unidentified residue) to leave a carbamate. As a final step of catalysis, the carbamate dissociates from the active site and spontaneously decomposes to ammonia and carbon dioxide.



Scheme 2

***In vivo* activation of urease apoprotein.** Even in the absence of nickel, urease apoprotein is synthesized in both prokaryotes and eukaryotes (66, 67). Thus, nickel ion appears not to be involved in transcriptional regulation of urease. The addition of nickel to intact *K. aerogenes* cells was shown to activate urease apoprotein. This slow and partial activation process was found to be independent of protein synthesis, but sensitive to a proton uncoupler, dinitrophenol, or an ATP synthase inhibitor, DCCD (52), indicating that *in vivo* activation is an energy-dependent process. *In vivo* activation of urease apoprotein also has been reported for several other bacteria including *P. mirabilis* (68), a purple sulfur bacterium (69), and a

cyanobacterium (70), as well as eukaryotes such as several algae (71) and soybean (67).

Accessory genes for urease activation in bacteria. Formation of the nickel-containing urease holoprotein *in vivo* has been shown to require several genes in addition to the structural genes encoding the urease subunits. For example, the *K. aerogenes* urease operon has four additional reading frames (ORF's) which were classified as accessory genes by the authors (39, 72). The *ureD* gene is located upstream of the *ureA, ureB,* and *ureC* genes encoding the urease subunits, and the *ureE, ureF,* and *ureG* genes follow the three structural urease genes. The same arrangement of genes has been found in several other microorganisms including *P. mirabilis* (73, 74), and a ureolytic strain of *E.coli* (75). The urease operon of *Bacillus* sp. strain TB-90 has a slightly altered organization of its urease gene cluster, where the location of *ureD* is downstream of *ureG* and additional genes, *ureH* and *urel*, are present (36). The urease gene cluster from *Helicobacter pylori* was shown to have nine genes in three blocks (76). Among them, two may have a regulatory role, two genes encode urease subunits and four genes appear to encode accessory proteins analogous to the counterparts of *K. aerogenes*. Transposon mutagenesis of the *H. pylori* urease gene cluster allowed the authors to identify *ureF, ureG* and *ureH* (homologous to *ureD* in *K.aerogenes*) as the essential accessory genes (76). A similar observation was made in studies with deletion mutants of the urease gene cluster of *Bacillus* sp. strain TB-90. The deletion of *ureE* caused somewhat impaired urease activity, whereas, deletions in *ureF, ureG,* and *ureD* abolished the enzyme activity (36). The importance of the accessory genes also has been shown in *K. aerogenes* urease formation (72). Inactive urease protein was synthesized in *E.coli* carrying the *K. aerogenes* urease gene cluster with

deletions in *ureD*, *ureF*, or *ureG*. The ureases from each deletion mutant turned out to have little or no amount of nickel. The defects of the deletion mutants, however, can be partially or fully repaired by complementation with the plasmid possessing each deleted gene. Furthermore, the *ureE* deletion mutant synthesizes urease enzyme with reduced activity which roughly corresponds to the reduced nickel content of the purified urease enzyme. All these results indicate that the accessory gene products are trans-acting factors essential for the incorporation of a functional nickel metallocenter into urease protein.

It is evident that elucidation of the functions of each accessory protein is essential for understanding the mechanism and control of nickel metallocenter formation. Although not fully understood, several roles of the proteins have been speculated. UreE purified from *K. aerogenes* has been shown to bind approximately six nickel ions per dimer with a K_d of ~10 μM (77). The authors suggested that UreE may bind nickel ion and act as the nickel donor to the urease apoprotein. UreF is expressed in very low amounts in *E. coli* containing *K. aerogenes* urease gene cluster. This protein has not been identified or purified except as a part of a protein complex (Chapter 6), which appears to have urease apoprotein, UreD, UreF and UreG. It is possible that UreF may play its unknown role only as a part of the complex. The function of UreG has not been determined, either. However, it is intriguing that the protein was revealed to have a P-loop motif (78) that is found in a variety of ATP- and GTP-binding proteins. This feature and the energy dependence for *in vivo* nickel ion incorporation suggest that UreG may bind ATP or GTP and couple its hydrolysis to the nickel incorporation event.

***In vitro* activation of urease apoprotein and the probable role of UreD.** *In vitro* activation of purified *K. aerogenes* urease apoprotein has been accomplished by simply providing CO_2 (half-maximal activation at ~0.2% CO_2) as well as nickel ion (Chapter 5). Based on the pH-dependent activation ($pK_a \geq 9$) and the precedent example of ribulose 1,5-bisphosphate carboxylase/oxygenase (reviewed by Hartman and Harpel, 79), it is proposed that CO_2 binds to apoprotein to form a ligand for nickel.

Prior to the successful activation of purified urease apoenzyme, *in vitro* activation of a UreD-urease complex was reported (80, Chapter 4). Since the poor expression of *ureD* in *E.coli* harboring the *K.aerogenes* urease gene cluster was suspected to be due to the weak ribosome-binding site, site-directed mutagenesis methods were used to overexpress the gene. UreD was found to co-purify with urease apoprotein in ion-exchange, size exclusion, and hydrophobic column chromatographies. The UreD-urease apoprotein complexes are able to be activated alone (Chapter 4), but also were shown to be activated in a CO_2 concentration-dependent manner (Chapter 5). In both the apoprotein and the UreD-apoprotein complex, activation appears to be accompanied by a non-productive reaction that results in enzyme inactivation. However, the presence of UreD evidently reduces the rate of this inactivation step (Chapter 5, Chapter 6). Thus, it was proposed that the UreD protein appears to function, at least in part, to prevent apoprotein from binding nickel non-productively in the absence of CO_2.

Eukaryotic ureases. Urease activities are present in many eukaryotes, including yeast (81), other fungi (82, 83), and plants. Urease from soybean (*glycine max*) is a well studied eukaryotic enzyme genetically. Four urease-related genes are identified. The *Eu1* locus encodes a urease structural gene that is expressed at very high levels in

the developing embryo (84, 85, 86). The partial amino acid sequences deduced from cloned genes apparently match those of the purified jack bean urease (87). An isozyme was found to be encoded by the *Eu4* locus and is constitutively expressed at low levels in all soybean tissues (88). In addition to the urease structural genes, accessory genes appear to be required for the functional plant enzymes. The defect in *Eu2* or *Eu3* gene loci result in deficient ureases (89). These loci appear to encode gene products that are required for a maturation of functional urease; for example, nickel ion incorporation.

Four complementation groups were identified for the formation of active urease in *Aspergillus nidulans* (83). It has been found that *ureA* encodes a urea transport protein, *ureB* is the single subunit urease enzyme, and *ureC* is essential for the formation of active urease, but the function is not known. The suppression of a *ureD* mutation by culture of this organism in the presence of 0.1 mM nickel sulfate (82) indicates that the gene product may play a role in the incorporation of the nickel cofactor. Four loci are also reported to be required for functional urease activity in *Neurospora crassa* (90) and in *Schizosaccharomyces pombe* (83).

Outline of this thesis

The following chapters describe my studies on characterization of *Klebsiella aerogenes* urease and *in vitro* activation of urease apoprotein. In Chapter 2, I present evidence that urease possesses at least one essential histidine residue per catalytic unit which may act as a general base [published in *J. Protein Chem.* **12**, 51-56 (1993) (61)]. In Chapter 3, I describe the generation of and characterization of ten histidine→alanine mutants by using site-directed mutagenesis. I identify histidine residues that appear to function in nickel ligation, substrate binding, and catalysis [published in *Protein Science.* **2:**1034-1041 (1993) (62)]. In Chapter 4, I describe studies related to *in vitro* activation of UreD-urease apoprotein complexes. One, two, or three UreD molecules are present per enzyme in different complexes and the UreD protein dissociates from the enzyme during activation [published in *PNAS* **91:**3233-3237 (1994) (80) with results related to the *in vitro* reconstitution of urease apoprotein in cell extracts by Mary Beth Carr]. In Chapter 5, I characterize apoprotein activation in the presence of CO_2 and nickel and compare the properties of this process with that for UreD-urease apoprotein complexes [submitted to *Science*]. Chapter 6 contains several sections. In section A, I describe the characterization of H219N/Q and H320N/Q mutant proteins. In section B, I present studies that further explore the possible role of UreD in the *in vitro* activation of urease. In section C, partial purification and characterization of UreD are described. In section D, I show evidence consistent with the existence of at least two different forms of UreD-urease protein complex containing the same number of UreD molecules bound per urease apoprotein. In section E, I show the presence of protein complexes containing urease apoprotein, UreD, UreF and UreG and describe its partial

characterization. In the last section, I suggest speculative models of urease apoprotein activation.

Additional studies that I have not included here involve several protein preparations for X-ray crystallographic analysis (urease holoprotein, H134A, H219A, H320A, UreE, and the three, enriched UreD-urease apoprotein complexes), variable temperature magnetic circular dichroism spectroscopy (urease holoprotein, H134A), and X-ray absorption spectroscopy (urease holoprotein, H134A). Finally, the construction of several plasmids that I have made is appended.

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CHAPTER 2

Diethylpyrocarbonate Reactivity of *Klebsiella aerogenes* Urease: Effect of pH and Active Site Ligands on the Rate of Inactivation

These studies were published in *J. Protein Chem.* 12, 51-56, 1993.

ABSTRACT

Reaction of *Klebsiella aerogenes* urease with diethylpyrocarbonate (DEP) led to a pseudo-first-order loss of enzyme activity by a reaction that exhibited saturation kinetics. The rate of urease inactivation by DEP decreased in the presence of active site ligands (urea, phosphate, and boric acid), consistent with the essential reactive residue being located proximal to the catalytic center. The pH dependence for the rate of inactivation indicated that the reactive residue possessed a pK_a of 6.5, identical to that of a group that must be deprotonated for catalysis. Full activity was restored when the inactivated enzyme was treated with hydroxylamine, compatible with histidinyl or tyrosinyl reactivity. Spectrophotometric studies were consistent with DEP derivatization of 12 mol of histidine/mol of native enzyme. In the presence of active site ligands, however, approximately 4 mol of histidine/mol of protein were protected from reaction. Each protein molecule is known to possess two catalytic units; hence, I propose that urease possesses at least one essential histidine per catalytic unit.

INTRODUCTION

Urease is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate; the carbamate spontaneously decomposes to form a second molecule of ammonia and carbonic acid (Andrews *et al.*, 1988; Mobley and Hausinger, 1989). The best studied bacterial urease is that from *Klebsiella aerogenes*: the native enzyme consists of three subunits (M_r = 60,304, 11,695, 11,086; Mulrooney and Hausinger, 1990) in apparent $\alpha_2\beta_4\gamma_4$ stoichiometry and contains two active sites, each of which possesses two tightly bound nickel ions (Todd and Hausinger, 1989). In addition to the requisite bi-Ni metallocenter, an essential cysteine residue has been suggested to be located proximal to the *K. aerogenes* active site (Todd and Hausinger, 1991a). The pH dependence of urease inactivation by disulfide and alkylating agents is consistent with a single essential thiol interacting with a second ionizable residue together yielding macroscopic pK_a values of <5 and 12. Active site labeling studies have identified the cysteine as residue 319 in the large subunit (Todd and Hausinger, 1991b), but the identity of the second ionizable residue is unknown. The pH dependence of urease catalysis is compatible with the presence of two additional ionizable groups participating in the hydrolytic reaction (Todd and Hausinger, 1987); one group ($pK_a \cong 6.55$) must be deprotonated and a second group ($pK_a \cong 8.85$) must be protonated for catalysis. Although the identity of these catalytic residues is unknown, histidine is a reasonable possibility for the group of lower pK_a. Furthermore, in a study comparing properties of urease holoenzyme and apoprotein Lee *et al.* (1990) noted that urease was inactivated by diethylpyrocarbonate (DEP). This result is also consistent with the presence of an essential histidine residue. Here, I follow up this observation to more carefully detail the DEP reactivity of urease and assess whether a histidine residue may be present at the active site.

MATERIALS AND METHODS

Materials. DEP, obtained from Sigma Chemical Co., was dissolved in ethanol immediately before use. DEP concentration was measured by reacting an aliquot with 10 mM imidazole (pH 7.0) and monitoring the absorbance at 230 nm using an extinction coefficient of 3,000 $M^{-1}cm^{-1}$ (Miles, 1977).

Enzyme Purification. Urease was purified to a specific activity of >2500 units/mg from *K. aerogenes* carrying plasmid pKAU19 (Mulrooney *et al.*, 1989) by procedures described previously (Todd and Hausinger, 1989). One unit of enzyme activity is defined as the amount of enzyme required to degrade 1 μmol of urea per min in the standard assay mixture which contained 50 mM urea, 0.5 mM EDTA, and 25 mM HEPES, pH 7.75, at 37°C. Linear regression analysis of the released ammonia, determined by conversion to indophenol, versus time yielded the initial rates. Protein was assayed by the method of Lowry *et al.* (1951).

Kinetics of Urease Inactivation. Inactivation reactions contained 0.5 mM EDTA, 80 mM HEPES, pH 7.0 buffer (or other buffers and pH values as indicated for the pH dependence studies) plus the indicated concentrations of reagent and inhibitor (if present). All reactions were performed at 37°C. Aliquots were removed at the indicated time points and diluted 200-fold into assay buffer. The stability of DEP in the various enzyme inactivation buffers was assessed in control experiments, and enzyme inactivation reactions were carried out over a sufficiently short period of time to minimize concerns over DEP hydrolysis.

Spectroscopy. All spectra were obtained by using a Gilford Response spectrophotometer. Spectral data were transferred to an IBM PC for calculation of difference spectra.

Recovery of Enzyme Activity by NH_2OH Treatment. Urease (140 nM) was inactivated with DEP (50 μM) for 1 min in 0.5 mM EDTA, 80 mM HEPES, pH 7.0 at 37°C. Excess DEP was reacted with imidazole (final concentration of 0.5 mM), and hydroxylamine was added to a concentration of 0.5 mM. The enzyme mixture was incubated at 37°C and aliquots were assayed for recovery of activity.

RESULTS AND DISCUSSION

Inactivation of Urease by DEP. The effect of DEP concentration on urease inactivation at pH 7 is shown in Figure 1. At each inhibitor concentration, a pseudo-first-order loss of activity was observed; i.e., ln V_t/V_o (where V_t is the velocity at time t and V_o is the initial velocity) is linear with time until less than 5% of the starting activity remains. Analogous studies were carried out at pH 6, and the observed rate constants for both experiments were plotted versus DEP concentration to yield the lines shown in Figure 2. At pH 6, high concentrations of DEP clearly demonstrated partial saturation of the inactivation rate. Saturation kinetics is expected for a reagent which reacts as illustrated in Scheme 1.

$$E + I \overset{K_d}{\rightleftharpoons} EI \xrightarrow{k_1} \text{inactive enzyme}$$

Scheme 1

For inactivators which follow this scheme (where k_1 is much slower that the rate of EI dissociation), the apparent rate of urease inactivation (k_{app}) obeys Equation 1.

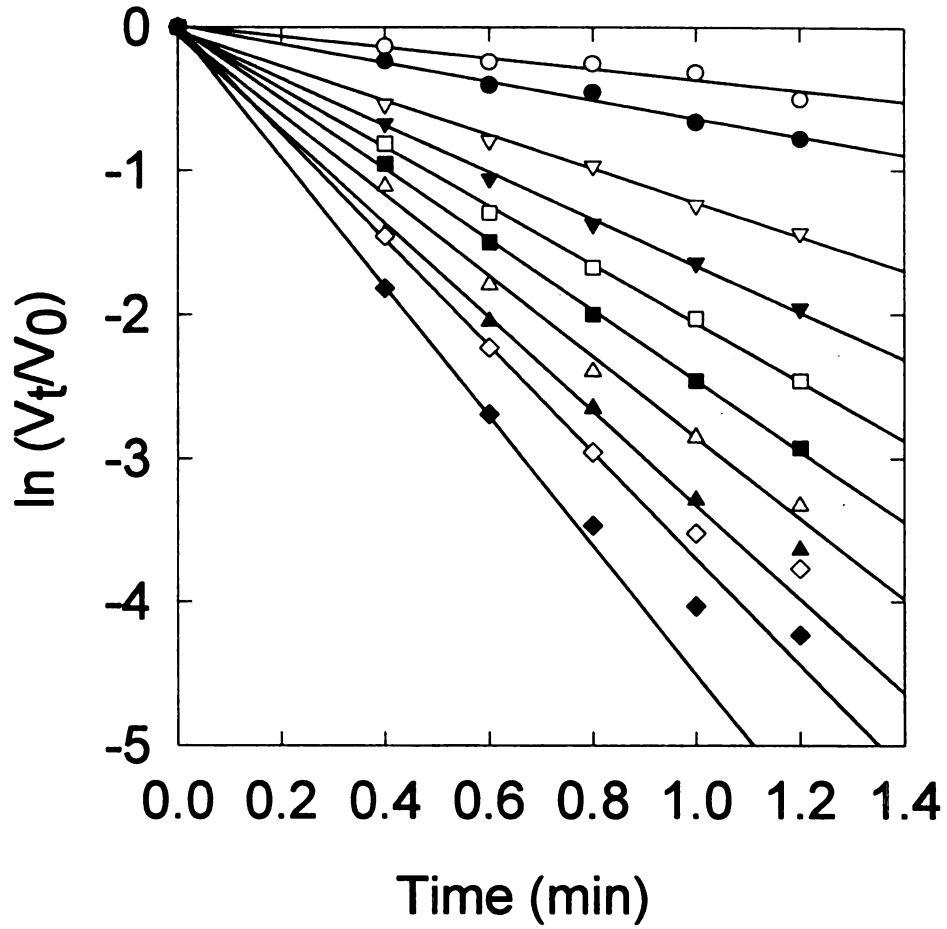

Figure. 1. **Pseudo-first-order urease activity loss in the presence of DEP.** The fraction of activity remaining is shown as a function of time for urease (140 nM) reacting with DEP at 5 (O), 10 (●), 20 (▽), 30 (▼), 40 (□), 50 (■), 60 (△), 70 (▲) 80 (◇), and 100 (◆) μM. All reactions were in 0.5 mM EDTA, 80 mM HEPES buffer at pH 7.0 and 37°C.

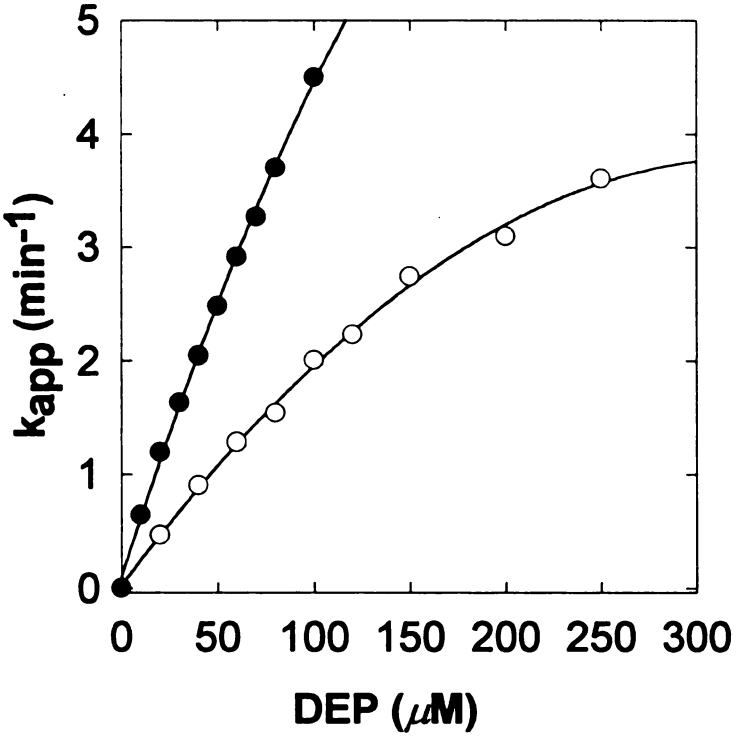

Figure. 2. **Effect of DEP concentration on pseudo-first-order rate of inactivation.** The pseudo-first order rate constants calculated from Figure. 1 (●) and analogous constants determined at pH 6.0 (○) were plotted as a function of DEP concentration.

$$k_{app} = \frac{k_1[I]}{[I] + K_d} \qquad (1)$$

At pH 6, K_d and k_1 were estimated to be 327 ± 36 μM and 8.3 ± 0.6 min^{-1}, yielding a second-order rate constant (k_1/K_d) of 25,400 M^{-1} min^{-1}. Non-linearity in the pH 7.0 data suggest that saturation also occurs here, however, the extremely rapid inactivation rate precluded estimation of the individual constants. Nevertheless, a value of 60,000 ± 2,000 M^{-1} min^{-1} was calculated for the second-order rate constant. These second-order rate constants surpass all other DEP-dependent second-order inactivation rates tabulated for diverse enzymes (Lundblad and Noyes, 1984), with the nearest similar rate being 10,380 M^{-1} min^{-1} at pH 7.2 for lactate dehydrogenase (Bloxham, 1981).

Effect of Ligands on the Rate of Urease Inactivation by DEP. Ligands which bind to the active site of urease were examined for their effect on the rate of inactivation by DEP. At 40 μM DEP, increasing concentrations of both borate and phosphate (competitive inhibitors of urease) were found to reduce the rate of inactivation (Figure. 3). At elevated phosphate concentrations, k_{app} was less than 20% that of the control; whereas, saturating levels of borate decreased k_{app} only by approximately 40%. These results can be accomodated by the model shown in Scheme 2 (where the ligands are represented by L).

$$\begin{array}{ccccc} E + I & \overset{K_d}{\rightleftharpoons} & EI & \xrightarrow{k_1} & \text{inactive enzyme} \\ + & & & & \\ L & & & & \\ \uparrow\downarrow K_L & & & & \\ EL + I & \overset{\alpha K_d}{\rightleftharpoons} & ELI & \xrightarrow{k_2} & \text{inactive enzyme} \end{array}$$

Scheme 2

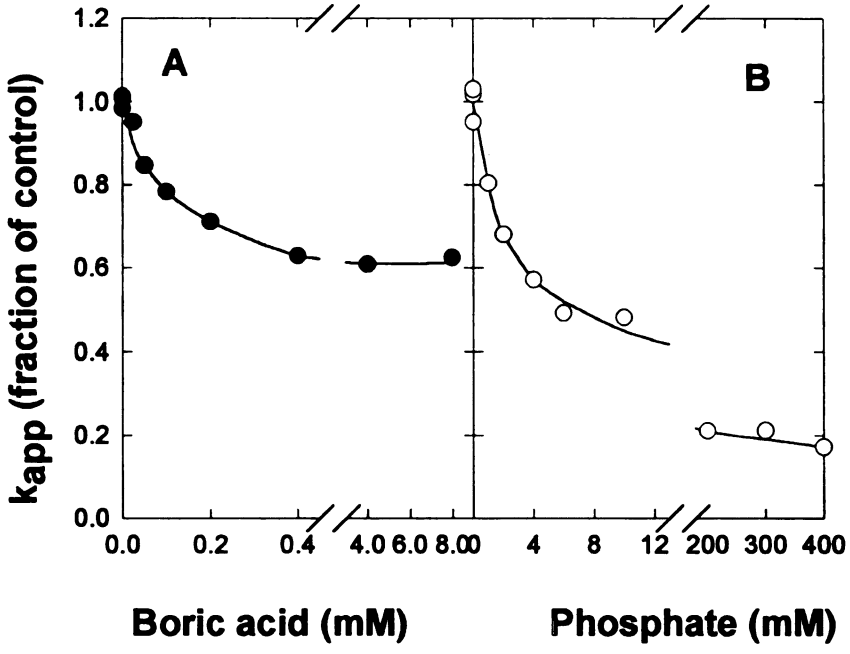

Figure. 3. **Effect of competitive inhibitors on the rate of urease inactivation by DEP.** The second-order rate constants for urease inactivation by DEP (40 μM) at pH 7.0 were determined in the presence of boric acid (panel A) or phosphate (panel B) at the indicated concentrations. The values shown are expressed as fraction of the inactivation rate in the absence of inhibitors.

