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ACTIVE SITE STUDIES ON *KLEBSIELLA AEROGENES* UREASE, A NICKEL-CONTAINING ENZYME

By

Matthew J. Todd

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ABSTRACT

ACTIVE SITE STUDIES ON *KLEBSIELLA AEROGENES* UREASE,
A NICKEL-CONTAINING ENZYME

By

Matthew J. Todd

Although ureases play important roles in microbial nitrogen metabolism and in the pathogenesis of several human diseases, little is known of the mechanism of urea hydrolysis. As a paradigm for microbial ureases, the enzyme from *Klebsiella aerogenes* was studied. This enzyme was purified 1070-fold and shown to consist of three polypeptides (estimated M_r = 72,000, 11,000, and 9,000) in an apparent $\alpha_2\beta_4\gamma_4$ quaternary structure, containing 4 mol nickel/mol urease. A tight-binding inhibitor was exploited to demonstrate the presence of two mol active site/mol enzyme, thus each catalytic unit ($\alpha\beta_2\gamma_2$) has two nickel. The heteropolymeric subunit structure was not expected since the well-studied plant urease from the jack bean is homopolymeric.

K. aerogenes urease is competitively inhibited by thiols, boronic acids, hydroxamates, phosphate and phosphoroamides. UV-visible absorbance studies demonstrated charge-transfer interactions between thiolate anions and

urease nickel ion, consistent with the presence of at least one nickel per catalytic unit. Comparison of K_i values for several thiolate inhibitors were consistent with electrostatic repulsion of anionic inhibitors by an anionic group on the enzyme near the thiolate binding site. Acetohydroxamate (AHA) and phenylphosphorodiamidate (PPD) were slow-binding competitive inhibitors with K_i values of 2.6 μM and 94 pM, respectively. Following initial binding to one nickel, the inhibitors were proposed to form complexes bridging the two urease active site nickel ions.

The kinetics of *K. aerogenes* urease thiol modification were studied using alkylating and disulfide reagents. Reactivity of the essential thiol was affected by substrate and competitive inhibitors, consistent with a cysteine located proximal to the active site. The atypical pH dependence for the rate of inactivation was interpreted as arising from an interaction between the thiol and a second (as yet unidentified) ionizing group. The competitive inhibitors phosphate and PPD (but not AHA) protected one mol thiol/mol catalytic unit from modification by 2,2'dithiodipyridine. Using ^{14}C-iodoacetamide, the essential cysteine was identified as Cys_{319}, an amino acid conserved among all known urease sequences.

to my wife and family

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As with any major work, the production of a dissertation is bound to inconvenience and even burden those around us. Recognizing this, I would like to acknowledge two individuals whose sacrifices helped to make it possible. Dr. Hausinger, not only as mentor, but also as a friend gave me much needed guidance and then retreated, allowing me to fail or succeed on my own. Janet, my wife, was willing to sacrifice her own time and energy, allowing me to pursue these studies. During most of our marriage, she has endured considerably less attention than she desired, without excess complaint, while reminding me that there is a "real" world, around which we should ideally focus our lives.

I would like to thank Scott Mulrooney for providing pKAU19, a plasmid which expresses urease to almost 10% of the cell protein, Mann Hyung Lee for one preparation of *K. aerogenes* urease, Dr. J. Breznek for the use of his scintillation counter, and Dr. J. Leykam for helpful discussions concerning peptide isolation strategy. Dr. J. Wilson and Dr. T. Deits gave insightful comments during preparation of chapters 2-4. The remaining committee members, Dr. C. Chang, Dr. J. Kaguni, and Dr. D. Lamport were available and willing to give guidance when needed.

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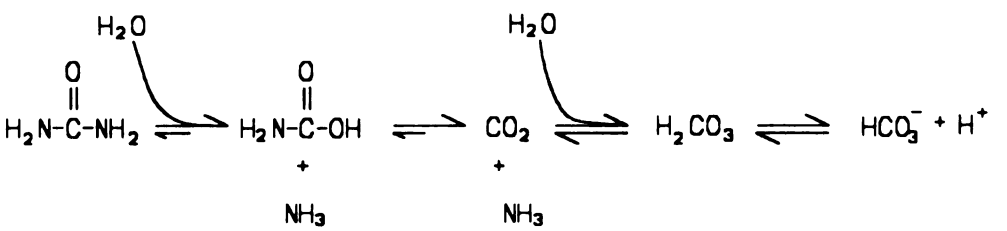
LIST OF ABBREVIATIONS

AHA, acetohydroxamic acid; BBA, benzene boronic acid; 4-Br-BBA, 4-bromobenzene boronic acid, CHES, 2-[N-cyclohexylamino]-ethanesulfonic acid; CAPS, 3-[cyclohexylamino]-1-propane-sulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTDP, 2,2'-dithiodipyridine; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; Gn·HCl, guanidine hydrochloride; HEPES N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; IAA, iodoacetate; IAM, iodoacetamide; β-ME, 2-mercaptoethanol; MMTS, methyl methanethiolsulfonate; MBPT, methyl butenyl phosphoric triamide; MES, 2-[N-morpholino]ethanesulfonic acid; MOPS, 3-[N-morpholino]propanesulfonic acid; NEM, N-ethyl maleimide; PPD, phenylphosphorodiamidate; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; TPCK, L-1-p-tosylamino-2-phenylethyl chloromethyl ketone; TFA, trifluoroacetic acid; TAPS, N-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; Tris·HCl, tris(hydroxymethyl)aminomethane·hydrochloride.

INTRODUCTION

Urea is a remarkably stable molecule: the half-life for the spontaneous degradation of urea is 3.6 years in aqueous solutions at 38°C (6). Urea is not, however, very stable in the environment. It is rapidly hydrolyzed to ammonia and carbamate through the action of the enzyme urease (EC 3.5.1.5) (5). Carbamate spontaneously degrades to another molecule of ammonia and carbon dioxide; the carbon dioxide is then hydrated to carbonic acid which will dissociate into H^+ and HCO_3^-. Two mol ammonia and one mol bicarbonate are the net products; thus urea hydrolysis results in a net increase in pH (Scheme 1).



Scheme 1

Ureases are found in bacteria, algae, filamentous fungi, yeasts, and plants (reviewed by Mobley and Hausinger; 55). Pathogenic ureolytic microorganisms have been isolated from humans, where they can colonize the urinary tract, the colon, or the stomach. Urease activity is also found in

soil, either associated with soil microbes, or as extracellular urease, probably arising from the lysis of soil microbes or the decomposition of plant tissues (61). The purpose of this chapter is to describe briefly several areas where microbial ureases affect the medical and agricultural community, then to review what is currently known about urease enzymology.

Medical significance- Urease is an important virulence factor in several pathogenic states, especially those associated with urinary tract infections. Ureolytic microorganisms in this environment will hydrolyze urea (at 0.4 M in human urine; 32) and cause a rise in urine pH which can lead to pyelonephritis (74). Elevated urine pH leads to supersaturation of the urine with respect to magnesium and calcium salts (32) which can precipitate forming stones; 15-20% of all kidney stones are associated with infections by ureolytic microorganisms (33). The extracellular polysaccharide of the bacterium is thought to provide an organic matrix necessary for stone initiation (51) and play a role in cementing the crystalline mineral components together (52). Growth of urinary calculi imbeds viable bacteria in interstices of the urinary stone (86), inhibiting antibiotic treatment of such an infection. Precipitation of urine salts also affects patients with long term urinary catheterization; 86% of catheterized patients have ureolytic bacteria present in their urine (56).

In addition to adverse effects caused by the elevation of pH, ureases release vast quantities of ammonia. Hyperammonemia (83), hepatic encephalopathy (78), and hepatic coma (76) are conditions caused by elevated levels of ammonia, which can not be removed from the system by the liver, either because the liver is damaged or because the organism is producing an excessive amount of ammonia. Ammonia is known to have many toxic effects (15), and the additional ammonia released by ureolytic infections can contribute to the overall nitrogen burden of the organism.

Treatment of stone-forming kidney infections, hyperammonemia, or ammonia toxicity may be more effective using urease inhibitors; studies using urease inhibitors ***in vitro*** (85) and ***in vivo*** (44, 53, 73) have demonstrated that calculus formation is reduced in urine lacking urease activity. Similarly, urease inhibitors have been demonstrated to reduce blood ammonia levels in patients with hepatic coma (83). The rational design of effective inhibitors will require detailed knowledge of the urease mechanism.

A second type of infection in which urease contributes to the pathogenicity of a bacterium occurs in the mucosal lining of the mammalian stomach (23). Colonization of this hostile environment by the acid sensitive bacterium, *Helicobacter pylori* occurs at intercellular junctions (10). *H. pylori* is the etiologic agent of type B gastritis (23, 31) and has been implicated in the formation of peptic ulcers (37); eradication of the infection relieves the symptoms

(23). *H. pylori* possesses an active urease which can rapidly hydrolyze serum urea, producing abnormal amounts of ammonia, initially thought to create a "cloud" of neutral pH immediately surrounding the bacterium (30). Current hypotheses to account for *H. pylori* pathology include: bacterial secretion of mucin digesting enzymes (80), interference with the passage of H^+ ions from the gastric glands to the lumen (37), and/or toxicity due to elevated ammonia concentrations, as mentioned above (15, 81). Supporting the role of urease as a virulence factor of *H. pylori*, Smoot et al. (81) demonstrated similar toxicity to human gastric adenocarcinoma cells grown with urea and either *H. pylori* or jack bean urease. As in the case of urinary tract infections, urease inhibitors may aid in combatting *H. pylori* infections.

Agricultural significance- The lack of fixed nitrogen in the soil is a major limitation to plant growth; therefore, fertilizers are used to supplement soil nitrogen levels when growing commercially important crop plants. Urea is a good source of nitrogen, because of its low cost, ease of use, and its high nitrogen content (3). Urea must be hydrolyzed by soil ureases before it can be assimilated into plant tissue; uncontrolled hydrolysis can lead to elevated soil pH, ammonia toxicity, and nitrogen loss as volatile NH_3 (up to 50% for submerged rice crops; 9). The simultaneous application of urease inhibitors and urea-based fertilizers could minimize crop damage and enhance the

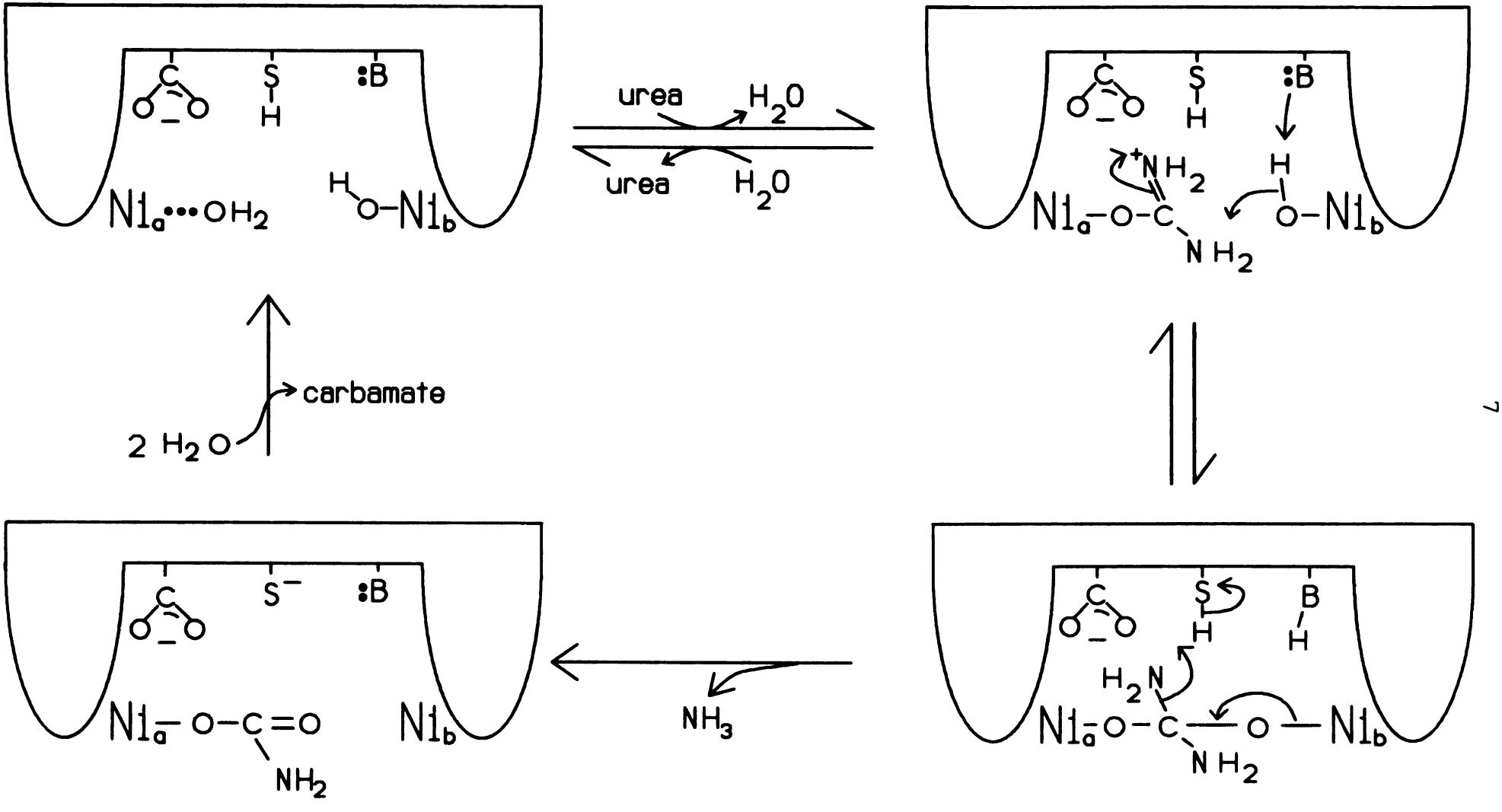
efficiency of nitrogen utilization (8, 9). Hydroxamic acids (69) and phosphoroamide derivatives (8, 16, 50, 61) retard urea hydrolysis in soils; however, an increase in plant yield has not yet been demonstrated. One problem regarding the application of urease inhibitors has been the accumulation of urea in leaves, resulting in leaf-tip necrosis (45). Obviously, further work in this field is of value.

Industrial significance- Ureases are also of practical use industrially. Inside a bioreactor, the delivery of nutrients to growing cells is diffusion limited; uniform growth of cells cannot occur if the cells at the site of nutrient input deplete the medium of nutrients. Attaching urease to the solid support in the bed of bio-reactors allowed Mak et al. (49) to use urea as a nitrogen source for growth of a photosynthetic algae, *Chlorella emersonii*. Reactors initiated with immobilized urease had a more uniform release of ammonia proximal to the growing cells and increased penetration of the gel matrix (48, 49). Ureases have also been used to remove urea waste from industrial effluents (G. Prabhakaran, personal communication), or from blood dialysate in artificial kidneys (67). The waste urea is hydrolyzed to NH_3, which can be easily removed by air stripping.

Plant ureases- Upon initiation of the current work, the only extensively studied urease was that from *Canavalia*

ensiformis, the jack bean plant (reviewed by Blakeley and Zerner; 6); few microbial ureases had even been purified. Jack bean urease has the distinction of being the first enzyme crystallized (84) and the first enzyme shown to possess nickel (19). Research on the active site of the plant enzyme allowed Zerner and colleagues to propose a mechanism for the hydrolysis of urea by urease (22), Scheme 2. Indeed, many of the initial results on *K. aerogenes* urease are interpreted in terms of this model in which each active site has two nickel ions: one binds to the carbonyl oxygen of urea, and the other binds a hydroxide for nucleophilic attack on the urea carbon. Elimination of one molecule of ammonia, followed by release of carbamate and binding of two molecules of water is thought to regenerate the resting enzyme. Although plant ureases have been found in seed, leaf and stem tissues, their role in plant nitrogen metabolism is not well understood (94).

Properties of purified ureases- In contrast to the homohexameric structure (M_r = 90,770; 88) of jack bean urease (6) and soybean urease (68), many microbial ureases have three distinct subunits, $\alpha, \beta,$ and γ, where α = 60-70 kDa, and β and γ are 8-12 kDa (39, 41, 59, 89), as first described for *K. aerogenes* urease (Chapter 2; 90). The β and γ subunits have been overlooked when analyzing certain enzymes by SDS PAGE using gels of less than 12% acrylamide (12, 82). In one organism, *H. pylori*, the β and γ subunits have



Scheme 2

undergone gene fusion into a single polypeptide of 30 kDa (46). Subunit ratios of urease enzymes have been variable among species as determined by integrated scanning densitometry, a method which assumes that each subunit has a similar affinity for the dye; therefore definitive comparisons of subunit ratios are meaningless. Genetic studies have confirmed the presence of three structural genes in *K. aerogenes* (60), *P. stuartii* (59), *P. vulgaris* (58), *P. mirabilis* (40), and *H. pylori* (46) and have shown that the three genes are highly honologous to the single plant gene, which probably arose via gene fusion. Furthermore, DNA sequence studies of Scott Mulrooney (60) suggested that three additional genes are involved in generation of active urease. One possible function of these additional genes is in nickel processing (47, 36); deletion of these accessory genes resulted in production of urease apoenzyme (60).

Nickel has been found in all ureases examined (35). Calculation of the amount of nickel per catalytic unit requires knowledge of the amount of nickel, and the catalytic unit molecular weight. Studies incorporating the above information have shown that the jack bean urease (19, 20) and the *K. aerogenes* enzyme (Chapter 2, 3; 90, 91) have 2 mol nickel/mol catalytic unit. For other bacterial ureases, 0.8-2.1 mol nickel/mol of large subunit has been calculated (reviewed by Mobley and Hausinger; 55) without determining the number of catalytic units per enzyme. Biophysical and spectroscopic analysis of the nickel center have only been

done with the jack bean and *K. aerogenes* enzymes. UV-visible spectroscopy of *K. aerogenes* urease in the presence of thiol inhibitors is described in chapter 3 (91), and compared to the jack bean enzyme results (4, 18). X-ray absorption spectroscopy (XAS) of jack bean (1, 14) and *K. aerogenes* (Scott and Hausinger, in preparation) ureases have demonstrated that the nickel is coordinated by a mixture of nitrogen and oxygen ligands; however the precise identity of these ligands remains unknown. Lee et al. (47) observed increased reactivity of *K. aerogenes* apo-urease towards diethylpyrocarbonate at pH 6.5, consistent with (at least partial) histidine ligation of the nickel. Magnetic susceptibility measurements are consistent with at least a portion of the nickel being paramagnetic for both *K. aerogenes* (appendix) and jack bean (17) ureases. In both cases, diamagnetism is observed in the presence of thiol compounds, indicating either that the the two nickel ions are antiferromagnetically coupled, or that the individual nickel ions have become low spin (S=0) (17). Low temperature Magnetic Circular Dichroism (MCD) spectroscopic studies on *K. aerogenes* (Michael Johnson, unpublished experiments) and jack bean (25) ureases show that the two nickel become antiferromagnetically coupled in the presence of thiols, suggesting that these inhibitors bridge the two nickel at the active site. In summary, the active site of urease includes a novel, poorly characterized bi-nickel metallocenter.

Kinetic parameters- The kinetic parameters of microbial ureases vary moderately and usually reflect the microorganisms particular ecological niche. For example, the K_m values for urea are considerable higher for enzymes purified from organisms which invade the kidney (2-20 mM; 55) where the concentration of urea is high (0.4-0.5 M; 32), than from organisms which colonize the stomach lining (24, 54) where the concentration of serum urea is 1.5-3.0 mM (54). Urease V_{max} is typically 1000-5000 μmol urea hydrolyzed min^{-1} mg^{-1} (reviewed by Mobley and Hausinger; 55). An important exception is the urease of *U. ureolyticum* (V_{max} ~ 90,000 μmol min^{-1} mg^{-1}; 82), which is at least 10-fold faster than any previously purified urease. The urease of this mycoplasma is thought to create an ammonia and H^+ gradient, and the organism may use this gradient to generate cellular energy (72). Most ureases have pH optima in the neutral to slightly basic range (55). *Lactobacillus fermentum,* however, has a urease with a pH optimum of 2 (41), which may reflect the ability of this organism to grow in acidic media.

Inhibition of urease- The inhibition of urease has many potential applications. For example, derivatives of hydroxamic acid have been used as chemotherapeutic agents to reduce blood ammonia levels in patients with hyperammonemia (83), and to reduce urine ammonia and pH in patients with urinary infections (33, 62, 73). Serious side effects

accompany the use of these compounds (27), and they have been suggested to be teratogenic and/or carcinogenic (73). Phosphoroamide derivatives (which are ~1000-fold more potent inhibitors) retard urinary stone growth (53, 85) and inhibit soil urease activity (16, 50, 61). In most instances where inhibition of urease is desired, the concentration of urea is present at levels which saturate the enzyme; therefore an ideal inhibitor would interact with urease by a non-competitive mechanism (57). Many determinations of the type of inhibition caused by these compounds reported non-competitive (26, 28, 29, 73) or mixed inhibition (28); other studies compared I_{50} values (the amount of inhibitor needed to obtain 50% inhibition of urease activity) without regard to the mechanism of inhibitor binding (27, 28, 34, 43). Acetohydroxamic acid and phenylphosphorodiamidate are slow-binding competitive inhibitors of *K. aerogenes* urease (Chapter 3, 91). To generate a useful comparison of the efficacy of these inhibitors using I_{50} values, one should also know the type of inhibition, the K_m and substrate concentration, and the enzyme concentration (11). Since these inhbiitors bind and dissociate from urease slowly, typical kinetic methods (derived assuming equilibrium or steady state conditions) simply reflect the amount of non-inhibited enzyme, thus appearing non-competitive (*i.e.* V_{max} is decreased, K_m is not affected).

The ionized form of hydroxamates are bidentate chelators of metals in solution and are used by some organisms as

siderophores (64). Ionized hydroxamates also inhibit metalloproteins (*e.g.* thermolysin, 65, 38) through bidentate binding to the metallocenter. Changes in the UV-visible spectra of jack bean urease are consistent with their interaction with the active site nickel ion(s) (21). In addition, UV-visible spectra of one hydroxamic acid bound to jack bean urease, trans-cinnamoyl hydroxamate, was consistent with the ionized form of the hydroxamic acid being present at the active site, leading Zerner and colleagues to propose that hydroxamates bind to the urease nickel in a bidentate manner (21). In contrast, ionized hydroxamic acids do not interact with horseradish peroxidase; monodentate binding to the active site heme is thought to be the cause of hydroxamate inhibition of this metalloenzyme (79). The binding of (neutral) acetohydroxamate to *K. aerogenes* urease is described in chapter 3 (91), and a distinct mechanism whereby the neutral hydroxamate bridges the two active site nickel (similar to the transition state of urea hydrolysis, Scheme 2) is proposed.

Phosphoramides bind to the zinc in metalloproteases (such as carboxypeptidase A and thermolysin) and inhibit by acting as transition state analogues (42). Less is known about the interactions of phosphoramidates with urease. Zerner and colleagues, studying the reactivation of the jack bean enzyme inhibited with PPD, MBPT, and phosphoric triamide (2), observed similar reactivation rates with all

three compounds. From this observation, derivatives of phosphoroamide were proposed to be alternative substrates of jack bean urease; all three being hydrolyzed to a common inhibitor, diamidophosphate. The slow-binding of phenylphosphorodiamidate to *K. aerogenes* urease involved saturation kinetics similar to those seen for AHA (chapter 3; 91). PPD was also proposed to bind as a transition state analogue. Although no product analysis was done in either the jack bean or the bacterial urease study, slowly processed substrates are known to behave kinetically as slow-binding inhibitors for several enzymes (57).

In addition to the potent inhibition observed by hydroxamates and phosphoramidates, many other compounds are known to inhibit urease by unknown mechanisms. In chapter 3 (91), evidence is presented that indicates thiols, phosphate, and boric and boronic acids are competitive inhibitors of *K. aerogenes* urease.

Active site residues- Elucidation of an enzyme mechanism is not complete without examination of the active site amino acids which participate in substrate binding, catalytic turnover, or metal ligation. Chapters 2-5 include experiments designed to detect these amino acids, including effects of pH on kinetic parameters, inhibitor and inactivator studies, and analysis of conserved sequences. Experiments using the well-studied plant urease demonstrated a slight increase in K_m upon deprotonation of an ionizable

group with pK_a = 6.5 (22). Activity (V_{max}) required deprotonation of the group with pK_a = 6.5 while retaining an enzyme group with pK_a = 9.0 in a protonated state. Analogous studies have not been reported for microbial ureases, except those described in chapter 2 (91), which characterize the pH dependence of *K. aerogenes* urease: one enzyme group (pK_a = 6.55) must be deprotonated, and a second enzyme group (pK_a = 8.85) must be protonated for activity (90). The affinity of enzyme for substrate is increased slightly below pH 6.5. While the identity of enzyme groups exhibiting these pK_a's is not known, amino acid specific reagents have been used to probe their reactivitiy.

Urease activity from plants (66, 71), algae (70) and bacteria (41, 63, 75, 92) is affected by thiol specific reagents. Dixon et al. (22) proposed that jack bean urease has an essential thiol acting as a general acid during catalytic turnover (Scheme 2). This essential thiol, Cys_{592} (87, 88), was reported to react with modifying reagents only in the ionized form with a pK_a = 9.0 (66). In contrast, the essential thiol in *K. aerogenes* urease interacts with some nearby amino acid, as yet unidentified, yielding a more complicated pH dependence of inactivation (Chapter 4; 92). The discrepancy between these two reports may lie in the methods used to determine the rate of essential thiol modification (see Chapter 4 for discussion); nevertheless, the analogous residue has been identified as the essential thiol in bacterial urease: Cys_{319} of the large subunit (Chapter 5; 93).