The rate of inactivation for a reaction that follows Scheme 2 is shown in Equation 2 (Carrillo *et al.*, 1981).

$$k_{app} = \frac{(k_1 K_L \alpha K_d + k_2[L]K_d)[I]}{\alpha K_d K_L (K_d + [I]} + K_d[L](\alpha K_d + [I]) \qquad (2)$$

The demonstration that active site ligands can decrease the rate of enzyme inactivation is consistent with DEP reacting with a residue proximal to the active site of urease.

A more detailed analysis of the results shown in Figure 3 highlights a problem with the simple model of Scheme 2. Values of 0.07±0.02 mM and 6±2 mM were graphically determined for the K_L of borate and phosphate; yet, these compounds were found to possess simple competitive inhibition (K_i) values of 0.223 mM and 50.4 mM at this pH value (pH 7.0). These results indicate that the binding step associated with K_L is distinct from that associated with K_i. Our data do not allow us to discriminate among models that could account for this behavior. The effects of ligands on urease inactivation by DEP contrasts with the results observed for alkylating and disulfide reagents (Todd and Hausinger, 1991a) where the K_L measured for the effect of ligands on inactivation matched the K_i of these inhibitors.

Urea (100 mM) also protected urease from modification by DEP (Figure 4). Although a portion of the urea was degraded under these experimental conditions, there was sufficient buffer capacity to prevent significant pH change and the urea concentration remained saturating during the short time required for the analysis.

Effect of pH on the Rate of Urease Inactivation. The effect of pH on the rate of urease inactivation by DEP is illustrated in Figure 5. The pK_a estimated from these data is 6.5±0.1. This value is identical to that of a urease residue which

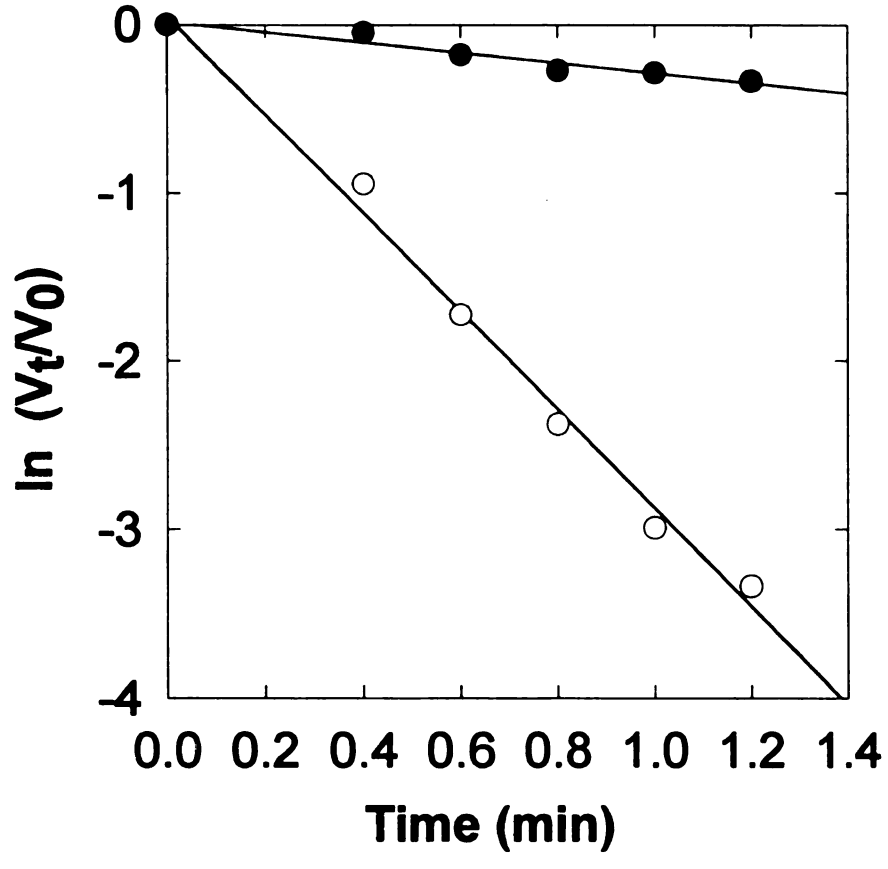

Figure. 4. **Effect of urea on the rate of urease inactivation by DEP.** Urease (140 nM) was inactivated by DEP (60 μM) in the presence (●) or absence (○) of urea (100 mM) at 37°C in 0.5 mM EDTA, 80 mM HEPES buffer, pH 7.0. The fractional activity remaining is shown as a function of time.

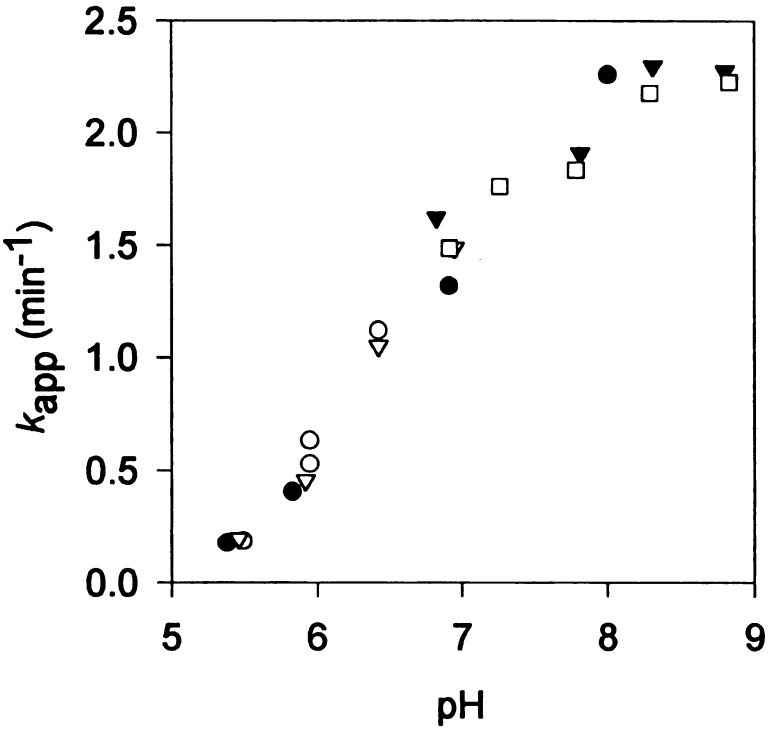

Figure. 5. **The pH dependence of DEP inactivation of urease.** The apparent second-order rate constants for urease inactivation with 25 μM DEP are plotted as a function of pH for reactions carried out at 37°C in 0.5 mM EDTA, 80 mM buffer: MES (○), MOPS (●), HEPES (▽), TAPS (▼), Tricine (□). At pH values less than 6, the reactions were carried out using the same conditions as above except that the concentration of DEP was 125 μM and the values were adjusted by using the equation (1).

must be deprotonated for catalysis (Todd and Hausinger, 1987). I propose that the DEP-reactive residue is the general base required for urea hydrolysis.

Although DEP has traditionally been viewed as a histidine-selective reagent, reaction with protein tyrosine, lysine, and other nucleophilic groups has been reported (reviewed by Miles, 1977; Lundblad and Noyes, 1984). In order to identify the nature of the amino acid that corresponds to the essential residue, I probed the stability of the modified residue and examined the inactivation reaction spectroscopically as detailed below.

Reactivation of DEP-modified Urease. Treatment of DEP-inactivated urease with 0.5 mM NH_2OH almost completely restored urease activity within 6 hr (Figure 6). The restoration of activity of DEP-treated urease with NH_2OH implies that the modification involved either histidine or tyrosine residues and suggests that lysine modification is not involved in activity loss (Miles, 1977; Lundblad and Noyes, 1984).

Spectrophotometric Analysis of Urease Reactivity with DEP. Urease (9.4 μM) was incubated in 80 mM HEPES and 0.5 mM EDTA, pH 7.0, containing 250 μM DEP for 30 min. The change in absorbance was monitored versus a reference cuvet containing urease in buffer without DEP (Figure 7). Modification of histidine residues leads to an increase in absorbance at 242 nm with an extinction coefficient of 3,200 $M^{-1}cm^{-1}$ (Lundblad and Noyes, 1984), whereas modification of tyrosine leads to a spectral decrease at 280 nm with an extinction coefficient of 1,310 $M^{-1}cm^{-1}$ (Mülhrad *et al.*, 1967). Our spectroscopic results were consistent with modification of ~12 histidine residues per mol of enzyme.

The rate of urease activity loss was compared to the kinetics of absorbance change at 242 nm for enzyme (4.2 μM), in 25 mM HEPES (pH 7.0) buffer, treated with 100 μM DEP (Figure 8, open circles). The experiment was repeated in the presence of 400 mM phosphate, a competitive inhibitor of urease (Figure 8, closed circles). Protection of approximately two histidine residues

correlated to protection of ~60% of urease activity, consistent with the presence of nearly four histidines protected by phosphate binding in fully active enzyme. In contrast, small changes observed at 280 nm did not correlate with changes in urease activity, precluding the presence of an essential tyrosine. It has been shown that there are two active sites per native urease molecule (Todd and Hausinger, 1989), hence, I conclude that there is at least one essential histidine residue proximal to each active site.

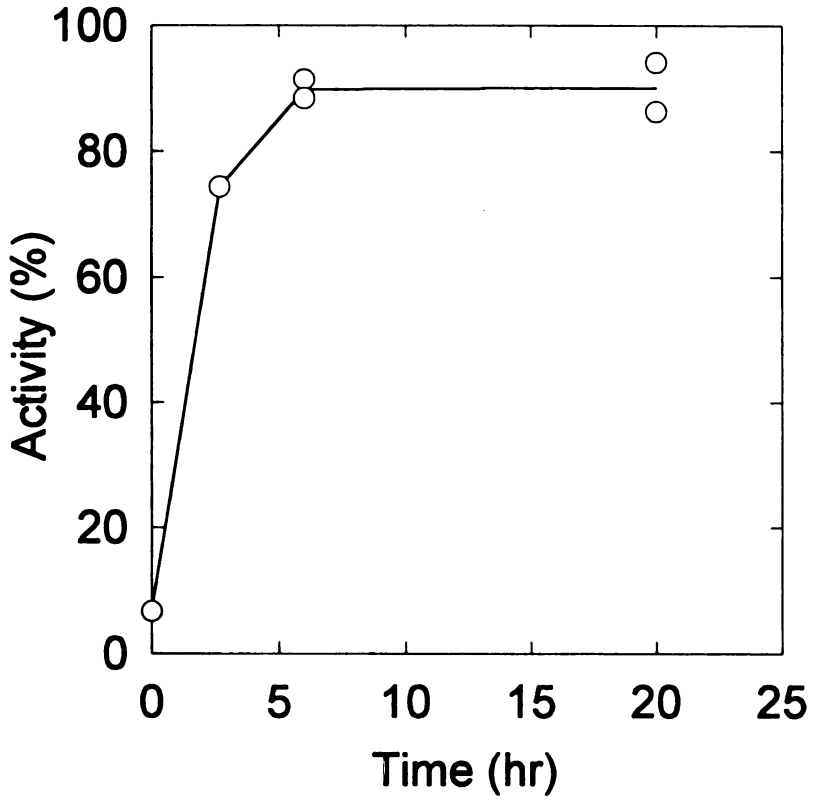

Figure. 6. **Reactivation of DEP-inactivated urease by hydroxylamine.** DEP-inactivated urease was incubated with 0.5 mM hydroxylamine at 37^{o}C and activity was monitored over time. The results are reported as percent of control (non-DEP-treated) urease subjected to the same conditions.

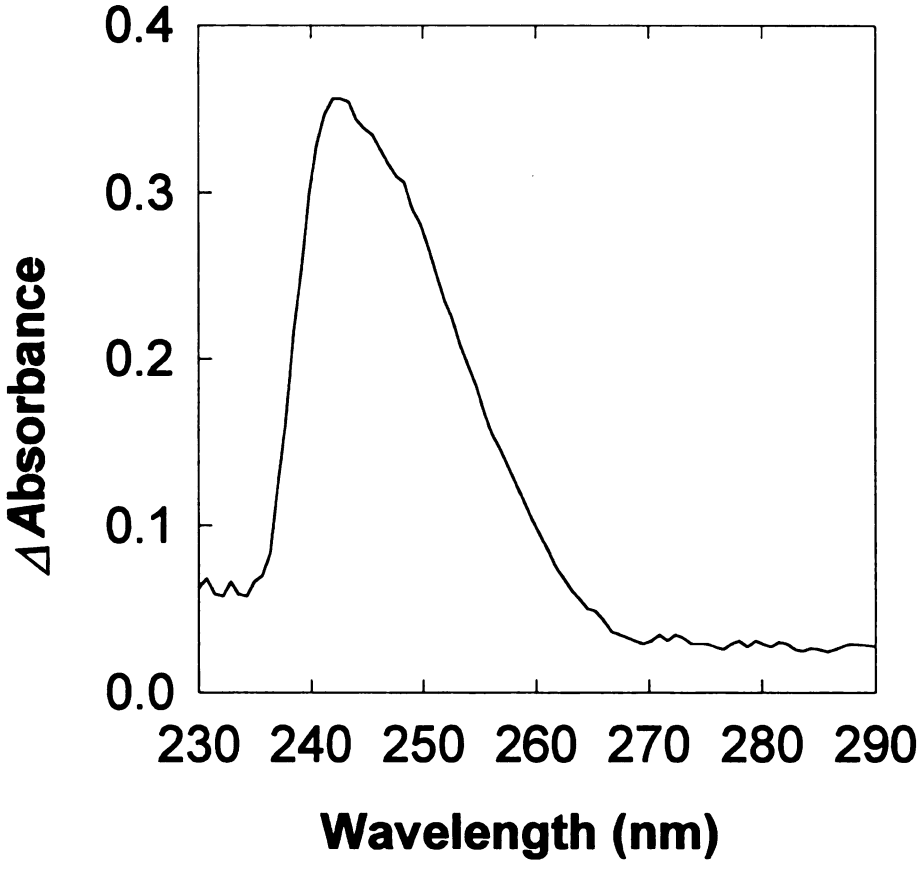

Figure. 7. **Spectroscopic analysis of urease modification by DEP.** Urease (9.4 μM) was incubated for 30 min with DEP (250 μM) in 0.5 mM EDTA, 80 mM HEPES buffer, pH 7.0 at 25^{o}C. The difference spectrum is shown for this sample minus untreated urease in same buffer.

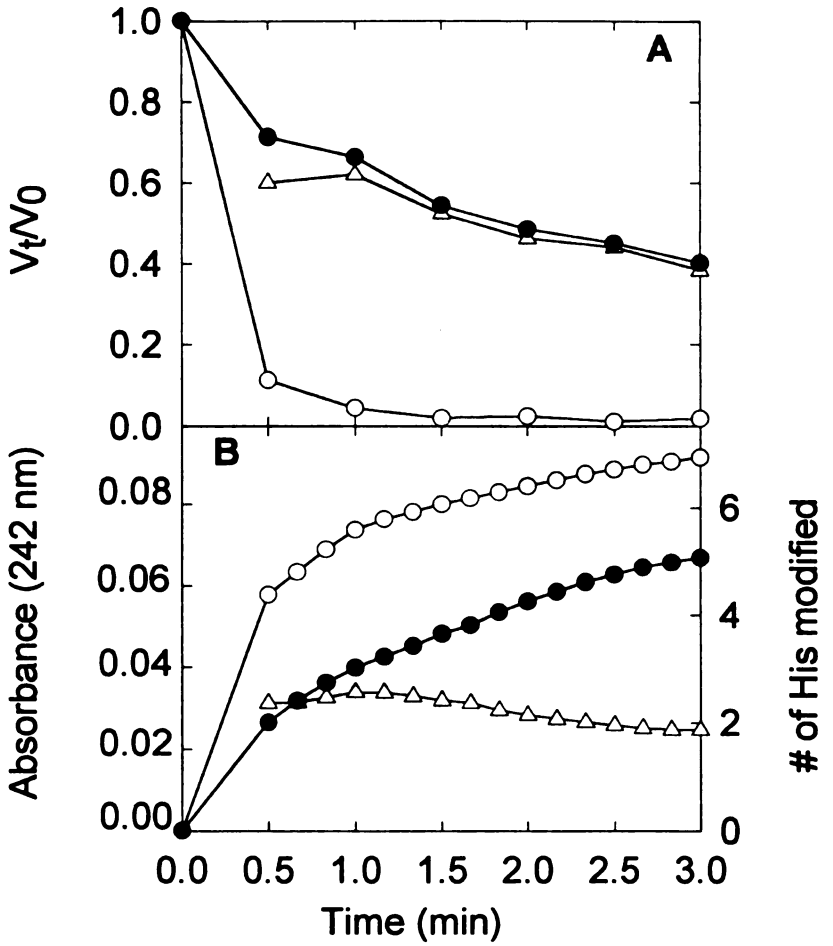

Figure. 8. **Kinetic comparison of activity and absorbance changes during modification by DEP.** Urease (4.2 μM) was incubated with DEP (100 μM) in 25 mM HEPES buffer, pH 7.0 at 25°C in the absence (○) or presence (●) of 400 mM phosphate. Changes in activity (A) and absorbance at 242 nm (B) were monitored, and differences between the phosphate-free and phosphate-containing samples were calculated (△).

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CHAPTER 3

Site-directed mutagenesis of *Klebsiella aerogenes* urease: Identification of histidine residues that appear to function in nickel ligation, substrate binding, and catalysis

These studies were published in *Protein Science* 2:1034-1041, 1993.

ABSTRACT

Comparison of six urease sequences revealed the presence of ten conserved histidine residues (H96 in the γ subunit, H39 and H41 in β, and H134, H136, H219, H246, H312, H320 and H321 in the α subunit of the *Klebsiella aerogenes* enzyme). Each of these residues in *K. aerogenes* urease was substituted with alanine by site-directed mutagenesis and the mutant proteins were purified and characterized in order to identify essential histidine residues and assign their roles. The γH96A, βH39A, βH41A, αH312A and α H321A mutant proteins possess activities and nickel contents similar to wild-type enzyme, suggesting that these residues are not essential for substrate binding, catalysis, or metal binding. In contrast, the αH134A, αH136A and α H246A proteins exhibit no detectable activity and possess 53%, 6%, and 21% of the nickel content of wild-type enzyme. These results are consistent with αH134, αH136 and αH246 functioning as nickel ligands. The αH219A protein is active and has nickel (~1.9% and ~80%, respectively, when compared to wild-type protein), but exhibits a very high K_m value (1100±40 mM compared to 2.3±0.2 mM for the wild-type enzyme). These results are compatible with αH219 having some role in facilitating substrate binding. Finally, the αH320A protein (K_m=8.3±0.2 mM) only displays ~0.003% of the wild-type enzyme activity, despite having a normal nickel content. Unlike the wild-type and αH219A ureases this mutant protein was not inactivated by diethylpyrocarbonate (DEP), consistent with αH320 being the DEP-reactive general base that facilitates catalysis.

INTRODUCTION

Urease (EC 3.5.1.5), found in a variety of plants and a broad range of bacterial species, is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form carbonic acid and two molecules of ammonia (Mobley & Hausinger, 1989). The best studied microbial urease is that from the enteric bacterium, *Klebsiella aerogenes*. The enzyme is comprised of three subunits [M_rs=60,304 (α), 11,695 (β), and 11,086 (γ) (Mulrooney & Hausinger, 1990)] and possesses two nickel ions per $\alpha\beta_2\gamma_2$ catalytic unit (Todd & Hausinger, 1987, 1989). Although *K. aerogenes* urease has been crystallized and the crystals shown to diffract to less than 2 Å (Jabri *et al.*, 1992), the three-dimensional structure of the enzyme has not yet been elucidated. Nevertheless, several structural features of the bacterial urease active site have been characterized and multiple essential roles for histidine residues have been implicated. For example, the pH dependence of enzyme activity is consistent with the presence of two chemical groups at the active site that participate in catalysis as a general base (pK_a=6.55) and a general acid (pK_a=8.85) (Todd & Hausinger, 1987). Chemical modification studies with the histidine-selective reagent DEP were compatible with a histidine residue serving as the general base (Park & Hausinger, 1993). Additional chemical modification and site-directed mutagenesis studies demonstrated the presence of an active site cysteine residue (C319 in the α subunit) in the *K. aerogenes* enzyme (Todd & Hausinger, 1991b; Martin & Hausinger, 1992). The pH dependence of urease inactivation by disulfide and alkylating reagents (Todd & Hausinger, 1991a) and the shift in pH optimum observed for C319A, C319S, and C319D mutant proteins compared to wild-type enzyme (Martin & Hausinger, 1992) were interpreted in terms of the cysteine residue ionically interacting with a second residue (X), together acting as a proton donor during catalysis.

Although the identity of X is unknown, the pH-dependent behavior of urease inactivation by thiol-specific chemical reagents is reminiscent of studies involving papain where a Cys-His ion pair has been characterized (Brocklehurst, 1987). Finally, Lee *et al.* (1990) compared the chemical reactivity of *K. aerogenes* holoenzyme and apoprotein toward DEP and found that more histidines are accessible to the reagent in the nickel-free protein. The enhanced DEP-reactivity of apoprotein is consistent with histidine residues participating as nickel metallocenter ligands in urease.

This study combines site-directed mutagenesis and enzyme characterization methods to identify several essential histidine residues in *K. aerogenes* urease and to define their roles. Comparison of the urease amino acid sequences from jack bean (Takishima *et al*, 1988), *Helicobacter pylori* (Clayton *et al.*, 1990; Labigne *et al.*, 1991), *Ureaplasma urealyticum* (Blanchard, 1990), *Proteus vulgaris* (Mörsdorf & Kaltwasser, 1990), *Proteus mirabilis* (Jones & Mobley, 1989) and *K. aerogenes* (Mulrooney & Hausinger, 1990) revealed the presence of ten conserved histidines: H96 in the γ subunit, H39 and H41 in β, and H134, H136, H219, H246, H312, H320 and H321 in the α subunit of the *K. aerogenes* enzyme. I substituted each of the conserved histidine residues in the *K. aerogenes* enzyme with alanine (a residue that contains a side chain which can not function as a metallocenter ligand or general base or general acid, can not participate in hydrogen bond interactions, and is smaller than the wild-type residue so that it will not cause steric disruption of the structure), purified the mutant proteins, and characterized their enzyme activities, nickel contents, and reactivities with DEP and iodoacetamide (IAM). The results suggest that histidine residues in the α subunit may play key roles with H134, H136, and H246 participating in the ligation of nickel, H219 facilitating the binding of substrate, and H320 acting as a general base in catalysis.

MATERIALS AND METHODS

Materials. DEP, obtained from Sigma Chemical Co., was dissolved in ethanol immediately before use. DEP concentration was measured by reacting an aliquot with 10 mM imidazole (pH 7.0) and monitoring the absorbance at 230 nm using an extinction coefficient of 3,000 $M^{-1}cm^{-1}$ (Miles, 1977). IAM (Aldrich Chemical Co.) was prepared in distilled water.