Aside from an essential cysteine, the mechanism of urea hydrolysis as proposed by Dixon et al., (Scheme 2) invokes two additional active site amino acids: a carboxylic acid, and an unidentified base. Evidence supporting an essential carboxylic acid at the jack bean urease active site is based on unpublished experiments measuring urease activity in the presence of triethyloxonium ion. Indirect evidence of an anionic residue near the active site of bacterial urease comes from the comparison of various thiol inhibitor K_i values (28, 34, 43, Chapter 3; 91) and comparison of the second order rate constants for several thiol modifying reagents (Chapter 4; 92). With regard to the general base, Sakaguchi et al. (77) have reported inactivation of jack bean urease by photo-oxidation of histidine residues using methylene blue; urease inhibitors prevented this inactivation. Lee et al., (47) have seen loss of *K. aerogenes* urease activity using diethylpyrocarbonate, a modifying reagent which reacts with histidine residues.

Comparison of published urease sequences from *K. aerogenes* (60), jack bean (88), *P mirabilis* (40), *P. vulgaris* (58), *U. ureolyticum* (7), and *H. pyloris* (46) demonstrate that ten histidine are conserved among these diversely represented species. One of these histidine residues may correspond to the general base described above. In addition, several of these histidines may serve as ligands to the nickel, as described above.

The following chapters describe my studies on *K. aerogenes* urease. First I purify the nickel-containing enzyme 1070-fold and show that this and several other microbial ureases are heteropolymeric (Chapter 2; published in *J. Biol. Chem.*, **262,** 5963-5967). I then demonstrate that some competitive inhibitors directly interact with the nickel center, and propose models for the interaction of these inhibitors with the bi-metal active site (Chapter 3; published in *J. Biol. Chem.*, **264,** 15836-15842). Finally, I describe the unusual reactivity of the essential cysteine in urease (Chapter 4; published in *J. Biol. Chem.*, **266,** 10260-10267), and I identify this amino acid as Cys_{319} in the α subunit (Chapter 5; 93).

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PURIFICATION AND CHARACTERIZATION OF THE NICKEL-CONTAINING MULTICOMPONENT UREASE FROM *KLEBSIELLA AEROGENES*

ABSTRACT

Klebsiella aerogenes urease was purified 1,070-fold with a 25% yield by a simple procedure involving DEAE-Sepharose, phenyl-Sepharose, Mono Q, and Superose 6 chromatographies. The enzyme preparation was comprised of three polypeptides with estimated M_r = 72,000, 11,000, and 9,000 in a $\alpha_2\beta_4\gamma_4$ quaternary structure. The three components remained associated during native gel electrophoresis, Mono Q chromatography, and Superose 6 chromatography despite the presence of thiols, glycols, detergents, and varied buffer conditions. The apparent compositional complexity of *K. aerogenes* urease contrasts with the simple well-characterized homohexameric structure for jack bean urease (Dixon, N. E., Hinds, J. A., Fihelly, A. K., Gazzola, C., Winzor, D. J., Blakeley, R. L., and Zerner, B. (1980) *Can. J. Biochem.* **58,** 1323-1334); however, heteromeric subunit compositions were also observed for the enzymes from *Proteus mirabilis*, *Sporosarcina ureae*, and *Selenomonas ruminantium*. *K. aerogenes* urease exhibited a K_m for urea of 2.8 ± 0.6 mM and a V_{max} of 2,800 ± 200 μmol of urea min^{-1} mg^{-1} at 37°C in 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid,

5.0 mM EDTA buffer, pH 7.75. The enzyme activity was stable in 1% sodium dodecyl sulfate, 5% Triton X-100, 1 M KCl, and over a pH range from 5 to 10.5, with maximum activity observed at pH 7.75. Two active site groups were defined by their pK_a values of 6.55 and 8.85. The amino acid composition of *K. aerogenes* urease more closely resembled that for the enzyme from *Brevibacter ammoniagenes* (Nakano, H., Takenishi, S., and Watanabe, Y. (1984) *Agric. Biol. Chem* **48,** 1495-1502 than those for plant ureases. Atomic absorption analysis was used to establish the presence of 2.1 ± 0.3 mol nickel/mol 72,000-dalton subunit in *K. aerogenes* urease.

Introduction

Urease is a nickel-containing enzyme which catalyzes the hydrolysis of urea to form carbon dioxide and ammonia (1,2). The archetype urease, isolated from jack bean, has been intensively studied since 1926 when Sumner first crystallized this protein (3). Jack bean urease (M_r = 590,000) is a hexamer of identical subunits (4) of known amino acid sequence (M_r = 90,790) (5). This plant enzyme contains two nickel ions per subunit (6), and the metal ions are apparently coordinated by oxygen and nitrogen ligands (7). Whereas soybean urease appears to be analogous in size, structure, and nickel content (8), microbial ureases are distinct from the jack bean enzyme. For example, ureases from *Brevibacter ammoniagenes* and *Bacillus pasteurii* are smaller in subunit size (M_r = 67,000 and 65,000) and native size (M_r = 200,000 and 230,000) and possess a single nickel ion per subunit (9,10). The *Selenomonas ruminantium* urease is also smaller (native M_r = 360,000), but like the plant enzymes it contains two nickel ions per subunit (M_r = 70,000) (11). Active site differences may also exist in the microbial and plant enzymes as shown by their differences in susceptibility to various inhibitors (12).

Herein we extend the comparison of plant and microbial ureases by characterizing the isolated *Klebsiella aerogenes*

enzyme. Previously, Friedrich and Magasanik (13) purified *K. aerogenes* urease 24-fold and examined the cellular regulation of this enzyme. In addition, Kamel and Hamed (14) described several properties of 150-fold-purified urease from the related organism, *Aerobacter aerogenes* PRL-R3. In this report, the nickel-containing *K. aerogenes* urease is intensively characterized and shown to have a novel urease structure comprised of three distinct polypeptide chains.

EXPERIMENTAL PROCEDURES

Bacterial growth conditions- Klebsiella aerogenes CG253, obtained from Boris Magasanik and Alexander Ninfa (Massachusetts Institute of Technology), was cultured at 37°C in minimal media which contained 10 g sucrose, 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.2 g $MgSO_2 \cdot 7H_2O$, 2.5 ml trace minerals, and 0.24 g urea (filter sterilized) per liter. The trace mineral solution contained, per 100 ml, 20 mg $ZnSO_4 \cdot 7H_2O$, 10 mg H_3BO_3, 10 mg $NaSeO_3$, 10 mg $NaMoO_4 \cdot 2H_2O$, 10 mg $NiCl_2 \cdot 6H_2O$, 5 mg $CuSO_4 \cdot 5H_2O$, 2 mg $AlK(SO_4)_2 \cdot 12H_2O$ and 1 ml H_2SO_4 (15). Large scale aerobic batch culture was performed by using a New Brunswick 25L Microferm fermenter with rapid agitation. The culture was grown to an absorbance (600 nm) of 1.8 and harvested by using a Pellicon Cassette system concentrator (Millipore). The cells (80 to 108 g wet weight per 22 L) were washed with 20 mM potassium phosphate, 1 mM

EDTA, 1 mM β-mercaptoethanol, pH 7.0 (abbreviated PEB) buffer and either used immediately or stored at -20°C.

Assays- Urease was assayed by measuring the rate of release of ammonia from urea. The released ammonia was converted to indophenol whose absorbance was monitored at 625 nm (16). Specific activity of urease was defined as μmol urea hydrolyzed min^{-1} mg^{-1} at 37°C in 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), 5.1 mM EDTA, pH 7.75 buffer containing 25 mM urea. Protein was assayed as described by Lowry et al. (17) with bovine serum albumin as the standard. In addition, the absorbance at 280 nm was used to quantitate highly purified samples of urease. An extinction coefficient of 8.5 at 280 nm was obtained for a 1% solution of the isolated enzyme, based on the protein concentration obtained by using the Lowry method. All UV/visible absorbance determinations were made by using a Gilford Response spectrophotometer.

Preparation of crude extract- The cells were suspended in an equal volume of PEB buffer containing 1.0 mM toluene sulfonyl fluoride and disrupted by two passages through a French pressure cell at 16,000 psi. In some cases, other protease inhibitors were used including bestatin, pepstatin, leupeptin, aprotinin, and phosphoramidone. Membranes and cellular debris were removed by centrifugation at 10,000 X g for 60 min at 4°C. All isolation procedures,

except that using the Fast Protein Liquid Chromatography (FPLC) system, were carried out at 4°C and using PEB buffer with the indicated additions.

SDS-polyacrylamide gel electrophoresis- The molecular weight for the urease polypeptides and the purity of samples were assessed by using SDS-polyacrylamide gel electrophoresis in a Hoefer SE 600 electrophoresis apparatus as described by Laemmli (18). The samples were denatured for 5 min at 100°C in 0.0625 M Tris buffer, pH 6.8 containing 3% SDS, 10% glycerol, and 5% β-mercaptoethanol. These samples were then electrophoresed at 30 mA constant current through a 3% or 4.5% acrylamide stacking gel and a 10%, 12% or 20% acrylamide running gel, each 1.5 mm in thickness. To improve molecular weight estimates for small polypeptides, 20% acrylamide gels possessed a 200:1 ratio of acrylamide to methylene bisacrylamide. In some cases, 5% to 15% acrylamide gradient gels were prepared to enhance the resolution of the entire range of peptides. Standards (Bio-Rad, Calbiochem, Sigma) used for comparison were: myosin, M_r = 200,000; β-galactosidase, M_r = 116,250; phosphorylase b, M_r = 92,500; jack bean urease, M_r = 90,790 (5); bovine serum albumin, M_r = 66,200; ovalbumin, M_r = 45,000; carbonic anhydrase, M_r = 31,000; chymotrypsinogen, M_r = 25,000; soybean trypsin inhibitor, M_r = 21,500; myoglobin, M_r = 16,950; α-lactalbumin, M_r = 14,200; lysozyme, M_r = 14,000; cytochrome C, M_r = 12,500; and myoglobin cyanogen bromide

fragments, M_r = 14,000, 8,160, 6,210, and 2,510. Gels were stained with Coomassie Brilliant blue or Silver stain (19) to detect proteins and with dansyl hydrazine to detect glycoproteins (20).

Native gel electrophoresis- Nondenaturing gel electrophoresis was carried out by using the buffers described by Laemmli (18) without SDS. Samples were electrophoresed at 35 mA constant current into the 3% acrylamide stacking gel and 5%, 7%, or 9% acrylamide running gel, 1.5 mm in thickness. Urease was detected in the gels either by a phenol red activity stain analogous to the cresol green stain described by Blattler et al. (21) or by the nitroblue tetrazolium activity stain described by Fishbein (22). Other proteins were detected by Coomassie brilliant blue or Silver stain methods.

Native molecular weight determination- The native molecular weight of *K. aerogenes* urease was estimated by two methods. Native gel electrophoresis was carried out on samples and standards by using several gels of varied acrylamide concentration (5% to 9%). The method of Zwaan was used to estimate the molecular weight by plotting the native protein size as a function of the ratio of percent migration versus tracking dye in the two gels (23). The standards used in the gel method included carbonic anhydrase, M_r = 31,000; ovalbumin, M_r = 45,000; ovalbumin dimer, M_r =

90,000; bovine serum albumin (BSA) monomer, M_r = 66,200; BSA dimer, M_r = 132,000; BSA trimer, M_r = 198,600; myosin, M_r = 200,000; and β-galactosidase, M_r = 465,000.

In addition, the molecular weight for native urease was deduced by using Superose 6 (1.0 x 30 cm, Pharmacia) gel filtration chromatography. Standards included thyroglobulin, M_r = 670,000; ferritin, M_r = 470,000; γ-globulin, M_r = 158,000; ovalbumin, M_r = 44,000; myoglobin, M_r = 17,000; and vitamin B-12, M_r = 1,350. Samples were eluted at 0.5 ml min^{-1} in PEB buffer containing 0.1 M KCl while monitoring the absorbance at 280 nm.

Stability studies- Urease (7 μg ml^{-1}) was incubated at 0-2°C in a 10-fold dilution of assay buffer containing various concentrations of detergents, glycols, thiols, salts, or mixtures of these substances. Aliquots from these incubations were diluted 200-fold into the routine assay buffer and assayed for activity.

pH Studies- The enzyme stability was assessed at pH values from 4 to 11 by incubating urease in buffers at various pH values for 30 min then assaying aliquots of the mixture in a 40-fold volume of the routine assay buffer containing 2.5 mM to 50 mM urea. The test buffers included acetate (pH 4-6), MES (pH 5-7), CHES (pH 8.5-10.5), or CAPS (pH 9-12) at a concentration of 50 mM. In addition, all buffers contained 10 mM EDTA.

The effects of pH on K_m (urea) and V_{max} were established by assaying urease activity in buffers containing 25 mM buffer, 5 mM EDTA and 2.5 mM to 50 mM urea, at the indicated pH values. The rate of urea hydrolysis was linear at each pH value at which the enzyme was stable. Buffers included the four mentioned above, HEPES (pH 6.5-9.0), and phosphate (pH 5.0-11.0).

Amino acid analysis- Protein samples were hydrolyzed *in vacuo* in 6 N HCl at 110°C for 24, 48, and 72 hr. Duplicate samples were analyzed by using a Beckman model 119CL automatic amino acid analyzer with ninhydrin detection system. The instrument was equipped with a model 126 data system for peak integration at 440 and 570 nm. Cysteine was quantitated as carboxymethyl cysteine for samples which were denatured, reduced and alkylated by using iodoacetic acid (2%). Threonine and serine values were extrapolated to zero time of hydrolysis. Tryptophan was quantitated spectrophotometrically by the absorbance at 280 nm; the extinction coefficient was taken to be 5700 $M^{-1}cm^{-1}$ for Trp and 1300 $M^{-1}cm^{-1}$ for Tyr.

Ni analysis- The nickel content of samples was quantitated by using a Perkin-Elmer PE-500 atomic absorption spectrophotometer equipped with an HGA 500 graphite furnace and an AS-1 autosampler. Samples were hydrolyzed in 1 M nitric

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acid, evaporated, and resuspended in 50 mM HNO_3. Nickel standards, which in some cases contained bovine serum albumin to provide a protein matrix, were treated identically to the urease samples. Aliquots of each sample (20 µl) were dried at 120°C, charred at 1200°C, and atomized at 2700°C. Nickel analysis was carried out using the instrument's background correction mode, and either peak height or peak area were monitored.

RESULTS

Urease purification- Extracts from 190 g of cell (wet weight) were chromatographed on a column (2.5 X 15 cm) of DEAE-Sepharose (Pharmacia) as shown in Figure 1. A 400 ml linear gradient from 0 M to 1 M KCl in PEB buffer was used to recover urease as a single peak of activity at approximately 0.45 M KCl. Fractions containing peak urease activity were adjusted to 1 M KCl and chromatographed on a column (2.5 X 15 cm) of phenyl-Sepharose (Pharmacia) by using step elution. The column was washed with 150 ml of 1 M KCl in PEB and activity was eluted with 100 ml PEB buffer. As illustrated in Figure 2, a broad peak of urease activity was obtained, well separated from the major peak of protein. The peak urease fractions were pooled, diluted with an equal volume of PEB buffer and chromatographed on a Mono Q column

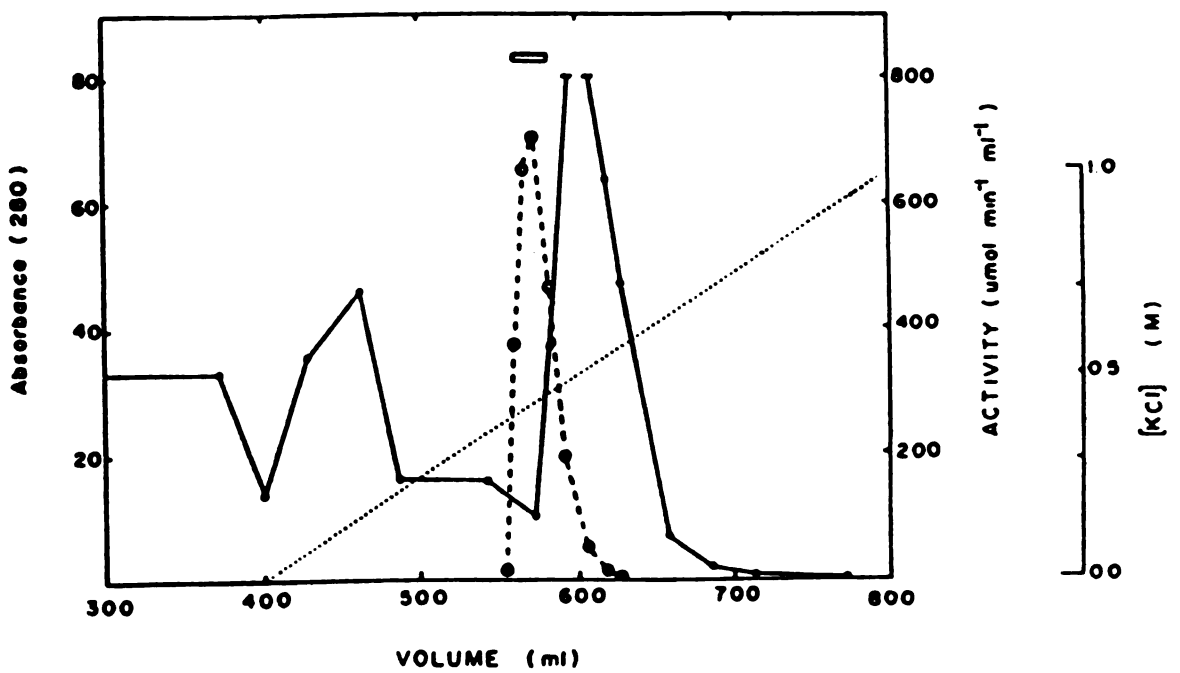

Figure 1. **DEAE-Sepharose chromatography of *K. aerogenes* urease.** Cell extracts were chromatographed on a column of DEAE-Sepharose by using a linear KCl gradient as indicated (· · · · ·). Fractions (5.5 ml) were monitored for absorbance at 280 nm after diluting aliquots 20-fold (●——●), and samples were assayed for urease activity (O - - - O). The pooled fractions are indicated by the bar.

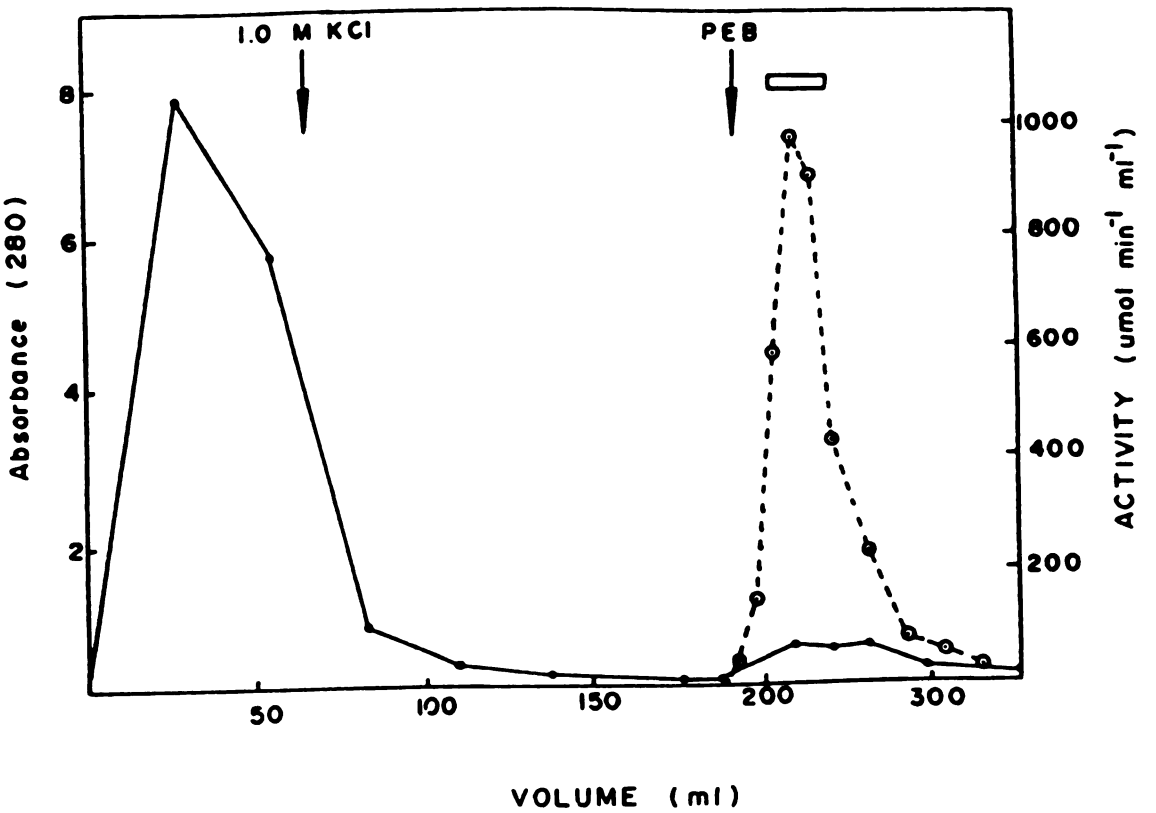

Figure 2. **Phenyl-Sepharose chromatography of *K. aerogenes* urease.** The pooled fractions from Figure 1 were adjusted to 1 M KCl and chromatographed on a column of phenyl-Sepharose by using step elution, decreasing the concentration of KCl as indicated while monitoring the absorbance at 280 nm (● —— ●). Samples (3 ml) were assayed for urease activity (O - - - O) and the pooled fractions are indicated by the bar.

(1.0 X 10 cm, Pharmacia) As shown in Figure 3, a KCl gradient was used to resolve urease from several contaminants. The Mono Q fractions containing the highest urease activity were concentrated to 0.2 ml by using a Centricon-10 (Amicon) and chromatographed on a column (1.0 X 30 cm) of Superose 6 (Pharmacia) as illustrated in Figure 4. This final purification step served to desalt the enzyme and to provide an estimate of the native molecular weight (*vide infra*). The purification of *K. aerogenes* urease is summarized in Table 1.

Table 1

Urease purification from *K. aerogenes*

Purification step	Specific Activity (mmole urea $min^{-1}mg^{-1}$)	Purification (fold)	Total Activity (mmol min^{-1})	Total Protein (mg)	Enzyme Recovery (%)
Cell extracts	2.06	1	19500	9460	100
DEAE-Sepharose	81.1	39	11800	145	60.5
Phenyl-Sepharose	574	279	5420	9.45	27.8
Mono Q	1170	568	5080	4.34	26.1
Superose 6	2200	1070	4770	2.17	24.5

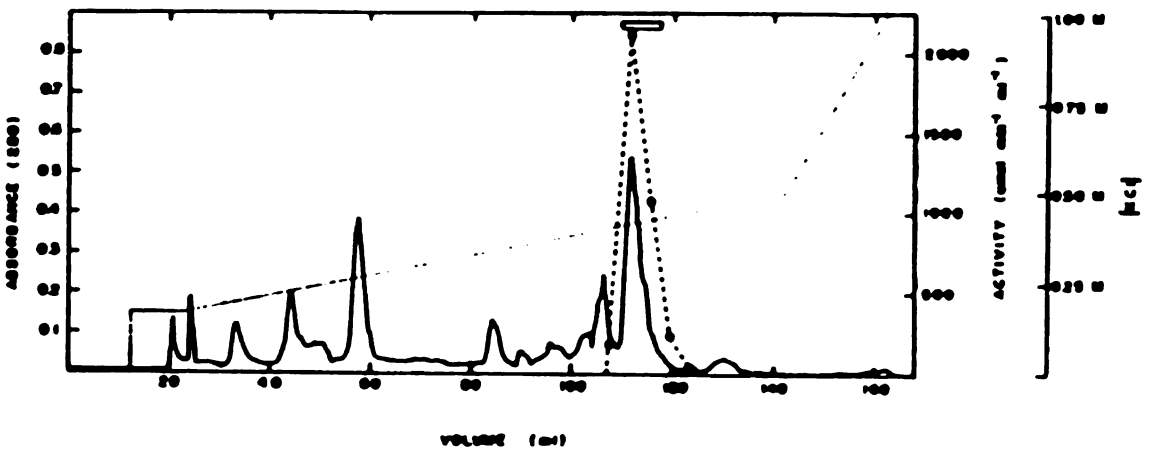

Figure 3. **Mono Q chromatography of *K. aerogenes* urease.** The pooled fraction from Figure 2 was subjected to Mono Q FPLC by using a KCl gradient as indicated (· · ·). The effluent was monitored for absorbance at 280 nm (——) and the fractions were assayed for urease activity (O - - - O). The pooled fractions are indicated by the bar.

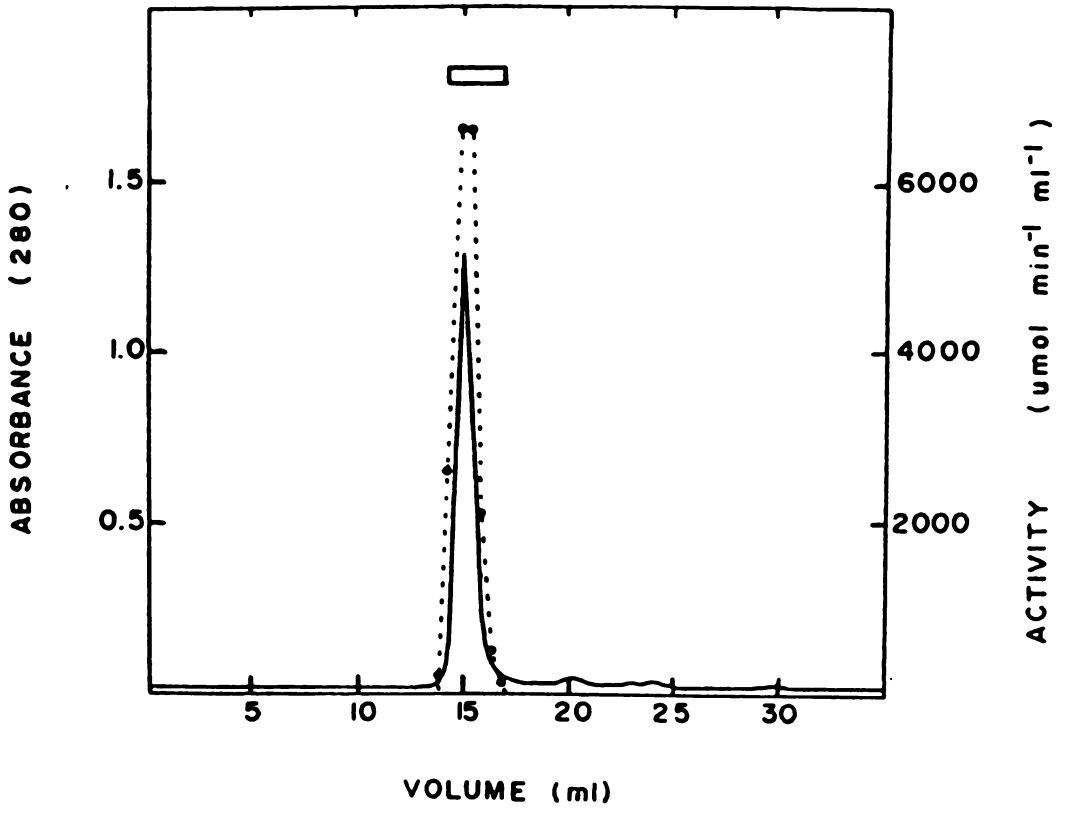

Figure 4. **Superose 6 chromatography of *K. aerogenes* urease.** The urease containing pool from Figure 3 was concentrated to 0.2 ml and chromatographed on a column of Superose 6 resin. The effluent was monitored for absorbance at 280 nm (——) and the fractions were assayed for activity (O---O).

SDS-polyacrylamide gel electrophoresis of K. aerogenes urease- *K. aerogenes* urease was found to contain three distinct polypeptides (M_r = 72,000 ± 2,000; 11,000 ± 2,000; and 9,000 ± 2,000) by using SDS-polyacrylamide gel electrophoresis (Figure 5). Gels comprised of less than 10% acrylamide did not resolve the smaller components from the bromophenol blue dye front; thus 12% or 20% acrylamide gels were used to estimate the molecular weight of the small components. The integrated intensities of the three bands from Coomassie blue stained gels were used to compare the component ratios. This method is suspect because individual polypeptides may have different affinities for the dye; nevertheless, this procedure yielded ratios for the large:small:smallest bands of 1:2:2. None of the components were glycopeptides, as demonstrated by a failure to stain using dansyl hydrazine, a carbohydrate specific stain.

Native molecular weight for K. aerogenes urease- By using the method of Zwaan (23) to analyze mobilities on non-denaturing gels, the native molecular weight for *K. aerogenes* urease was estimated as 190,000 ± 20,000 daltons. This value was not significantly altered if the enzyme was incubated at room temperature in 1.0 M KCl, 5% SDS, 5% Triton X-100, 50% ethylene glycol, 50% glycerol, 50% sucrose, 5% β-mercaptoethanol, 20% dimethylsulfoxide, or mixtures of these substances prior to electrophoresis. Superose 6 gel filtration chromatography was used as a

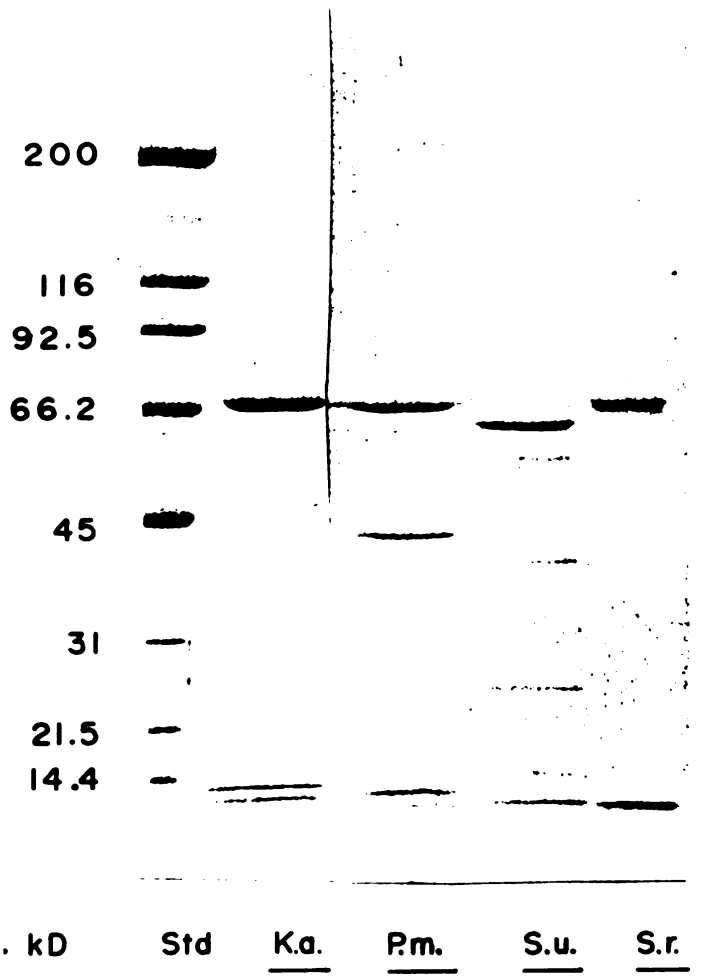

Figure 5. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of several microbial ureases.** Standard proteins (2 µg) and ureases from *K. aerogenes* (K. a.), *P. mirabilis* (P. m.), *S. ureae* (S. u.), and *S. ruminantium* (S. r.) (8 µg each) were denatured and electrophoresed on a 5-15% gradient gel, and then stained with Coomassie Brilliant Blue. Details of these methods are described under "Experimental Procedures".

second method to estimate the native molecular weight for urease. Assuming that the ratio of molecular weight to Stoke's radius is similar for the enzyme and the standards, the *K. aerogenes* urease molecular weight is 260,000 ± 50,000 daltons.

Attempts to resolve the three urease components- The presence of three polypeptide components in *K. aerogenes* urease is unprecedented when compared with previously published urease preparations. To examine this discrepancy, intensive efforts were carried out to further resolve the enzyme by electrophoretic and chromatographic techniques. Two-dimensional gel electrophoresis, in which the urease band from native gel electrophoresis was subsequently denatured and run on an SDS polyacrylamide gel, was used to demonstrate that all three polypeptides remain associated during native gel electrophoresis. Furthermore, prior incubation of urease in the presence of 1 M salt, 5% SDS, 5% Triton X-100, 50% ethylene glycol, 50% glycerol, 50% sucrose, 5% β-mercaptoethanol, 20% dimethylsulfoxide, or mixtures of these substances had no apparent effect on component association as demonstrated by native gel electrophoresis. Ureolytic fractions obtained by using Superose 6 chromatography equilibrated with PEB buffers containing 0.5 M KCl, 0.1% SDS, or 3% β-mercaptoethanol consistently possessed all three components upon SDS-polyacrylamide gel electrophoretic analysis. Mono Q chromatography of active

urease under a variety of buffer conditions (pH values from 6.0 to 9.0, the use of 20 mM β-mercaptoethanol, and elution with 0.5% triton X-100 in the buffers) also was incapable of resolving the three urease components. Thus, the three polypeptides remained associated under all conditions at which the enzyme activity was stable. Resolution of the large polypeptide has only been achieved by Superose 6 gel filtration chromatography in the presence of 6 M guanidine-HCl; however, enzyme activity was lost under these conditions.

Enzyme stability studies- Attempts to resolve the three urease components included the use of rather harsh conditions; yet, the enzyme retained activity in most of these experiments. The stability of *K. aerogenes* urease was quantitatively examined under varied buffer conditions at 0-2°C, as summarized in Table 2. The enzyme was found to retain nearly full activity for over 200 h despite the presence of salts, glycols, thiols, and detergents. Glycols, and perhaps 1 M KCl, enhanced the ureolytic activity at initial times; however 20-30% of the initial values were lost after 200 h, resulting in little net change from the control. The enzyme activity appeared to be more stable in Triton X-100 than in SDS; nevertheless, the enzyme could tolerate high concentrations of both detergents. Incubations containing β-mercaptoethanol exhibited an initial drop in activity, consistent with competitive inhibition by

Table 2

Stability of *K. aerogenes* urease

Buffer additions[2]	Percent activity remaining[1]		
	0 h	24 h	200 h
None	100	106	111
1 M KCl	119	100	97
1 M KCl, 20% glycerol	131	107	91
20% glycerol	133	115	104
20% ethylene glycol	131	119	106
200 mM β-ME[3]	92	104	97
1 % SDS	95	118	61
1 % SDS, 200 mM β-ME	89	99	95
5 % Triton X-100	85	112	102
5 % Triton X-100, 200 mM β-ME	76	103	94

[1] Values (accurate to ± 10% are relative to the control at zero time. Incubations were at 0-2°C.

[2] Control incubation buffer was comprised of 5 mM HEPES, 0.1 mM EDTA, pH 7.75.

[3] β-ME, β-mercaptoethanol.

this compound (see below). Thiol oxidation may lead to the increase in activity for these samples after 24 h.

Kinetic parameters- The rate of urea hydrolysis was monitored as a function of urea concentration from 1 mM to 50 mM urea. Simple Michaelis-Menten kinetics were followed; however, substrate inhibition was observed if the urea concentration was increased to 100 mM. The data was analyzed by the method of Wilkinson (25) to yield a K_m for urea of 2.8 ± 0.6 mM and a V_{max} of 2800 ± 200 μmol urea degraded min^{-1} mg^{-1} at 37°C in 25 mM HEPES, 5.0 mM EDTA buffer, pH 7.75. Some enzyme samples contained β-mercaptoethanol, which was a competitive inhibitor of *K. aerogenes* urease; however, the concentration of this compound in the assay conditions led to negligible inhibition.

pH Studies- *K. aerogenes* urease retained full activity when assayed under standard conditions following a 30 min incubation at 37°C in buffers ranging from pH 5 to 10.5; however, the enzyme was rapidly inactivated outside of this range.

The effect of pH on the kinetic constants for urease was determined in buffers at various pH values. The K_m for urea was nearly constant over the pH range at which the enzyme is stable; i.e., the K_m varied slightly from ca. 2 to 4 mM. In contrast, the V_{max} exhibited an optimum from pH 7.25 to 8.25. The effect of pH on log V_{max}/K_m is shown in

Figure 6. The slope is 1 below the optimum and -1 above the optimum with inflection points at pH values of 6.55 ± 0.10 and 8.85 ± 0.10 (26).

Amino acid analysis- The amino acid composition of *K. aerogenes* urease is shown in Table 3. Also shown for comparison are the compositions reported for ureases from *B. ammoniagenes* (9), jack bean (5), and soy bean (8). The relatedness of *K. aerogenes* urease to these other proteins was examined by using the method of Cornish-Bowden (27) in which the difference index (DI), the compositional divergence (D) and the Marchalonis and Weltman index ($S\Delta Q$) were calculated.

Atomic absorption analysis- Purified *K. aerogenes* urease was demonstrated to contain nickel by atomic absorption analysis. Three independent preparations of urease were found to contain 2.1 ± 0.3 mol Ni/mol 72,000 dalton subunit when compared to a standard curve which was prepared as described in Methods.

DISCUSSION

The studies reported here extend the work of Friedrich and Magasanik with *K. aerogenes* urease (13) and of Kamel and Hamed with urease from the related microbe, *A. aerogenes*

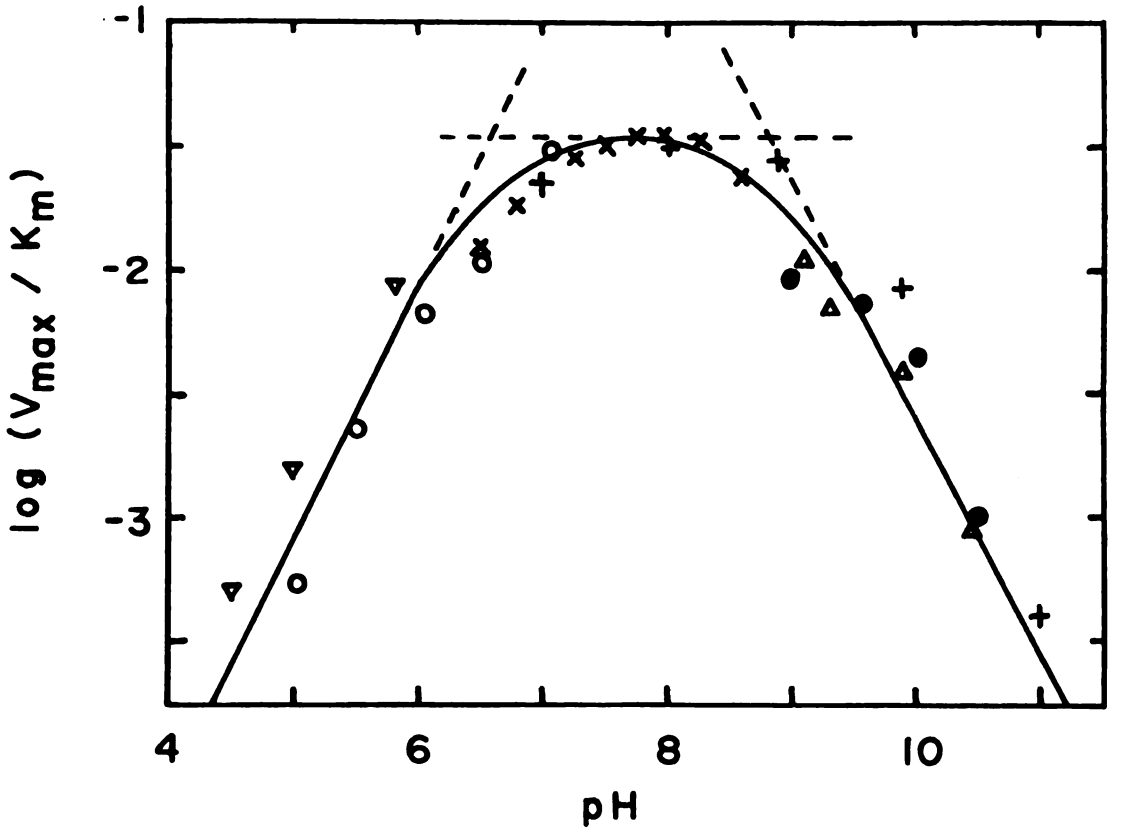

Figure 6. **The effect of pH on log V_{max} / K_m for *K. aerogenes* urease.** Urease V_{max} and K_m values were calculated and the log of the ratio is plotted here in arbitrary units versus the pH of the assay buffer. The buffers include acetate (O), MES (∇), HEPES (X), CHES (Δ), CAPS (●), and phosphate (+).

Table 3
Amino Acid Compositions of Ureases

Amino Acid	*K. aerogenes*[a]	*B. ammoniagenes*[b]	Jack bean[c]	Soybean[d]
Cys	1.44	0.38	1.79	0.47
Asx	9.23	11.60	10.60	12.76
Thr	6.90	7.23	6.55	5.28
Ser	6.05	3.64	5.48	5.79
Glx	10.32	10.22	8.22	10.09
Pro	5.50	4.66	5.00	6.09
Gly	12.35	9.68	9.40	10.35
Ala	10.45	10.68	8.80	7.44
Val	7.46	7.80	6.55	6.34
Met	1.68	1.75	2.50	2.04
Ile	5.72	7.13	7.86	6.16
Leu	6.92	7.46	8.21	8.17
Leu	6.92	7.46	8.21	8.17
Tyr	2	1.17	2.5	2.43
Phe	2.57	3.02	2.86	4.23
His	3.00	2.91	2.98	2.09
Lys	3.44	4.87	5.71	5.64
Arg	3.96	4.89	4.52	4.62
Trp	0.99	0.90	0.48	NR[e]
Related indices[f]				
D1	0	8.1	9.58	10.62
D	0	0.052	0.057	0.065
SΔQ	0	26.8	32.33	41.73

[a] Values represent the mole percent of each amino acid.
[b] Taken from (9).
[c] Taken from (5).
[d] Taken from (8).
[e] NR, not reported.
[f] Relatedness indices calculated for each urease compared to the *K. aerogenes* urease using the method of Cornish-Bowden (27).

PRL-R3 (14). *K. aerogenes* urease was purified 1070-fold to homogeneity by standard chromatographic techniques, similar to the procedure used to isolate *S. ruminantium* urease (11). The final specific activity achieved for the *K. aerogenes* enzyme (2200 μmol min^{-1} mg^{-1}) is among the highest reported for any bacterial urease and approaches that of the jack bean enzyme, about 3500 μmol min^{-1} mg^{-1} or 93 katal/liter $/A_{280}$ (where a katal is the amount of enzyme which degrades 1 mol of urea/s in a defined pH-stat assay) (1). For comparison, 24-fold-purified enzyme obtained by Friedrich and Magasanik had an activity of 45.4 μmol min^{-1} mg^{-1} (13), and 150-fold-purified *A. aerogenes* urease had an activity of 690 μmol min^{-1} mg^{-1} (14). The K_m for urea of 2.8 mM determined for purified *K. aerogenes* urease was 4 times that determined by Friedrich and Magasanik of 0.7 mM (13); however, different strains were employed. Similarly, the K_m was 2-fold greater with this enzyme compared to the 1.48 mM K_m determined for *A. aerogenes* urease (14). The V_{max} calculated for *K. aerogenes* urease was 2800 μmol min^{-1} mg^{-1}.

A novel feature of *K. aerogenes* urease is the presence of three polypeptide components in the active enzyme. One of these species (M_r = 72,000) is similar in size to many other microbial ureases, whereas the two smaller components (M_r = 11,000 and 9,000) have not been described in any other urease enzyme. Intensive efforts were used to resolve the larger polypeptide from the smaller components; however, the three species remained associated under all conditions

which retained urease activity (presence of salt, glycols, thiols, detergents, and mixtures of these substances). These results may indicate that all three polypeptides are subunits of *K. aerogenes* urease. The three polypeptides are unlikely to be proteolytically derived from a common precursor found in cell extracts, because a variety of protease inhibitors (toluenesulfonyl flouride, bestatin, pepstatin, leupeptin, aprotinin, phosphoramidone, and EDTA) had no apparent effect on the SDS-PAGE gel profile for the purified enzyme. The two smaller polypeptides run at the dye front in gels containing less than 10% acrylamide, and these species can be easily overlooked. Indeed, we have found that the *S. ruminantium* urease, previously thought to possess a singly 70,000-dalton polypeptide (11), also exhibits two small polypeptides on 20% acrylamide gels. Furthermore, in our most highly purified samples of urease from *Proteus mirabilis* (provided by Julie M. Breitenbach) and *Sporosarcina urea* (provided by Rick Ye) we have observed a large subunit and two small polypeptides (Figure 5). Thus, one large and two small polypeptides may be generally associated with microbial ureases. Recent studies involving the cloned urease gene from *Providencia stuartii* support this hypothesis (28). Mobley *et al.* (28) found that there may be at least two polypeptides peptides (M_r = 73,000 and 25,500) associated with urease in this microbe by using transposon mutagenesis. In contrast to the above microbial enzymes, we have not observed any small polypeptides

associated with jack bean urease. Further efforts are clearly required to establish the roles for each of the three *K. aerogenes* urease components.

The quaternary structure and native molecular weight of *K. aerogenes* urease were not precisely established. From the integrated intensities of the gel scan profiles, an approximate stochiometry for the three components was found to be 1:2:2. This result, combined with the native molecular weight determined by gel electrophoresis and Superose 6 gel filtration chromatography, would indicate a native urease structure (M_r = 224,000) containing 2 mol of the 72,000-dalton peptide, 4 mol of the 11,000-dalton peptide and 4 mol of the 9,000-dalton component. These results contrast significantly with the simple homohexameric structure of jack bean urease and the suggested homopolymeric structures (trimer, tetramer, pentamer, and hexamer) reported for other microbial ureases (9, 10, 11, 29, 30).

Stability studies of ureases from both jack bean (1) and *Arthrobacter oxydans* (31) showed irreversible loss of activity below pH 4.5. Similar results were observed with the *K. aerogenes* enzyme; in addition, a high pH inactivation occurred at pH 10.5 and above. As in the case of the jack bean urease (32), the K_m for urea is nearly constant throughout the entire pH range over which the enzyme is stable. *K. aerogenes* urease exhibited a V_{max}/K_m dependence on pH which is most easily interpreted by assuming a catalytic requirement for a deprotonated active site group

with a pK_a = 6.55 and a protonated group with a pK_a = 8.85. Very early work with jack bean urease indicated similar simple behavior with pK_a values of 6.1 and 9.2 (33); however, subsequent studies suggest a more complex behavior (32). In contrast to *K. aerogenes* urease, the urease from *A. aerogenes* exhibited very complex pH behavior and was suggested to possess three functional groups with pK_a values of 5.6, 6.65, and 8.1 (14).

The amino acid composition for *K. aerogenes* urease was determined and compared to that of jack bean, soy bean, and *B. ammoniagenes* ureases. *K. aerogenes* urease was not related to the plant enzymes according to the "weak" test of Cornish-Bowden (27); *i.e.* the relatedness indices exceeded the recommended limits for a sequence of 840 amino acids. Surprisingly, the two microbial urease compositions also fail to meet the weak test for relatedness; however, the D1, D, and SΔQ values indicated that *K. aerogenes* urease was more closely related to the bacterial than the plant enzymes.

K. aerogenes urease has joined the growing list of nickel-containing enzymes which include other ureases, methylcoenzyme M reductase, carbon monoxide dehydrogenase, and certain hydrogenases (35). Thus far, nickel has been shown to be present in ureases from jack bean (6), soy bean (8), *S. ruminantium* (11), *B. ammoniagenes* (9), *B. pasteurii* (10), and *A. oxydans* (30). Furthermore, indirect evidence of nickel-dependent growth is consistent with the presence

of nickel in ureases from other plants (34) and from *S. ureae* (31), *Aspergillus nidulans* (36), *Phaeodactylum tricornutum* (37) and *Tetraselmis subcordiformis* (37). The nickel stochiometry is clearly 2 mol nickel/mol subunit in the jack bean and soybean enzymes, whereas either 1 mol (9,10) or 2 mol of nickel (11) per subunit has been found for the bacterial ureases. The *K. aerogenes* urease results are most consistent with 2 mol nickel/mol of the 72,000-dalton subunit. The environment of nickel and its role in urease hydrolysis have not been determined.

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Competitive Inhibitors of *Klebsiella aerogenes* Urease: Mechanism of Interaction with the Nickel Active Site

ABSTRACT

We examined several compounds for their mechanisms of inhibition with the nickel-containing active site of homogeneous *Klebsiella aerogenes* urease. Thiolate anions competitively inhibit urease and directly interact with the metallocenter, as shown by UV-visible absorbance spectroscopic studies. Cysteamine, which possesses a cationic β-amino group, exhibited a high affinity for urease (K_i = 5 μM), whereas thiolates containing anionic carboxyl groups were uniformly poor inhibitors. Phosphate monoanion competitively inhibits a protonated form of urease with a pK_a of less than 5. Both the thiolate and phosphate inhibition results are consistent with charge repulsion by an anionic group in the urease active site. Acetohydroxamic acid (AHA) was shown to be a slow-binding competitive inhibitor of urease. This compound forms an initial E·AHA complex which then undergoes a slow transformation to yield an E·AHA* complex; the overall dissociation constant of AHA is 2.6 μM. Phenylphosphorodiamidate, also shown to be a slow-binding competitive inhibitor, possesses an overall dissociation constant of 94 pM. The tight binding of

phenylphosphorodiamidate was exploited to demonstrate the presence of two active sites per enzyme molecule. Urease contains 4 mol nickel/mol enzyme, hence there are two mol nickel ion/catalytic unit. Each of the two slow-binding inhibitors are proposed to form complexes in which the inhibitor bridges the two active site nickel ions. The inhibition results obtained for *K. aerogenes* urease were compared with inhibition studies of other ureases and are interpreted in terms of a model for catalysis proposed for the jack bean enzyme (Dixon, N. E., Riddles, P. W., Gazzola, C., Blakeley, R. L., and Zerner, B. (1980) *Can. J. Biochem.* **58**, 1335-1344.)