Site-directed mutagenesis. For γH96A, βH39A and βH41A, a 1.4-kbp *Sac*I-*Sma*I fragment of the pKAU17 (Mulrooney *et al.,* 1989) was subcloned into M13 mp18, and mutagenized by the method of Kunkel *et al.* (1983). For α H134A, αH136A, αH219A, αH246A, αH312A, αH320A and αH321A, a 1.1-kbp *Bam*H1-*Sal*I fragment of the same plasmid was used. Uracil-containing, single-stranded template DNA was prepared from *E. coli* CJ236 (*dut*1 *ung*1 *thi*-1 *rel*A1/pCJ105[*cam*^{r} F']). Mutagenized phage were isolated in *E. coli* MV1193 (Δ[*lacI-pro*AB] *rpsL thi endA spcB*15 *hsdR*4 Δ[*srl-rec*A]306::Tn10[*tet*^{r}] F'[*traD*36 *pro*AB$^+$ *lacI*^{q}*lacZ*ΔM15]). The following oligonucleotides were synthesized by using an Applied Biosystems Model 394 DNA synthesizer at the Michigan State University Macromolecular Structural Facility: AATCGGGTTGGCAACGGTGAC, GAAATGGTAGGCCGAACCGAC, CTCGGCGAAAGCGTAGTGCG, CTGAAGATCGCTGAGGACTGG, GGTCGCCCTGGCCAGCGACACC, GGATCGATACCGCTATTCACTG, ACCCATATTGCCTGGATCTGT, CCATCGATGAAGCTCTCGATATG, ATGGTCTGCGCCCATCTGGAC, GTCTGCCACGCTCTGGACCCG. These primers were used to alter the ten conserved histidine codons to encode alanine at each position. Site-directed mutants were identified by DNA sequencing, and subcloned back into pKAU17

on a 1.1-kbp *Sac*I-*Mlu*I fragment for γH96A, βH39A, and βH41A and a 0.8-kbp *Mlu*I-*Bsm*I fragment for the other mutations. These regions were completely sequenced by using Sequenase 2.0 (United States Biochemicals) and the single-strand DNA method of Sanger *et al.* (1977) to ensure that no other mutations had been introduced into M13. In one case, sequence analysis demonstrated that a recombination event had occurred and an alternate clone that possessed the desired muation was chosen for further studies. After subcloning, the mutated sequences were again confirmed by double-strand DNA sequencing methods (Sambrook *et al.*, 1989).

Enzyme purification. Ureases were purified from *E. coli* DH5 carrying pKAU17 or the site-directed mutants of pKAU17 by procedures described previously (Todd & Hausinger, 1989), except that cells were grown in LB medium containing 1 mM $NiCl_2$. As noted by Lee *et al.* (1992) and Martin and Hausinger (1992), the wild-type enzyme isolated from cells grown under these conditions does not exhibit the maximum specific activity observed for enzyme isolated from *K. aerogenes* [pKAU19] cells (2,500 units mg^{-1}, Todd & Hausinger, 1989). This decreased activity does not result from a difference in nickel content, but rather may be related to problems arising from the high levels of urease biosynthesis or to unknown host-dependent effects (Lee *et al*, 1992). Because urease synthesis in cells containing the mutated plasmids is similar to that in cells producing wild-type enzyme, it is reasonable to directly compare their relative specific activities and nickel contents. Purification of inactive mutant proteins (αH134A, αH136A, α219A, αH246A, and αH320A) was monitored for urease-containing fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10-15% polyacrylamide gradient gels and the buffers described by Laemmli (1970). Sample purities were determined

by using a gel scanner (AMBIS Inc.) and the measured values were used for correction of enzyme activities and nickel contents.

Assay of enzyme activity. The urease activities for wild-type and mutant proteins except αH219A and αH320A were assayed in 25 mM HEPES, pH 7.75, 0.5 mM EDTA, and 50 mM urea. The αH219A protein activity routinely was measured by using 1 M urea; however, for monitoring activity loss during thermal stability and chemical modification studies a concentration of 100 mM urea was used. The αH320A activity was assayed in 25 mM HEPES, pH 6.75 buffer containing 0.5 mM EDTA and 50 mM urea. One unit of enzyme activity is defined as the amount of enzyme required to degrade 1 μmol of urea per min at 37°C. Linear regression analysis of the released ammonia, determined by conversion to indophenol (Weatherburn, 1967), versus time yielded the initial rates. Calculation of kinetic constants made use of the method of Wilkinson (1961). Protein was assayed by the method of Lowry *et al.* (1951).

Nickel quantitation. The nickel content of purified urease was assayed by using a computer-controlled Varian Spectra AA-400Z graphite furnace atomic absorption spectrophotometer with Zeeman background correction as previously described (Lee *et al.*, 1992). For calculation of the number of nickel ions per catalytic unit, a M_r of 105,866 for the $\alpha\beta_2\gamma_2$ unit was used.

Thermal stability. Cell extracts containing wild-type and mutant urease proteins were incubated in 20 mM phosphate, pH 7.0 buffer containing 1 mM EDTA, and 1 mM β-mercaptoethanol at 50°C, 60°C, and 70°C. At the indicated times, aliquots were removed and the remaining activities were assayed.

Inactivation by DEP. Inactivation reactions were performed at 37°C in 1 mM EDTA, 50 mM HEPES, pH 7.0 buffer (or other buffers and pH values as described) plus the indicated concentrations of DEP. For wild-type and αH219A proteins, aliquots were removed at the indicated time points and diluted at least 100-fold into assay buffer. For the αH320A protein, aliquots (100 μl) were quenched with imidazole (final concentration of 5 mM) and incubated for 5 min on ice prior to measuring enzyme activity. The stability of DEP in the various enzyme inactivation buffers was assessed in control experiments, and enzyme inactivation reactions were carried out over a sufficiently short period of time to minimize concerns over DEP hydrolysis. The pK_a values for inactivation of wild-type and αH219A proteins were calculated by fitting data sets to the following equation (Cousineau & Meighen, 1976) by using linear-least-squares methods:

$$\frac{1}{k_{app}} = \frac{1}{(k_{app})_{max}} + \frac{[H^+]}{(k_{app})_{max} K_a}$$

Inactivation by IAM. Purified urease was incubated in 1 mM EDTA, 50 mM HEPES, pH 7.75 buffer (or other buffers and pH values as described) plus the indicated concentrations of IAM at 37°C. Aliquots were taken at 30-min intervals and assayed for urease activity as previously described (Todd & Hausinger, 1991a).

RESULTS

Initial characterization of mutant proteins. *Escherichia coli* cells containing the *K. aerogenes* urease genes on plasmid pKAU17 or derivative

plasmids with His→Ala substitutions were grown under culture conditions that led to high level synthesis of the wild-type and mutant ureases. The wild-type and mutant proteins were highly purified (Figure 1) and the specific activities, K_m values, and nickel contents were determined (Table 1). The γH96A, βH39A, βH41A, αH312A and αH321A proteins have K_m values, specific activities, and nickel contents that are similar to the wild-type enzyme, indicating that these histidine residues are not likely to be important for substrate binding, catalysis, or nickel ligation. In contrast, the other five mutant proteins exhibit significant changes in their properties, consistent with important roles for the αH134, α H136, αH219, αH246, and αH320 residues. These mutant proteins fall into three classes, each of which is described separately below.

The αH134A, αH136A and αH246A proteins are inactive and possess approximately 50% of the normal metal content (αH134A) or the near absence of nickel (αH136A and αH246A). These results are compatible with αH134, αH136 and αH246 functioning as nickel ligands in the enzyme. It remains unclear whether the latter two residues may bridge the two metal atoms at the active site so that neither nickel can bind in the absence of these double ligands, or if the increased lability of one active site nickel atom by loss of a ligand leads to lability of the second metal ion. Similarly, it is unknown whether the 50% nickel content of the αH134A protein represents a case where one nickel is incorporated into each catalytic unit randomly between the two nickel sites or whether one of the two nickel sites is filled while the other remains unoccupied due to the loss of an essential ligand.

The αH219A protein exhibits a very high K_m value for urea coupled with a large decrease in specific activity (although the enzyme activity could not be assayed under saturating conditions, the calculated V_{max} value was about 3% of that found in the wild-type protein). The altered properties of the αH219A protein apparently are not due to changes in nickel content because, at most, 20% of the nickel was lost in this mutant protein compared to wild-type enzyme.

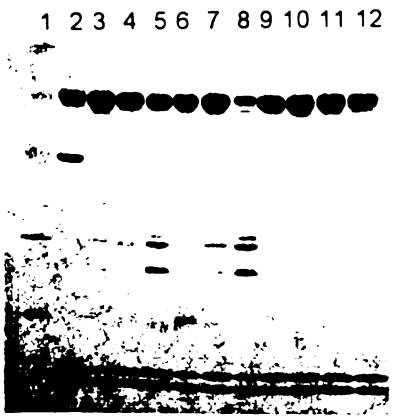

Figure. 1. Denaturing gel electrophoretic analysis of partially purified urease proteins. Samples were run on a 10-15% polyacrylamide gradient gel and stained with Coomassie brilliant blue. The percent purity for each sample was assessed by using an AMBIS gel scanner. Lane 1: molecular weight markers (phosphorylase *b*, M_r of 92,500; bovine serum albumine, M_r of 66,200; ovalumin, M_r of 45,000; carbonic anhydrase, M_r of 31,000; soybean trypsin inhibitor, M_r of 21,500; and lysozyme, M_r of 14,400). The protein designations and percent purities for the samples are indicated for lane 2: γH96A (72%), lane 3: βH39A (96%), lane 4: β H41A (90%), lane 5: αH134A (70%), lane 6: αH136A (80%), lane 7: αH219A (93%). lane 8: αH246A (52%), lane 9: αH312A (>98%), lane 10: αH320A (>98%), lane 11: αH321A (98%), and lane 12: Wild-type (88%).

Table 1. *Characteristics of wild-type and mutant Klebsiella aerogenes ureases*[a]

Urease	K_m (*mM*)	Specific activity (*U/mg*)	(*%*)	Nickel content (*#/catalytic unit*)	(*%*)
Wild-type	2.3±0.2	1900	100	2.1	100
γH96A	1.9±0.2	1700	90	2	95
βH39A	1.5±0.2	1500	79	2.1	100
βH41A	1.4±0.2	1300	68	2.5	120
αH134A	--	<0.001	--	1.1	53
αH136A	--	<0.001	--	0.13	6
αH219A	1100±40[b]	36[b,c]	1.9	1.7	80
αH246A	--	<0.001	--	0.44	21
αH312A	1.6±0.2	1800	95	2.2	105
αH320A	8.3±0.2[d]	0.051[b,e]	0.0027	2.3	110
αH321A	2.0±0.2	1700	90	2.4	114

[a] Determined for enzyme purified by DEAE-Sepharose and Mono-Q column chromatographies as illustrated in Fig. 1, except where indicated.

[b] Determined by using enzyme purified only with DEAE-Sepharose column chromatography.

[c] Determined by using 1 M urea instead of the standard assay.

[d] Determined for cell extract.

[e] Determined by using pH 6.75 buffer instead of the normal 7.75 buffer.

Rather, the high K_m value of the αH219A protein suggests that the αH219 residue is somehow important to substrate binding. For example, the histidine residue may facilitate urea binding by forming a hydrogen-bond with the substrate. Alternatively, however, the results are compatible with a model in which the imidazole group simply props open the substrate binding site and maintains its accessibility.

The αH320A protein was found to have a very significant decrease in specific activity and a moderate increase in K_m value compared to wild-type enzyme. The observed kinetic changes were not correlated to the protein's nickel content, consistent with αH320 having a role in urease activity other than in substrate binding or nickel ligation.

Thermal stabilities of mutant ureases. Of those mutant ureases that possess activity, all but the αH312A protein exhibited significant reductions in thermal stability compared to the wild-type enzyme (Table 2). These results are consistent with the γH96, βH41, βH39, αH219, αH320, and αH321 residues participating in ion-pair or hydrogen-bond interactions in the native protein. Loss of these interactions leads to protein destabilization at temperatures ≥ 50°C but not at the growth temperature of 37°C. The reason for the enhanced stability of the αH312A mutant protein over wild-type enzyme is unclear.

pH dependence of mutant urease activities. The pH optimum of the wild-type enzyme (Figure 2A) at around pH 7.75 is retained in the active mutant proteins γH96A, βH39A, βH41A, αH219A, αH312A, and αH321A, as illustrated for the αH219A protein (Figure 2B). The results are consistent with the wild-type general base and general acid groups being retained in these mutant proteins. In contrast, the αH320A protein exhibited a significantly shifted pH optimum at

Table 2. *Thermal stabilites of wild-type and mutant ureases*[a]

	Incubation					
	50°C		60°C		70°C	
Urease	1 hr	2 hr	1 hr	2 hr	1 hr	2 hr
Wild-type	79	70	67	45	13	3
γH96A	74	61	33	12	<1	<1
βH39A	66	53	9	<1	<1	<1
βH41A	64	51	45	21	<1	<1
αH219A[b]	83	72	43	16	<1	<1
αH312A	80	76	85	70	42	22
αH320A[c]	85	74	8	<2	<3	<3
αH321A	65	58	30	12	<1	<1

[a] Values are expressed as percent of that for control samples that were not subjected to high temperature incubation. In each case, cell extracts were incubated in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM β-mercaptoethanol at the indicated temperatures for up to 2 hr and the activities were measured by using the standard assay conditions, except where indicated.

[b] Activities for the αH219A protein were measured by using a concentration of 100 mM urea.

[c] Assays for the αH320A protein were carried out at pH 6.75.

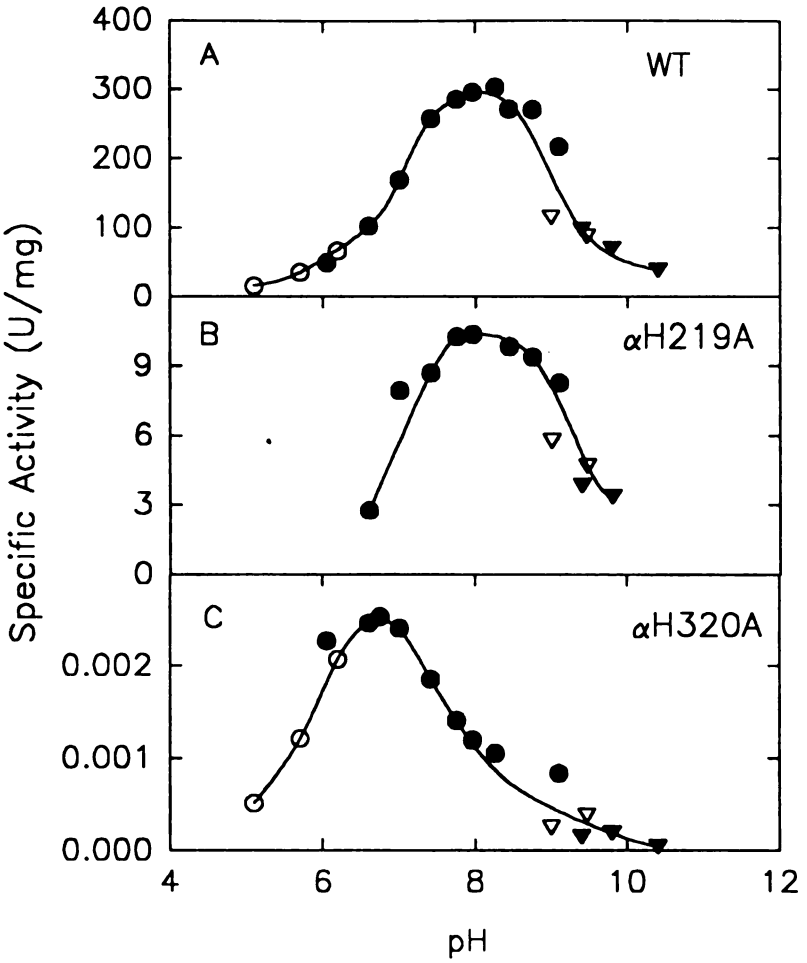

Figure 2. The pH dependence of urease activity. Analysis using **A**: Cell extracts of *E. coli* DH5[pKAU17], **B**: Purified αH219A enzyme, **C**: Cell extracts containing the αH320A mutant protein. The reaction mixtures contained urea (50 mM for wild-type enzyme and H320A protein or 1 M for the αH219A protein), 0.5 mM EDTA and the following buffers at a concentration of 25 mM: MES(○), HEPES (●), CHES (▽), and CAPS (▼).

around 6.75 (Figure 2C). Hence, one of the residues serving as a general base or general acid may have been mutated in the αH320A protein or the mutation may have indirectly shifted one of these pK_a values.

Inactivation of mutant ureases by DEP. The properties of the αH219A and αH320A mutant proteins were consistent with these two histidine residues playing important roles in the enzyme other than metallocenter ligation. Because chemical modification studies of urease using DEP had provided evidence for an essential histidine residue acting as a general base in the native enzyme (Park & Hausinger, 1993), the DEP reactivities of these two mutant proteins were assessed. While the wild-type enzyme was rapidly inactivated by 50 μM DEP (Figure 3), both the αH219A and αH320A proteins appeared to retain full enzyme activity after the same treatment (data not shown). At 1 mM DEP, however, the αH219A protein was inactivated in a pseudo-first-order process, whereas the αH320A protein continued to be resistant to inactivation by DEP. Further characterization of DEP inactivation of the αH219A protein demonstrated that the rate was pH-dependent (Figure 4) with the pattern for inactivation of the αH219A (pK_a=6.8) and wild-type (pK_a=6.5) proteins being nearly identical. These data are inconsistent with αH219 serving as the general base for catalysis in the wild-type enzyme. Rather, the reduced level of DEP reactivity observed for the αH219A protein may be due to the same features that account for the high K_m value of this enzyme. The structure of DEP shares some similarity to that of urea, hence, the binding of this reagent to the active site correspondingly may be reduced in affinity. Alternatively, if αH219 is important for maintaining access to the catalytic site the reduced reactivity in the mutant protein may derive from partial closure of this region. In contrast to the DEP reactivity of the αH219A protein, the resistance to inactivation by DEP

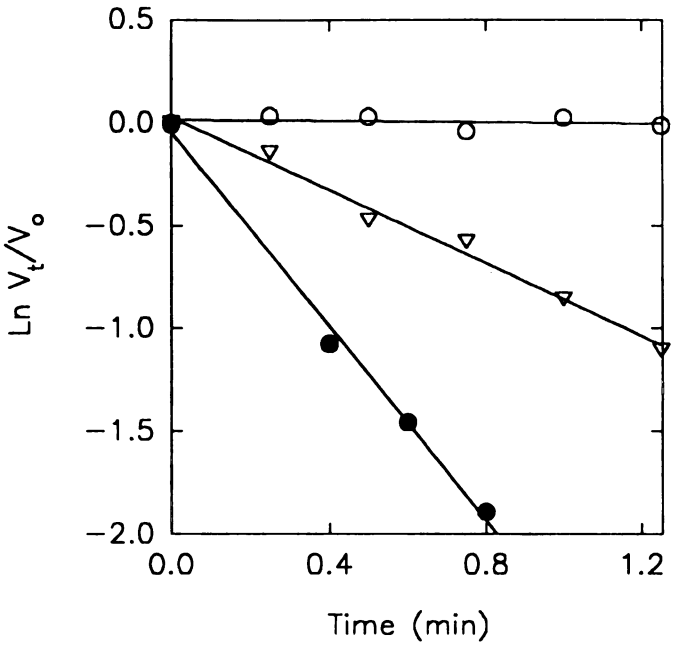

Figure 3. Kinetics of urease inactivation by DEP. Purified wild-type (●), α H219A (▽), and αH320A (○) proteins were treated with DEP (50 μM, 1 mM, and 1 mM, respectively) in 1 mM EDTA and 50 mM HEPES (pH 7.0) buffer. The natural logarithm of V_t/V_0 (where V_t is the velocity at time t and V_0 is the initial velocity) is shown as a function of time.

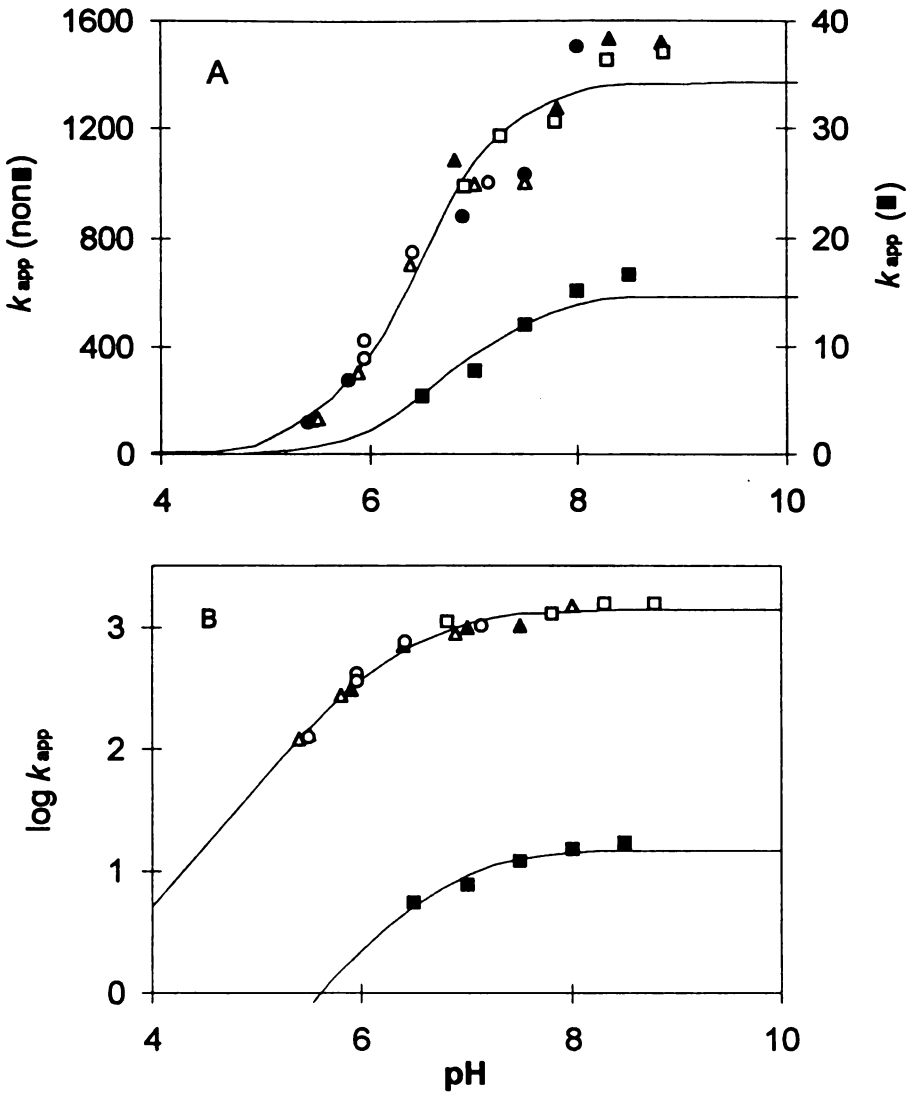

Figure 4. The pH dependence of DEP inactivation of wild-type and αH219A ureases. A: Apparent second-order rate constants ($M^{-1}sec^{-1}$) for urease inactivation with DEP (left axis for wild-type enzyme and right axis for the αH219A enzyme) are plotted as a function of pH. B: The same data plotted as log k_{app} versus pH. The reactions were carried out in 1 mM EDTA and 50 mM concentrations of the following buffers: MES (○), MOPS (●), HEPES (Δ), Tricine (□) or TAPS (▲) for wild-type and MOPS (■) for H219A urease.

displayed by the αH320A protein is compatible with αH320 serving as the target of DEP in the native enzyme; i.e., the general base that facilitates catalysis.

Inactivation of mutant proteins by IAM. To further explore the roles of the αH219 and αH320 residues, the chemical reactivities of the mutant proteins towards IAM were assessed. This reagent had been used previously (Todd & Hausinger, 1991a) to provide evidence that a thiol group is ionically coupled to another residue, X, together acting as the proton donor in catalysis. Both the α H219A and αH320A proteins were inactivated by incubation with IAM (Figure 5). A much higher concentration of the alkylating reagent is required for inactivation of the αH219A urease than for wild-type enzyme or the αH320A protein. Because IAM structurally resembles urea, part of this effect may arise from decreased affinity of the reagent for the active site (prior to reaction with the cysteine residue) due to the same features that lead to the high K_m value in this protein. Again, however, the results are compatible with a model in which the active site is simply less accessible in the αH320A protein. The pH dependence of αH320A inactivation by IAM could not be examined because of the low activity of the protein. In contrast, the pH dependence for αH219A protein was measured and shown to be similar to that in the wild-type enzyme (Figure 6). This result would not be expected if the αH219 residue was equivalent to X in the Cys-X ion pair that has been proposed to occur in this protein (Todd & Hausinger, 1991a).

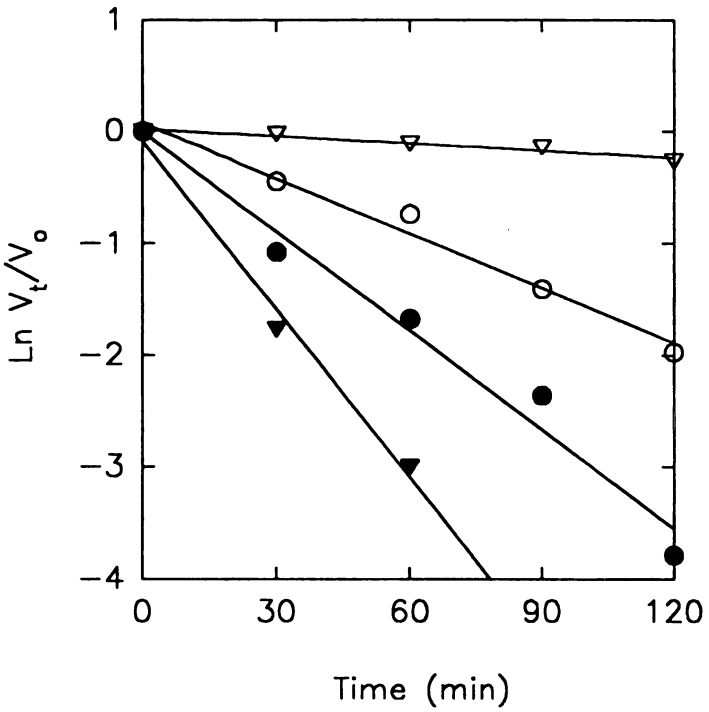

Figure 5. Kinetics of urease inactivation by IAM. Purified wild type (●), αH219A (▽), and αH320A (○) proteins were treated with 40 mM IAM and purified α H219A (▼) protein was treated with 400 mM IAM in 1 mM EDTA and 50 mM HEPES (pH 7.75) buffer. The natural logarithm of V_t/V_0 (where V_t is the velocity at time t and V_0 is the initial velocity) is shown as a function of time.

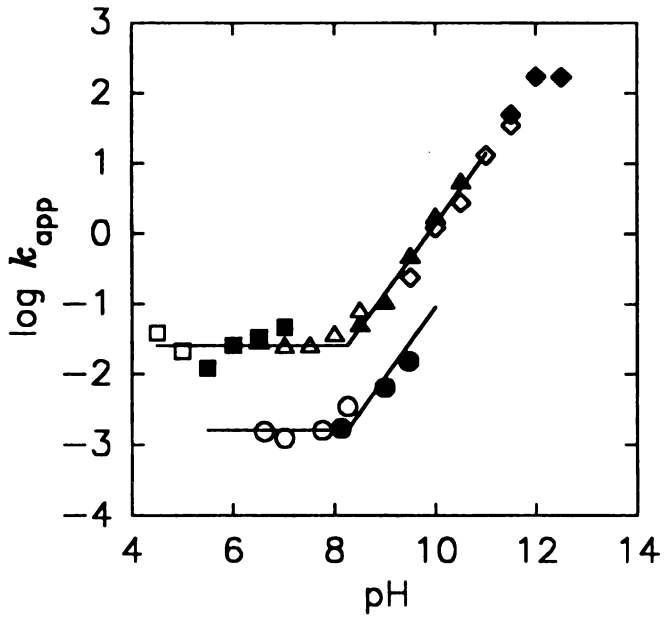

Figure 6. The pH dependence of IAM inactivation of wild-type and αH219A ureases. Apparent second-order rate constants ($M^{-1}sec^{-1}$) for urease inactivation with IAM (20 mM for wild type enzyme and 120 mM for the αH219A enzyme) are plotted as a function of pH. The reactions were carried out either in 1 mM EDTA and 80 mM of acetate (□), MES (■), HEPES (△), CHES (▲), CAPS (◇), and phosphate (◆) buffers for wild-type enzyme or in 1 mM EDTA and 50 mM HEPES (○) and CHES (●) buffers for the αH219A urease.

DISCUSSION

Dixon *et al.* (1980) proposed an elegant model for the hydrolysis of urea by the bi-nickel active site of jack bean (*Canavalia ensiformis*) urease that serves as an excellent framework for discussion of *K. aerogenes* urease site-directed mutagenesis. In their model, one nickel ion is suggested to coordinate a water molecule and a second nickel ion coordinates hydroxide ion. Urea is proposed to displace the water molecule and bind in *O*-coordination to nickel as the Ni··$^{-}$O-C(-NH_2)=$NH_2^{\oplus}$ resonance structure with electrostatic stabilization by a nearby carboxyl group. A general base is hypothesized to activate the nickel-coordinated hydroxyl group that carries out a nucleophilic attack on the urea carbon. The resulting tetrahedral intermediate is thought to decompose to form carbamate and ammonia with the participation of a nearby thiol group acting as a general acid. Subsequently, carbamate dissociates and spontaneously is converted to carbon dioxide and a second molecule of ammonia. According to the Dixon model, two nickel ions, a carboxyl group, a general base and a general acid are required for the jack bean enzyme activity. In independent studies with jack bean urease, Sakaguchi *et al.* (1983) provided evidence from photo-oxidation studies of the enzyme in the presence of methylene blue and active site-directed inhibitors that histidine residues play essential roles at the catalytic site. Furthermore, Takishima *et al.* (1988) identified the reactive cysteine residue in the enzyme and found it to be located in a region that was rich in histidine residues.

In studies with *K. aerogenes* urease, known to be ~60% identical in sequence to the jack bean enzyme (Mulrooney & Hausinger, 1990), the Dixon model has undergone further elaboration. The chemical reactivity of the general base ($pK_a \cong 6.5$) that appears to facilitate catalysis (Todd & Hausinger, 1987) was

shown to be compatible with that of a histidyl group (Park & Hausinger, 1993). The thiol group that participates in proton donation was identified (Todd & Hausinger, 1991b; Martin & Hausinger, 1992) and shown to function as an ion pair with another residue (X) that could reasonably be accounted for by a histidine group (Todd & Hausinger, 1991a). Finally, the enhanced DEP reactivity of apoprotein over holoenzyme (Lee *et al.*, 1990) is consistent with at least partial metallocenter ligation by histidyl residues. The multiple potential roles for histidine groups in the protein led to the experiments described above.

My results are consistent with the participation of three histidyl residues (αH134, αH136, and αH246) in nickel coordination, one histidine residue (α H320) serving as the general base in catalysis, and one residue (αH219) somehow facilitating substrate binding. The latter residue may stabilize urea binding by hydrogen-bond formation [similar to the ionic stabilization of bound urea by a postulated carboxyl group in the model of Dixon *et al.* (1980)], or perhaps αH219 may act by maintaining accessibility to the catalytic site. No evidence was obtained for the presence of a conserved histidine acting as residue X in a Cys-X pair that functions as a general acid. Further efforts toward elucidating the three-dimensional structure of *K. aerogenes* urease by x-ray crystallographic methods (c.f., Jabri *et al.*, 1992) will establish the validity of several of the roles for histidine residues that have been proposed here.

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CHAPTER 4

In Vitro Activation of Urease Apoprotein and Role of UreD as a Chaperone Required for Nickel Metallocenter Assembly

These studies, less those shown in Fig. 1 and combined with urease activation studies of cell extracts by Mary Beth Carr, were published in *Proc. Natl. Acad. Sci. USA* 80, 396-3965, 1994.

ABSTRACT

The formation of active urease in *Klebsiella aerogenes* requires the presence of three structural genes for the apoprotein (*ureA*, *ureB*, and *ureC*), as well as four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) that are involved in functional assembly of the metallocenter in this nickel-containing enzyme. Partial activation of urease apoprotein was observed after adding nickel ion to extracts of *Escherichia coli* cells bearing a plasmid containing the *K. aerogenes* urease gene cluster or derivatives of this plasmid with deletions in *ureE*, *ureF*, and *ureG*. In contrast, extracts of cells containing a *ureD* deletion derivative failed to generate active urease, thus highlighting a key role for UreD in the metallocenter assembly process. Site-directed mutagenesis methods were used to overexpress *ureD* in the presence of the other urease genes, and UreD was found to co-purify with urease. A molecule of native urease apoprotein is capable of binding 0, 1, 2, or 3 molecules of UreD, consistent with a trimeric structure of urease catalytic units. The UreD-urease apoprotein complexes are competent for activation by nickel, with the level of activity obtained being directly related to the number of UreD bound per urease molecule. Activation of the UreD-urease complexes is rapid and accompanied by UreD dissociation. I propose that UreD is a chaperone protein which stabilizes a urease apoprotein conformation that is competent for nickel incorporation.

INTRODUCTION

Urease (EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form carbonic acid and two molecules of ammonia. Until recently, the best characterized urease was that from jack bean (1). This enzyme was the first ever crystallized (2) and the first shown to possess nickel (3). However, because of the ease of cell growth and genetic manipulation in bacteria compared to plants and the medical importance of bacterial urease, our understanding of the procaryotic enzyme has outpaced knowledge of the plant enzyme (4). Importantly, the active site residues of plant and bacterial ureases are highly conserved and both enzymes possess a novel bi-nickel metallocenter.

Assembly of the urease bi-nickel metallocenter appears to be a complex process requiring the action of several accessory gene products. For example, deletion analysis of the *Klebsiella aerogenes* urease gene cluster revealed that one gene (*ureD*) located directly upstream and three genes (*ureE*, *ureF*, and *ureG*) found directly downstream of the three genes (*ureA*, *ureB*, and *ureC*) encoding the subunits of the bacterial urease are involved in the functional assembly of the urease metallocenter (5, 6). Homologs of the accessory genes have been found in several bacterial species (e.g., 7-9) and an essential role for auxiliary genes in forming active urease has been confirmed in these and many other bacteria. Genetic studies are consistent with a similar requirement for urease accessory genes in the fungi *Neurospora crassa* (10), *Schizosaccharomyces pombe* (11), and *Aspergillus nidulans* (12, 13). Finally, two loci (*Eu2* and *Eu3*) that appear to encode genes associated with urease maturation factors such as nickel emplacement have been identified in soybean plants (14). Thus, accessory genes involved in metallocenter assembly may be a universal component of urease activation.

The detailed roles for the urease accessory genes are unknown. The histidine-rich *K. aerogenes* UreE protein has been purified and shown to reversibly bind six nickel ions per dimer (15); hence, it is reasonable to suggest that UreE

serves as the nickel donor to urease apoprotein. UreG contains a P-loop motif that is found in many nucleotide-binding proteins (16); thus, this protein may be involved in coupling nucleotide hydrolysis to the assembly process. In this regard, *in vivo* evidence is consistent with an energy-dependence for urease activation (17). Furthermore, the sequence of *K. aerogenes* UreG was shown (18) to be approximately 25% identical to that of the *Escherichia coli hypB* gene product (19). The *hyp* operon encodes a *hy*drogenase *p*leiotropic operon that is required for functional activation of hydrogenase. A mutation in *hypB* was shown to be complemented, in part, by the addition of high levels of nickel (20), consistent with a role for this protein in nickel processing. Of potential significance to the function of UreG, purified HypB protein has been shown to bind guanine nucleotides and to hydrolyze GTP (21). In contrast to UreE and UreG, the sequences for UreD or UreF offer no insight into their function and these proteins have not been purified or characterized.

Here I demonstrate the ability to activate urease apoprotein in vitro and present evidence supporting a model in which UreD serves as a urease-specific chaperone during this process.

MATERIALS AND METHODS

Site-Directed Mutagenesis of the *ureD* Ribosome Binding Site. In order to enhance expression of *ureD*, the ribosome binding site and initiation codon of this gene were changed from CACGGCACCGTG to GAGGGCACCATG by two rounds of site-directed mutagenesis. A 0.82-kb *Eco*RI-*Sac*I fragment of plasmid pKAU17 containing a urease gene cluster from *K. aerogenes* (22) was subcloned into M13 mp18 and mutagenized by the method of Kunkel *et al.* (23). Uracil-containing single-stranded template DNA was prepared from *Escherichia coli* CJ236 (*dut1 ung1 thi-1 rel*A1/pCJ105[*cam*^{r}F']). Using a CACGGTGCCCTCCAATGTTGC oligonucleotide primer, the phage DNA was

mutagenized and mutant phage were isolated in *E. coli* MV1193([*lacI-proAB*] *rpsL thi endA spcB*15 *hsdR*4 [*srl-recA*]306::Tn10[*tet*^{r}] F'[*traD*36 *proAB*$^+$ *lacI*^{q}*lacZ*M15]). Uracil-containing single-stranded DNA was again prepared and similarly mutagenized with the primer GTGGTAACATGGTGCCCTC. The site-directed mutants were identified by sequence analysis using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland OH) and the single-strand DNA sequence method of Sanger *et al.* (24). The region was completely sequenced to ensure that no other mutations had been introduced into M13. The mutated *Eco*RI-*Sac*I region was subcloned back into pKAU17 to generate pKAUD2 and the sequence was confirmed by double-stranded DNA sequencing methods (25).

Cell Growth and Disruption. *E. coli* DH5 carrying pKAUD2, pKAU17, pKAU22ΔD-1 (*ureD* deletion derivative of pKAU22, ref. 6) or pKAUDABC (*ureEFG* deletion derivatives of pKAU17, see appendix) were grown in LB medium to stationary phase. The harvested cultures were resuspended in 20 mM phosphate (pH 7.0), 0.1 mM EDTA, and 0.1 mM 2-mercaptoethanol buffer and disrupted by three passages through a French pressure cell (American Instrument Co., Silver Spring MD) at 18,000 lb/in^2. Cell extracts were obtained after centrifugation (100,000 x *g* for 60 min) at 4°C. The ability to activate urease apoenzyme in *E. coli* [pKAUD2] extracts was stable for over 1 month when samples were stored on ice and adjusted to 20% glycerol and 0.8 mM dithiothreitol.

Purification of UreD-Urease Apoprotein Complex. *E. coli* DH5[pKAUD2] cell extracts from a 2 L culture were chromatographed on a column (2.5 x 20 cm) of DEAE-Sepharose at 4°C in PEG buffer [16 mM phosphate (pH 7.0), 0.8 mM EDTA, 20% glycerol, 0.8 mM 2-mercaptoethanol, 0.8 mM dithiothreitol]. The proteins were eluted in PEG buffer with a linear salt gradient to 1.0 M KCl, and UreD was found to co-elute with urease apoprotein at ~0.45 M KCl. UreD-containing fractions were pooled, dialyzed against PEG buffer, and applied to a Mono-Q HR 10/10 column equilibrated in the same buffer. A linear salt gradient to 1 M KCl in PEG buffer again eluted the UreD in the same fractions as urease.

A portion of the pooled fractions containing UreD was adjusted to 1 M KCl by addition of PEG buffer containing 2 M KCl and applied to a phenyl-Superose HR 5/5 column equilibrated in this buffer. A linear gradient to 0 M KCl in PEG buffer was used for elution. Samples were monitored for the presence of UreD and urease apoprotein by using polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by using the buffers of Laemmli (26) and included a 13.5% polyacrylamide running gel and a 4.5% polyacrylamide stacking gel. Nondenaturing gels utilized the same buffers without SDS and used 7.5% and 3% acrylamide running and stacking gels. Gels were stained with Coomassie brilliant blue or, in the case of selected native gels, blotted onto nitrocellulose, probed with anti-*K. aerogenes* urease antibodies (22), and developed by using anti-rabbit IgG-alkaline phosphatase conjugates (27). Band intensities were measured by using a gel scanner (Ambis, Inc.). For calculation of the ratio of UreD to UreC, M_r values of 30,000 and 60,300 were used for these peptides.

Urease Activity Assays. Urease activities were assayed in 25 mM Hepes (pH 7.75) and 0.5 mM EDTA buffer containing 50 mM urea. Linear regression analysis of the released ammonia, determined by conversion to indophenol (28), versus time yielded initial rates. One unit of activity is defined as the amount of enzyme required to degrade 1 mmol urea per min at 37°C. Protein concentrations were assessed by the spectrophotometric assay of Lowry *et al.* (29) or, in the case of samples containing Hepes buffer, the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond CA).

RESULTS

***In Vitro* Activation of Urease Apoprotein.** *E. coli* [pKAU17] carries the wild-type *K. aerogenes* urease gene cluster. The urease apoprotein in extracts from

these cells (grown in the presence of nickel-free medium) is partially activated by incubation with $NiCl_2$ at 20~500 μM (Fig. 1). Although *in vivo* activation of urease apoprotein has been described [e.g., (17)], data obtained by Mary Beth Carr of this lab (not shown) and that shown in Fig. 1 represent the first evidence for *in vitro* activation of this enzyme. The highest specific activity values observed in these studies (40 U/mg protein) account for activation of only about 10% of the urease that is present [i.e., when grown in the presence of 1 mM $NiCl_2$ *E. coli* [pKAU17] cell extracts typically possess specific activity values of 300-400 U/mg (17, 30)]. The leveling off of activity is not due to simultaneous formation and inactivation of active enzyme because purified urease holoprotein is completely stable under the incubation conditions used for activation of the cell extracts. Alteration of the activation conditions (varying the pH, buffer composition, ionic strength, and $NiCl_2$ concentration) failed to further improve the extent of activation and the process is hindered by elevated nickel concentrations (4 mM) and high (> 8.0) or low (< 7.0) pH.

Analogous urease apoprotein activation experiments were carried out using extracts from cells that were defective in *ureD* or *ureEFG* (Fig. 1). Surprisingly, deletion of *ureEFG* (Fig. 1) has no significant effect on the *in vitro* activation process. This finding is intriguing because Δ*ureF* or Δ*ureG* cultures possess no detectable urease activity even when provided with 1 mM nickel ion in the growth medium (6). Of greatest significance, urease apoprotein in cell extracts from a Δ*ureD* mutant was not competent for activation under these activation conditions. These results highlight a key role for UreD in the urease activation process.

Purification of UreD-Urease Apoprotein Complex. Characterization of UreD from *E. coli* [pKAU17] is hampered by very low level expression of the *ureD* gene (6). We circumvented this problem by using site-directed mutagenesis to improve the ribosome binding site and to provide an ATG initiation codon for this gene in plasmid pKAUD2. An intense band of UreD is observed on SDS-polyacrylamide gels for extracts of *E. coli* [pKAUD2] (Fig. 2, lane 2) that is not

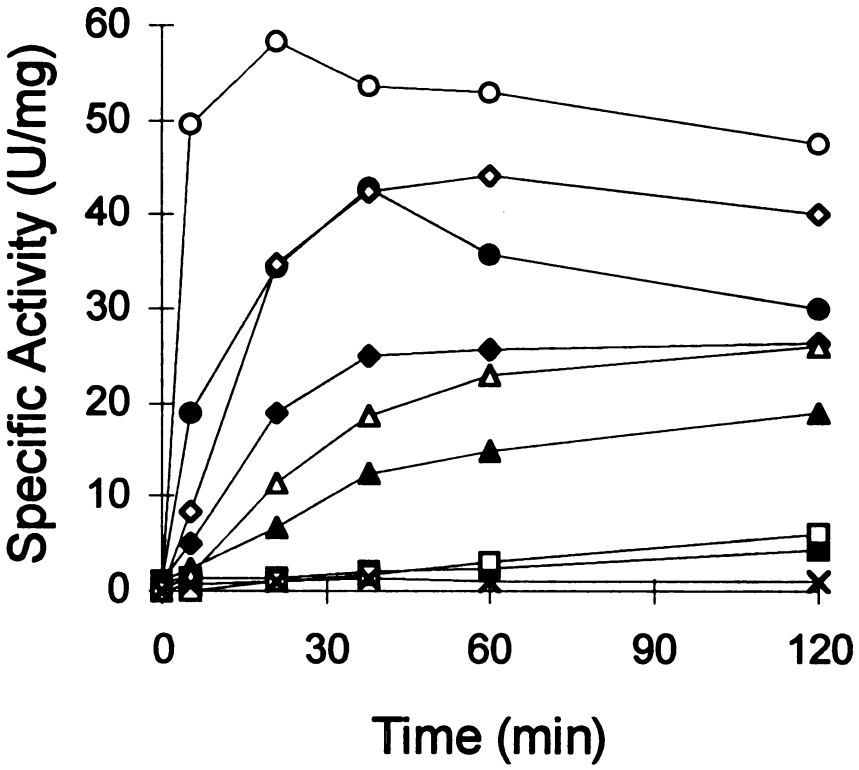

Fig. 1. *In vitro* activation kinetics of urease apoprotein. Cell extracts (2 mg/ml) from *E. coli* [pKAU17] (closed symbols) or Δ*ureD* (x), Δ*ureEFG* (open symbols) deletion derivatives were incubated in 25 mM HEPES (pH 7.9)/0.08 mM EDTA/ 0.08 mM 2-mercaptoethanol containing 20 (square), 50 (triangle), 100 (diamond), or 500 (circle, x) μM $NiCl_2$ at 37°C. Aliquots were withdrawn and assayed for urease activity and protein.

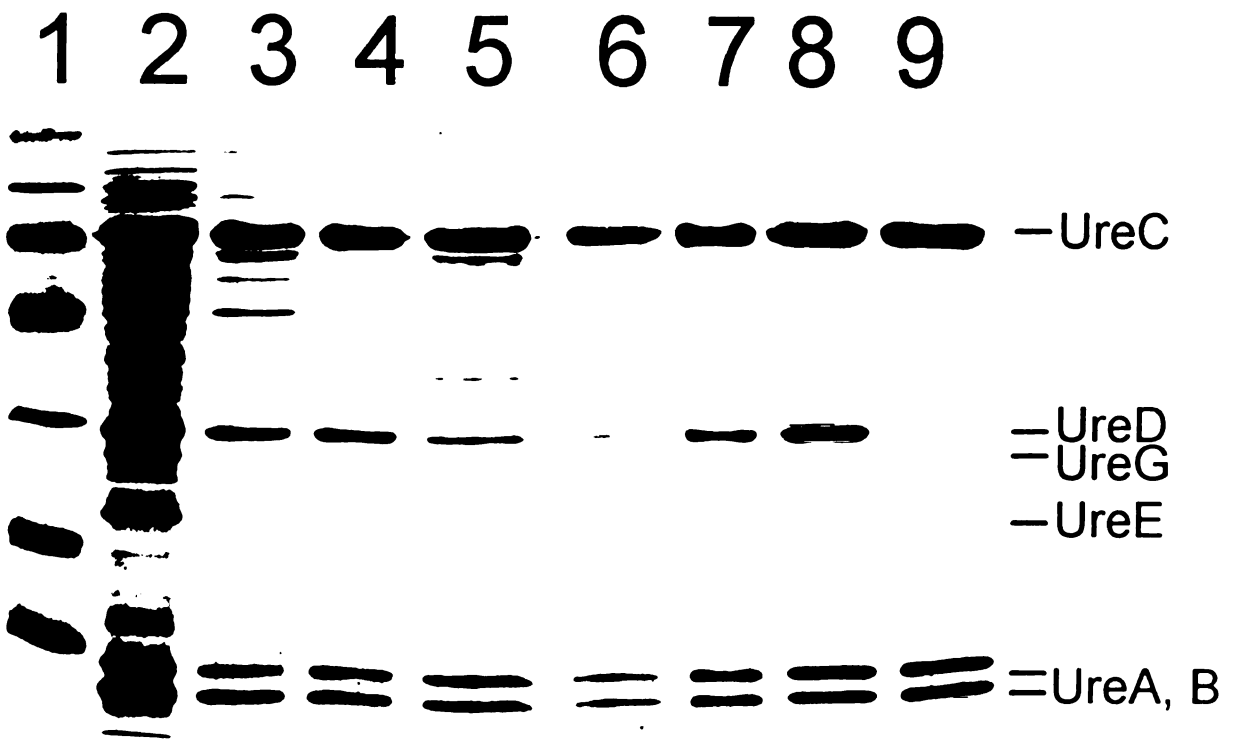

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of UreD and urease fractions. Lanes: 1, molecular weight markers (phosphorylase *b*, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400); 2, cell extracts of *E. coli* [pKAUD2]; 3, DEAE-Sepharose pool; 4, Mono-Q pool; 5-8, phenyl-Superose fractions FT and 1-3 (see Fig. 3); 9, urease.

present in extracts of *E. coli* [pKAU17] (not shown). Using extracts from *E. coli* [pKAUD2] cells that were grown in the absence of nickel, UreD was found to co-elute with urease during two steps of ion exchange chromatography and during hydrophobic interaction chromatography on phenyl-Superose HR 5/5. The latter step resolves the UreD/urease species into four pools that were termed flow-through (FT) and fractions 1, 2, and 3 (Fig. 3). UreD and urease apoprotein are unlikely to be present as a complex in the FT fraction because these proteins are resolved using phenyl-Sepharose CL-4B chromatography (not shown). In contrast, fractions 1, 2, and 3 do appear to represent distinct UreD-urease apoprotein complexes.