INTRODUCTION

Urease (urea amidohydrolase E. C. 3.5.1.5) is a nickel-containing enzyme that hydrolyzes urea to form carbamate and ammonia; carbamate spontaneously degrades to CO_2 and a second molecule of ammonia (1-3). The best characterized urease is the enzyme purified from the plant *Canavalia ensiformis* (jack bean) (1,2). Jack bean urease is a homohexameric enzyme (subunit M_r = 90,770; 4) which contains 2 mol nickel/mol subunit (5). Zerner and colleagues have proposed a model for jack bean urease catalysis in which one nickel coordinates the oxygen atom of urea, polarizing the carbonyl group, and a second nickel coordinates hydroxide ion, the catalytic nucleophile. Three amino acid residues are also proposed to be located at the active site (6): a carboxyl group, a sulfhydryl group, and an unidentified base. Despite extensive investigation the detailed mechanism of catalysis has not been established and the structure of the metallocenter is unknown.

In contrast to the wealth of studies on the jack bean enzyme, much less in known about catalysis by microbial ureases (3). Clear structural differences from the plant enzyme have been observed; for example, the bacterial urease of *Klebsiella aerogenes* possesses three subunit types (M_r = 72,000, 11,000, and 9,000) in an $\alpha_2\beta_4\gamma_4$ stoichiometry (7)

and contains 4 mol nickel/mol enzyme (7). Further characterization of microbial urease is important because infectious ureolytic microorganisms can contribute to the development of urinary stones, pyelonephritis, gastric ulceration, catheter encrustation, and other pathogenic conditions (3). In addition, elevated levels of soil microbial urease activity can decrease the efficiency of urea fertilizers (8) and retard seed germination and seedling growth (9). Thus, the study of urease inhibitors may have medical or agronomic significance, as well as providing insight into the urease catalytic mechanism.

Here we characterize the inhibition of purified *K. aerogenes* urease by thiols, phosphate, acetohydroxamate and phenylphosphorodiamidate. We compare these results to previous bacterial urease inhibition studies, and we discuss our results in terms of the catalytic model proposed for jack bean urease.

EXPERIMENTAL PROCEDURES

Materials- Sources are: acetohydroxamic acid, bovine serum albumin, cysteamine, cysteine methyl ester, β-mercaptoethanol (Sigma); phenylphosphorodiamidate (ICN Biomedicals Inc.); urea (enzyme grade) (U. S. Biochemical Corp.); ethane thiol, 3-mercapto-2-butanol, 2-mercaptopropionate, 3-mercaptopropionate, methylthioglycolate, 2-propane thiol,

3-mercapto-1,2-propanediol (Aldrich); cysteine (Fisher); cresol red (Allied Chemical and Dye Corp.).

Enzyme purification- Urease was purified from *K. aerogenes* (currently *K. pneumoniae)* strain CG 253 carrying the recombinant plasmid pKAU19 (10) by procedures essentially identical to those described previously (7). Since the initial specific activity was much higher in this overproducing organism, the final gel filtration step was not required for maximal specific activity (2500 μmol min^{-1} mg^{-1}). The K_m value for urease was reevaluated to be 2.4 mM at pH 7-9 rather than 2.8 mM as reported initially (7). The K_m decreases moderately to 0.72 mM at pH 5-6. The enzyme appeared homogenous by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Enzyme concentrations were based on a molecular mass of 224 kDa (7).

Assays- Unless otherwise noted, urease was assayed in the presence of 5 mM EDTA, 50 mM HEPES, pH 7.75 and 50 mM urea at 37°C (standard assay mixture). Reactions were initiated by addition of small aliquots ($\leq$ 0.1 ml) of enzyme to standard assay mixtures ($\geq$ 2.0 ml) at 37°C. Portions of the assay mixtures were withdrawn at intervals and the ammonia was converted to indophenol (11) which was quantitated by measuring the absorbance at 625 nm. Linear regression analysis of the ammonia concentrations versus time gave the initial rate.

Alternative assays were used to measure urea hydrolysis when compounds interfered with the indophenol ammonia assay. Cysteine inhibition was examined by using an ammonia microelectrode (Microelectrodes Inc.). Standard assays with varying urea and cysteine concentrations were run, and the reaction was terminated by addition of 0.1 ml of 2.0 M HNO_3 to 0.8 ml aliquots of these assays. A low pH was chosen to terminate the reaction because urease is irreversibly inactivated below pH 4.5 and because the ammonia exists as the nonvolatile ammonium ion at low pH values. Ammonia concentration was determined immediately after aliquots were made 1.0 M in KOH. Millivolt readings were converted to concentration by comparison to an NH_4Cl standard.

A spectrophotometric assay was employed to examine the effect of thiourea on urease activity. Urease was added to a 1.0 ml cuvette at 37°C containing 10 mM Tris·HCl pH 7.7, 80 µg/ml cresol red, and varied concentrations of urea and thiourea. Urea hydrolysis was monitored by the increase in the absorbance at 573 nm, which was linear with time between 0.8 and 1.6 absorbance units. Initial rates were calculated by comparison to absorbance changes in a control to which aliquots of KOH were added.

Protein was assayed by the method of Lowry (11) with bovine serum albumin as the standard.

Spectroscopy- All spectra were obtained by using a Gilford Response spectrophotometer with a 1.0 cm

microcuvette (25°C). Spectral data were transferred to an IBM-PC to calculate difference spectra. Enzyme samples were extensively dialyzed prior to scanning to remove the β-ME present during enzyme storage.

K_i Determinations- For inhibitors which equilibrate rapidly with urease, initial rates were determined in the presence of four different inhibitor concentrations at each of seven urea concentrations (50, 10, 5, 3.3, 2.5, 1.667, and 1.25 mM). Data were plotted according to Lineweaver-Burk (13), and best fit lines were calculated by using the method of Wilkinson (14). K_i was determined from replots of slope or apparent K_m values *versus* inhibitor concentration (15). The response of K_i to changes in pH was investigated by using 100 mM MES (pH 5.00-6.75) or 100 mM HEPES (pH 6.75-8.50) buffers, with 5 mM EDTA, inhibitor and urea (as indicated above).

Inhibitor Binding Rates in the Absence of Substrate- Urease was added to 10 mM EDTA, 100 mM HEPES, pH 7.75 at 37°C containing the indicated concentrations of slow binding, competitive inhibitor. At regular intervals, small aliquots were diluted 40-fold into the standard assay mixture, and activities were determined. Linear regression analyses of the initial rates provided correlation coefficients $r > 0.995$, indicating that further inhibitor binding did not occur and that very little enzyme-inhibitor

complex dissociated during the assays. Apparent rate constants were calculated by linear regression analyses of the data plotted as $\ln(V_t/V_0)$ *versus* time (V_t = initial velocity after t min preincubation with inhibitor and V_0 = initial velocity of enzyme in the absence of inhibitor).

Progress Curves in the Presence of Inhibitor- Progress curves for the rate of urea hydrolysis in the presence of inhibitors were obtained by measuring the ammonia produced as a function of time (P_t). For each experiment, 25-50 time points were taken. A curve-fitting program (see below) was used to fit the data points to:

$$P_t = P_0 + V_f t + (V_i - V_f)(1 - e^{-k_{app} t}) k_{app}^{-1} \quad (1)$$

where P_0 = product present at time 0, V_f = steady state velocity, V_i = initial velocity, and k_{app} = apparent rate constant (16).

Dissociation Rate- The rate of dissociation for the enzyme-inhibitor complex (which was <2% active) was measured by diluting 10,000-fold into an assay mixture at 37°C containing the indicated concentrations of urea in 10 mM EDTA, 100 mM HEPES, pH 7.5. The concentration of AHA during the assay was less than 0.1 μM, and the concentration of PPD was less than 0.1 nM. Progress curves for the recovery of

activity were monitored for several hours and fit to Equation 1.

Dissociation of AHA-urease was also determined for the isolated urease-AHA complex at 37°C, following removal of excess AHA on a Bio-Gel P-6 (0.7 x 14.0 cm) gel filtration column. Dissociation was followed by periodically diluting a small aliquot into the standard assay mixture and comparing the activity to an appropriate control. The rate of dissociation (k) was obtained by fitting the fraction activity recovered as a function of time (F_t) to Equation 2.

$$F_t = F_0 + (1 - F_0)(1 - e^{-kt}) \qquad (2)$$

Statistical Methods--Results are provided as the mean ± S.D., where applicable. The fitting software (17) is available free of charge to those with a modem. The BASIC program uses the Marquardt Algorithm (18) to fit by least-squares a set of data points to a curve. The user supplies a function and the partial derivatives with respect to each parameter to be fit.

Quantitation of Active Sites- The graphical method of Ackerman-Potter (19) was used to determine the number of active sites per native enzyme molecule. Urease (0.0-10.0 nM) in 100 mM HEPES, 10 mM EDTA, 0.1 mM β-ME, pH 7.75 was incubated with 0, 5, or 10 nM PPD at 0°C. Aliquots were

analyzed for activity at 37°C after 2, 24, and 96 h by using the standard assay mixture.

RESULTS

Thiol Inhibition- Several thiol compounds were shown to be competitive inhibitors of urease, with apparent K_i values as provided in Table 1. To determine whether the protonated thiol or the thiolate anion was the actual inhibitory species, the pH dependence of cysteamine inhibition was characterized (Figure 1). Log K_i responded linearly to pH changes, with a slope of -1 at pH values below the cysteamine pK_a of 8.1 and with a slope of 0 at pH values above this pK_a. This behavior is consistent with the deprotonated thiol acting as the inhibitory species.

To compare relative inhibition within the series of thiols in Table 1 the pH dependence of each urease inhibitor should be determined. However, in this initial analysis, the K_i values were simply recalculated on the basis of the actual thiolate anion concentrations at pH 7.75 by using published pK_a values. Of the compounds tested, the thiolate with the lowest K_i value (5.0 μM) is cysteamine, which has a positively charged β-amino group at pH 7.75. In contrast, thiol compounds containing an anionic carboxyl group are poor inhibitors at pH 7.75 (*e.g.* 3-mercaptopropionate and cysteine, with K_i values of >500 μM and 9600 μM,

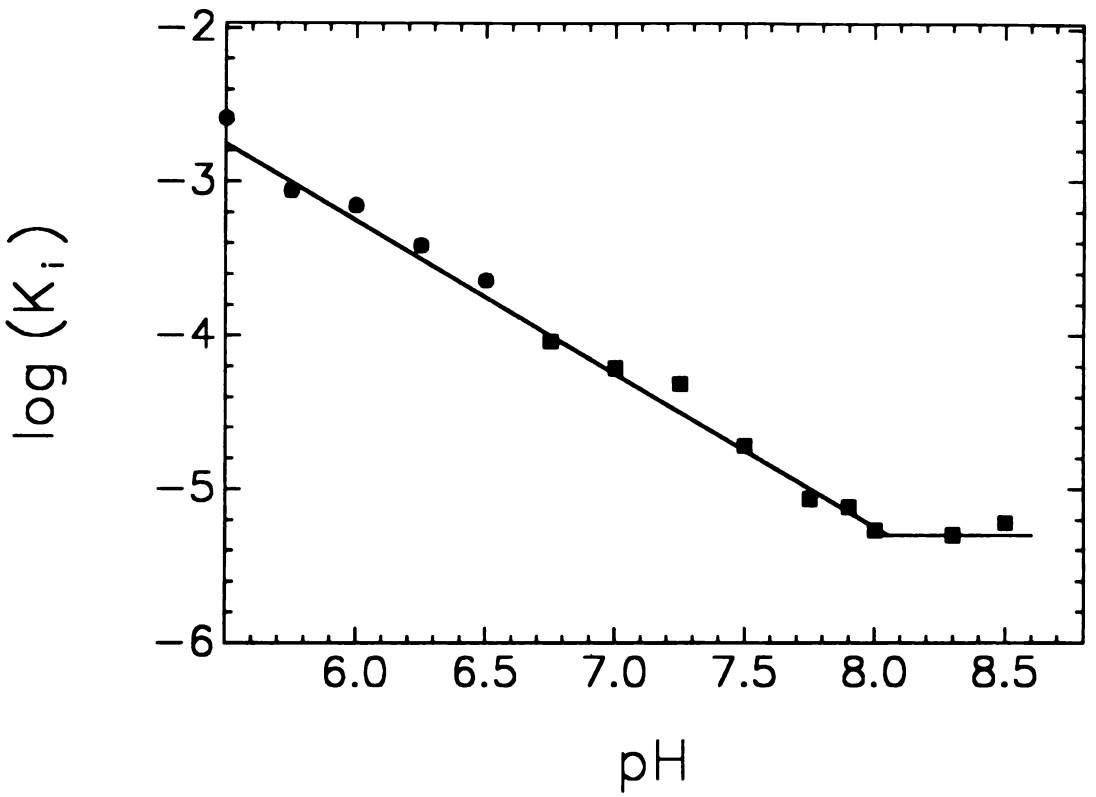


Figure 1. **pH dependence of cysteamine inhibition of urease.** The K_i values for competitive inhibition of urease by cysteamine were determined in MES (●--●) or HEPES (■--■) buffers and are plotted as log(K_i) *versus* pH.

Table 1

Thiol Inhibition of K. aerogenes urease

Thiol	Total Inhibitor K_i^a	Thiolate anion		Ref.
		K_i^b	pK_a	
	mM	*μM*		
cysteamine	0.010	5.0	8.1	20
β-mercaptoethanol	0.55	7.7	9.6	20, 21
3-mercapto-1,2-propane diol	1.7	21	9.66	22
ethanethiol	21	37	10.5	20
cysteine methyl ester	0.12	110	6.53	20
3-mercapto-2-butanol	13.5	190		
methylthioglycolate	1.6	850		7.7
2-propane thiol	> 100	> 140	10.6	21
cysteine	95	9600	8.7	20
3-mercaptopropionate	> 100	> 500	10.05	20, 21, 23

a) Based on total inhibitor concentration regardless of species present at pH 7.75.

b) Thiolate anion K_i values at pH 7.75 are calculated based upon the literature pK_a values.

respectively). Esterification of the carboxyl group enhances binding as demonstrated by cysteine methyl ester with a K_i of 110 μM. Thiols with uncharged side chains possess intermediate K_i values ranging from 7.7 μM to 190 μM. The ability of 3-mercapto-2-butanol to inhibit urease indicates that the active site must be accessible to secondary thiols.

Spectral interactions of β-ME with urease- In the absence of thiols, *K. aerogenes* urease displays an absorption spectrum (Figure 2) which includes a peak at 406 nm (ε = 2,240 $M^{-1}cm^{-1}$, based on M_r = 224,000). This spectrum is essentially pH-independent (data not shown). Binding of thiolate anions enhances the absorbance in four regions between 800 and 300 nm with the exact wavelength depending upon the thiolate structure. For example, the difference spectra maxima were 322, 374, 432, and 750 nm for β-ME, *versus* 339, 387, 425, and 750 nm for cysteamine. These spectral interactions are similar to those seen with known thiolate-nickel complexes (*e.g.* nickel-substituted metalloenzymes or model compounds; 24).

A double reciprocal plot (Figure 3) of the absorbance changes *versus* β-ME concentration provided the binding constant (K_d = 0.38 ± 0.12 mM) and the extinction coefficients for the thiolate-nickel complex ($\varepsilon_{322\ nm}$ = 2230 ± 220 M^{-1} cm^{-1}, $\varepsilon_{374\ nm}$ = 1060 M^{-1} cm^{-1}, $\varepsilon_{432\ nm}$ = 530 ± 80 M^{-1} cm^{-1}). The slight upward curvature at low β-ME concentrations in Figure 3 is a result of the high protein concentration (0.25

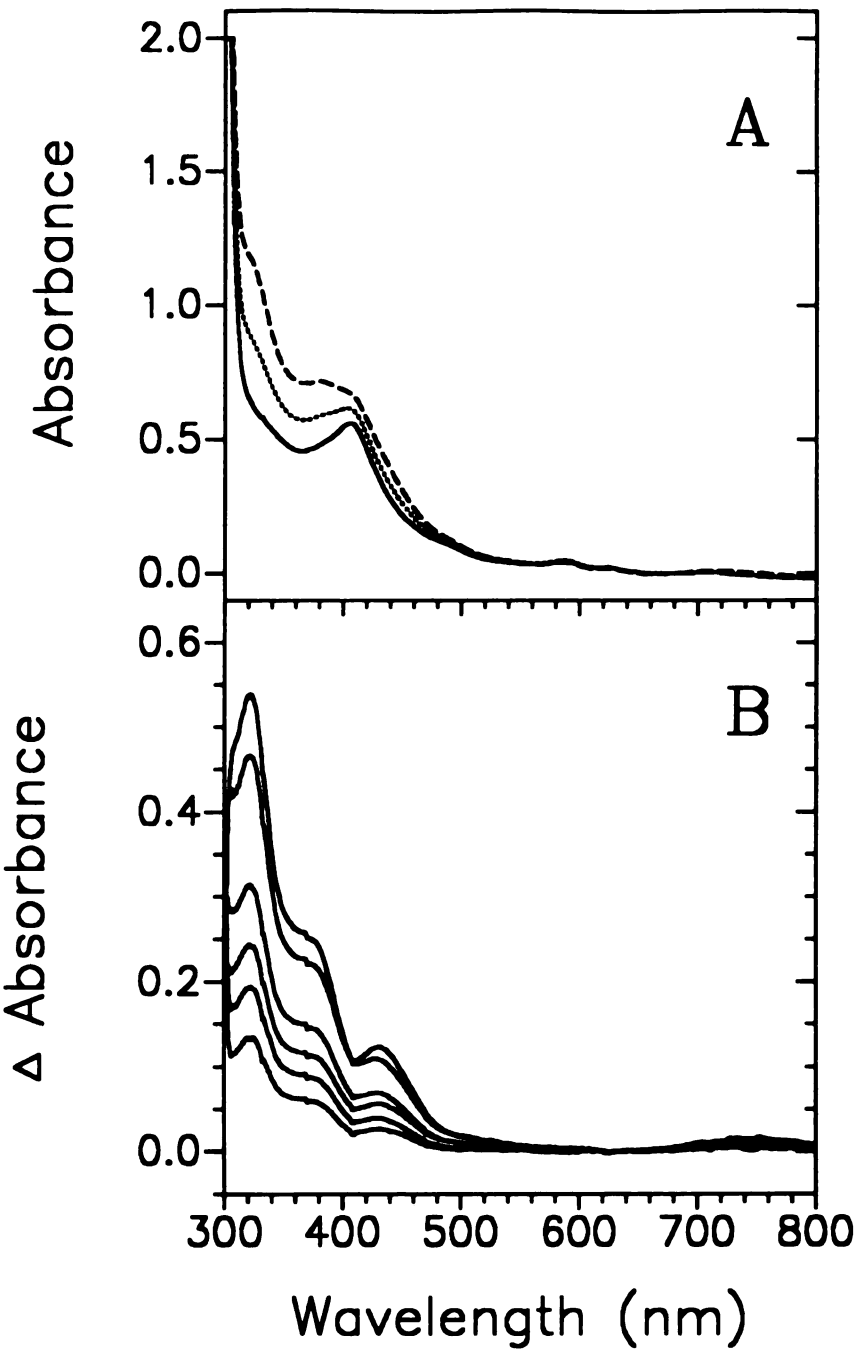


Figure 2. **UV-visible spectra of urease in the presence of thiol.** *A,* Spectra of urease (56 mg/ml) in 10 mM EDTA, 100 mM HEPES, pH 7.75, at 25°C are shown in the presence of 0.0 mM (——), 0.33 mM (· · · ·), and 5.0 mM (----) β-ME. *B,* Difference spectra of urease in the presence of 0.2, 0.25, 0.33, 0.5, 1, or 5 mM β-ME were calculated *versus* the control (0.0 mM β-ME, 56 mg/ml).

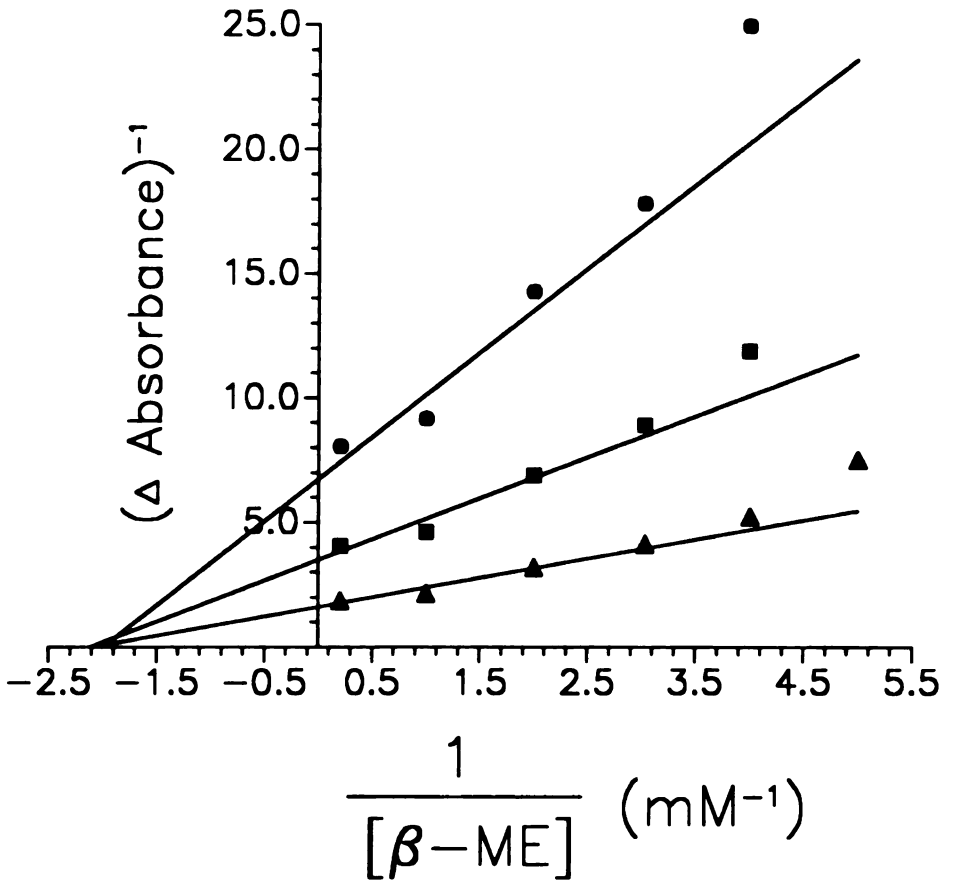


Figure 3. **Double-reciprocal plot of 1/Δ absorbance *versus* 1/[β-ME].** The inverse change in absorbance at 25°C was plotted as a function of 1/[β-ME] at $A_{432\ nm}$ (●), $A_{374\ nm}$ (■), and $A_{322\ nm}$ (▲).

mM) required to observe these absorbance changes. A Dixon plot (15), used when the enzyme and inhibitor concentrations are of the same magnitude, yielded the same K_d value (data not shown). The spectrally determined K_d value is identical to the K_i determined kinetically for β-ME inhibition of urea hydrolysis at pH 7.75 (0.55 ± 0.08 mM).

Phosphate Inhibition- Lineweaver-Burk plots demonstrated that phosphate competitively inhibits urease at pH values from 5.0 to 7.0 (data not shown). Urease is labile at pH values below 5.0 and phosphate inhibition was not examined. Above pH 7.0 phosphate inhibition was not purely competitive, possibly due to the effects of high ionic strength. Within the pH range examined, values of log K_i are pH dependent (Figure 4), exhibiting a slope of 1 from pH 5.0 to 6.3 and a slope of 2 from pH 6.3 to 7. Thus, phosphate inhibition of urease appears to require protonation of two ionizable groups with pK_a values of 6.3 and <5.

Acetohydroxamate inhibition- Progress curves for urea hydrolysis in the presence of AHA are shown in Figure 5. These curves demonstrate a gradual decrease in urease activity in the presence of AHA. The concentration of both AHA and urea appear to affect the rate of inhibitor binding. These initial results are consistent with slow-binding competitive inhibition. Such inhibitors are thought to follow the mechanism shown below (16), where inhibitor (I) competes

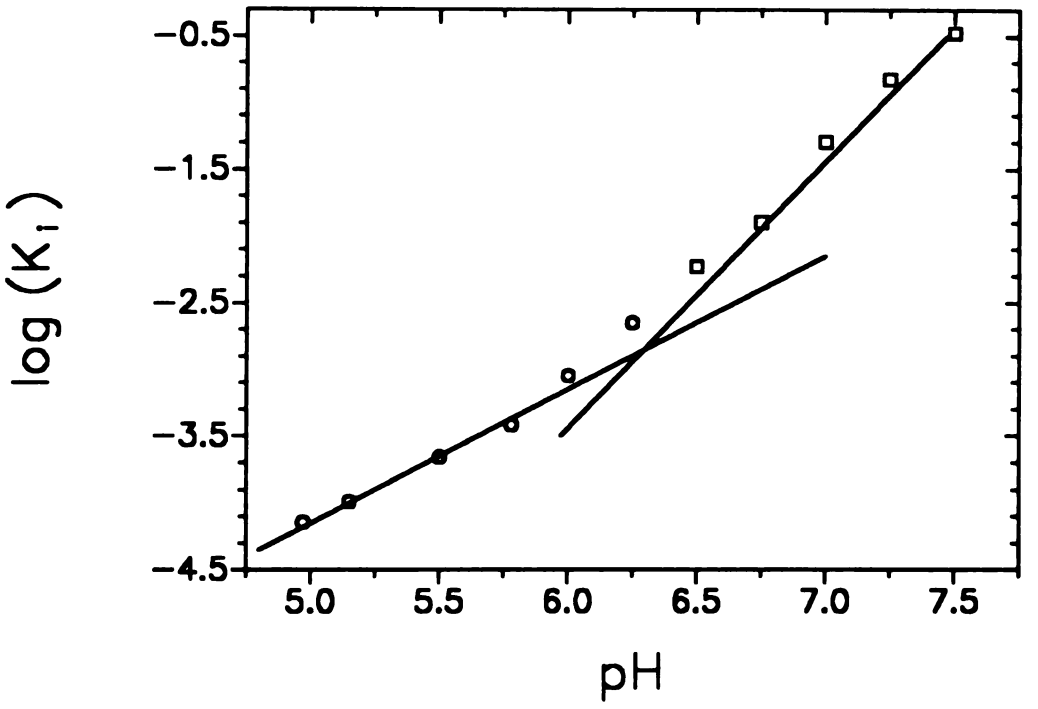


Figure 4. **pH dependence of phosphate inhibition.** The K_i for competitive inhibition of urease by total phosphate species was determined in MES (O) or HEPES (□) buffers and is shown as log (K_i) *versus* pH.