The ratio of UreD to urease apoprotein in the phenyl-Superose fractions is not uniform as demonstrated by SDS-polyacrylamide gel analysis (Fig. 2, lanes 6-8). Given that urease is a trimer of catalytic units (P. A. Karplus, personal communication), our gel scanning results are consistent with the presence of 1.0-1.3, 2.2-2.5, and 3-3.2 molecules of UreD per molecule of native urease in fractions 1, 2, and 3. Native gel electrophoresis of the four phenyl-Superose pools (Fig. 4, lanes 4-7) clearly demonstrates that four distinct urease apoprotein species are present. The FT fraction is comprised almost entirely of form I, a species that migrates identically with urease apoprotein or urease holoprotein (Fig. 4, lane 8). Fractions 1, 2, and 3, in contrast, are comprised predominantly of urease apoprotein species of decreasing mobilities that are labeled forms II, III, and IV (Fig. 4, lanes 5-7). Form III appears to predominate in the DEAE-Sepharose and Mono-Q pools (Fig. 4, lanes 2 and 3), and is the major fraction found on the phenyl-Superose column (Fig. 3). Mobility differences among the four urease species on native gels are likely to arise primarily from differences in native size. Gel filtration chromatography results are consistent with this hypothesis; when the Mono-Q pool was chromatographed on a Superose 6 column and the fractions analyzed by native gel electrophoresis, the overlapping order of elution was form IV, form III, form II, and form I (data not shown). Thus, we conclude that the four species observed upon phenyl-

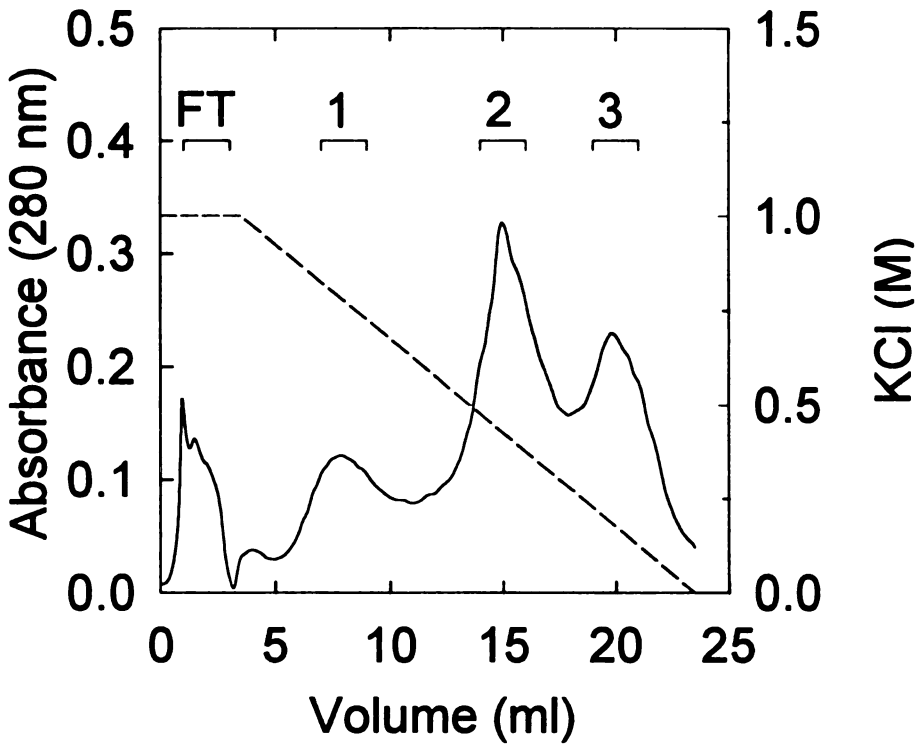

Fig. 3. Phenyl-Superose resolution of UreD-urease complexes. Starting with extracts from *E. coli* [pKAUD2] cells that overexpress *ureD*, UreD was found to co-elute with urease apoprotein during chromatography on DEAE-Sepharose and Mono-Q resins. The latter pool was resolved into four fractions by chromatography on phenyl-Superose. Elution was monitored by absorbance at 280 nm, and the pooled samples are denoted flow-through (FT) and fractions 1, 2, and 3.

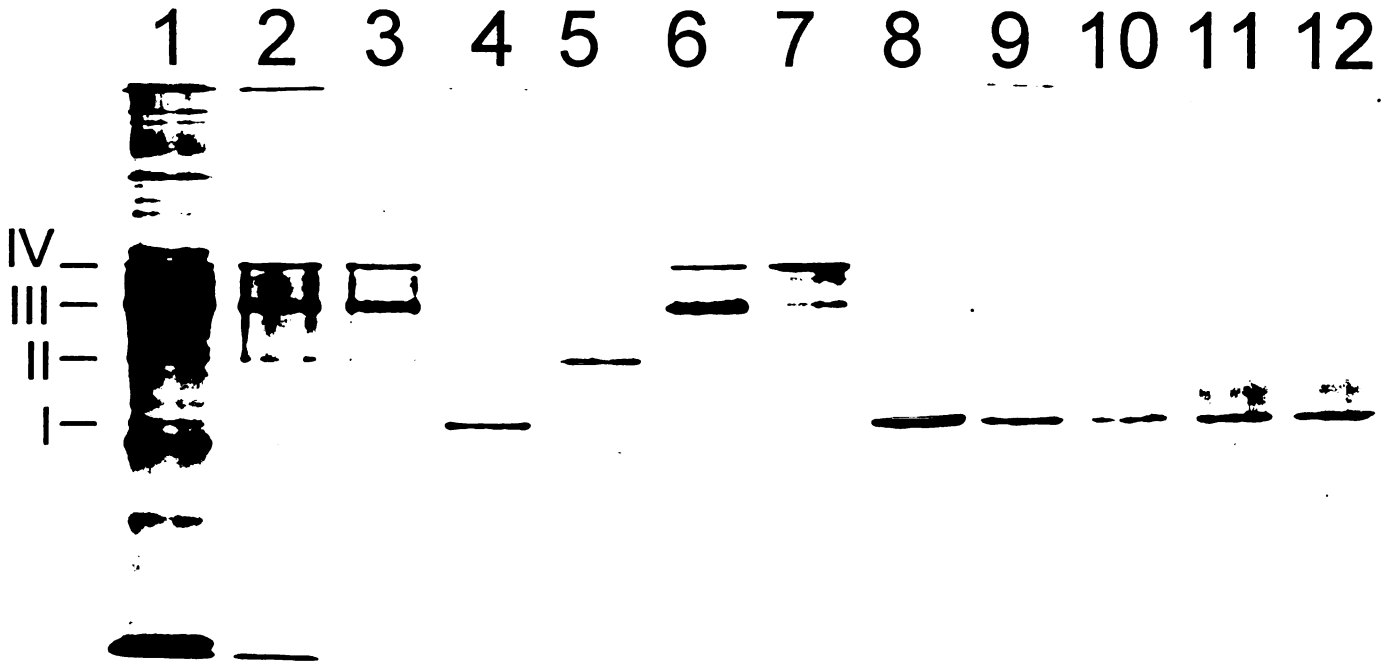

Fig. 4. Native polyacrylamide gel electrophoretic analysis of urease fractions. Lanes: 1-8 are equivalent to lanes 2-9 of Fig. 2; 9-12 are the same four phenyl-Superose fractions as in lanes 4-7, but after incubation of the samples for 100 min at 37°C in the presence of 0.1 mM $NiCl_2$.

Superose chromatography and resolved by native gel electrophoresis arise from the presence of different numbers of UreD (0, 1, 2, or 3) complexed to the native urease enzyme.

Activation of UreD-Urease Apoprotein Complexes. When adjusted to 0.1 mM $NiCl_2$, urease apoprotein is activated to different extents in each of the four phenyl-Superose pools (Fig. 5). Importantly, the greater the ratio of UreD to urease apoprotein, the greater is the final specific activity of the preparation. The small amounts of activation observed in the FT fraction are likely to arise from trace contamination with the form II species that contain UreD. The activation process for the UreD-urease complexes is rapid. The highest level of activity reached (~550 U/mg protein) accounts for approximately 30% of full activation for the urease apoprotein that is present in the predominantly form IV species [assuming one UreD per catalytic unit (M_r of 113,000) and a specific activity of 2,500 U/mg protein for fully active urease (6)]. This level of activation is even more impressive when one considers that full activation of urease is not observed in *E. coli* [pKAU17] cells grown in the presence of 1 mM $NiCl_2$ [with typical specific activity values of 1,500-1,900 U/mg (6, 30)]. These results are consistent with the presence of a UreD-urease complex playing a central role in urease activation.

Upon treatment with nickel ion, the form II, III, and IV species observed in native gels collapse to the form I species for each of the phenyl-Superose pools (Fig. 4, lanes 9-12). These results are consistent with dissociation of UreD during the activation process. A band corresponding to UreD is not observed in the native polyacrylamide gels, perhaps due to the inability of the highly insoluble protein to enter the gel.

Rapid and high level *in vitro* urease apoprotein activation (to ~200 U/mg total protein) similarly was observed in cell extracts of the *E. coli* [pKAUD2] cells upon addition of nickel ion. If urease is estimated to account for approximately 20% of the protein in these cell extracts (see Fig. 2, lane 1), this result allows me to calculate a specific activity for urease of about 1,000 U/mg in this sample.

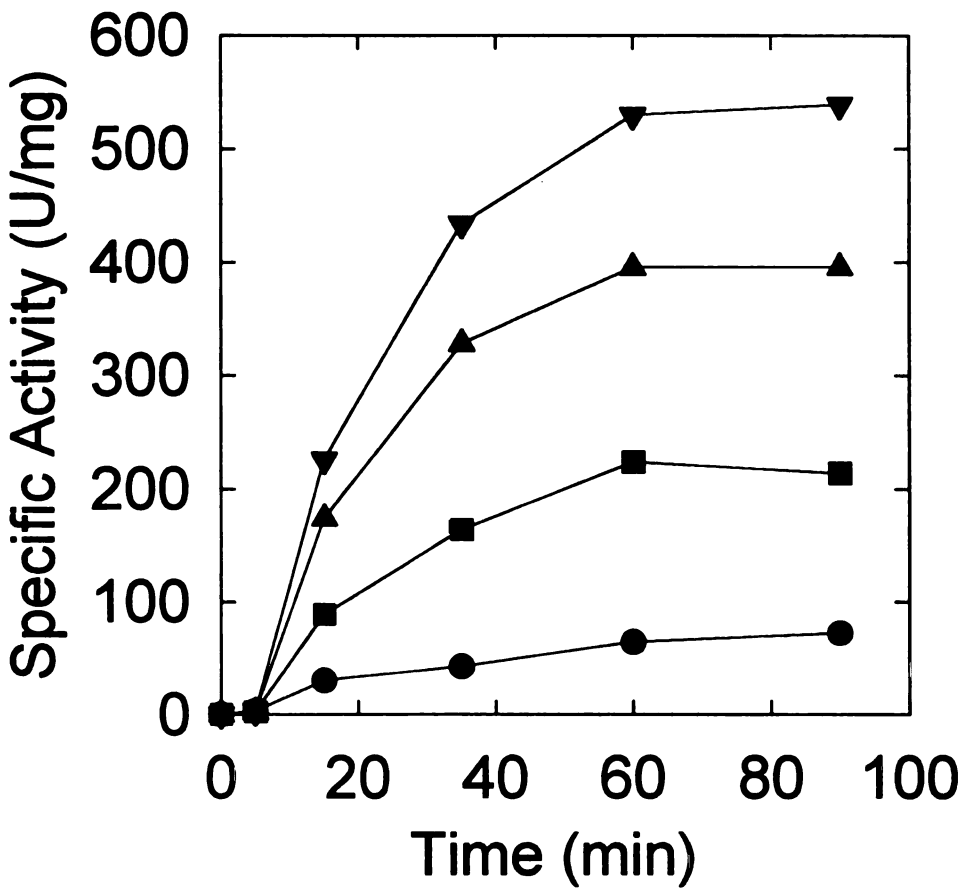

Fig. 5. Activation kinetics of the UreD-urease complexes. The four phenyl-Superose pools [FT (●) and fractions 1 (■), 2 (▲), and 3 (▼)] from Fig. 3 were incubated at 37°C in the presence of 0.1 mM $NiCl_2$ in 25 mM Hepes (pH 7.9), 0.15 M KCl and aliquots were removed and assayed for urease activity over time.

Optimal activity was observed from pH 7.6 to 8.2 and using 0.5 mM $NiCl_2$. Phosphate strongly inhibits the activation process at concentrations greater than 20 mM. Surprisingly, growth of *E. coli* [pKAUD2] in the presence of 1 mM $NiCl_2$ yields cell extract activities that are very low (3-15 U/mg), equivalent to 1-5% of the activity found in *E. coli* [pKAU17] cell extracts. It is possible that this result may relate, at least in part, to the low level expression of *ureG* that is observed in these cells compared to cells containing pKAU17. Addition of nickel to these low activity cell extracts results in rapid and high level activation, similar to the extracts from cells obtained by nickel-free conditions.

Using anti-urease antibodies and Western blot analysis of cell extracts electrphoresed through a native gel, the three UreD-urease complexes from *E. coli* [pKAUD2] were shown to collapse almost entirely to the form I (UreD-free apoprotein or holoprotein) species (Fig. 6, lanes 4-5) after incubation with 0.3 mM nickel. More significantly, the presence of at least two UreD-urease complexes (forms II and III) was established in *E. coli* [pKAU17] cells that do not overexpress *ureD*. Furthermore, nickel addition to these cell extracts also was shown to lead to diminishment of the bands corresponding to these complexes (Fig. 6, lane 2-3).

DISCUSSION

I propose that urease apoprotein activation occurs by the mechanism illustrated in Fig. 7. [This simplified model does not indicate that two nickel are incorporated per catalytic unit or that three catalytic units are present per native enzyme]. UreD is proposed to be a chaperone protein that stabilizes a conformation of urease that is competent for nickel incorporation. This role is reminiscent of that suggested for NifY in activation of apodinitrogenase by the iron-molybdenum cofactor (31, 32) and for MelC1 in incorporating copper into tyrosinase (33, 34). The *in vitro* source of nickel in the proposed urease

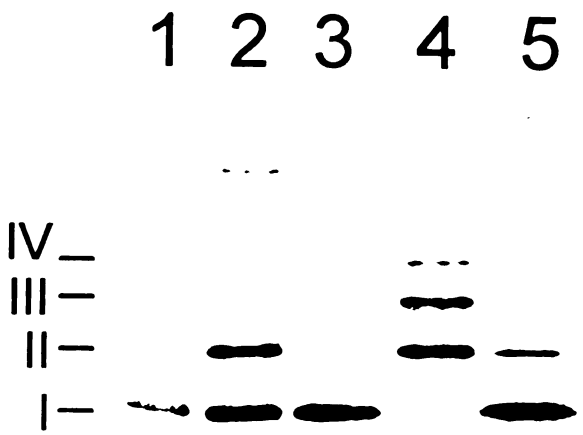

Fig. 6. Western blot comparison of cell extracts before and after nickel activation. Samples were activated by incubation for 100 min in 0.3 mM $NiCl_2$, 25 mM Hepes (pH 7.9). Lanes: 1, purified urease; 2, cell extracts of *E. coli* [pKAU17]; 3, same as 2 after activation; 4, *E. coli* [pKAUD2] cell extracts; 5, same as 4 after activation.

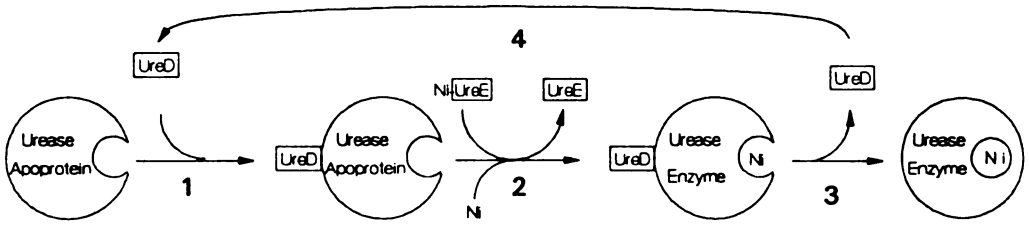

Fig. 7. Simplified model illustrating the functional role for UreD in urease apoprotein activation.

metallocenter assembly mechanism is $NiCl_2$, whereas, nickel bound to UreE may fill this role *in vivo* (15). The roles of UreF and UreG in the activation process remain unclear, however, it is reasonable to speculate that they may function in assembly of the UreD-urease complex (step 1), in facilitating interactions between the complex and UreE (step 2), by enhancing the level of activation during UreD release (step 3), or in recycling of UreD (step 4). The energy-dependence of activation that was observed *in vivo* (17), but not *in vitro* (above), and the low level of activation observed in *E. coli* [pKAU17] cell extracts can be interpreted in terms of this model. For example, it is possible that UreD recycling requires energy, whereas single turnover of the UreD-urease complex does not. The levels of *in vitro* activation that we observe are consistent with generation of active enzyme only from the UreD-urease complexes, with no recycling of UreD. It is possible that, in the absence of sufficient UreD, partially folded urease that is newly released from the ribosome undergoes a final conformational change to bury the nickel binding site. Our *in vitro* conditions are inadequate to allow the folded apoprotein to revert to the partially unfolded state that binds UreD and is capable of activation; however, this species does appear to be slowly activated *in vivo* (17). This working model may help to focus future efforts to understand urease metallocenter assembly.

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CHAPTER 5

Carbon Dioxide is Required for *in vitro* Assembly of the Urease Nickel Metallocenter

These studies were submitted to *Science*.

ABSTRACT

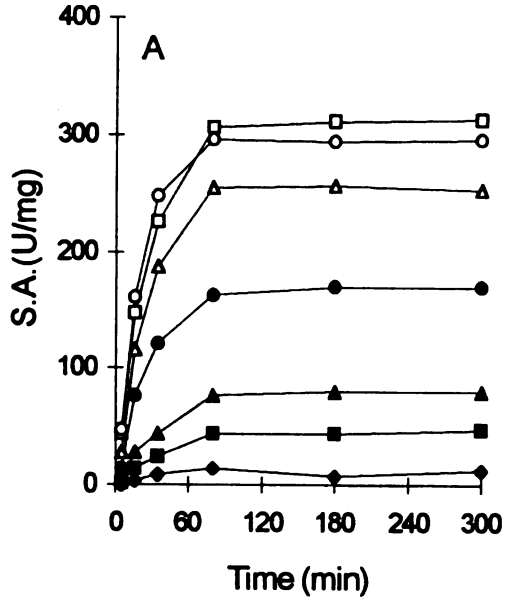
In vivo assembly of the urease metallocenter is known to require four accessory proteins: UreD, postulated to be a urease-specific molecular chaperone, UreE, a Ni(II)-binding protein, and UreF and UreG whose functions remain obscure. *In vitro* activation of purified *Klebsiella aerogenes* urease apoprotein has now been accomplished by simply providing CO_2 (half-maximal activation at ~0.2% CO_2) in addition to nickel ion. Activation requires that CO_2 be incorporated into urease in a pH-dependent reaction ($pK_a \geq 9$). CO_2 concentration also affects the level of activation for UreD-urease apoprotein complexes. I propose that CO_2 binding to urease apoprotein generates a ligand that facilitates productive nickel binding.

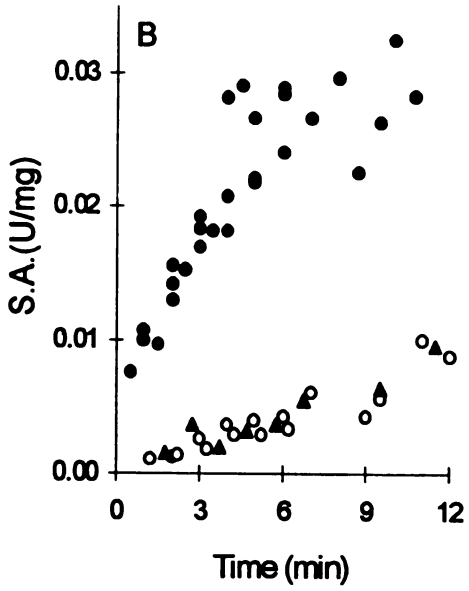
Urease, the first enzyme ever crystallized (1) or shown to possess nickel (2), is found in selected plants, fungi, and bacteria where it plays important roles in environmental nitrogen transformations and as a virulence factor in certain pathogenic microbes (3). The protein contains a dinuclear Ni(II) active site (4) where each metal atom has a Ni(imidazole)$_x$(N,O)$_{5-x}$ (x = 2 or 3) coordination environment according to X-ray absorption spectroscopic analysis (5). *In vivo* assembly of this novel metallocenter in *Klebsiella aerogenes* (6) involves the participation of four accessory gene products: UreD, UreE, UreF, and UreG (7). UreD has been postulated to function as a molecular chaperone that stabilizes a urease apoprotein conformation which is competent for nickel incorporation (8). Evidence consistent with this hypothesis includes the ability to purify several forms of a UreD-urease apoprotein complex [(urease)$_3$UreD$_N$, where N = 1, 2, or 3], the demonstration that the complexes can be partially activated by addition of nickel ions (increasing levels of activation correlate to increasing N), and the finding that UreD dissociates from urease during activation. UreE has been proposed to serve as a nickel donor for urease activation (9). This suggestion was based on the ability of the protein to bind approximately six nickel ions per dimer (K_d ~10 μM) with rather high specificity. The roles for UreF and UreG in urease activation are unknown, but they are absolutely essential for activation *in vivo*. Of related interest, UreG is homologous in sequence to HypB, a GTP-binding protein that is required for incorporation of nickel into hydrogenase (10, 11). Here, I demonstrate that carbon dioxide is required for *in vitro* activation of urease apoprotein. I detail several properties of this system, propose a mechanism for the *in vitro* process, and discuss how UreD may assist in urease activation.

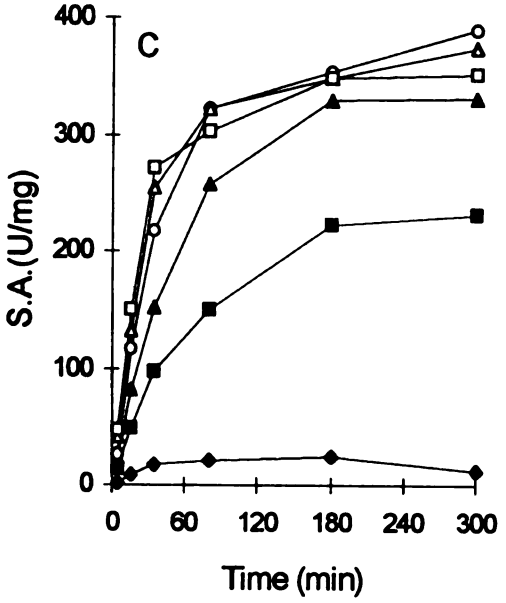
Purified *K. aerogenes* urease apoprotein can be activated in the absence of any accessory protein by incubation with nickel ion in the presence of bicarbonate-containing buffers (Fig. 1A). Because bicarbonate is in equilibrium with dissolved carbon dioxide in solution, it becomes imperative to determine which species is the actual activating factor. By using $NaHCO_3$ from stock solutions that were adjusted to

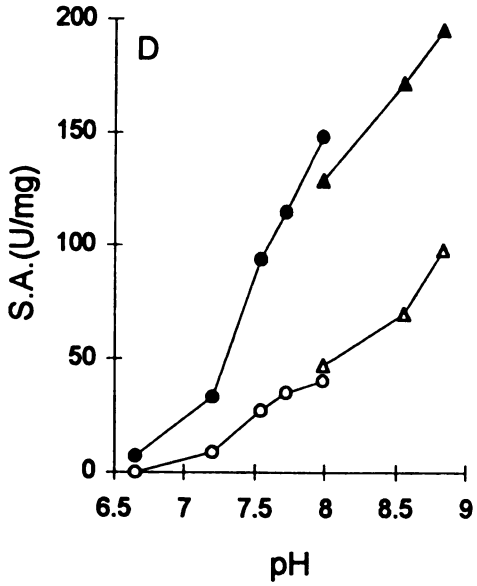
pH 4.2 or pH 8.5 [the dissolved CO_2 concentration is much greater in the former (12)], urease activation was demonstrated to be carbon dioxide-dependent (Fig. 1B). Additional support for this conclusion was obtained from experiments in which carbonic anhydrase was included in the activation mixture. The initial rapid activation (complete by ~5 min) observed for sample amended with the low pH $NaHCO_3$ stock solution is eliminated in the presence of carbonic anhydrase due to immediate hydration of dissolved CO_2 at the pH of the assay (Fig. 1B). The bicarbonate/CO_2 concentration affects the extent of activation (Fig. 1A); the maximal specific activity (~310 U/mg) in this illustratration accounts for activation of approximately 12.5% of the urease apoprotein that was present, but activation of up to 30% was observed in another apoprotein preparation. Half-maximal activation of urease apoprotein at pH 8.3 occurs at approximately 10 mM bicarbonate concentration, corresponding to about 0.063 mM or 0.2% CO_2 (13) [for comparison, the CO_2 concentration in the atmosphere is approximately 0.03% (12)]. The activating CO_2 molecule becomes part of the enzyme as shown by measuring incorporation of ^{14}C-$NaHCO_3$ (14). The labeling accounted for incorporation of 0.48 bicarbonate carbon atoms per protein molecule. The specific activity generated in this experiment (241 U/mg) is much less than 48% of that expected for fully active enzyme (~2500 U/mg, 15); thus, CO_2 incorporation does not equate to enzyme activation and other factors are important. The nickel ion concentration dependence for urease apoprotein activation was assessed using buffer containing 100 mM $NaHCO_3$ (Fig. 1C). The activation rate appears to saturate at approximately 60 μM nickel ion. This value is only ~six-fold higher than the protein concentration or only 3-fold higher than that needed to load the bi-nickel site of the protein. Importantly, prior incubation of urease apoprotein with nickel ion followed by addition of bicarbonate failed to yield active enzyme. This non-productive interaction between apoprotein and nickel ion may account for the low extent of activation described above. Activation of urease apoprotein under an atmosphere of 0.3% CO_2 exhibited

Fig. 1. *In vitro* activation of urease apoprotein. Urease apoprotein was purified from *Escherichia coli* DH5 cells bearing plasmid pKAU22*ureD-1*, containing the *K. aerogenes* gene cluster deleted in *ureD*, as previously described (7). Enzyme activity was assayed in 25 mM HEPES, 0.5 mM EDTA, 50 mM urea (pH 7.75). The specific activity of urease was calculated based on linear regression analysis of the rate of released ammonia, determined by conversion to indophenol (21), and protein concentration determination (22). One unit of urease activity is defined as the amount of enzyme required to degrade 1 mmol of urea per min at 37°C. **(A)** Apoprotein (0.8 mg/ml or 9.6 μM final concentration) was added to activation buffer [100 mM Hepes (pH 8.3), 150 mM NaCl, 10 mM EDTA] containing 100 μM $NiCl_2$ and 0 (◆), 1 (■), 3 (▲), 10 (●), 30 (△), 100 (□) and 200 (○) mM $NaHCO_3$ at 35°C. Aliquots were removed over time to monitor for urease activity. The results are representative of four independent experiments. **(B)** Apoprotein (as above) was placed in activation buffer containing 200 μM $NiCl_2$ at 0°C. $NaHCO_3$ was immediately added (final concentration of 1 mM) from 4 mM stock solutions that were prepared at pH 4.2 (●) or 8.5 (▲) and timed aliquots were removed for assay. An identical low pH $NaHCO_3$ stock solution experiment was carried out in the added presence of carbonic anhydrase (0.2 mg/ml) (○). The figure represents the combined results of three separate experiments. **(C)** Apoprotein (as above) was incubated at 35°C in activation buffer containing 100 mM $NaHCO_3$ and $NiCl_2$ was added to a concentration of 10 (◆), 20 (■), 30 (▲), 40 (○), 60, (△), or 100 (□) μM. Aliquots were removed and assayed at the indicated timepoints. The results are representative of four independent experiments. **(D)** Activation buffers containing 100 mM Hepes (○ or ●) or Ches (△ or ▲), 100 μM $NiCl_2$, and 0.2 mg/ml carbonic anhydrase (added to speed up the rate of equilibration) were incubated at 35°C in the presence of 0.3% CO_2 atmosphere. After a 30 min incubation, apoprotein (as above) was added and aliquots were removed for assay at 45 (open symbols) and 120 (closed symbols) min. The indicated pH values were measured at the conclusion of the experiment. The results are representative of two independent experiments.


A
400
300
200
100
0
S.A.(U/mg)
0
60
120
180
240
300
Time (min)


B
0.03
0.02
0.01
0.00
S.A.(U/mg)
0
3
6
9
12
Time (min)


C
400
300
200
100
0
S.A.(U/mg)
0
60
120
180
240
300
Time (min)


D
200
150
100
50
0
S.A.(U/mg)
6.5
7
7.5
8
8.5
9
pH

a clear pH dependence (Fig. 1D) with buffers of increasing pH leading to greater rates of activation. The pK_a of the activation process is 9 or greater; higher pH values could not be examined because of the excessive amounts of bicarbonate that would be present in the reaction mixture.

Urease activation kinetics, in which the extent of reaction is governed by the CO_2 concentration and the rate is controlled by the nickel ion concentration, can be accomodated by either an interaction of CO_2 with apoprotein prior to metal ion binding or formation of a metal ion/CO_2 complex that binds to the apoprotein. There exists a well characterized biological precedent for the former mechanism involving ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). This magnesium-dependent enzyme has been shown to be activated by reaction of a lysine residue with CO_2, where the resulting carbamate serves as a metal ligand (reviewed in 16). A similar activation step may occur in 1-aminocyclopropane-1-carboxylate (ACC) oxidase, a ferrous ion-dependent enzyme (17). As we observe for urease, the activation rates for these enzymes are enhanced at higher pH (18, 19). I propose that metallocenter assembly into urease apoprotein occurs by a pH-dependent reaction between a protein sidechain and an activating CO_2 molecule to generate a ligand that facilitates productive nickel binding. Unlike Rubisco and ACC oxidase, however, urease activation is not reversed by addition of metal ion chelators. We speculate that urease activation may include a protein conformational change that serves to trap the metallocenter.

The ability to partially activate urease apoprotein in the absence of any accessory protein compelled me to re-evaluate the previous suggestion that UreD functions as a molecular chaperone (8); thus, we examined the effect of CO_2 on activation of a mixture of UreD-urease apoprotein complexes [$(urease)_3UreD_N$, where N = 0, 1, 2, and 3; $\langle N \rangle \cong 1.6$]. The extent of activation was shown to depend on the bicarbonate concentration (Fig. 2A) and the rate was found to depend on nickel ion concentration (Fig. 2B) with concentration dependences and kinetics that closely resemble data obtained for urease apoprotein alone (Fig. 1A and 1C).

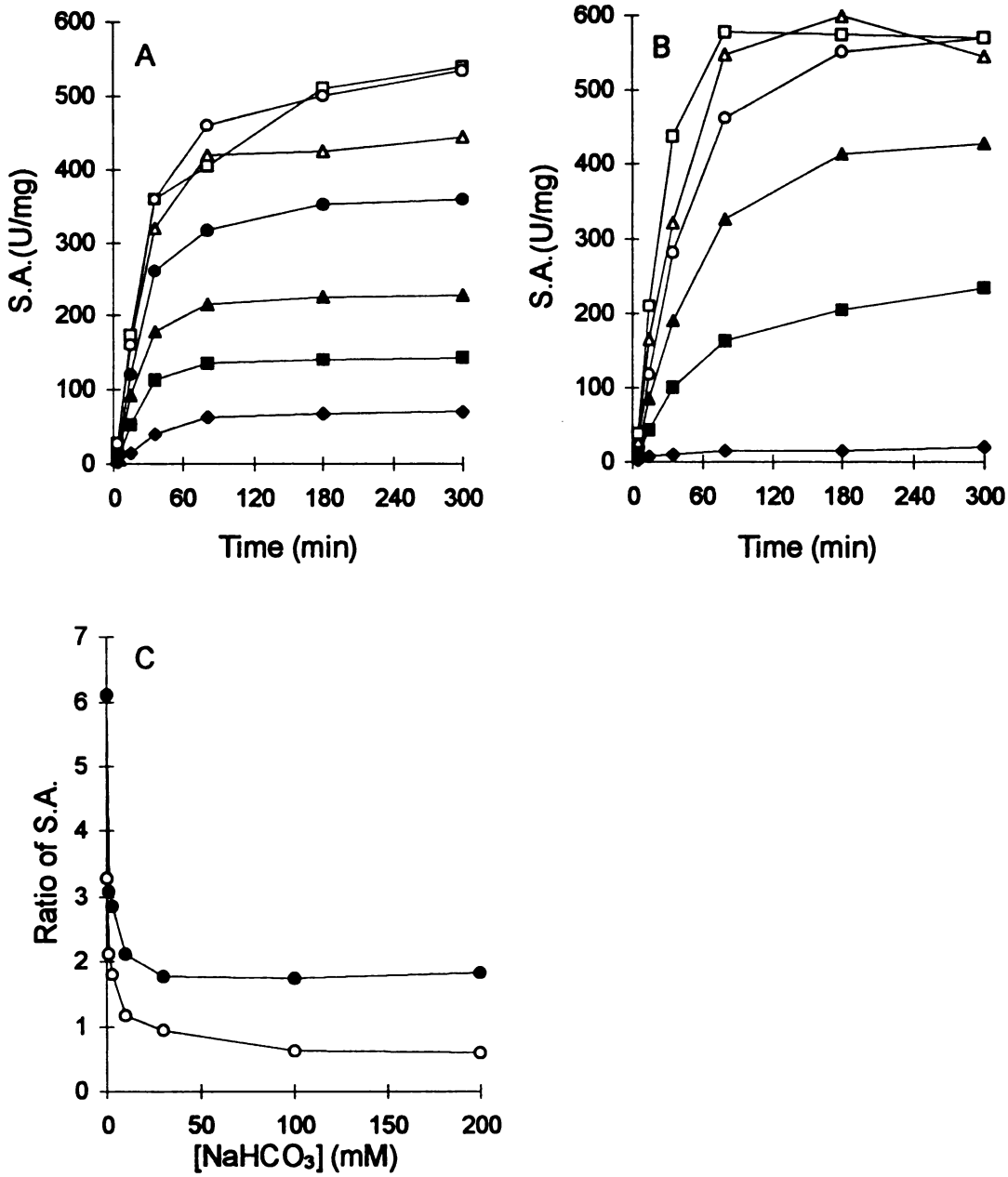


Fig. 2. *In vitro* activation of UreD-urease apoprotein complex. A mixture of the UreD-urease apoprotein complexes was enriched through the Mono Q HR 10/10 step of purification as previously described (8). The **(A)** bicarbonate dependence of activation and **(B)** nickel dependence of activation for the protein complex (0.8 mg/ml) was carried out as described for the apoprotein alone (Fig. 1A and Fig. 1C, respectively). The ratio of the specific activities that are generated during activation of the UreD-urease apoprotein and the urease apoprotein are compared as a function of bicarbonate concentration for 4 min (O) and 300 min (●) of activation.

Furthermore, 0.51 carbon molecules were incorporated into urease from ^{14}C-$NaHCO_3$ treatment of the ureD-urease apoprotein complex mixture, again in close agreement with the urease apoprotein studies. However, the final level of activation for the UreD-urease complex mixture is higher than for the urease apoprotein alone, with the effect most pronounced at the lowest bicarbonate concentrations (Fig. 2C). Indeed, the indicated ratios for activation are underestimated by a factor of 1.19 if one converts U/mg total protein to U/mg urease protein for the experiments that include UreD (20). In order to compare the effect of UreD on initial activation rates, the activities at 4 min were compared for the UreD-urease apoprotein complex and the apoprotein (Fig. 2C). The presence of UreD appears to accelerate the rate at low bicarbonate concentrations, but at concentrations above ~50 mM the rate is reduced in the presence of UreD. Like apoprotein, the UreD-urease apoprotein complex was shown to form a non-productive nickel-urease species; however, the presence of UreD appears to reduce the rate of this inactivation step. For example, 10 min incubation of urease apoprotein with 100 μM nickel ion prior to adding bicarbonate reduces its ability to be activated to 8.8% of that in which nickel ion and bicarbonate are provided simultaneously, whereas, UreD-urease apoprotein complex that is treated in a similar manner retains 25% of its ability to be activated. These results confirm the importance of UreD to urease activation at low concentrations of CO_2 and suggest a mechanism for this protein. I propose that UreD functions, at least in part, to minimize non-productive binding of nickel to apoprotein that lacks bound CO_2; i.e., the protein species that is expected to predominate at low CO_2 concentrations. This function, if verified by further studies, may still be considered that of a molecular chaperone in which UreD controls the proper sequence of activation steps so that CO_2 binds prior to nickel ion.

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6. *K. aerogenes* urease is comprised of three subunits (M_r 11,086, 11,695, and 60,304) arranged in a $(\alpha\beta\gamma)_3$ structure containing three active sites (4). Protein or urease concentrations indicated here refer to the $\alpha\beta\gamma$ catalytic unit.
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13. Calculation of the dissolved CO_2 concentration was based on the equation $[CO_2] = C_T[H^+]^2/(K_{a1}K_{a2} + K_{a1}[H^+] + [H^+]^2)$, where C_T is 10 mM, $[H^+]$ is 5.01×10^{-9} (for pH = 8.3), and values for pK_{a1} and pK_{a2} were 6.0 and 9.74 (interpolated from Tables 2.1 and 2.2 of ref. 12), respectively. Conversion from mM to % concentrations used Henry's law with pK_H estimated to be 1.61 (interpolated from the same source).
14. Urease apoprotein (0.5 mg/ml final concentration) was incubated in the standard activation buffer containing 100 μM $NiCl_2$ and 50 mM $NaHCO_3$ (1.44 mCi/mmole) for 9 h, quenched with EDTA (5 mM final concentration), and

chromatographed using a Mono Q 10/10 column as previously described (15). Following chromatography, the enzyme specific activity was measured and the specific radioactivity was determined using a scintillation counter with measurement of the stock $NaHCO_3$ radioactivity used as a control. Analogous experiments were carried out with a mixture of the UreD-urease apoprotein complexes at 0.79 mg/ml.

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20. For converting U/mg total protein to U/mg urease protein for the UreD-urease complexes, the measured specific activity was multiplied by M_r $(urease)_3UreD_N$ and divided by M_2 $(urease)_3$, where $(urease)_3$ has M_r 249,300 and UreD has M_r 29,807.
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CHAPTER 6

RELATED STUDIES, AND PROSPECTS FOR FURTHER RESEARCH

A. Characterization of αHis-219 and αHis-312 mutant proteins

The αHis-219 residue was suggested to be important for substrate binding, based on the high K_m value of the αH219A mutant protein (1). Two possible mechanisms to account for this result include (i) stabilization of urea binding by hydrogen-bond formation and (ii) maintenance of catalytic site accessibility by protein configurational support involving the imidazole group. Reductions in the rates of urease inactivation by alkylating agents and diethylpyrocarbonate (DEP) are consistent with diminished active site accessibility of the αH219A mutant protein. To further examine the function of this residue, I constructed and partially characterized glutamine (αH219Q) and asparagine (αH219N) mutant proteins. Unlike the alanine mutant, these changes may still enable hydrogen-bonding to take place, and these residues have more of a resemblance to histidine in terms of shape and size. Both mutant proteins have lower K_m values (~ 175 mM for αH219N or ~227 mM for αH219Q) compared to the alanine mutant protein (~ 2100 mM) (Table 1); however, the values are still higher than that of the wild-type protein (~ 2.25 mM). The αH219N protein, like αH219A, had 10~20-fold effects on the rate, whereas the αH219Q protein possessed a rate that was only ~1/2 that of the wild-type enzyme. The optimal pH for catalysis was found to be ~6.2 in the αH219N protein and ~6.5 in the αH219Q protein (Fig. 1A and B), compared to a value of 7.75 in the wild-type and H219A samples. The role of the αHis-219 residue remains unclear. Further characterization, e.g. selected chemical inactivation studies of these mutant proteins, may be useful to allow us to further define the function of this amino acid. Furthermore, additional synthesis of mutant proteins with bigger amino acids, such as tryptophan, in place of the αHis-219 residue may be necessary to confirm the effect of amino acid side chain size on substrate binding.

αHis-320 was proposed to act as a general base in catalysis (1). Evidence consistent with this hypothesis includes the rapid inactivation of the

Table 1. Characteristics of wild-type and selected mutant *Klebsiella aerogenes ureases*[a].

	K_m[c]	k_{cat}[c]		Assay[d]
Urease[b]	(mM)	U/mg	%WT	pH, buffer
Wild-type	2.25±0.04	296±4	100%	7.75, HEPES
H219A	2092±634	14±3	5%	7.75, HEPES
H219N	175±8	11.6±0.2	4%	6.2,MES
H219Q	227±21	134±6	45%	6.5,MES
H320A	10.9±0.6	0.0049±0.0002	0.0017%	6.2,MES
H320N	7.4±0.5	0.0043±0.0001	0.0015%	6.2,MES
H320Q	10.6±1.0	0.0024±0.0001	0.0008%	6.2,MES

[a] Determined for cell extracts. SDS-polyacrylamide gel electrophoretic analysis revealed that the urease expression levels were nearly identical in all constructs except H219N, that appears to have approximately half the urease protein of the others.

[b] For site-directed mutagenesis, a 1.1-kbp *Bam*H1-*Sal*I fragment of the pKAU17 was used as described previously (Chapter 3). The following oligonucleotides were used: GAAGATCCAAGAGGACTGG (H219Q), CTGAAGATCAATGAGGACTGG (H219N), GGTCTGCCAGCATCTGGAC (H320Q), GATGGTCTGCAACCATCTGG (H320N).

[c] Measured by using 50, 100, 130, 170, 250, and 1000 mM urea for the His-219 mutant proteins or 2, 2.5, 3.5, 5, 10, and 50 mM for other proteins.

[d] Assays were carried out in 25 mM buffer containing 0.5 mM EDTA.

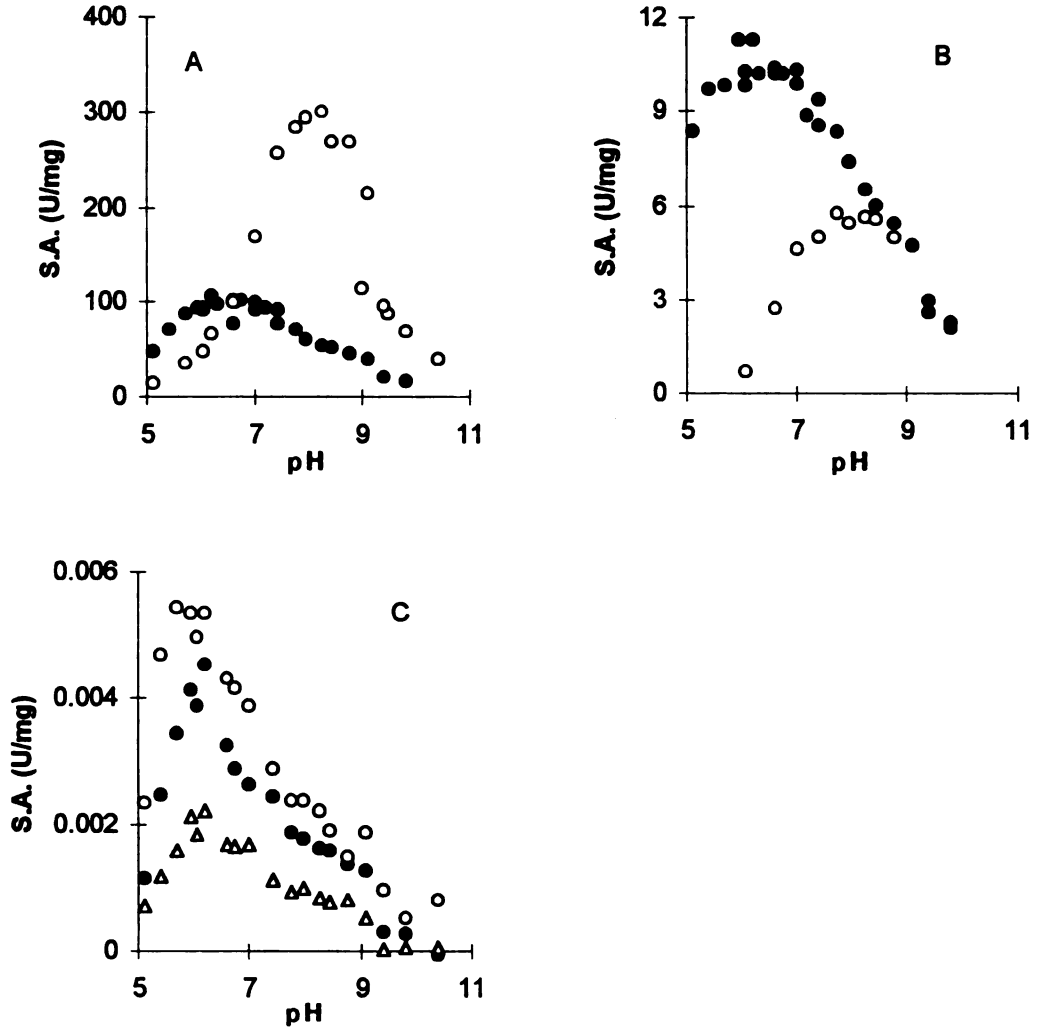


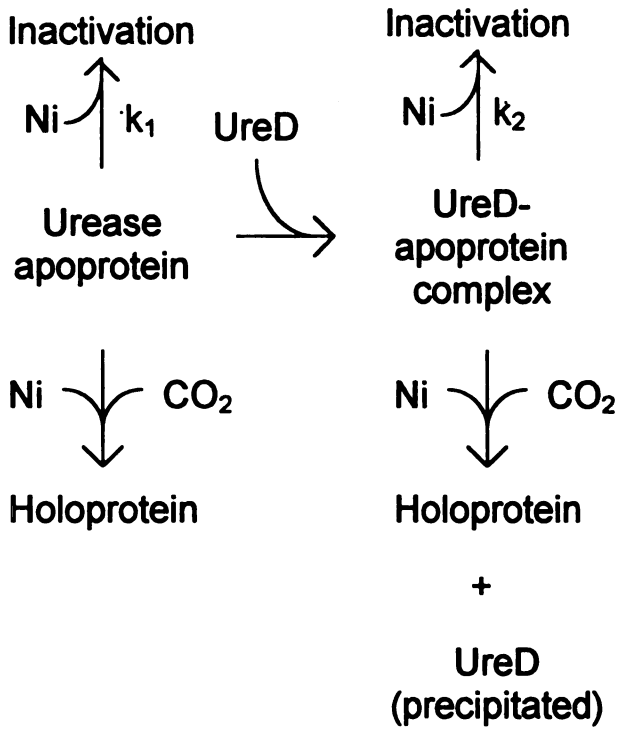
Fig. 1. The pH dependence of wild-type and mutant urease activity of cell extracts. The activities were measured for extracts of *E. coli* DH5 cells carrying pKAU17 or mutant plasmids that encode the following proteins: (A) wild-type (○) and the αH219Q mutant (●); (B) αH219A (○) and αH219N (●); (C) αH320A (●), αH320Q (△), and αH320N (○). The reaction mixtures contained urea (50 mM for wild-type enzyme and H320A, Q, and N proteins or 1 M for the αH219A, Q, and N proteins), 0.5 mM EDTA, and the following buffers at a concentration of 25 mM: MES (pH 5.1~6.19), HEPES (6.0~9.1), and CAPS (9.4~10.4).

wild-type enzyme by DEP (2), a histidine-selective modifier, and the resistance of αH320A mutant protein to inactivation by DEP (1). Two additional mutant proteins, αH320N and αH320Q, were synthesized to further examine this proposal. All three mutant proteins (αH320A, αH320Q, αH320N) have similar K_m values, specific activities, and pH dependencies in catalysis (Table 1, Fig. 1C). These results indicate that the histidine residue is probably not important for steric or hydrogen bonding interactions, rather these results are indirectly consistent with the role of αHis-320 as a general base for catalysis, as suggested previously (1).