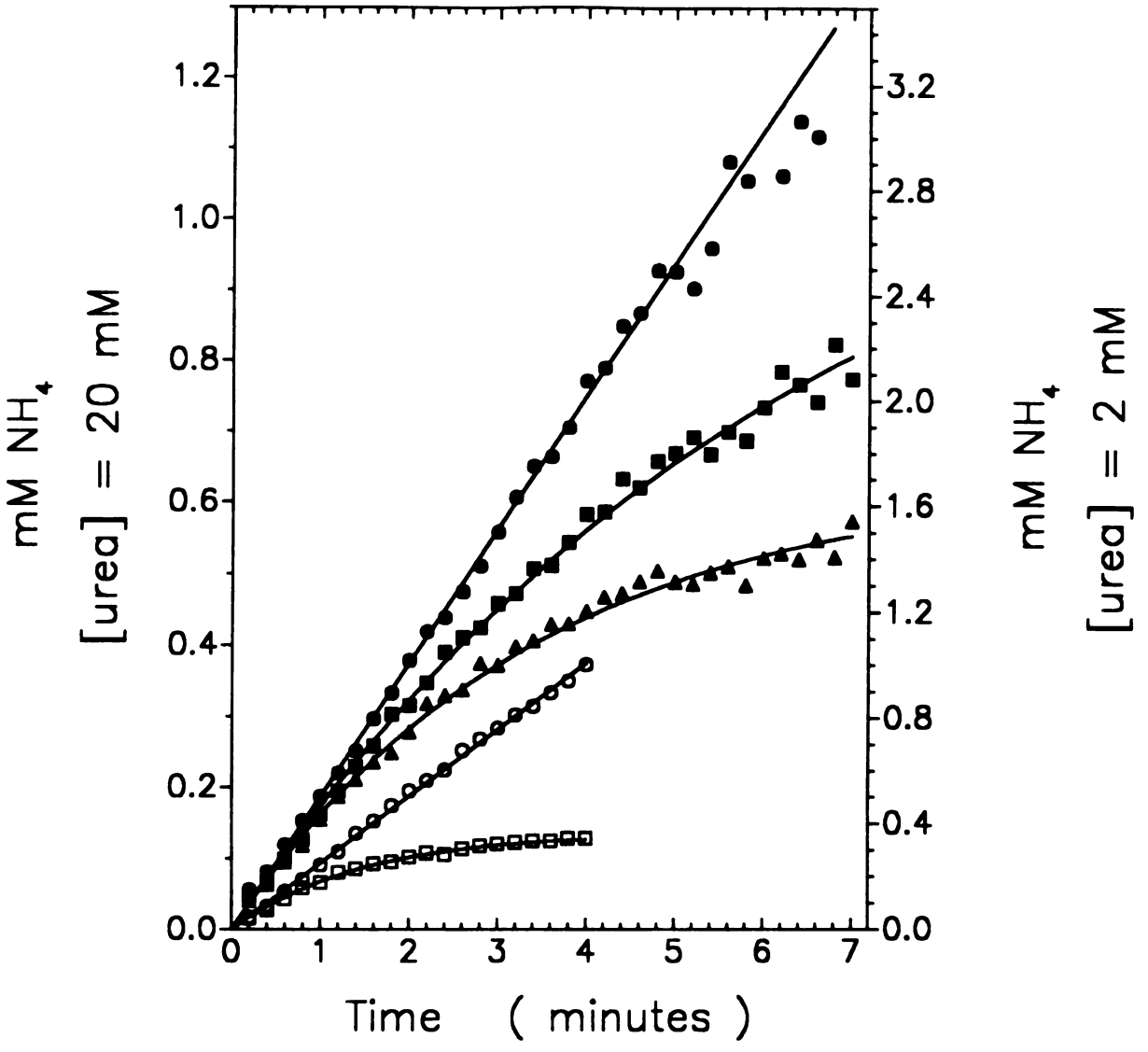
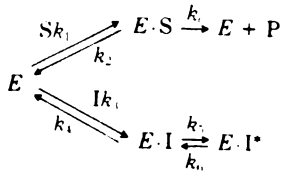


Figure 5. **Urease progress curves in the presence of AHA.** Urease was assayed at 37°C in 10 mM EDTA, 100 mM HEPES, pH 7.75 containing 20 mM urea (*solid symbols*) or 2 mM urea (*open symbols*), and 0.0 mM (*circles*), 1.5 mM (*squares*), or 3.0 mM (*triangles*) AHA. The reactions were initiated by addition of urease (with 20 mM urea, [urease] = 140 pM; with 2 mM urea, [urease] = 280 pM), and the product, ammonia, was monitored. The curves were fit as described under "Experimental Procedures."

with substrate (S) for enzyme (*E*) to form an initial enzyme-inhibitor complex (*E*·I), which is slowly transformed to a more stable complex (*E*·I*).



Scheme I

A series of experiments was designed to test this mechanism and to define the rate constants.

The effect of AHA concentration on the apparent rate of inhibitor binding in the absence of substrate is shown in Figure 6. At each inhibitor concentration, a first order loss of activity was observed. When the apparent rate constants derived from these and other experiments were replotted as a function of AHA concentration, they yielded a hyperbolic curve (Figure 6, *inset*). Morrison and Walsh (16) have shown that this behavior is expected for an inhibitor which reacts as illustrated in Scheme I. The apparent rate of inhibitor binding (k_{app}) follows Equation 3,

$$k_{app} = k_6 + k_5 \frac{\frac{[I]}{K_i}}{\frac{[I]}{K_i} + 1 + \frac{[S]}{K_m}} \qquad \text{(Eq. 3)}$$

where S = substrate, $K_m = (k_2 + k_c)/k_1$ = Michaelis constant, and $K_i = k_4/k_3$. Expanding from this equation, one can show that if

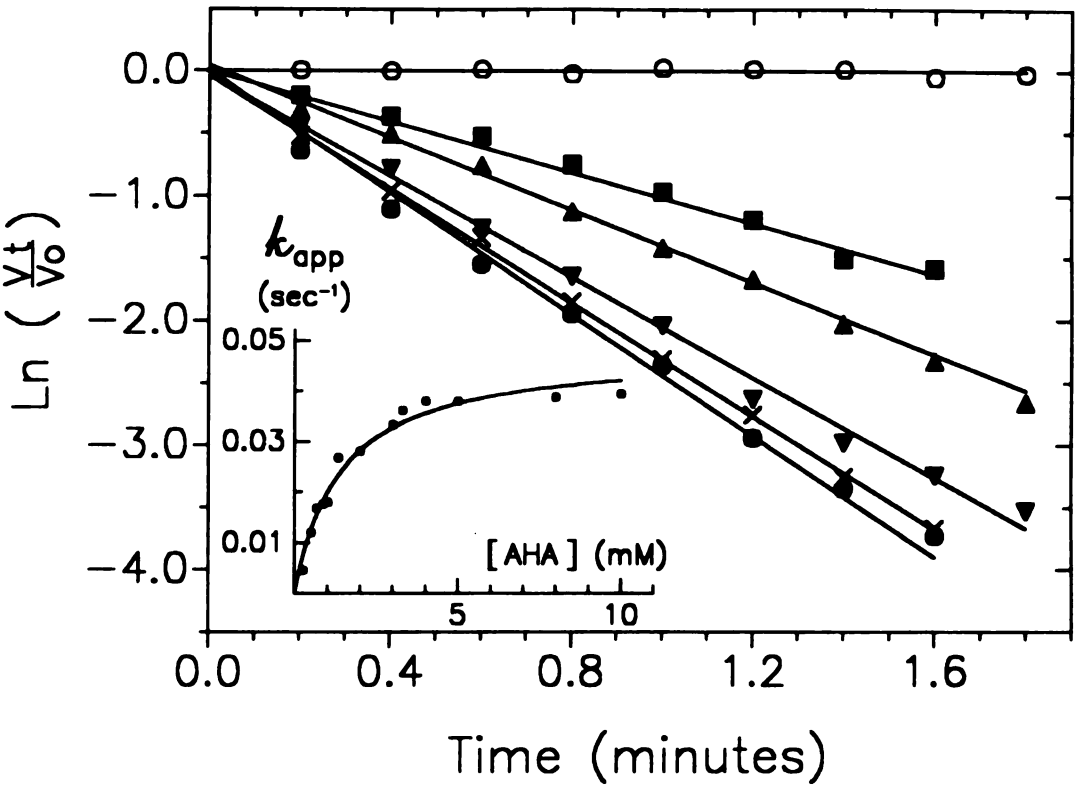


Figure 6. **Rates of AHA binding in the absence of substrate.** $\ln(V_t/V_o)$ is shown as a function of time for urease (3.5 nM) incubated at 37°C and pH 7.75 in the presence of 0.0 mM (O), 1.0 mM (■), 2.0 mM (▲), 3.0 mM (▼), 5.0 mM (X), or 10.0 mM (●) AHA. *Inset,* apparent binding rates are displayed as a function of AHA concentration.

$$k_6 << k_5 \quad \frac{[I]}{[I] + K_i\left(1 + \frac{[S]}{K_m}\right)} \qquad \text{(Eq. 4)}$$

then

$$k_{app} \sim k_5 \quad \frac{[I]}{[I] + K_i\left(1 + \frac{[S]}{K_m}\right)} \qquad \text{(Eq. 5)}$$

Hence,

$$\frac{1}{k_{app}} \sim \frac{1}{k_5} + \frac{K_i\left(1 + \frac{[S]}{K_m}\right)}{k_5\ [I]} \qquad \text{(Eq. 6)}$$

and a double reciprocal plot of $1/k_{app}$ *versus* 1/[I] should yield a straight line. The data obtained using AHA exhibit this behavior (Figure 7). The *y* intercept provides a value for k_5 = 0.047 ± 0.002 s^{-1} and the *x* intercept provides a value for K_i = 1.4 ± 0.2 mM.

Figure 7 also shows the result from a similar experiment carried out in the presence of β-ME, a competitive inhibitor of urease (see above). β-ME competes with AHA binding; the K_i value (0.26 ± 0.09 mM) is in reasonable agreement with the K_i for competitive inhibition of urea hydrolysis by β-ME (K_i = 0.55 ± 0.08 mM).

To demonstrate that AHA competes directly with urea, rate constants for AHA binding at several urea

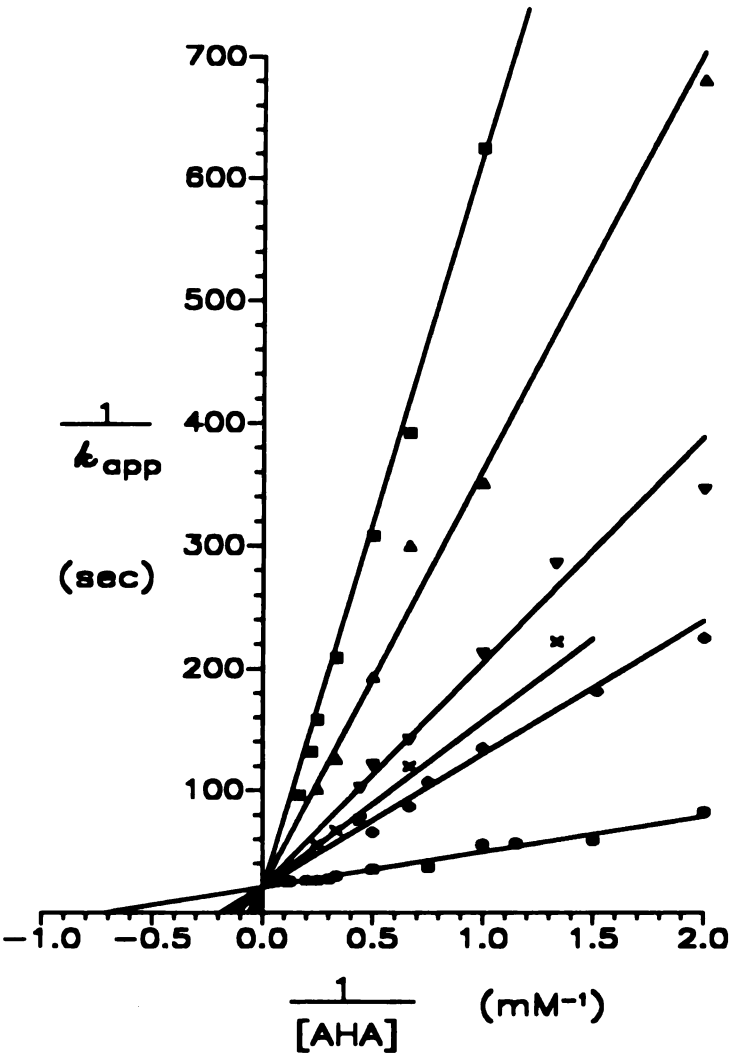


Figure 7. **Double reciprocal plot of $1/k_{app}$ *versus* 1/[AHA].** Data from Figure 6 (*inset*) are replotted as $1/k_{app}$ *versus* 1/[AHA] (●). Analogous experiments were carried out in the presence of 1.0 mM β-ME (X). Also shown are apparent rate constants for AHA binding from experiments such as Figure 5 for assays containing 5 mM (◆), 10 mM (▼), 15 mM (▲), and 20 mM (■) urea.

concentrations were obtained from progress curves such as those shown in Figure 5. The initial velocity did not appear to be affected by the presence of inhibitor; however, k_{app} responded hyperbolically to increasing AHA levels at each urea concentration (data not shown), similar to the response of k_{app} seen in the absence of substrate (Figure 6, *inset*). Double reciprocal plots (Figure 7) show that urea competes with AHA binding, and this K_i of 1.6 ± 0.2 mM is in close agreement with the kinetically determined K_m value of 2.4 ± 0.4 mM.

The rate of $E \cdot I^*$ dissociation, k_6, was determined by diluting the AHA-urease complex (or an appropriate control) into assay mixtures and measuring urea hydrolysis at 37°C as a function of time (Figure 8). The resulting progress curves (fit to Equation 1 with $V_f = V_o$) yielded values for k_6 of 9.4 ± 1.3 x 10^{-5} s^{-1} in 40 mM urea and a k_6 of 9.0 ± 0.8 x 10^{-5} s^{-1} in 5 mM urea.

An independent determination of k_6 was achieved by separating the AHA-urease complex from excess AHA by gel filtration and measuring activity as a function of time at 37°C. The k_6 determined by using Equation 2 was 7.8 ± 0.6 x 10^{-5} s^{-1}, in close agreement with the result obtained in the presence of substrate. Thus the approximation of Equation 4 is valid for the AHA and urea concentrations used to study the binding rates. Also consistent with the proposed mechanism, substrate concentration did not significantly affect k_6.

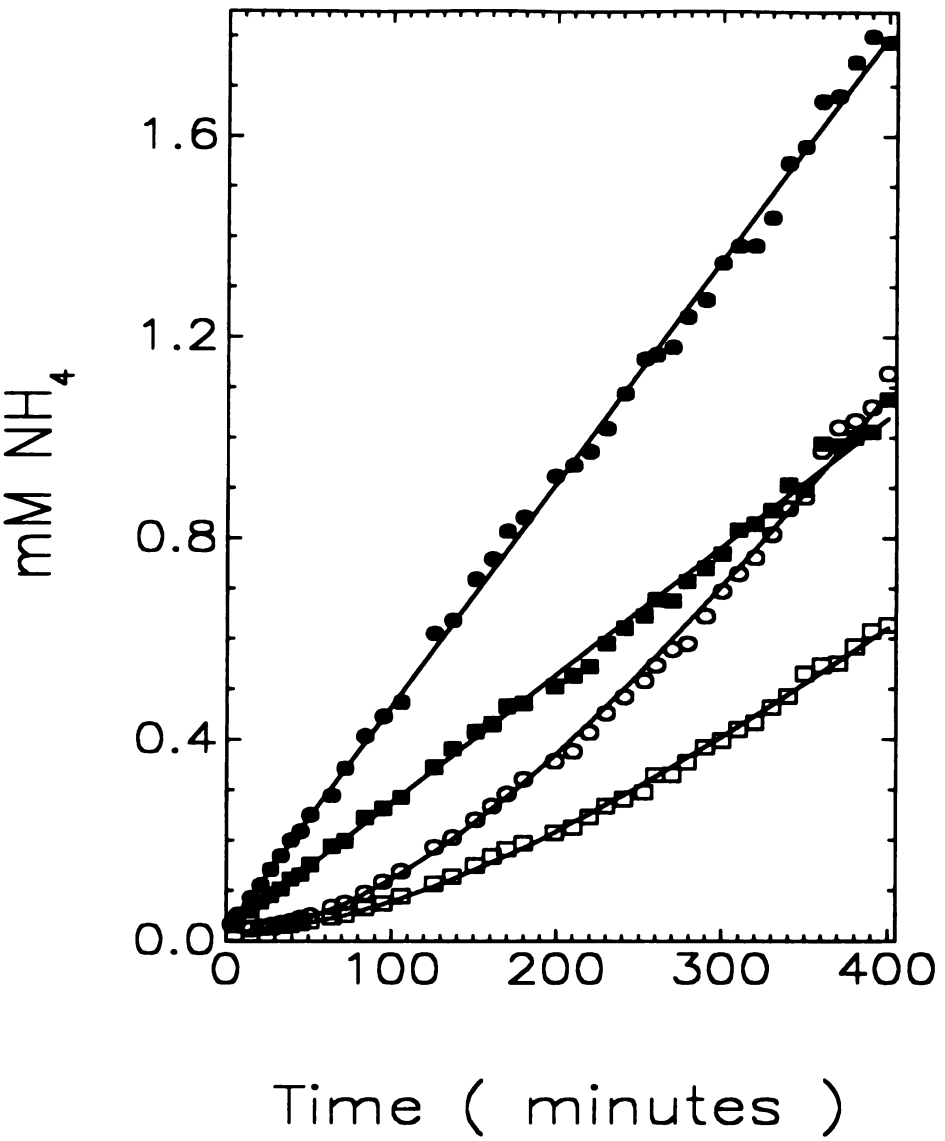


Figure 8. **Progress curves for AHA dissociation.** Urease which had been incubated with 1.0 mM AHA (*open symbols*) or buffer (*solid symbols*) was diluted 10,000-fold and assayed in the presence of 40 mM urea (*circles*) or 5 mM urea (*squares*) at 37°C and pH 7.75. The final concentration of urease was 8.1 pM. Curves were fit as described under "Experimental Procedures."

The overall inhibition constant for a slow-binding competitive inhibitor as expressed by Morrison and Walsh (16) is

$$K_i^* = K_i \frac{k_6}{k_6 + k_5} \qquad \text{(Eq. 7)}$$

For AHA inhibition of *K. aerogenes* urease, the K_i^* = 2.6 ± 0.4 μM.

In other experiments (data not shown), the kinetic constants for AHA binding were examined as a function of temperature and pH. K_i remained constant as the temperature varied between 37°C and 0°C, whereas k_5 decreased 72 fold (k_5 = 6.45 ± 0.36 x 10^{-4} s^{-1} at 0°C). The values for k_5 and K_i were nearly unchanged when measured at pH 6.75, 7.75, and 8.25; however, k_6 increased at lower pH values (*e.g.* k_6 = 17.2 x 10^{-5} at pH 6.50), decreasing the overall affinity of AHA for urease (Equation 7). The resulting increase in K_i^* could explain the apparent decrease in AHA inhibition seen by Hase and Kobashi (25) at low pH.

PPD Inhibition- Urease inhibition by PPD was also found to exhibit slow-binding kinetics. PPD experiments essentially identical to those described above for AHA were carried out yielding K_i = 95 ± 10 nM, k_5 = 0.074 ± 0.005 s^{-1}). As with AHA, competition for PPD binding by urea (K_i = 1.43 ± 0.15 mM) was in reasonable agreement with the kinetically determined K_m of urea (2.4 ± 0.4 mM). The rate

of urease-PPD dissociation (determined by assaying the complex in 250 mM urea) was k_6 = 4.7 x $10^{-5}s^{-1}$, thus the approximation of Equation 5 is valid for PPD. Using the K_i, k_5 and k_6 values determined above, Equation 7 gives K_i^* = 94 ± 10 pM.

Quantitation of Active Sites- The tight binding inhibition of urease by PPD was exploited to quantitate the number of active sites per native enzyme molecule by using the graphical method of Ackerman-Potter (19). As illustrated in Figure 9, incubation of PPD with urease led to a shift in the position of the velocity *versus* enzyme concentration curve. Extrapolation of the linear portion of the curve to zero velocity demonstrates that 5.0 nM PPD completely inhibits 2.3 nM enzyme and that 10.0 nM PPD completely inhibits 4.3 nM enzyme. Assuming that 1 mol PPD binds/mol active site, the enzyme contains 2.2 ± 0.2 catalytic sites/224 kDa. Previous work (7) had demonstrated that each mol of enzyme contained 4 mol of nickel, thus two nickel ions are present per catalytic unit. This metal content per active site is identical to that reported for jack bean urease (26).

Other Inhibitors- Boric acid and phenyl borate were found to be simple competitive inhibitors of *K. aerogenes* urease (K_i = 0.33 mM and 11.5 mM, respectively). These values are several fold greater than those reported for *Proteus mirabilis* urease (27). As in the *P. mirabilis*

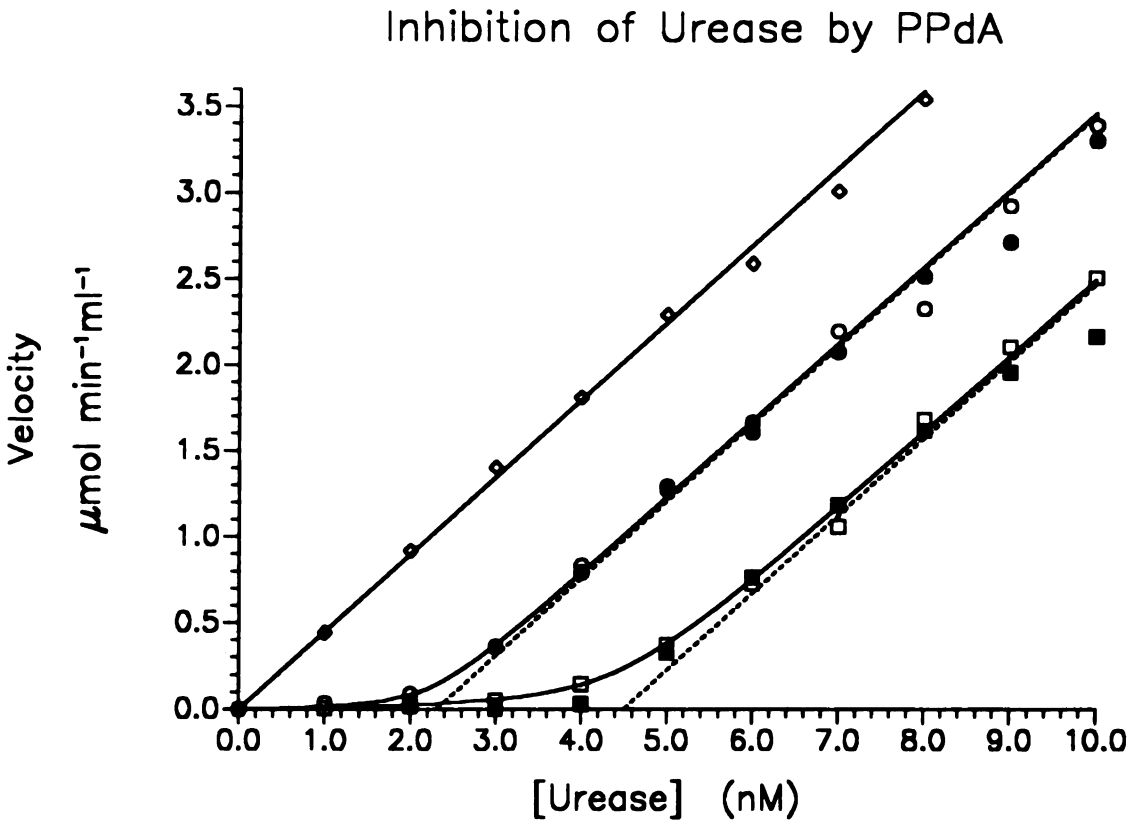
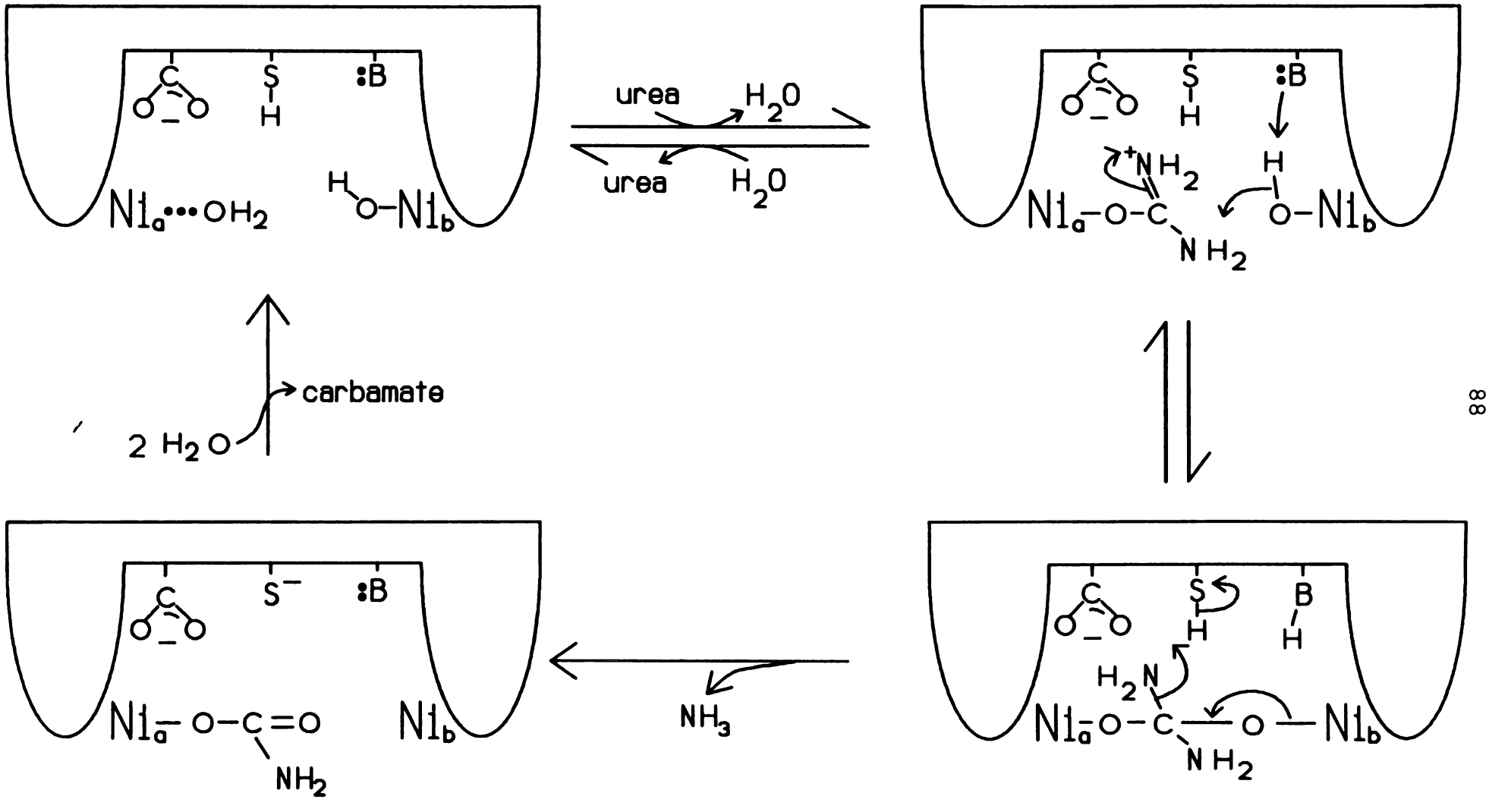


Figure 9. **Quantitation of urease active sites.** Ureolytic activity at 37°C is shown as a function of enzyme concentration following incubation at 0°C with PPD at 0.0 nM (*diamonds*), 5.0 nM (*circles*), or 10.0 nM (*squares*), for 2.0 h (*open symbols*) or 96 h (*solid symbols*). Curves shown are for data points fit to 2-h time points according to Williams and Morrison (20).

urease studies, the pH dependence of boric acid inhibition was consistent with trigonal $B(OH)_3$ being the inhibitor, rather than tetrahedral $B(OH)_4^-$. Thiourea is not an inhibitor of *K. aerogenes* urease despite its structural similarity to urea. In addition, since no pH increase was observed when urease was incubated in the presence of thiourea (100 mM), it is probably not an alternative substrate either. Acrylamide, acetamide, thioacetamide and acetate exhibited no significant inhibition at 500, 100, 20, and 20 mM respectively.

DISCUSSION

As a framework for discussion of the *K. aerogenes* urease inhibition results, we relate these studies to the Zerner model (6) of the jack bean urease bi-nickel active site, shown in Scheme 2. In the resting jack bean enzyme, one nickel is suggested to coordinate a water molecule, and a second nickel coordinates hydroxide. Urea displaces the water molecule and the illustrated resonance structure is thought to coordinate to nickel such that the positively charged nitrogen is electrostatically stabilized by a nearby carboxylate anion. A general base is proposed to activate the nickel-coordinated hydroxyl group for nucleophilic attack on the urea carbon to form a tetrahedral intermediate. Decomposition of this intermediate and release of ammonia is



Scheme 2

thought to include general acid catalysis by a nearby thiol group. Finally, carbamate is released with regeneration of resting enzyme (6). Evidence for the active site carboxyl and thiol groups in jack bean urease is primarily derived from inactivation studies involving triethyloxonium ion and alkylating agents (1).

If the above scheme is applicable to the bacterial enzyme, ureolysis would require the participation of several catalytic groups that may be sensitive to pH: a carboxylic acid, a general base, a thiol, and the nickel-coordinated hydroxide group. We have previously reported (7) that *K. aerogenes* urease exhibited a V_{max}/K_m dependence on pH (over the range pH 4.5-11) which was most easily interpreted by assuming a catalytic requirement for a deprotonated active site group with a pK_a = 6.55 and a protonated group with a pK_a = 8.85. The K_m of urea is only moderately affected by pH: *i.e.* a small decrease in K_m is observed upon protonation of a group with pK_a = 6.3 (K_m = 2.4 mM at pH 7-9 and K_m = 0.72 at pH 5-6). The bacterial urease pH dependence of catalysis is reasonably consistent with the Zerner model, but specific group assignments for each pK_a cannot be made.

Thiol Interactions with Urease- Each of a series of thiol compounds was shown to be a competitive inhibitor of *K. aerogenes* urease. We have demonstrated that the thiolate anions are the actual inhibitory species by examining the effects of pH on cysteamine inhibition. In addition, we

used UV-visible spectroscopy to demonstrate direct interaction of the thiolate anions with the nickel center in bacterial urease, as evidenced by the development of charge transfer bands. The demonstration of nickel-sulfur interactions using a competitive inhibitor, where the spectrally observed K_d is identical to the K_i, provides strong support for a mechanism where urea also binds to the nickel metallocenter, However, the data do not allow us to distinguish which nickel (Ni_a or Ni_b) coordinates the thiolate. Support for the presence of a negative charge at the urease active site was obtained from the survey of different thiol inhibitors (Table 1). Thiolates which possess a negatively charged carboxylate group were significantly poorer inhibitors than uncharged or positively charged thiolates. Thus, the thiolate anion ($R\text{-}S^-$) inhibition is consistent with the model shown in Scheme 3, where $E\text{-}X^-$ is an anionic active site group.

$$E\text{-}X^-$$

$$Ni \cdots {}^-S\text{-}R \qquad \text{Scheme 3}$$

Scheme 3, derived from the bacterial urease thiolate inhibition studies, has obvious similarities to portions of the jack bean urease model (Scheme 2), where a carboxylate is present at the active site. Using jack bean enzyme, Zerner and co-workers (24, 28) also characterized the spectral interactions associated with β-ME inhibition of jack bean urease. Differences exist between the bacterial and

plant urease β-ME spectroscopic results, both in the absorption peak positions (322, 374, and 432 nm *versus* 324, 380, and 420 nm) and in the extinction coefficients at these wavelengths (1115, 530, and 265 M^{-1} cm^{-1} vs 1550, 890, and 460 M^{-1} cm^{-1}), perhaps due to slight differences in the geometry of the active site. (The extinction coefficients are based on the concentration of active sites in order to allow comparison.)

Phosphate Inhibition- Phosphate competitively inhibits *K. aerogenes* urease in a pH dependent manner. The inhibition data are consistent with a requirement for protonation of two ionizable groups with pK_a values of <5 and 6.3. These pK_a values could be associated with the inhibitor, with groups on the enzyme, or with both the enzyme and the inhibitor; thus four simple mechanisms for phosphate inhibition can be considered. In mechanism 1, both pK_a values are associated with phosphate, which is known to have pK_{a1} near 2 and pK_{a2} near 7 (these values can be affected by temperature, ionic strength and buffer composition). In such a mechanism the actual inhibitor is H_3PO_4 and the binding of inhibitor is unaffected by the protonation state of the enzyme. If H_3PO_4 were the true inhibitory species, a K_i = 120 nM would be estimated. There is no obvious reason why neutral H_3PO_4 should possess such high affinity for urease, and it appears unlikely that an active site nucleophile could attack H_3PO_4 to form a pentavalent intermediate at low

pH. Hence, we are disinclined to accept this mechanism. In mechanism 2, both pK_a values are associated with protonation of enzyme groups, and the inhibitor is $H_2PO_4^-$. *K. aerogenes* urease is thought to possess an active site group with a pK_a = 6.55 (see above), which is in good agreement with the inhibitor pK_a = 6.3. The protonation state of this active site group changes the K_m for uncharged urea only 3-fold, but may have a more significant effect on binding the negatively charged inhibitor. Furthermore, the jack bean urease active site model (Scheme 2) includes a carboxylate which could correspond to the group with a $pK_a < 5$. This mechanism requires that the two phosphate pK_a values be outside the pH range studied. Phosphate monoanion as the inhibitor is consistent with the observed thiolate anion inhibition; however, it is unclear why two enzyme groups must be protonated for highest affinity in the phosphate case. Perhaps there is electrostatic repulsion of the delocalized charge on $H_2PO_4^-$ by two negatively charged groups at the active site. In mechanism 3, the higher pK_a is due to an enzyme group and the lower pK_a is due to phosphate. This mechanism would require the pK_{a2} of phosphate to be above 7 and the actual inhibitor to be H_3PO_4. As in mechanism 1 there is no compelling reason for H_3PO_4 to possess such high affinity for the active site of protonated enzyme. Finally, mechanism 4 assigns the lower pK_a to an enzyme group and the higher to pK_{a2} of phosphate. The actual inhibitor in such a mechanism would be $H_2PO_4^-$. As in mechanism 2 the phosphate

monoanion may bind in a manner similar to the thiolate anions. The affinity is enhanced when a single enzyme group with a pK_a <5 (*e.g.* the carboxylate in the Zerner model is protonated; electrostatic repulsion of the phosphate monoanion would then be decreased.

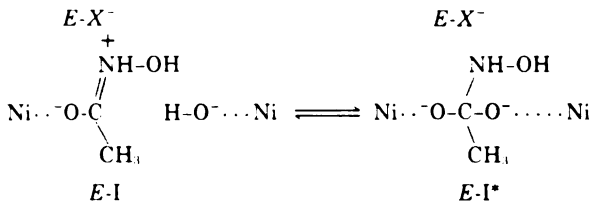
Since phosphate inhibition of urease is significant at low pH values (*e.g.* at pH 6.5, K_i = 6 mM for the total phosphate concentration), care must be taken when using phosphate buffer. Inhibition by buffer probably accounts for previously reported irregularities in curves showing urease activity as a function of pH (29, 30). Competitive inhibition of jack bean urease by phosphate was described as early as 1949 (31); however, the pH dependence of inhibition has never been examined for the plant enzyme.

Acetohydroxamic Acid Inhibition- Hydroxamic acids have been reported to inhibit urease by competitive, non-competitive, mixed, or irreversible mechanisms (Reviewed in Ref. 3). Most investigators, recognizing the time-dependent nature of hydroxamate inhibition, have preincubated enzyme with inhibitor for a fixed time and reported I_{50} values (in the μM range), where I_{50} is the inhibitor concentration which leads to 50% loss of activity. In many cases, it is unclear whether equilibrium was reached during these preincubation periods (*e.g.* to attain 95% equilibration of *K. aerogenes* urease with 10 μM AHA requires approximately 3.5 h). In addition, I_{50} values do not provide any insight into

the inhibitory mechanism. If equilibrium conditions were reached and if the type of inhibition was known, then the overall dissociation constant for inhibitor binding could be related to the reported I_{50} values by taking into account the substrate and enzyme concentration (32, 33). Our full time course studies have resolved the mechanism of AHA inhibition, concluding that AHA is a slow-binding competitive inhibitor of *K. aerogenes* urease. Furthermore, we have determined the rate constants for inhibitor binding and dissociation, the ratio of which (k_5/k_6) is a useful measure of efficacy when comparing inhibitors (16).

The initial $E{\cdot}I$ complex may involve coordination of the AHA anion (pK_a = 8.7) to the metallocenter, analogous to that proposed for other anions (*e.g.* thiols, phosphate). If this mechanism is correct, one would expect K_i to be pH-dependent, yet we observed no significant change in K_i from pH 6.75 to 8.25. An alternative mode of AHA binding, similar to the model proposed by Zerner and colleagues (6) for urea binding (Scheme 2), would involve a resonance form of AHA coordinating to the nickel and stabilized electrostatically through interactions with a nearby active site anion (Scheme 4). The rate of $E{\cdot}I$ formation is probably not diffusion limited; rapid $E{\cdot}I$ formation would be evident in progress curve experiments (Figure 5) as changes in initial velocity which depend on AHA concentration (16). Rather, we find that at a particular urea concentration the initial velocities are the same (within 10%) for all AHA concentrations.

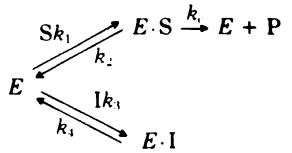
The slow transition which forms the $E \cdot I^*$ complex may arise from protein conformational changes or from inhibitor coordinational changes at the active site. For example, bidentate binding of AHA to the metallocenter may occur in the enzyme as reported for hydroxamates with free metal ions (36). An intriguing alternative is that AHA may bridge the two nickel ions in the urease active site. For example, nucleophilic attack by nickel-coordinated hydroxide may lead to the following dead end complex.



Scheme 4

Preliminary pH dependence studies of AHA inhibition are consistent with such a model. AHA affinity decreases at lower pH as would be expected if a catalytic nucleophile is required for complex formation. Also consistent with an event involving the nickel center, the UV-visible spectrum of jack bean urease is slowly perturbed upon AHA binding (34).

Zerner and colleagues have studied the kinetics of jack bean urease inhibition by AHA (34, 35). Although they have not demonstrated direct competition between AHA and urea, the enzyme could not simultaneously bind β-ME and AHA (35). Thus, they proposed the following mechanism:



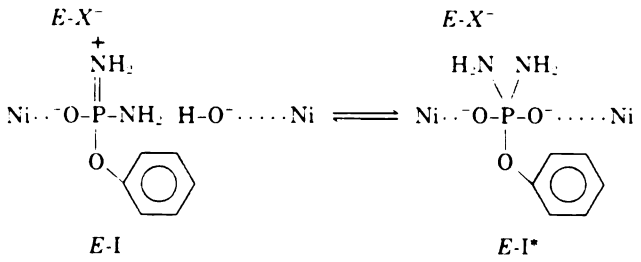
Scheme 5

where k_3 = 42 $M^{-1}s^{-1}$, k_4 = 76 x $10^{-5}s^{-1}$, and the equilibrium dissociation constant $K_i = k_4/k_3$ = 19.8 μM at pH 7.12 and 38°C (35). These kinetic constants for the plant enzyme are similar to k_5/K_i (35 $M^{-1}s^{-1}$), k_6 (8.7 x 10^5s^{-1}), and K_i^* (2.6 μM) as determined for AHA inhibition of *K. aerogenes* urease at pH 7.75 and 37° C, except that the value of k_6 for *K. aerogenes* urease is about 10-fold less than the corresponding value of k_4 for the jack bean enzyme. This in turn decreases the K_i^* for the bacterial enzyme about 10-fold compared to the K_i of the plant enzyme. The mechanisms of AHA binding to bacterial and plant ureases appear to differ (Scheme 1 *versus* Scheme 5). The distinction between the two kinetic mechanisms arises from the ability to observe a limiting apparent binding rate (k_{app}) at high inhibitor concentrations using the bacterial enzyme. The k_{app} for AHA binding to jack bean urease was linear up to 8 mM AHA (35), whereas we observed a hyperbolic response to increasing AHA concentration with the *K. aerogenes* enzyme (Figure 3). Zerner and colleagues (2) show a model in which AHA inhibits the jack bean enzyme through bidentate coordination to one nickel ion.

Phenylphosphorodiamidate Inhibition- PPD inhibition of *K. aerogenes* urease also conformed to the slow-binding competitive mechanism of Scheme 1; however, the K_i and K_i^* values were much smaller for PPD inhibition (95 nm and 94 pM)

than for AHA (1.5 mM and 2.6 μM). Previous studies of microbial urease inhibition by phosphoroamides were limited to determination of I_{50} values, which were generally in the nanomolar range (3).

In analogy to the postulated mechanisms for urea (Scheme 2) and AHA (Scheme 4) binding, the ***E***·I complex for PPD may involve the structure shown in Scheme 6. The very



Scheme 6

low K_i value for PPD (95 nM) may be a result of electrostatic stabilization and structural similarity to the tetrahedral intermediate postulated to occur during urea hydrolysis. The slow transformation which yields the very stable ***E***·I* complex may arise from nucleophilic attack by nickel-coordinated hydroxide to form a pentavalent structure. As in the case of slow-binding inhibition by AHA, the inhibitor may bridge the two active site nickel ions. The parallel nickel-bridging mechanisms forming the ***E***·I* complex for AHA and PPD are supported by the similarity of the k_5 and k_6 for these inhibitors, although the PPD complex is more stable.

Two kinetic studies have been carried out analyzing the effect of phosphoroamide compounds on jack bean urease

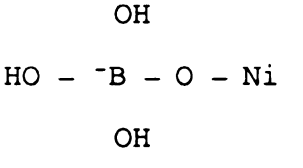
according to Scheme 5 (28, 37). Phosphoroamidate ($H_3N\text{-}PO_3^{2-}$) was deduced to possess K_i = 1.9 mM and a dissociation rate of $8.4 \times 10^{-4} s^{-1}$ at 25°C and pH 7.11 (28). These values do not compare well with the bacterial urease PPD inhibition, perhaps because of the highly anionic nature of phosphoroamidate. In contrast, N-acyl phosphoric triamides may inhibit jack bean urease in a manner similar to that observed for PPD inhibition of *K. aerogenes* urease. A second order rate constant for jack bean urease inhibition by methyl butenyl phosphoric triamide was calculated to be 4×10^4 $M^{-1}s^{-1}$ (37). This rate compares reasonably well to the corresponding value for k_5/K_i (7.8×10^5 $M^{-1}s^{-1}$) determined for PPD inhibition of *K. aerogenes* urease. Furthermore, the dissociation rates at 38°C, pH 7.01, of inhibitor-jack bean urease complexes for PPD, methyl butenyl phosphoric triamide, and phosphoric triamide were each $3.5 \times 10^{-5} s^{-1}$ (37). This rate corresponds well to our value for $k_6 = 4.7 \times 10^{-5}$ s^{-1}. Because the three phosphoroamides yielded identical dissociation rates in the jack bean study, the authors suggested that each of these compounds is hydrolyzed by urease to a common inhibitor, diamidophosphate (37). Although no product analysis were presented in the jack bean urease study, Zerner and colleagues propose a mechanism in which the diamidophosphate product bridges the two nickel ions. Since slowly processed substrates are known to behave kinetically as slow-binding inhibitors for several enzymes (16),

we can not exclude PPD hydrolysis by *K. aerogenes* urease in our experiments.

Boric and Boronic Acids--Boric and boronic acids were earlier reported to be competitive inhibitors of *P. mirabilis* urease (27) and now are shown to inhibit *K. aerogenes* urease in a similar manner. The pH dependence of inhibition was consistent with trigonal $B(OH)_3$ being the inhibitor rather than tetrahedral $B(OH)_4^-$. As discussed elsewhere (27), boronic acids are thought to inhibit several proteases by reacting with an active site serine group.

$$\text{Ser-OH} + B(OH)_3 \longrightarrow \text{Ser-O-}B(OH)_3^- \qquad \text{Scheme 7}$$

Thus, a reasonable scheme for urease inhibition by boric acid is formation of the following complex, which is consistent with the Zerner model involving nucleophilic attack by hydroxide coordinated to nickel.



Scheme 8

Such a complex may not be able to form a bridge between the two nickel ions, thus borate does not exhibit slow-binding kinetics. Analogous mechanisms involving nucleophilic attack by nickel-coordinated hydroxide on an inhibitor could also be envisioned for formation of the initial AHA and PPD

complexes. However, because AHA and PPD are analogs of urea, we favor mechanisms shown in Schemes 4 and 6 for these inhibitors.

In summary, the bacterial urease inhibition results are consistent with most aspects of a model previously proposed (6) for the active site of jack bean urease (Scheme 2). The illustrated enzyme-inhibitor complexes are proposed as working models to stimulate further studies.

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REACTIVITY OF THE ESSENTIAL THIOL

OF *KLEBSIELLA AEROGENES* UREASE:

EFFECT OF pH AND LIGANDS ON THIOL MODIFICATION

ABSTRACT

The kinetics of *Klebsiella aerogenes* urease inactivation by disulfide and alkylating agents was examined and found to follow pseudo-first-order kinetics. Reactivity of the essential thiol is affected by the presence of substrate and competitive inhibitors, consistent with a cysteine located proximal to the active site. In contrast to the results observed with other reagents, the rate of activity loss in the presence of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) saturated at high reagent concentrations, indicating that DTNB must first bind to urease before inactivation can occur. The pH dependence for the rate of urease inactivation by both disulfide and alkylating agents was consistent with an interaction between the thiol and a second ionizing group. The resulting macroscopic pK_a values for the two residues are <5 and 12. Spectrophotometric studies at pH 7.75 demonstrated that 2,2'-dithiodipyridine (DTDP) modified 8.5 ± 0.2 mol thiol/mol enzyme, or 4.2 mol thiol/mol catalytic unit. With the slow-tight binding competitive inhibitor

phenylphosphorodiamidate (PPD) bound to urease, 1.1 ± 0.1 mol thiol/mol catalytic unit was protected from modification. PPD-bound, DTDP-modified urease could be reactivated by dialysis, consistent with the presence of one thiol per active site. Analogous studies at pH 6.1, using the competitive inhibitor phosphate, confirmed the presence of one protected thiol per catalytic unit. Under denaturing conditions, 25.5 ± 0.3 mol thiol/mol enzyme (M_r = 211,800) were modified by DTDP.

INTRODUCTION

Urease [urea amidohydrolase (EC 3.5.1.5)] is a nickel-containing enzyme that catalyzes the hydrolysis of urea to ammonia and carbamate; the carbamate spontaneously hydrolyzes to form a second molecule of ammonia and carbonic acid (2 15). The enzyme from the plant *Canavalia ensiformis* (jack bean) is the best characterized urease; however, the role of the plant protein is unclear (29). Dixon et al. (6) proposed a model for the hydrolysis of urea by jack bean urease in which one nickel coordinates the oxygen atom of urea, polarizing the carbonyl group, and a second nickel coordinates hydroxide ion, the catalytic nucleophile. Three amino acid residues were suggested to participate in catalysis: a carboxyl group, a sulfhydryl group, and an unidentified base. Consistent with the presence of an essential thiol group, both alkylating and disulfide reagents were shown to inhibit the enzyme (1, 7, 19). The essential thiol exhibited a pK_a of 9.15, and at pH 7.7 reacted more slowly than several other thiols which were not required for activity (19).

In contrast to the jack bean enzyme, very little is known about the thiol reactivity of microbial ureases. Characterization of the bacterial enzyme is essential because of its important role in cellular nitrogen

metabolism and certain pathogenic states (reviewed by Mobley and Hausinger, 15). Ureases from *Brevibacterium ammoniagenes* (18), *Ureaplasma urealyticum* (22), and a bovine ruminal population (13) have been shown to be inhibited by alkylating reagents and *p*-chloromercuribenzoate; however, the kinetics of activity loss and other properties of the reacting group were not analyzed.

We demonstrate that *Klebsiella aerogenes* urease has an essential thiol and examine the pH dependence of its reactivity with alkylating and disulfide reagents. We also examine the effect of substrate and several competitive inhibitors on the rate of inactivation to assess whether this essential thiol is at the active site. Finally, we characterize spectroscopically the reactivity of both essential and non-essential thiol groups in urease.

EXPERIMENTAL PROCEDURES

Enzyme purification- K. aerogenes urease was purified to a specific activity >2500 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ from strain CG 253 carrying the recombinant plasmid pKAU19 (17) by using procedures previously described (26). For spectroscopic analysis of urease thiols, the final enzyme purification step was carried out with buffers containing DTT rather than β-ME. One unit of urease activity is defined as 1

μmole urea degraded per minute in the standard assay mixture, which contained 50 mM urea, 5 mM EDTA, 50 mM HEPES, pH 7.75 at 37°C. Linear regression analysis of the released ammonia, determined by conversion to indophenol (28), versus time yielded the initial rates. Protein was assayed by the method of Lowry (12).