B. Function of UreD in urease apoprotein activation

Activation of urease apoprotein requires CO_2 as well as nickel (3). UreD was previously shown to form three protein complexes with urease apoprotein (4), and a mixture of these complexes is also competent for activation in a CO_2-dependent manner (3). In both cases, the activation is apparently accompanied by a non-productive inactivation process; however, the presence of UreD appears to reduce the rate of this inactivation step. Based on this finding, the UreD protein was proposed to function, at least in part, to keep apoprotein from binding nickel non-productively in the absence of CO_2, as illustrated in Scheme 1, where k_2 is reduced compared to k_1. This hypothesis was further tested by characterizing the activation process for the partially resolved complex species.

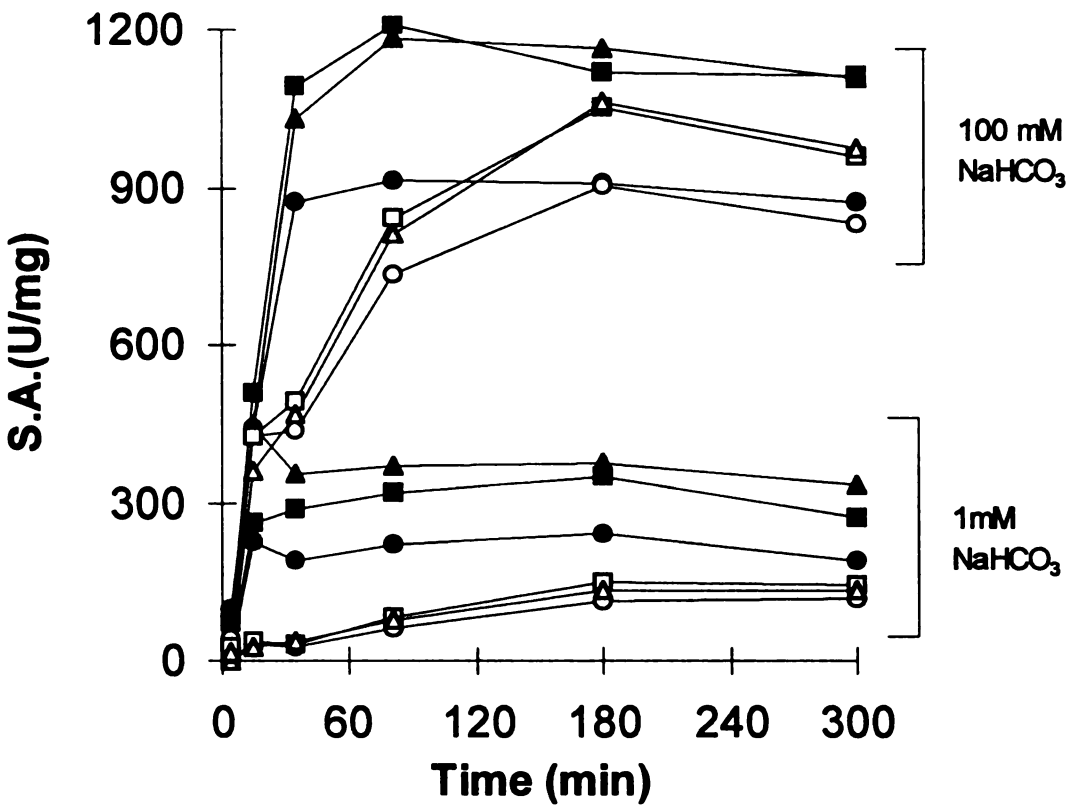
A molecule of urease apoprotein can associate with 1, 2, or 3 molecules of UreD (4). Each of the forms of UreD-urease apoprotein complex was enriched by chromatography of the mixture on a phenyl-Sepharose column. The urease activation kinetics look similar in all three pools (Fig. 2). At low concentrations (1 mM) of HCO_3^- (therefore, low concentrations of CO_2) the extent of activation for each complex was limited even in the presence of saturating concentrations (100 μM) of nickel ion (Fig. 2). Importantly, the presence of increasing numbers of UreD per urease apoprotein leads to higher activation levels in the low



<Scheme 1>

bicarbonate studies. In contrast, the level of activation reached near its maximum in the presence of high concentration (100 mM) of HCO_3^- regardless of concentration (20 vs. 100 μM) of nickel ion, and the effect of UreD number was less pronounced. The results are consistent with CO_2 being the limiting factor in

Fig. 2. *In vitro* activation of the three partially purified UreD-urease apoprotein complexes. The protein complexes were purified as previously described, with minor modifications (4). *E. coli* DH5[pKAUD2] cell extracts from a 2 L culture were chromatographed on a column (2.5 x 20 cm) of DEAE-Sepharose at 4°C in PEDG buffer [18 mM phosphate (pH 7.0), 0.09 mM EDTA, 10% glycerol, 0.09 mM dithiothreitol]. The proteins were eluted in the same buffer with a linear salt gradient to 1.0 M KCl. The protein complex-containing fractions were pooled, dialyzed against PEDG buffer, and applied to a Mono-Q HR 10/10 column equilibrated in the same buffer. The protein was eluted with a linear salt gradient to 1 M KCl in PEDG buffer. The pooled fractions containing the mixture of protein complexes was adjusted to 1 M KCl by addition of PEDG buffer containing 2 M KCl and applied to a phenyl-Sepharose HP (Pharmacia) column (2.6 x 10 cm) equilibrated in this buffer. A linear gradient to 0 M KCl in PEDG buffer was used to partially resolve the protein complexes into three pools. Samples were monitored for the presence of the UreD-urease apoprotein complexes by using native polyacrylamide gel electrophoresis. Pool 1 (eluted at 0.23~0.19 M KCl) contains apoprotein (19% of total protein), 1UreD-apoprotein (47%), 2UreD-apoprotein, (27%), and 3UreD-apoprotein (7%) (averaging to 1.00 UreD per apoprotein molecule). Apoprotein was undetectable in pool 2 (eluted at 0.11~0.08 M KCl) which possesses 1UreD-apoprotein (16% of total protein), 2UreD-apoprotein (58%), and 3UreD-apoprotein (26%) (averaging to 2.00 UreD per apoprotein molecule). In pool 3 (eluted at 0 M KCl) only 2UreD-apoprotein and 3UreD-apoprotein were detectable (14% and 86% of total protein, respectively. averaging to 2.83 UreD per apoprotein molecule). Each pool was again chromatographed on a Mono-Q HR 10/10 column, as described above. The three species of UreD-apoprotein complex (0.2 mg/ml or 2.4 μM final concentration; circle: pool 1, square: pool 2, triangle: pool 3) were added to activation buffer [100 mM Hepes (pH 8.3), 150 mM NaCl, 10 mM EDTA] containing 20 (open symbols) or 100 (closed symbols) μM $NiCl_2$ and 1 or 100 mM $NaHCO_3$ at 35°C. Aliquots were removed over time to monitor for urease



activity. Enzyme activity was assayed as described elsewhere (1). In order to convert the specific activity values based on total protein (urease plus UreD) into values of urease specific activity, the rates were multiplied by factors of 1.12 for pool1, 1.24 for pool 2, and 1.34 for pool 3.

urease apoprotein activation and with the presence of UreD serving a more important role at low CO_2 levels.

Each of the UreD-urease apoprotein complexes lost their ability to be activated by incubation in the presence of 100 μM $NiCl_2$ prior to addition of bicarbonate. For example, an 80 min incubation with nickel almost completely abolished the ability of these proteins to be activated (Fig. 3). A 10 min incubation reduced the level of final activation to only 25~30% of the control samples (Fig. 3). Importantly, the number of UreD bound per apoprotein affected the extent of inactivation with increasing protection observed with increasing UreD molecules bound per apoprotein.

The initial activation rates of each complex were examined under four different conditions. When the activation was carried out at saturated concentrations of HCO_3^- (100 mM) and $NiCl_2$ (100 μM), the rate in the pool 1 sample was the fastest (Fig. 4A). This suggests that an increasing number of UreD per urease apoprotein may hinder the rate of activation under these conditions. However, the rate of pool 3 activation becomes the fastest and that of pool 1 the slowest (Fig. 4B) when high concentrations of $NiCl_2$ (100 μM) are used in the presence of low concentrations of CO_2 (no addition of $NaHCO_3$). The difference apparently increases (Fig. 4C) when the concentration of $NiCl_2$ is even higher (200 μM). From these results it can be hypothesized that either (i) the complex is better at incorporating CO_2 than the apoprotein alone under low concentration of CO_2 or (ii) the presence of UreD may protect the urease apoprotein from undergoing nickel-induced inactivation. A low concentration of $NiCl_2$ (20 μM) results in pool 1 exhibiting the fastest activation among the three pools even in the presence of low CO_2 (Fig. 4D). This result is compatible with hypothesis (ii); *i.e.*, UreD may somehow prevent the nonproductive inactivation of urease apoprotein.

C. Characterization of UreD

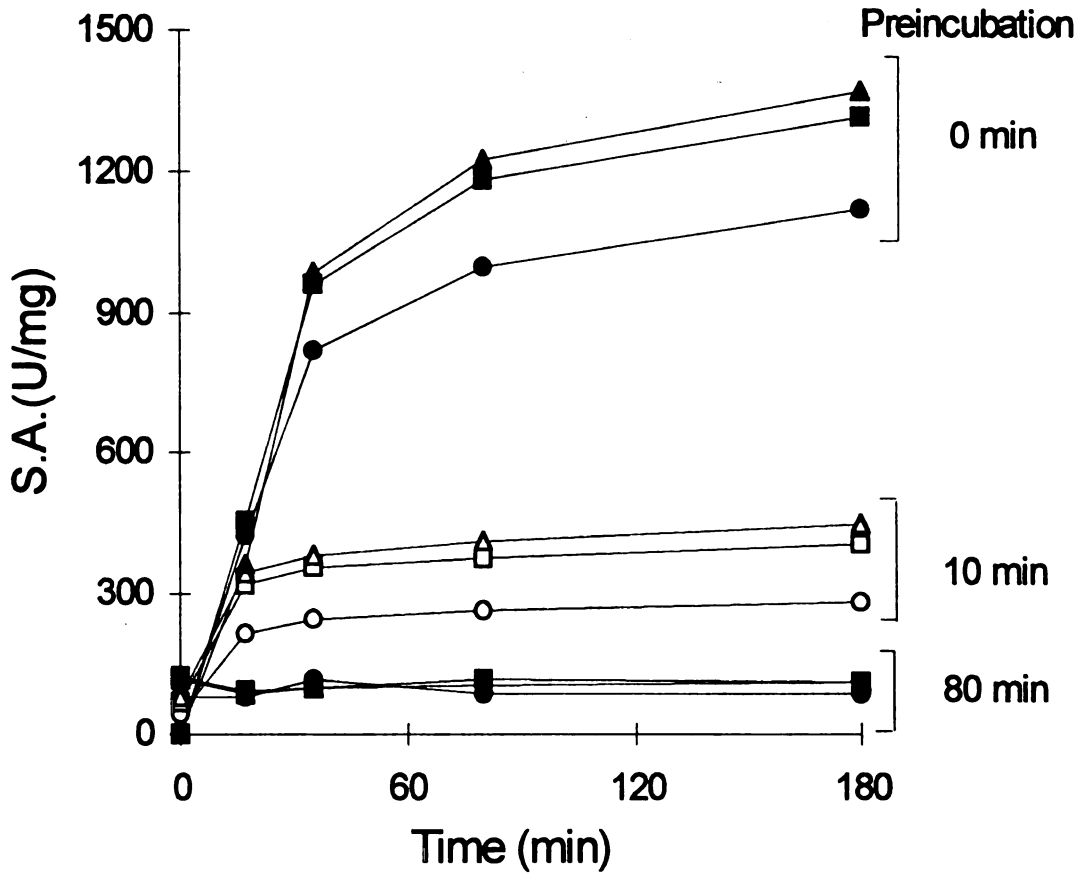


Fig. 3. Inactivation of the three UreD-urease apoprotein complexes by incubation with nickel prior addition of $NaHCO_3$. The UreD-urease apoprotein complexes (0.2 mg/ml; circle: pool 1, square: pool 2, triangle: pool 3, see Fig. 2 for details) were incubated with the activation buffer containing 100 μM $NiCl_2$ for 0, 10, or 80 min. $NaHCO_3$ (100 mM, final concentration) was added at time 0 and aliquots were removed over time to monitor for urease activity. Calculation of urease specific activities was done as described in Fig. 2.

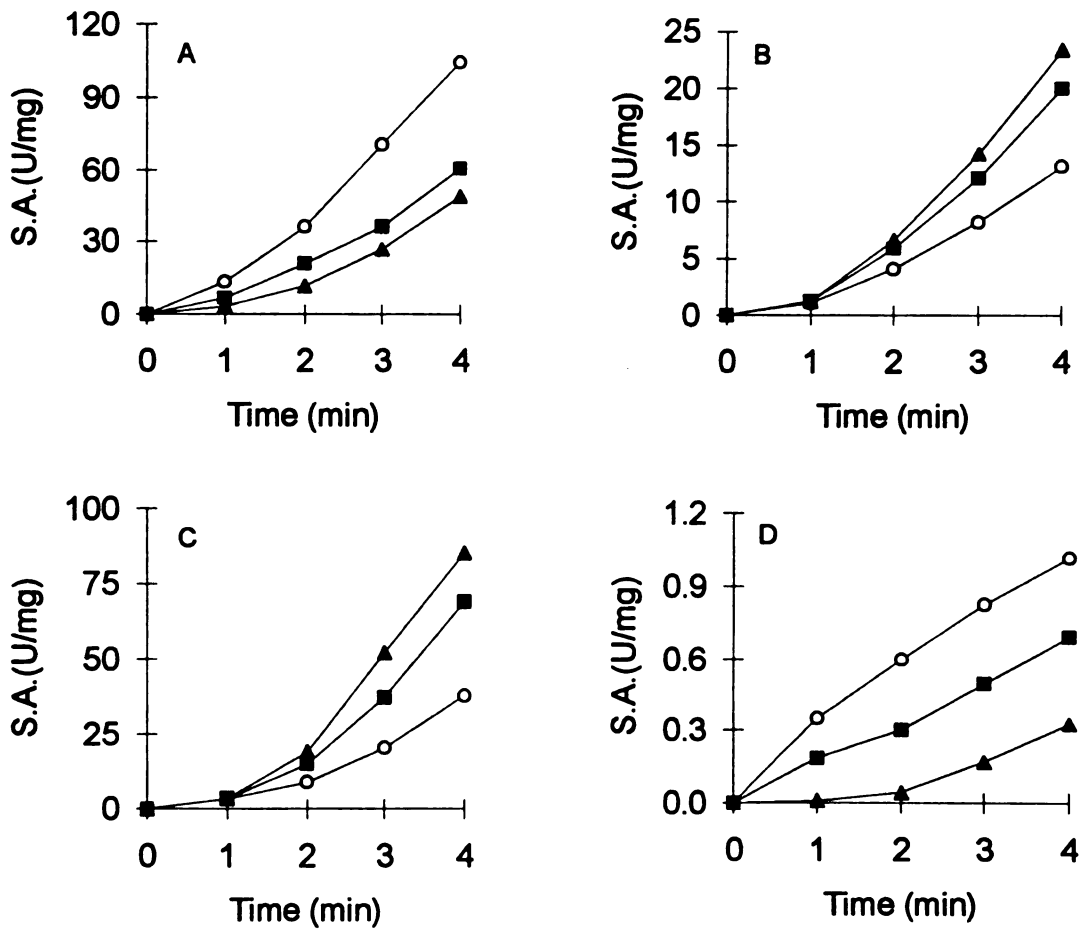


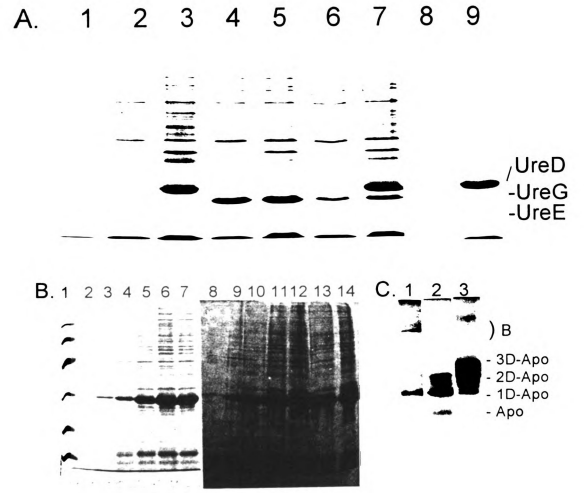
Fig. 4. Initial activation rates of UreD-urease apoprotein complexes. The UreD-urease apoprotein complexs (0.2 mg/ml; ○, pool1, ■, pool 2, ▲, pool 3, see Fig. 2 for details) were added to the activation buffer containing (A) 100 μM $NiCl_2$ and 100 mM $NaHCO_3$, (B)100 μM $NiCl_2$ and no added $NaHCO_3$, (C) 200 μM $NiCl_2$ and no added $NaHCO_3$, or (D) 20 μM $NiCl_2$ and no added $NaHCO_3$, all at 35°C. Aliquots were removed over time to monitor for urease activity. Specific activity corrections were done as described in Fig. 2.

UreD has been purified only as one of three UreD-urease apoprotein complexes (4). Further characterization of UreD is required to test the validity of its suggested function as a molecular chaperone in apoprotein activation (3, 4). In this section, I describe the partial characterization of UreD and my efforts to examine the formation of the UreD-urease apoprotein complex.

pKAUD6 (see Appendix) was constructed to contain only the *ureD* gene without the urease structural genes or other accessory genes. Samples prepared from *E.coli* DH5 containing this plasmid possess large amounts of UreD, which, however, was found only in the insoluble fraction (Fig. 5A, lane 3). The presence of other accessory gene products (UreE, UreF, and UreG) does not appear to be helpful for maintaining solubility of UreD (Fig. 5A). Several detergents tested (such as NP-40, Tween-20, and Triton X-100) at up to 3% concentration do not solubilize the protein. The protein was found to be capable of solubilization in the presence of urea (~6 M) or guanidine-HCl (~4 M) (Fig. 5B). This result suggests that UreD forms an inclusion body when synthesized in large amounts. It is unclear whether low level synthesis of UreD may result in a soluble protein. The denaturant (8 M urea or 4 M guanidine HCl)-solubilized protein was diluted into buffer alone or into buffer containing 1% detergent and found to be insoluble under these conditions (data not shown)

To test whether another accessory gene (*ureE, ureF* or *ureG*) is required for formation of the UreD-urease apoprotein complex, plasmid pKAUDABC containing only *ureD, ureA, ureB* and *ureC* (see Appendix) was constructed. *E.coli* containing this plasmid was shown to possess UreD-urease species (Fig. 5C), indicating that formation of the UreD-urease apoprotein complexes does not require other accessory genes. The cell extracts prepared from *E. coli* [pKAUDABC] appear to have a similar level of ability to be activated *in vitro* as that from wild-type extracts (Chapter 4, Fig. 1). This result suggests that other accessory proteins (UreE, UreF, and UreG) are not essential for forming a urease apoprotein species that is capable of activation *in vitro*.

Fig. 5. Characterization of UreD. (A) Samples were prepared from *E.coli* cells containing pKAUD6 (lane 2, 3), pKAUG1 (lane 4, 5), pKAUDG1 (lane 6, 7), or pKADEFG (lane 8, 9) (see Appendix for maps). Whole disrupted cells (lane 3, 5, 7, 9) or the supernatants (lane 2, 4, 6, 8) of the cell extracts after centrifugation at 15,000 *g* for one hour were analyzed on SDS- polyacrylamide (12%) gel electrophoresis. Lane 1 is molecular weight markers (phosphorylase *b*, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400). (B) UreD present in *E. coli*[pKAUD6] cells was tested for solublization in buffer containing 25 mM Hepes (pH 7.9), 150 mM NaCl, 1 mM EDTA, and 10% glycerol supplemented with 3, 4, 5, 6, 7, and 8 M urea (lane 2~7), or 1, 2, 3, 4, 5, and 6 M guanidine HCl (lane 8~13). After incubation at 0°C for 30 min, the cell extracts were centrifuged for 5 min at 15,000g. The supernatants were analyzed on SDS- polyacrylamide (13.5%) gel electrophoresis. Lane 1 is molecular weight markers. Disrupted cells are shown in lane 14. (C) Western blot comparison of cell extracts of *E. coli* [pKAU17] (lane 1), *E. coli* [pKAUDABC] (lane 2) and cell extracts of *E. coli* [pKAUD2] (lane 3). Cell extracts were run on 6% native gel and probed with anti-urease antibody (5).


A. 1 2 3 4 5 6 7 8 9
UreD
-UreG
-UreE
B. 1 2 3 4 5 6 7 8 9 10 11 12 13 14
C. 1 2 3
) B
- 3D-Apo
- 2D-Apo
- 1D-Apo
- Apo

D. Possible role of UreF

The *ureF* gene is absolutely essential for the formation of active urease *in vivo* (6). This gene is expressed at very low levels, apparently due to a weak Shine-Dalgarno (SD)-sequence (ribosome-binding site) in front of the structural gene (7). I made two site-directed mutants (pKAUF1 and pKAUF2, Fig. 6) which have changes in their ribosome binding site regions leading to matches with 3 or 5 positions compared to the consensus SD-sequences. However, the desired high level expression of UreF was not observed in cell extracts from these mutants. Although the reason for continued low level expression of *ureF* is not clear, there is still a prospect for the high level synthesis of UreF if the environment around the *ureF* gene is altered, e.g., if the other genes are deleted.