Kinetics of urease inactivation- Inactivation reactions contained 80-100 mM buffer, 8 mM EDTA, plus the indicated reagent concentrations. All thiol modification reagents were dissolved in H_2O, except DTDP and DTNB, which were dissolved in 100% HPLC grade methanol. Although low concentrations of methanol were found to have no apparent effect on urease activity, reaction series using these reagents were kept at a constant concentration of methanol, usually <2%. Inactivation reactions were initiated by addition of a small amount of enzyme (<1% by volume) and aliquots were periodically diluted 50-fold into the standard assay for activity determination. This dilution effectively halted any further inactivation during the assay as shown by the linearity of the initial rate determination. Pseudo-first-order rate constants were obtained from plots of ln(percent activity remaining) versus time. The pH of the inactivation reactions was taken immediately following completion of the assays.

Progress curves in the presence of urease inactivators were typically carried out using 5 ml reactions

containing 8 mM EDTA, 80 mM HEPES, pH 7.75 at 37°C, and 0.10 ml aliquots were analyzed periodically for ammonia. Experiments in the absence of urea demonstrated a pseudo-first-order loss of initial velocity consistent with

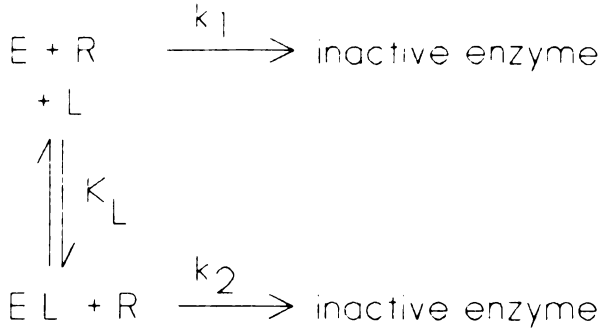
$$V_t = V_o \, e^{-k_{app} R t} \qquad (1)$$

where V_o = initial velocity of urease in the absence of modifying reagents, R = concentration of modifying reagent, and k_{app} = pseudo-first-order rate constant for loss of urease activity. Pseudo-first-order rate constants in the presence of urea were obtained by fitting the product, ammonia, produced as a function of time (P_t) to:

$$P_t = P_o + \frac{V_o}{k_{app}} (1 - e^{-k_{app} t}) \qquad (2)$$

where P_o = product at time t = 0. Except for DTNB, these rate constants were proportional to the concentration of inactivating reagent at all urea concentrations tested, showing that loss of activity is a second-order process, even in the presence of saturating concentrations of substrate (50 mM urea).

Kitz and Wilson (9) have shown that the rate of amino acid modification in the presence of ligands can be represented by:



(Scheme 1)

where E = enzyme, R = modifying reagent, L = ligand, k_1 = second-order rate constant for the inactivation of free enzyme, k_2 = second-order rate constant for inactivation of ligand-bound enzyme, and K_L = equilibrium dissociation constant for ligand binding. If the concentration of modifying reagent is much greater than the concentration of enzyme, the effect of the ligand on the apparent pseudo-first-order rate constant, k_{app}, will be given by:

$$k_{app} = [R]\, k_1 \frac{(K_L + \frac{k_2}{k_1}[L])}{K_L + [L]} \qquad (3)$$

such that k_{app} will be dependent on the ratio of k_2 to k_1: if $k_2 > k_1$, the ligand increases k_{app}; and if $k_2 < k_1$, then the ligand decreases k_{app}, providing complete protection of the susceptible amino acid as k_2 approaches zero. Pseudo-first-order rate constants for urease inactivation in the presence of ligands were fit to equation 3 using the Markfit program (see below).

Reactivation of DTNB- or DTDP-inactivated urease- Urease was incubated with 2 mM DTNB or with 0.5 mM DTDP for 15 min, then diluted 200-fold into 5 mM EDTA, 50 mM HEPES, pH 7.75 at 37°C containing various concentrations of DTT. Aliquots were periodically diluted into the standard assay mixture to measure the activity. Initial velocity data (V_t) were fit to the following expression for the pseudo-first-order recovery of activity:

$$V_t = V_o + V_{max} (1 - e^{-k_{app} t}) \quad (4)$$

where V_o = initial velocity of DTDP- or DTNB-modified urease, V_{max} = maximum velocity recovered, and k_{app} = apparent pseudo-first-order rate constant for the reactivation of disulfide-inactivated urease by DTT.

Spectroscopic analysis- Phosphate and dithiothreitol, used to stabilize urease during storage, were removed by ultrafiltration using an Amicon Centricon-30 microconcentrator (5 X 1:10 dilution). Spectroscopic data were obtained by using a Gilford Response spectrophotometer with 1.0-cm microcuvettes, at 37°C, assuming that 2-thiopyridine had $\varepsilon_{343\ nm} = 7030\ M^{-1}cm^{-1}$ (8). Independent determinations of $\varepsilon_{343\ nm}$ were done using each of the buffer solutions by two methods: aliquots of DTDP were added to cuvettes containing 1 mM β-ME or 1 mM cysteine, or aliquots of β-ME were added to cuvettes containing 0.5 mM

DTDP. Identical results were obtained by both methods, confirming the value of 7030 for $\varepsilon_{343\ nm}$ of 2-thiopyridine. The total number of urease thiols was determined by reacting urease with DTDP (0.5 mM) in the presence of 5.5 M guanidine·HCl, 10 mM EDTA, 0.1 M Tris·HCl, pH 7.9. The maximum absorbance at 343 nm was attained within 30 seconds and did not change after an additional 30 minutes. The concentration of protein was determined by the method of Lowry (12), and the molar concentration of urease was calculated based on M_r = 211,800 (16).

The rates of reaction for the accessible thiols in native enzyme were assessed by scanning three cuvettes for each reaction: a buffer blank, DTDP control, and DTDP + 6-8 μM urease. Spectral data were transferred to an IBM PC to correct for the absorbance increase due to spontaneous degradation of the thiol reagent (<0.1% spontaneously hydrolyzed in 30 minutes at pH 9.0). During the time scan, aliquots were removed for assay using the standard assay mixture. For all reactions involving native enzyme, DTDP was added last, allowing ample time for enzyme and competitive inhibitor (when present) to equilibrate. Prior to DTDP addition, activity measurements of urease and AHA or urease and PPD showed >99.9% inhibition.

Curve fitting- Curve fitting made use of a BASIC program termed Markfit (23) which employs the Marquardt

algorithm (14) to fit by non-linear least squares a set of data points to a rate equation.

RESULTS

Inactivation of urease by thiol-specific reagents- Homogeneous *K. aerogenes* urease exhibited a pseudo-first-order loss of activity in the presence of disulfide and alkylating reagents. As illustrated for DTDP, pseudo-first-order rate constants were directly proportional to the concentration of inactivating reagent (Figure 1) allowing calculation of the apparent second-order rate constant. Similar results were obtained for several other reagents, and their second-order rate constants for the loss of ureolytic activity are provided in Table 1.

Inactivation of urease by DTNB is also a pseudo-first-order process; however, the apparent rate constant reaches a limiting value at high concentrations of DTNB. Preliminarily, the data were fit to the following model.

$$E + R \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ER \xrightarrow{k_3} \text{inactive enzyme}$$

(Scheme 2)

The apparent pseudo-first-order rate constant for an inactivator that exhibits a binding step follows

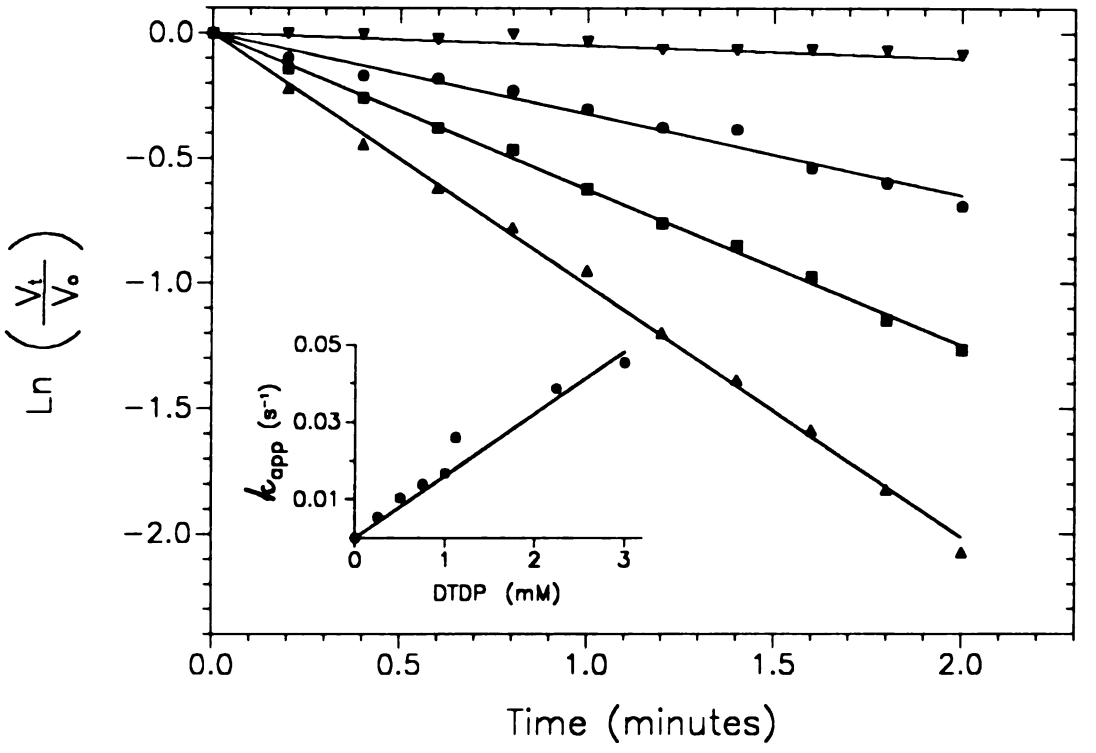


FIGURE 1. **Pseudo-first-order urease activity loss in the presence of DTDP.** The fraction of activity remaining is shown as a function of time for urease [4.4 nM], reacting with DTDP at 0 (▼), 0.25 (●), 0.5 (■), or 1.0 mM (▲). All reactions were in 8 mM EDTA, 80 mM HEPES at pH 7.75, and 37°C. INSET: Determination of the second-order rate constant for urease inactivation by DTDP.

Table 1

Second-order rate constants for loss of urease activity by thiol-specific reagents[a]

Inactivator	Concentration examined	Rate constant ± Standard error
	(mM)	($M^{-1}s^{-1}$)
Alkylating reagent		
IAA	10-25	0.002
IAM	1-200	0.026 ± 0.001
NEM	0.050-0.200	4.76 ± 0.19
Disulfide reagent:		
DTNB[b]	0.1-5.0	7.2
DTDP	0.01-4.5	22 ± 5
Cystine	0.5-1.0	0.077 ± 0.004
Cystamine	0.25-1.0	27 ± 1
MMTS	0.025-0.100	710 ± 50

[a] Values determined in 8 mM EDTA, 80 mM HEPES, pH 7.75 buffer at 37°C.

[b] Inactivation of urease by DTNB is not a simple second order reaction. The pseudo-first-order rate can be saturated at high concentrations of DTNB (Scheme 2); therefore, the apparent second-order rate constant shown is

$$k_{app} = \frac{k_1 k_3}{k_2} .$$

$$k_{app} = \frac{k_3 \, k_1 \, R}{k_1 \, R + k_2 + k_3} \qquad (5)$$

under steady state conditions (9). Since the inactivation step occurs much slower than the rapid equilibrium between enzyme and modifying reagent, $k_3 << k_2$, and

$$\frac{1}{k_{app}} = \frac{1}{k_3} + \frac{k_2}{k_1 \, k_3 \, R} \qquad (6)$$

Therefore under steady state conditions, double reciprocal plots of $1/k_{app}$ versus 1/[R] yield a straight line. The results for DTNB inactivation of urease fit this pattern as shown in Figure 2. Values for k_3 and $k_2/k_1 = K_i$ were calculated to be 0.0065 s^{-1} and 0.9 mM, respectively.

To test whether thiol modification released the tightly bound nickel ions of urease, enzyme modified by IAM, DTDP, or MMTS was dialyzed to remove any low molecular weight compounds, then subjected to atomic absorption analysis as previously described (25). Enzyme samples which had lost >90% activity retained essentially 100% of the original amount of nickel.

Reactivation of urease inactivated by disulfide reagents- Urease which was inactivated by DTNB slowly recovered activity after dilution into buffer containing DTT, as illustrated in Figure 3. Data for reactivation at three DTT concentrations were fit as described in

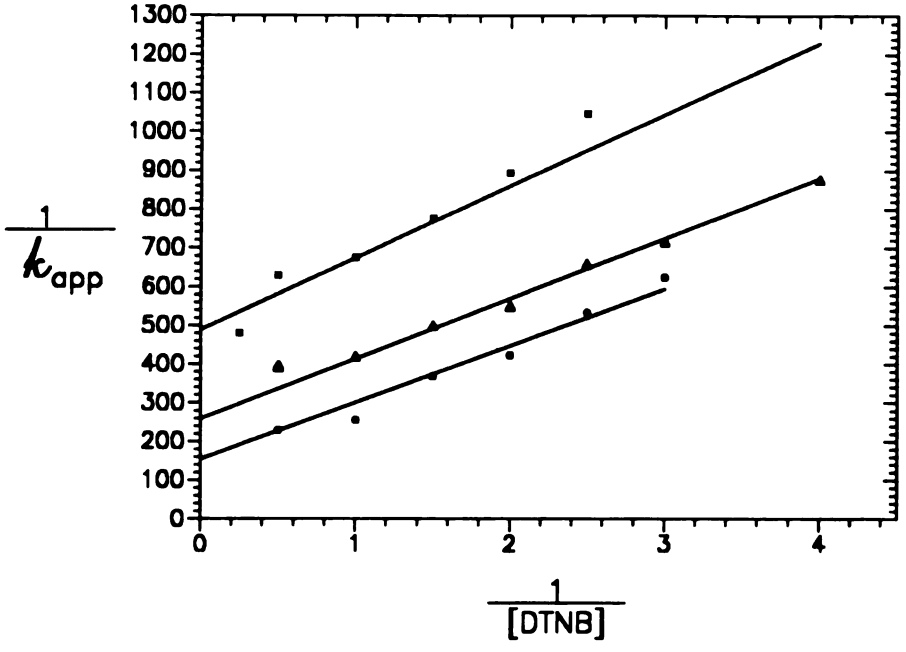


FIGURE 2. **Saturation of the apparent rate of urease modification by DTNB: the effects of urea and borate.** Pseudo-first-order rate constants for the modification of urease by DTNB were obtained from plots similar to Figure 1. k_{app}^{-1} (s^{-1}), obtained at pH 7.75 in 8 mM EDTA, 80 mM HEPES, 37°C, is shown as a function of $[DTNB]^{-1}$ (mM^{-1}) for urease (●), urease in the presence of 2 mM (▲) or 5 mM (■) urea.

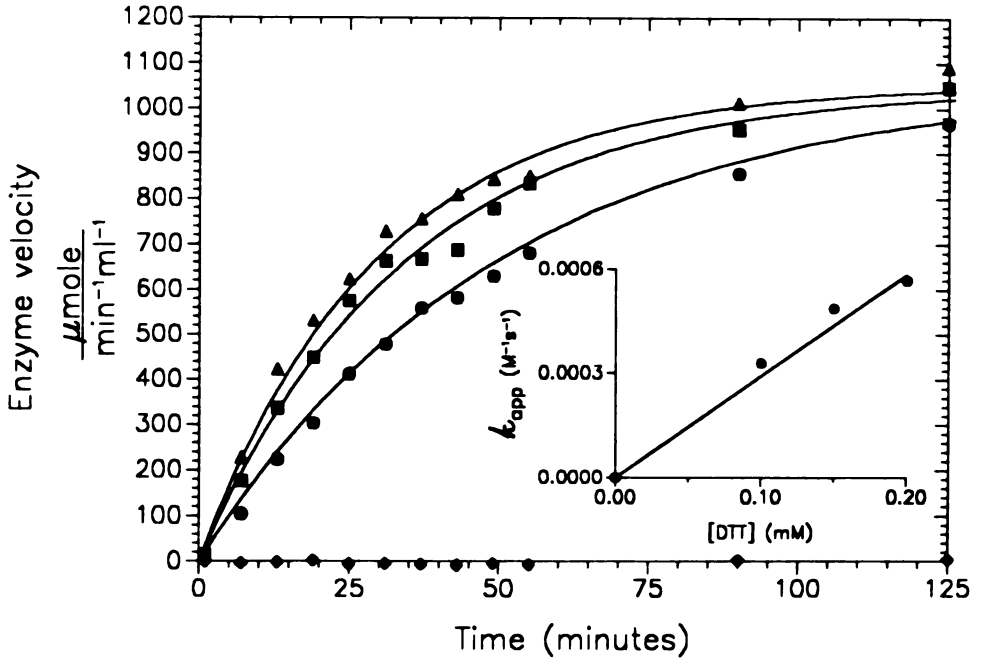


FIGURE 3. **Reactivation of DTNB-inactivated urease.** Urease (17 nM), previously inactivated by DTNB, was incubated in the presence of 8 mM EDTA, 80 mM HEPES pH 7.75 with 0 (♦), 0.1(●), 0.15 (■), or 0.2 (▲) mM DTT at 37°C. The initial velocity was determined at the indicated times by using the standard assay. INSET: Determination of the second-order rate constant for the reactivation of DTNB-modified urease by DTT.

Experimental Procedures, and the second-order rate constant for urease reactivation (3.1 ± 0.2 $M^{-1}s^{-1}$) was calculated from regression of the slope of k_{app} versus DTT concentration. Similar DTT reactivation results were obtained for enzyme inactivated with DTDP (second-order reactivation rate constant = 2.7 ± 0.3 $M^{-1}s^{-1}$).

In contrast to the recovery of activity in the presence of DTT, no activity was recovered when DTDP-modified enzyme was treated with excess KCN (500 mM) for 120 minutes at 37°C. Cyanolysis did occur as shown spectrophotometrically by the release of 4 mole 2-thiopyridine/mole enzyme. Addition of DTT (1 mM) to cyanide-treated DTDP-inactivated urease did not release additional 2-thiopyridine.

Effect of ligands on the rate of thiol modification- Having shown that thiol-reactive reagents inactivate *K. aerogenes* urease, experiments were designed to test whether ligands which bind to the active site of urease alter the rate of inactivation.

The effect of two competitive inhibitors of urease on the rate of inactivation by IAM is shown in Figure 4. Phosphate was previously shown to be a competitive inhibitor of urea hydrolysis, with a K_i that depended on pH in a complex manner (26). Since phosphate binds with higher affinity to urease at lower pH, the effect of phosphate on urease inactivation was examined at pH 6.1 in MES buffer

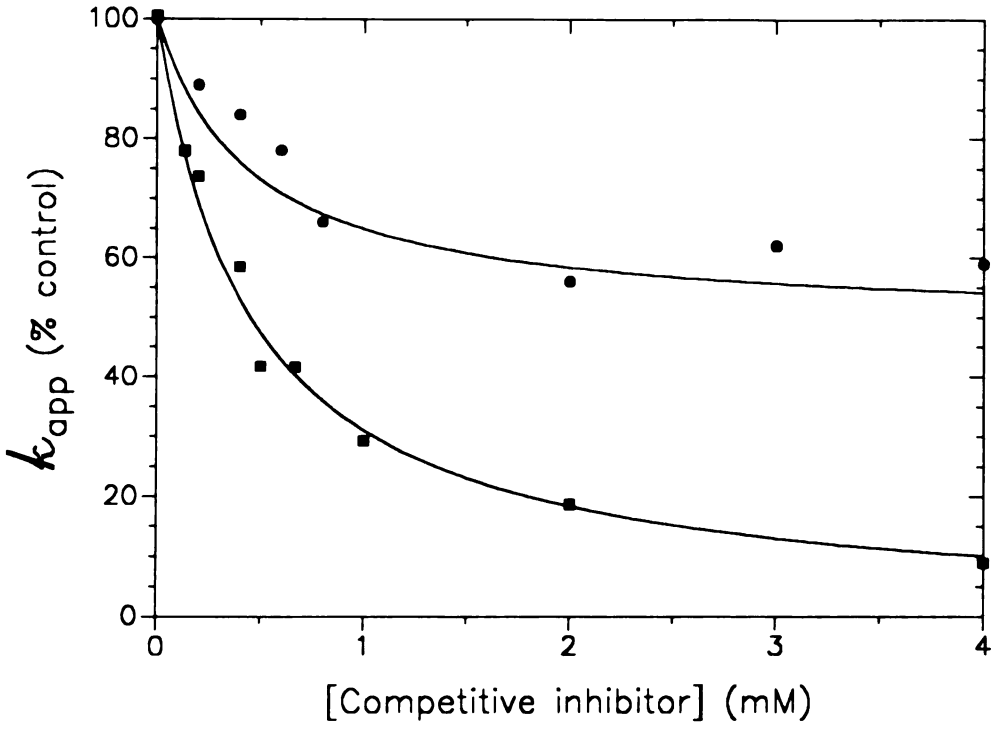


FIGURE 4. **Effect of the competitive inhibitors boric acid and phosphate on the rate of urease inactivation by IAM.** The second-order rate constants for urease inactivation by IAM were determined in the indicated concentrations of phosphate (■) and boric acid (●), and are expressed as percent of the inactivation rate in the absence of inhibitors. Reaction conditions were 8 mM EDTA, 80 mM buffer (MES, pH 6.1 for phosphate inhibition, HEPES pH 7.7 for borate inhibition) at 37°C.

where the $K_{i\,(phosphate)}$ = 1 mM. Increasing concentrations of phosphate decreased the apparent urease inactivation rate; at saturating phosphate concentrations k_{app} was less than 2% that of the control. Increasing concentrations of boric acid also reduced the apparent rate of inactivation; however, saturating levels of borate decreased k_{app} by only two-thirds. This difference in behavior between phosphate and borate is not due simply to a pH effect, because borate slowed the rate of urease inactivation by IAM to the same extent at pH 6.0, 7.75, and 9.5 (data not shown).

Values for k_2/k_1 (Scheme 1), the ratio of inactivation rates for ligand-bound and free enzyme, and K_L, the ligand dissociation constant, were calculated from the above studies. These values are provided in Table 2, which also lists values obtained in analogous IAM inactivation experiments using the inhibitors BBA and 4-Br-BBA and values for the inhibition of urease inactivation using the disulfide reagent DTDP. While 4-Br-BBA parallels borate in providing partial protection of the essential thiol, BBA has little effect on urease inactivation by IAM, and actually increased the rate of urease inactivation by DTDP (Table 2). The effect of the ligand urea on the rate of urease inactivation by DTDP was assessed by analysis of progress curves. Urea also provided partial protection of the essential thiol (Table 2), with the apparent second-order rate constant decreasing about 2.5-fold.

Table 2

Effect of Urease Ligands on the Rate of Inactivation by Thiol-Specific Reagents[a]

Ligand	K_i or K_m for urea hydrolysis (mM)	Inactivator: IAM $\frac{k_2}{k_1}$[b]	IAM K_L (mM)	DTDP $\frac{k_2}{k_1}$	DTDP K_L (mM)
Borate	0.34	0.32	0.30	0.49	0.45
BBA	10	0.8	11	2.6	13
4-Br-BBA	0.37	0.24	0.34	n.d.[c]	n.d.
Phosphate[d] (pH 6.1)	0.8	<0.02	0.50	<0.02	0.43
urea[e]	2.5	n.d.	n.d.	0.4	3

[a] All reactions were done in 8 mM EDTA, 80 mM buffer (pH 6.1, MES; pH 7.75, HEPES) at 37°C. The pseudo-first-order rate of activity loss was measured as a function of inhibitor concentration, then rate constants were fit to equation 3.

[b] k_2 and k_1 are defined in Scheme 1.

[c] n.d. not determined.

[d] Initial attempts to fit phosphate data to equation 3 showed k_2 less than 2% k_1, so K_L was obtained by fixing $k_2 = 0$.

[e] The pseudo-first-order rate for activity loss in the presence of urea was obtained from progress curves in 8 mM EDTA, 80 mM HEPES, pH 7.75, at 37°C and various concentrations of DTDP. Pseudo-first-order rate constants were obtained by fitting data to equation 2, then these pseudo-first-order rate constants were fit to equation 3.

Ligands were also examined for their effects on the rate of activity loss in the presence of DTNB, which was shown above to exhibit saturation of the apparent rate at high reagent concentrations, consistent with Scheme 2. Progress curves for ureolysis in the presence of DTNB yielded rates for activity loss which when plotted as k_{app}^{-1} vs $[DTNB]^{-1}$, were parallel. Saturating concentrations of substrate decreased the maximum rate, k_3, 10-fold, and also decreased the concentration of DTNB required to reach half the maximum rate, k_2/k_1, by 10-fold.

The effect of pH on k_{app}- Figure 5 illustrates the effect of pH on the rate of *K. aerogenes* urease inactivation by IAM and DTDP. Above pH 12, the rate does not appear to be affected by changes in pH. Between pH 8.5 and 12, plots of log k_{app} vs pH have a slope of +1. Between pH 8 and 5, the rate of modification is not affected by pH changes; lower values of pH led to rapid denaturation of urease. The pH dependence for DTNB inactivation is complicated because of the saturation observed at high DTNB concentrations (as described above). In terms of the reaction illustrated in Scheme 2, the k_2/k_1 (or K_i) of DTNB appeared to be nearly independent of pH, whereas k_3 for DTNB modification of urease behaved similarly to the other inactivators (Figure 6). Inactivation by DTNB was examined only below pH 9.5 due to reagent instability.