While comparing UreD-urease complex samples from cells containing the intact gene cluster and various deletion mutants, a difference was observed that provides evidence for a role of UreF in complex formation. The major UreD-urease apoprotein complex in cell extracts prepared from *E. coli* [pKAU17] elutes earlier from a Sepharose 6 column than does the apoprotein, consistent with a larger hydrodynamic radius for UreD-urease than for urease alone (data not shown). Similar elution patterns are observed for extracts from cells carrying pKAUΔE-1 (Fig. 7A) or pKAUΔG-1 (Fig. 7C) (*i.e.*, *ureE* and *ureG* deletion mutants). Surprisingly, the UreD-apoprotein complex in extracts from cells carrying pKAUΔF, a *ureF* deletion mutant, elutes at the same position as the apoprotein (Fig. 7B). This result suggests that the UreF protein may be involved in conversion of a highly compact form of UreD-urease apoprotein complex to a less compact structure where these forms are not resolved by native polyacrylamide gel electrophoresis. It is possible that such a conformational change is related to the decreased level of *in vitro* urease activation observed in *E. coli* (pKAUΔF) cell extracts compared to a control sample or to samples possessing *ureD* or *ureG* deletions (4).

pKAU17 ··· CAGCCACTAGCATGTC···
pKAUF1 ··· CAGAGACTAGCATGTC···
pKAUF2 ··· CAGGAGCTAGCATGTC···

Fig. 6. Site-Directed Mutagenesis of the *ureF* Ribosome Binding Site. In order to enhance expression of *ureF*, the ribosome binding site of this gene was changed as shown above (underlined sequences) by site-directed mutagenesis methods. A 1.8-kb *Bam*H1-*Avr*II fragment of plasmid pKAU17 containing the urease gene cluster from *K. aerogenes* was subcloned into M13 mp18 and mutagenized by the method of Kunkel *et al.* (8). Uracil-containing single-stranded template DNA was prepared from *Escherichia coli* CJ236 (*dut1 ung1 thi-1 rel*A1/pCJ105[*cam*^{r}F']). Using GACATGCTAGTCTCTGTGAGCGTG (pKAUF1) or GTCGACATGCTAGCTCCTGTGAGCGTGGTG (pKAUF2) oligonucleotide primers, the phage DNA was mutagenized and mutant phage were isolated in *E. coli* MV1193([*lacI-pro*AB] *rpsL thi endA spcB*15 *hsdR*4 [*srl-rec*A]306::Tn10[*tet*^{r}] F'[*traD*36 *pro*AB$^+$ *lacI*^{q}*lacZ*M15]). The site-directed mutants were identified by sequence analysis using Sequenase 2.0 (U.S. Biochemical Corp., Cleveland OH) and the single-strand DNA sequence method of Sanger *et al.* (9). The region was completely sequenced to ensure that no other mutations had been introduced into M13. The mutated *BamH1-AatII* region was subcloned back into pKAU17 to generate pKAUF1 and pKAUF2 and the sequence was confirmed by double-stranded DNA sequencing methods. Putative ribosome-binding sites and the start codon are highlighted.

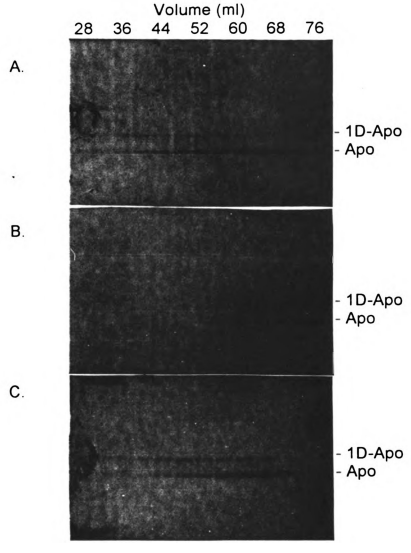


Fig. 7. Western blot comparison of fractions from Sepharose 6 (Pharmacia) column chromatography of selected cell extracts. Cell extracts were prepared from (A) *E.coli* [pKAU17ΔE-1], (B) [pKAU17ΔF], and (C) [pKAU17ΔG-1] and applied to a Sepharose 6 column (2.6 x 50 cm). Fractions (4 ml) were collected and aliquots were analyzed on a 6% native gel, transferred to nitrocellulose membrane, and detected by using anti-urease antibody and an alkaline phosphatase-conjugated goat anti-rabbit-IgG.

E. Presence of a complex containing UreD, urease apoprotein, UreF, and UreG

In addition to the three UreD-urease apoprotein complexes described earlier (4), faint bands of much lower mobility that contain anti-urease antibody cross-reactive material are detected in cell extracts from *E. coli* DH5 containing pKAU17 (wild-type), pKAU17ΔE-1 and pKAUD2 (abbreviated as D^+, leading to high level synthesis of UreD) in western blotting studies using anti-urease antibody (lane 1 and 3 of Fig. 5C, and lane 1 and 3 of Fig. 8). The bands are designated B in the figures because they comprise a second or B series of complexes that are distinct from the faster migrating UreD-urease complexes already discussed. Importantly, analogous bands were not detected in the *ureEFG* deletion mutant (lane 2, Fig. 5C) or in *ureD, ureF*, or *ureG* deletion mutants (lane 2, 4, 5 of Fig. 8). It is very intriguing that cells found to be activation-competent *in vivo* possess the B complexes, whereas *ureD, ureF*, or *ureG* deletion mutants, which are not competent for *in vivo* activation, do not.

The B complexes were enriched from extracts of cells containing pKAU17 and pKAUD2 by using DEAE-Sepharose and Mono-Q FPLC column chromatographies. The species do not separate from the UreD-urease apoprotein complexes when using Sepharose 6 size-exclusion column chromatography. This was unexpected because the B complexes migrate much more slowly than the UreD-urease apoprotein complexes during the native polyacrylamide gel electrophoresis. Although overlapping, the peak fractions of the UreD-urease complexes elute before the peak fractions of the B complexes (Fig. 9A). When the same fractions are examined by denaturing gel electrophoresis (Fig. 9B), it is clear that the fractions containing the B complexes possess several bands in addition to those corresponding to urease and UreD. One of these additional bands was demonstrated to be UreG by a western blotting experiment using anti-UreG antibody (not shown). The other band below

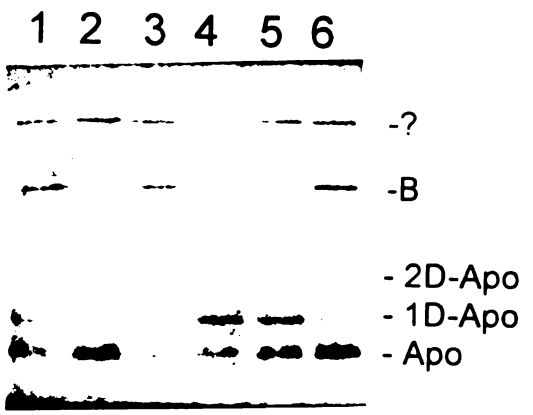
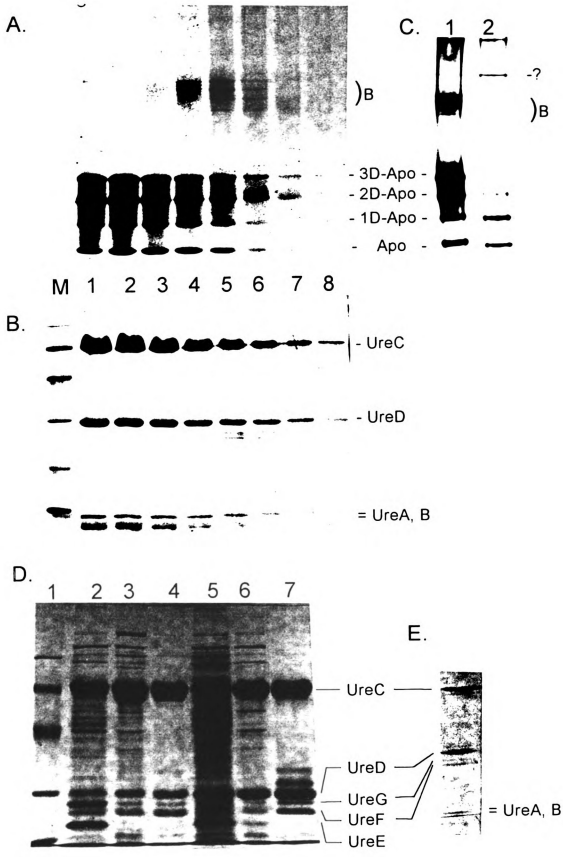


Fig. 8. Western blot comparison of cell extracts. Lane 1, *E. coli* [pKAU17]; Lane 2, *E. coli* [pKAU22ΔD-1]; Lane 3, *E. coli* [pKAU17ΔE-1]; Lane 4, *E. coli* [pKAU17ΔF]; Lane 5, *E. coli* [pKAU17ΔG-1]; Lane 6, *E. coli* [pKAUF1] (see Fig. 6). The cell extracts were analyzed on a 5% native gel, transferred to a nitrocellulose membrane, and probed with anti-urease antibody.

Fig. 9. Partial purification and characterization of the urease-containing B complexes. Cell extracts from *E. coli* DH5 containing pKAU17 or pKAUD2 were chromatographed on a column of DEAE-Sepharose at 4°C in PEDG buffer. The proteins were eluted in the same buffer with a linear salt gradient to 1.0 M KCl. The B protein complex-containing fractions were pooled, dialyzed against PEDG buffer, and applied to a Mono-Q HR 10/10 column equilibrated in the same buffer. The proteins were eluted with a linear salt gradient to 1 M KCl in PEDG buffer with the B complexes eluting at ≈0.5 M KCl. (A) Native polyacrylamide gel electrophoretic analysis of Mono-Q fractions eluting from 0.43 to 0.51 M KCl from D^+ cells possessing elevated levels of UreD. (B) SDS-polyacrylamide gel electrophoretic analysis of the same Mono-Q fractions. M represents molecular weight markers (see Fig. 5). (C) Nondenaturing polyacrylamide gel electrophoretic analyses of Mono-Q pool of B-complex-containing fractions from *E. coli* DH5 [pKAUD2] (lane 1) or *E. coli* DH5 [pKAU17] (lane 2). (D) SDS-polyacrylamide gel electrophoretic analysis of selected pools containing B complexes. Lane 1, molecular weight markers. Lane 2, 3, and 4: cell extracts, DEAE-Sepharose pool, and Mono-Q pool of *E. coli* DH5 [pKAU17], respectively. Lane 5, 6, and 7: cell extracts, DEAE-Sepharose pool, and Mono-Q pool of *E. coli* DH5 [pKAUD2]. (E) 2D gel electrophoretic analysis of the B complex. The native gel portion containing the B complexes of the Mono-Q pool of *E. coli* DH5 [pKAUD2] was excised, denatured, and eletrophoresed using an SDS-polyacrylamide gel.


A.
)B
- 3D-Apo -
- 2D-Apo -
- 1D-Apo -
- Apo -
C.
1
2
-?
)B
M 1 2 3 4 5 6 7 8
B.
- UreC
- UreD
= UreA, B
D.
1 2 3 4 5 6 7
E.
UreC
UreD
UreG
UreF
UreE
= UreA, B

UreG on the gel was excised and shown to have an N-terminal amino acid sequence that agrees with that deduced for UreF from the DNA sequence (7). As found for extracts of cells carrying pKAUD2, the pooled fractions from cells containing pKAU17 exhibit bands corresponding to the B complexes (Fig. 9C) and these fractions also appear to possess the UreF and UreG peptides (Fig. 9D). When the bands corresponding to the B complexes were excised from a native gel and re-electrophoresed on a denaturing gel, both the UreG and UreF bands were present as well as the urease subunits and UreD (Fig. 9E). Thus, I suggest that the B complexes are composed of UreD, urease apoprotein, UreF, and UreG.

Urease apoprotein that is associated with the B complexes is apparently capable of activation (Fig. 10). Upon incubation of the D^+ Mono-Q fraction with 100 μM $NiCl_2$ (when this experiment was carried out, the involvement of CO_2 in the activation process was not known), the protein was found to undergo a change such that when analyzed by native gel electrophoresis the mutiple B complex bands collapsed (Fig. 10A) in a manner which is reminiscent of UreD-urease apoprotein activation (Chapter 4, Fig. 4). Enzyme activity was measured in individual gel slices (Fig. 10B). As expected, significant levels of activity were associated with the region associated with the UreD-urease apoprotein complexes (region 1, 2, 3, and 4 in Fig. 10A) (Fig. 10B). Significant activity was also detected in region 6 containing the B complex (Fig. 10B). Enzyme activation in the region appears to be complete at 30 min (Fig. 10C). The K_m value determined for the enzyme in the slice of gel was ~10 mM which is a little higher than that of urease (~3 mM, measured in the same way, or ~2.25 mM, measured by using urease in solution). ATP, GTP, or high salt (up to 1 M KCl) does not dissociate the B complex into its components (data not shown).

Purification of the complex will be essential for its further characterization.

F. Urease apoprotein activation: possible scheme

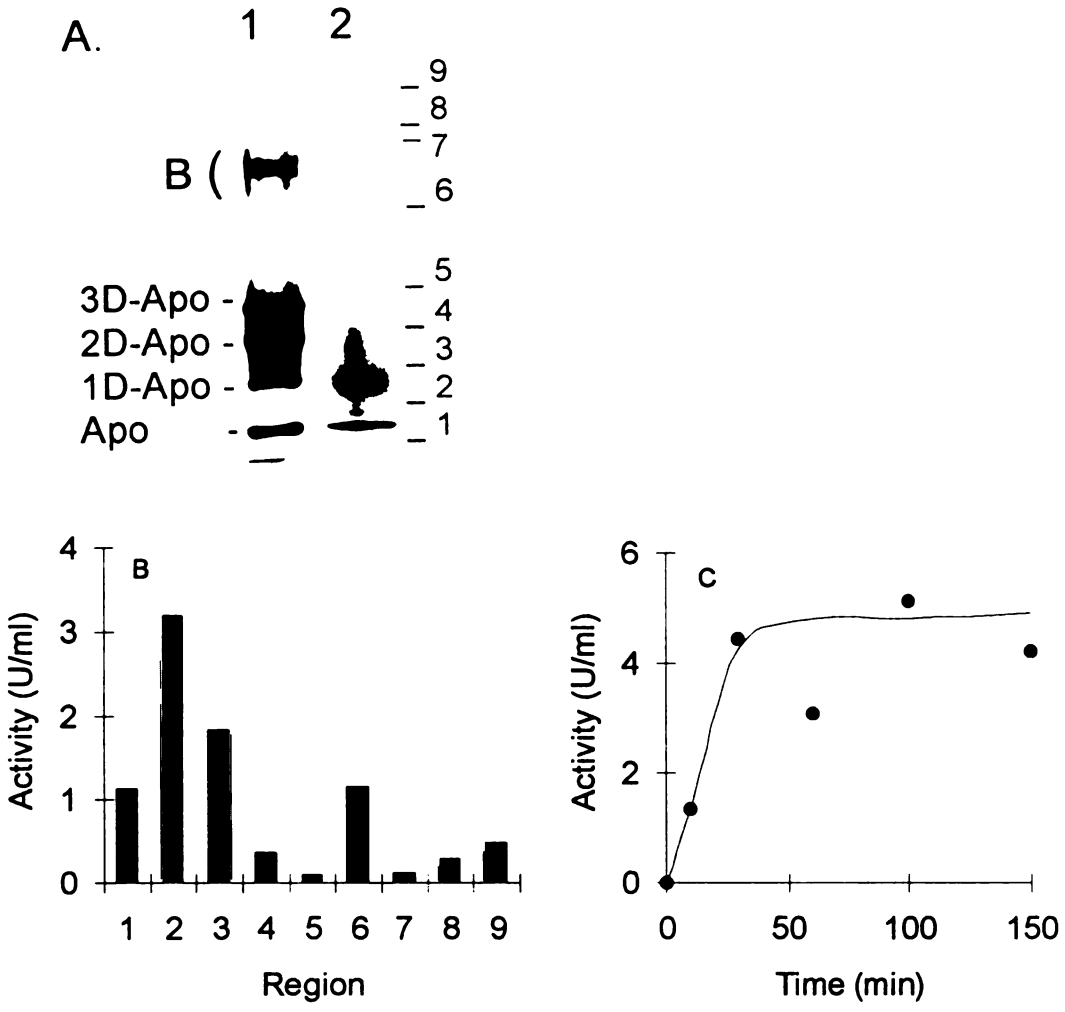
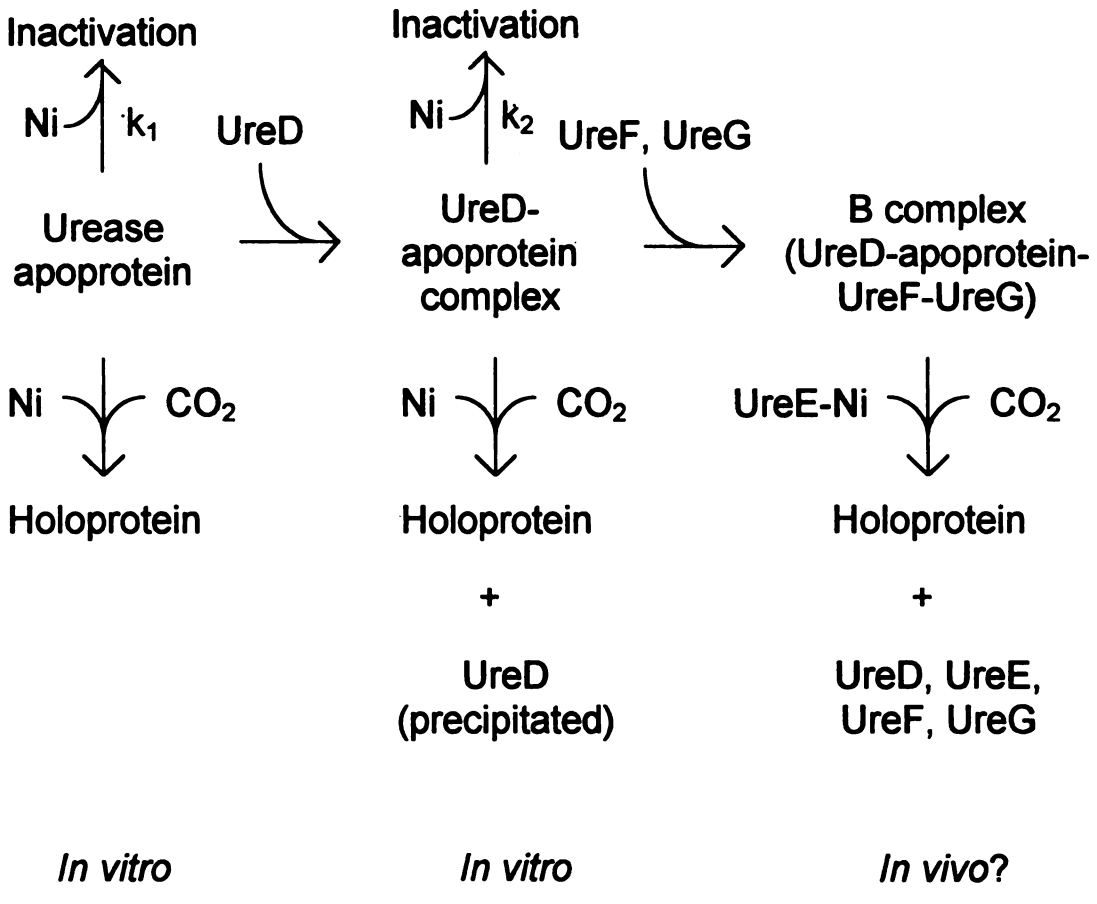


Fig. 10. Activation of urease apoprotein associated with the B complexes. (A) The Mono-Q pool of *E. coli* DH5 [pKAUD2] was incubated for 0 or 100 min (lane 1 and lane 2, respectively) at 37°C in the presence of 0.1 mM $NiCl_2$ and analyzed by native gel electrophoresis. (B) The same sample was incubated as described in (A) for 30 min and the urease activities were measured for 9 gel slices, cut as shown in A. (C) The effect of incubation time on the level of activity was measured in the gel slice corresponding to region 6.

Urease apoprotein alone can be activated *in vitro* by providing nickel in the presence of CO_2. UreD-urease apoprotein is also activation-competent *in vitro*, but the presence of UreD and urease subunits is not sufficient for forming active enzyme in the cell. The activation of urease *in vivo* absolutely requires UreF and UreG, as well as UreD (6). It is reasonable that conditions *in vivo* should be different from that found *in vitro*; for instance, the concentration of nickel or CO_2 may be limited *in vivo*. The accessory gene products may serve to provide these activating factors. For example, UreE is known to bind nickel ion with high specificity suggesting that the protein may be the nickel donor. The B complexes are composed of UreD, UreF, UreG and urease apoprotein. It is intriguing that the B complexes have been found only in the activation-competent wild-type, UreD-overexpressor (D^+), and UreE deletion mutant cells. From these results, I propose that the B complexes are required for *in vivo* activation of urease, as illustrated in Scheme 2.


Inactivation
Ni
k1
UreD
Urease apoprotein
Ni
CO2
Holoprotein
In vitro
Inactivation
Ni
k2
UreF, UreG
UreD-apoprotein complex
Ni
CO2
Holoprotein
+
UreD (precipitated)
In vitro
B complex (UreD-apoprotein-UreF-UreG)
UreE-Ni
CO2
Holoprotein
+
UreD, UreE, UreF, UreG
In vivo?

<Scheme 2>

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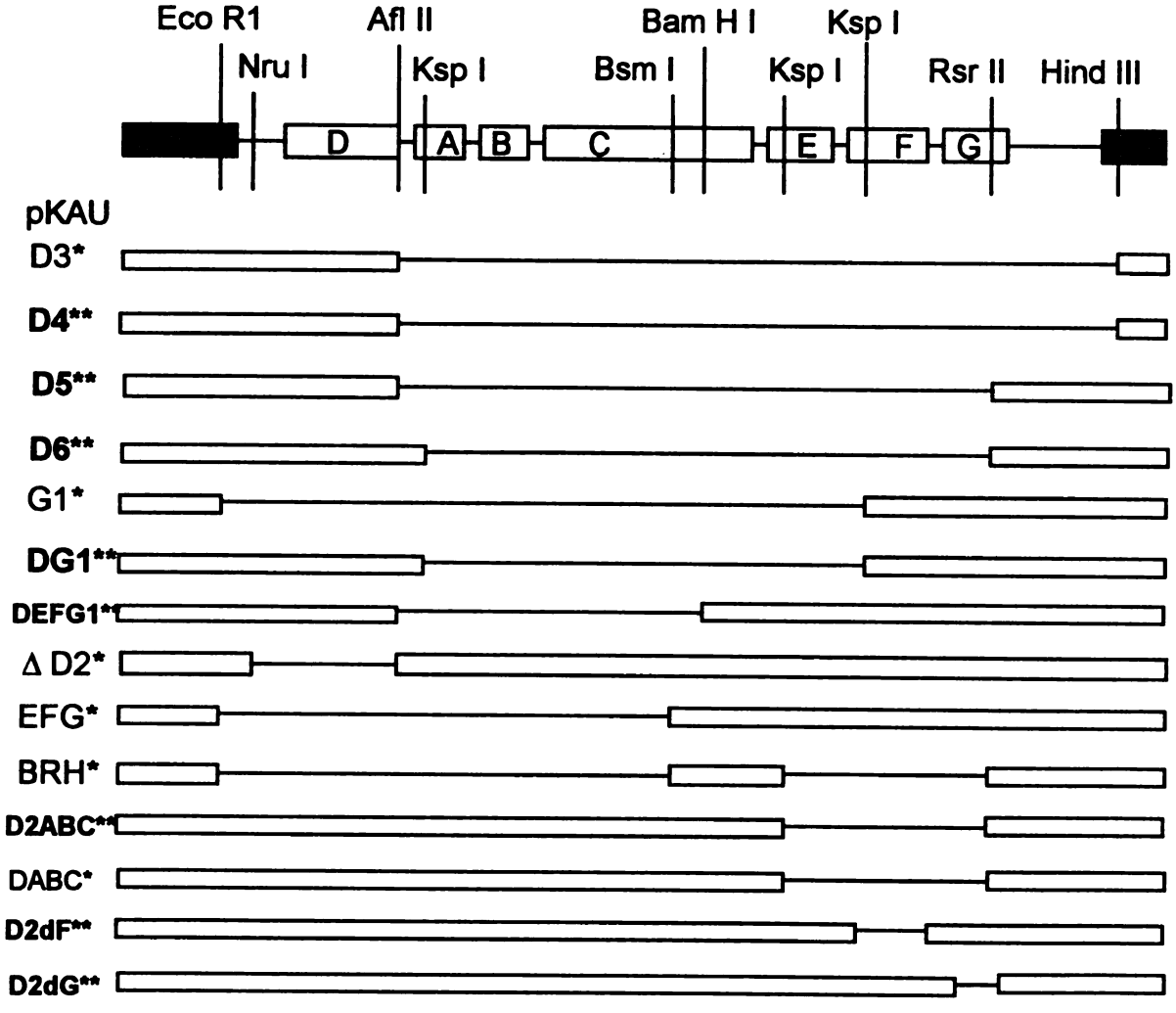
Appendix

pKAU17, pKAUD2 and their deletion mutants



* Derived from pKAU17

** Derived from pKAUD2

——— : Deletion

■ : pUC8

□ : Urease gene