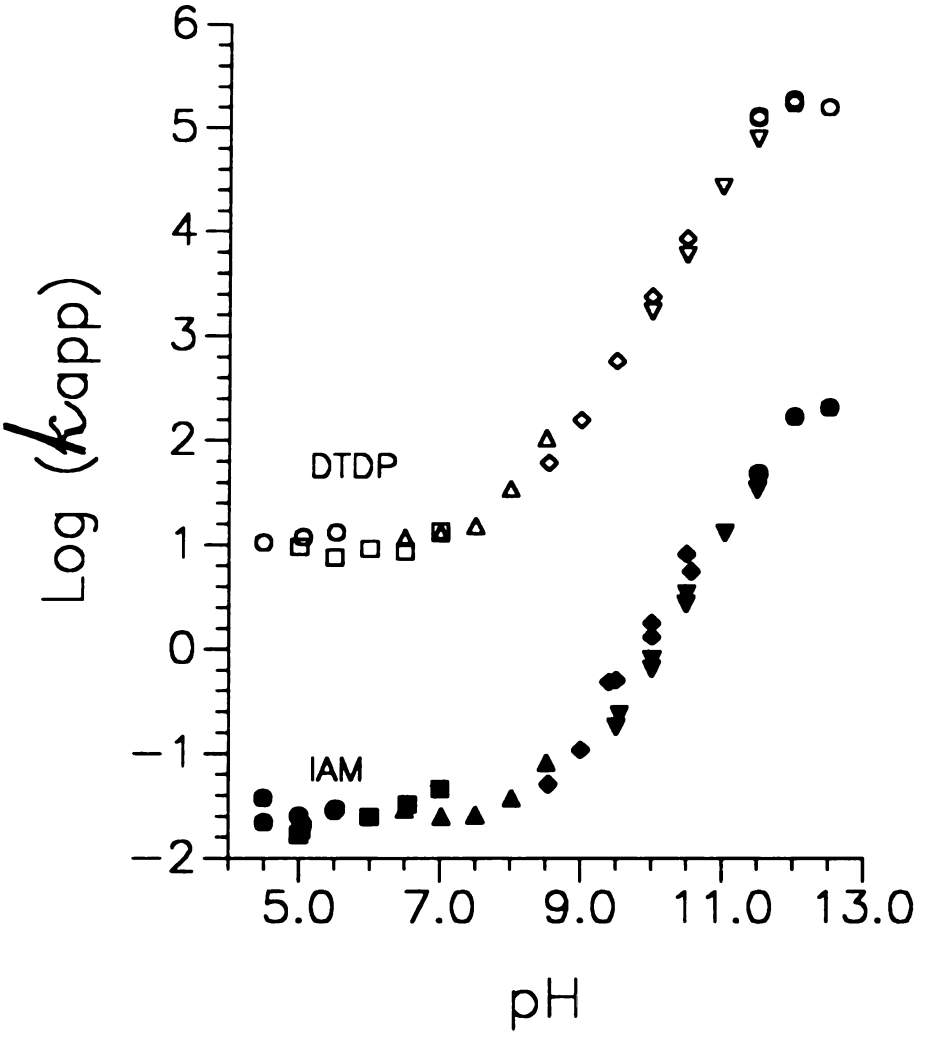


FIGURE 5. **The pH dependence of IAM and DTDP inactivation of urease.** The log of the apparent second-order rate constants in ($M^{-1}s^{-1}$) are shown as a function of pH for DTDP (*open symbols*) or IAM (*closed symbols*) for reactions carried out in 8 mM EDTA, 100 mM buffer: acetate (*circles*), MES (*squares*), HEPES (*triangles*), CHES (*diamonds*), CAPS (*inverted triangles*), or phosphate (*circles*).

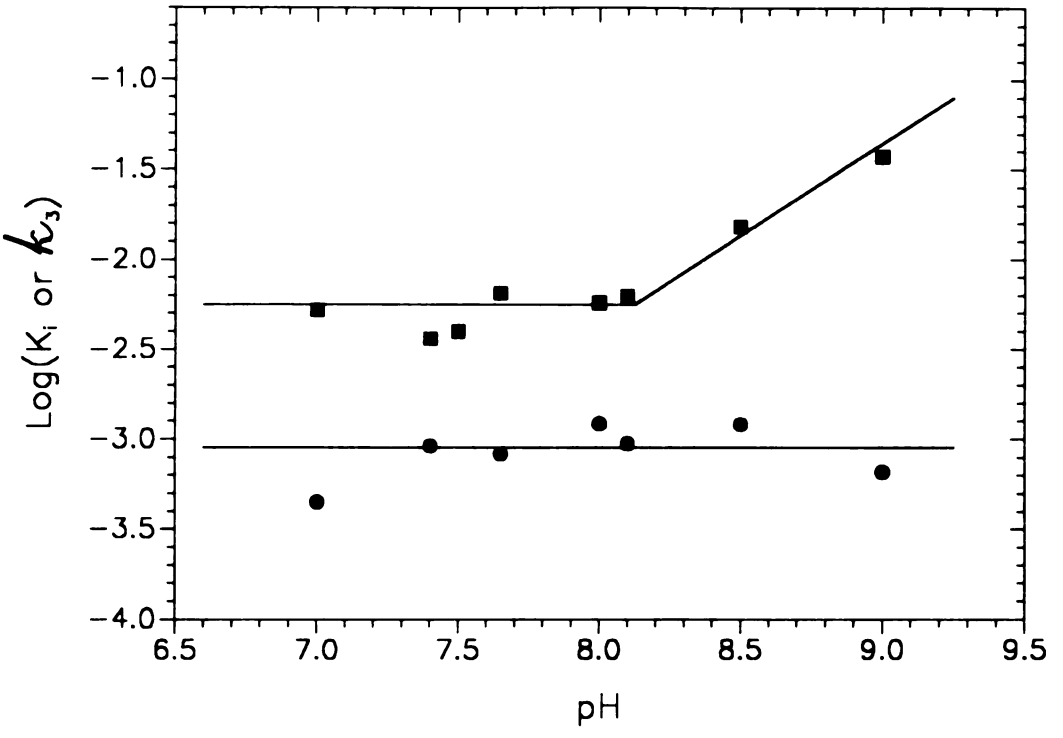


FIGURE 6. **Effect of pH and DTNB concentration on the pseudo-first-order rate of urease activity loss.** The rate of urease inactivation by DTNB was determined in 8 mM EDTA, 80 mM buffer, at 5 or more concentrations of DTNB at 37°C using MOPS, HEPES, TAPS, and CHES. K_i and k_3 (Scheme 2) were determined by non-linear least squares analysis of the pseudo-first-order rate constants versus DTNB concentration; log K_i (M) (●) and log k_3 (s^{-1}) (■) are shown as a function of pH.

Spectrophotometric quantitation of urease thiols- In addition to the essential thiol group, other cysteine residues may react with disulfides or alkylating agents without loss of urease activity. The total number of urease thiols and the number and reactivity of accessible thiols in native enzyme were probed by spectrophotometric methods.

DTT treated urease (free from low molecular weight thiols) was incubated with DTDP in the presence of 5.5 M guanidine. The number of thiol equivalents was divided by the molar protein concentration to demonstrate that 23.2 ± 0.3 mol thiol/mol denatured enzyme could react (assuming the native molecule possesses an $\alpha_2\beta_4\gamma_4$ stoichiometry with M_r = 211,800).

Figure 7a shows progress curves for the reaction of DTDP with urease at pH 7.75. The release of 2-thiopyridine corresponded to the reaction of 8.5 ± 0.2 mol thiol/mol enzyme. The DTDP-reactive cysteines were converted to mixed disulfides with 2-thiopyridine, rather than to cystine residues, as shown by DTT-dependent release of > 8.3 ± 0.3 mol 2-thiopyridine/mol of the isolated, inactivated enzyme with concomitant recovery of activity. When urease was equilibrated with the slow, tight-binding competitive inhibitor PPD prior to reaction with disulfide reagents, only 6.3 ± 0.4 mol thiol/mol enzyme reacted. Urease modified in the presence of PPD recovered over 70% of its original activity following prolonged dialysis versus 1 mM EDTA, 50 mM phosphate, pH 6.5, at 4°C, (at 1:20,000 volumes, with several

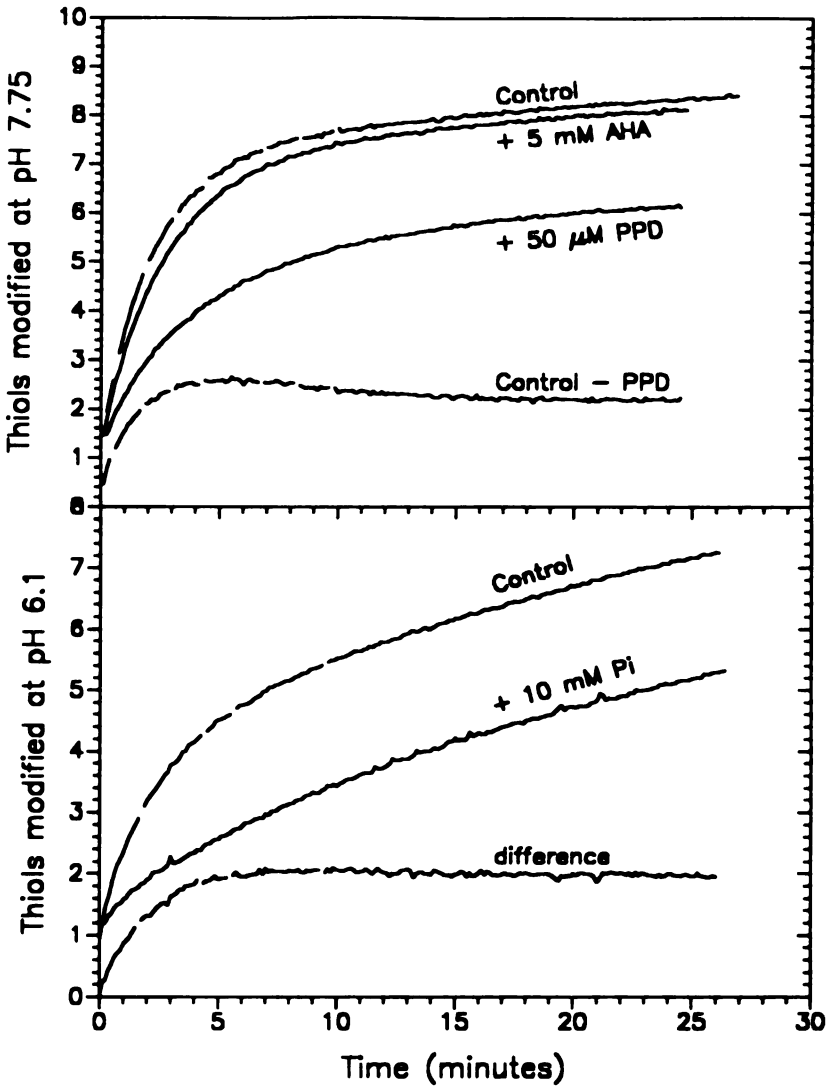


FIGURE 7. **Spectroscopic progress curves for the reaction of urease with DTDP.** Absorbance at 343 nm was converted into the number of urease thiols reacting and is shown as a function of time. DTT-treated urease (6-8 µM) was reacted with 0.5 mM DTDP in 8 mM EDTA, 80 mM buffer (HEPES, pH 7.75 or MES pH 6.1) in a 1.0 cm micro-cuvette. Samples include: **A)** urease at pH 7.75, urease equilibrated with 10 mM AHA or 50 µM PPD, and the absorbance difference between control and PPD inhibited urease, and **B)** urease at pH 6.1, urease equilibrated with 10 mM phosphate, and the difference scan.

buffer changes). In contrast to PPD, in the presence of the slow binding inhibitor AHA, 8.1 ± 0.1 mol thiol/mol enzyme were accessible to modification by DTDP, and prolonged dialysis resulted in recovery of <2% the original activity. Figure 7b illustrates the DTDP reactivity of urease at pH 6.1 in the presence and absence of phosphate. Phosphate protects 2.0 ± 0.2 mole thiol/mole enzyme from modification by DTDP. An initial rapid phase was followed by a slow, steady increase (perhaps due to low pH-induced denaturation), so that after 30 min, 8.5 ± 0.5 urease thiols had reacted.

Figure 8 compares the percent activity of urease as a function of the number of urease thiols modified at pH 6.1 and 7.75 according to the method of Tsou with $i = 1$ (27). Tsou plots with $i > 1$ did not fit the data and are not shown for clarity. The essential thiols reacted more rapidly than non-essential thiols at pH 6.1, possibly as a result of the plateau observed in the pH dependence studies. The slowest phase of urease modification by DTDP at pH 6.1 is not correlated to modification of the essential thiol, as > 95% of the activity is lost by the time 5 thiols have reacted.

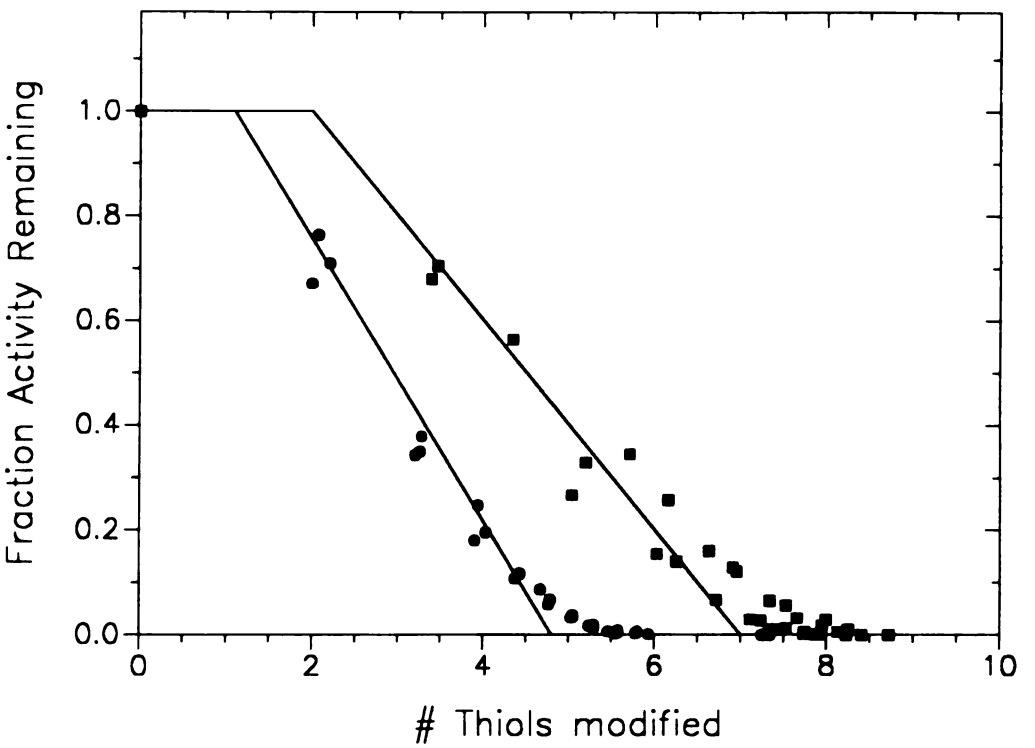


FIGURE 8. **Activity as a function of urease modification.** At periodic intervals during the scans in Figure 7, 8.5 pmoles of urease was withdrawn to assay under standard conditions. Fraction activity remaining ($a^{1/i}$ where $i = 1$) is shown as a function of total number of thiols modified at pH 6.1 (●), and pH 7.75 (■), according to the method of Tsou (27).

DISCUSSION

Demonstration of an essential thiol in K. aerogenes urease- Reaction of urease with either disulfide reagents or alkylating reagents led to at least a 10,000-fold decrease in activity, consistent with the presence of at least one essential enzyme thiol. The decrease in activity is probably not a steric effect, since cyanolysis of the bulky 2-thiopyridine group to yield the small uncharged cyanide derivative failed to increase ureolytic activity. Similarly, modification of urease thiols with MMTS, resulting in the small, uncharged methylthio- derivative, led to the loss of >99.9% of activity. Inactivation was not accompanied by a loss of nickel ion, as shown by atomic absorption analysis. Furthermore, DTT-dependent release of either 5-thio-2-nitrobenzoate or 2-thiopyridine led to 100% recovery of the initial activity.

Comparison of the second-order rate constants for urease inactivation by the eight thiol-modifying reagents in Table 1 indicates that reagents containing anionic groups react with urease much more slowly than corresponding neutral reagents, and the positively charged cystamine reacts over 300-fold faster than the zwitterionic cystine. These observations are consistent with electrostatic effects caused by an anionic group proximal to the essential

thiol. We previously suggested the presence of an anionic group at the active site of *K. aerogenes* urease based on comparison of the K_i values for a series of competitive inhibitors (26). These two observations may be related if the essential thiol is at the urease active site.

Is the essential thiol at the active site? Loss of urease activity by thiol-specific reagents may be due to modification of an active site thiol, or to modification of a non-active site residue, resulting in a conformational perturbation. In an attempt to discriminate between these two possibilities, the effects of urease active site ligands on the rate of inactivation was explored.

Four competitive inhibitors of urease and the enzyme substrate, urea, were shown to affect the rate of enzyme inactivation, as summarized in Table 2. Whereas urea, boric acid, and 4-Br-BBA afforded only partial protection of the essential thiol, phosphate completely protected the enzyme against inactivation by either IAM or DTDP. In all cases, the effects were achieved at concentrations corresponding to K_i or K_m of the ligand. These results are consistent with the essential thiol being at the urease active site. Since urea provides only partial protection from inactivation, the essential thiol may not be directly involved in urea binding, but could participate in catalysis. These results are consistent with a model for the

jack bean urease active site in which a thiol is proposed to serve as a proton donor (6).

pH dependence of urease inactivation by IAM and DTDP- Roberts et al. (21) have shown that the intrinsic reactivity of a thiolate anion with MMTS is >5 X 10^9 times that of the protonated thiol. Similar results were obtained by Bednar (3) for the reaction of thiols with NEM. Thus, the apparent pseudo-first-order rate constant for either disulfide exchange or alkylation can be calculated by

$$k_{app} = \frac{K_a k_1}{[H^+] + K_a} \qquad (7)$$

where k_1 is the second-order rate constant for reaction with the thiolate anion, and K_a is the acid dissociation constant of the amino acid which is modified. A plot of $\log(k_{app})$ versus pH for thiols exhibiting this behavior would possess a slope of 1 below the pK and a slope of 0 above the thiol pK. The pH dependence of urease inactivation by IAM and DTDP is inconsistent with this simple mechanism; *i.e.* the reactivity of the essential thiol is pH independent below pH = 8 for both reagents in Figure 5.

One mechanism to explain the data in Figure 5 invokes a greatly enhanced reactivity of protonated reagent towards the enzyme thiolate anion. Brocklehurst et al. (4) demonstrated that protonated DTDP (pK = 2.2) reacts with thiolate anions 1500-fold faster than the neutral

disulfide. For urease, the equation describing loss of ureolytic activity by this mechanism would be:

$$k_{app} = k_1 [E \cdot S^-] [R] + k_2 [E \cdot S^-] [R\ H^+] \quad (8)$$

with $k_1 = 10^5\ M^{-1}s^{-1}$ and $k_2 = 10^{11}\ M^{-1}s^{-1}$. This explanation is implausible for DTDP, since k_2 is faster than the diffusion rate.

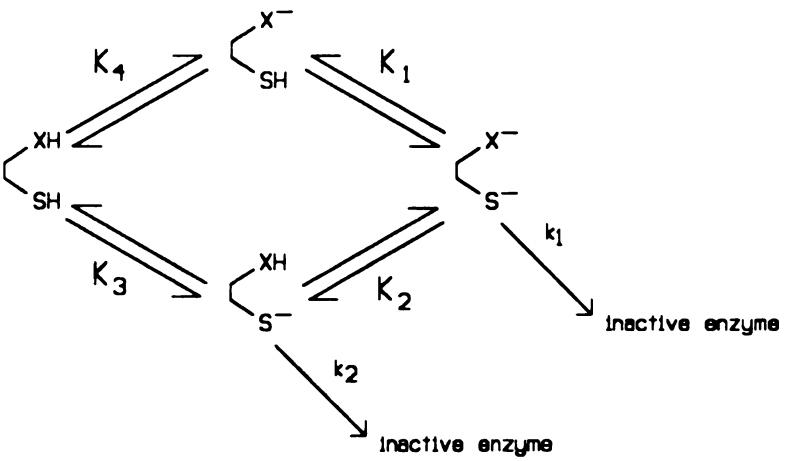
A second mechanism consistent with the results in Figure 5 requires the presence of two essential thiols in urease, similar to the mechanism proposed for the reaction of the two active site thiols in thioredoxin reductase (10). In this model, one urease thiol (with a very high $pK_1 = 12$) reacts rapidly (k_1); a second thiol reacts more slowly (k_2) and has a low pK_2 (< 5). The apparent inactivation rate constant is given by:

$$k_{app} = \frac{k_1 \frac{K_1}{[H^+]} + k_2}{1 + \frac{[H^+]}{K_2} + \frac{K_1}{[H^+]}} \quad (9)$$

A key point of this model is that inactivation is caused by modification of predominantly one thiol at pH values above 8 and by modification of a second thiol below pH 8. The uniform effect of boric acid on IAM inactivation at pH 6.0, 7.75, and 9.5 appear to preclude this model. If two distinct thiols were reacting at different pH values, one would not expect that a competitive inhibitor would affect

the rates equivalently by decreasing the k_{app} for modification of either thiol by a factor of 3.

Our best interpretation of the urease inactivation results is shown in Scheme 3 (modified from Roberts et al., 21), which assumes that the essential cysteine interacts with a second ionizing group (**X**).



(Scheme 3)

Two forms of the enzyme can react with IAM or DTDP with second-order rate constants indicated by k_1 and k_2. The observed macroscopic equilibrium constants K_I and K_{II} can be related to the individual microscopic equilibrium constants of Scheme 3 by equations 10 and 11 (Roberts et al., 1986).

$$K_I = K_3 + K_4 \tag{10}$$

$$K_{II}^{-1} = K_1^{-1} + K_2^{-1} \tag{11}$$

The apparent rate constant for inactivation can then be represented by

$$k_{app} = \frac{\frac{k_1 K_{II}}{[H^+]} + \frac{k_2 K_3}{K_I}}{1 + \frac{[H^+]}{K_I} + \frac{K_{II}}{[H^+]}} \qquad (12)$$

When the data in Figure 5 were fit to equation 12, values for pK_I, pK_{II}, k_2K_3/K_I, and k_1 were <5, 12, 0.032 $M^{-1}s^{-1}$, and 230 $M^{-1}s^{-1}$, for IAM, and < 5, 12, 11 $M^{-1}s^{-1}$, and 2.0 X 10^5 $M^{-1}s^{-1}$ for DTDP. Values for k_2 alone could not be determined because K_3 and K_4 (Scheme 3) are unknown. Characterization of the individual equilibrium constants would require additional experiments; *e.g.* potentiometric titrations of these residues could be carried out analogous to studies involving the active site thiol and histidine of papain (11). Nevertheless, referring to Scheme 3 and the results in Figure 5, the ionization state for **X** must affect the thiol pK such that either pK_1 approaches 12 when **X** is deprotonated or pK_3 approaches 5 when **X** is protonated.

An alternative but related model also invokes the presence of a second ionizing group (**X**); however, here a single intermediate form of the enzyme exists in which the two residues are hydrogen-bonded. The apparent rate constant for inactivation can again be represented by equation 9.

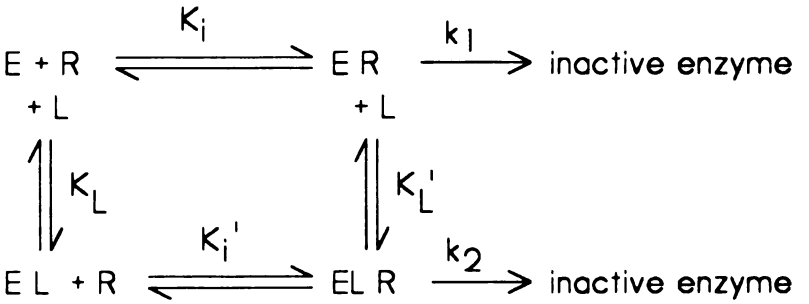
In summary, the pH dependence of bacterial urease inactivation is most consistent with a single essential thiol interacting with a second ionizable residue

(together yielding pK_a values of < 5 and 12). By contrast, Norris and Brocklehurst (19) concluded that the essential thiol in jack bean urease has a pK_a = 9.15 and does not react with another ionizing group. They examined the essential thiol using DTDP as a spectrophotometric probe after first blocking the surface thiols with NEM, DTDP or DTNB. Although activity measurements were not reported in their pH dependence studies, the spectroscopic reactivity results yielded no plateau in the low pH end of the log rate versus pH plot, down to pH 7.0. However, the jack bean urease study was carried out using phosphate buffers below pH 8. Figure 4 illustrates that phosphate decreases the rate of inactivation of the bacterial enzyme. Furthermore, we have shown (26) that phosphate inhibition is pH dependent, with phosphate becoming a more potent inhibitor as the pH decreases. Thus, a low pH plateau in the jack bean study may have been masked by phosphate-dependent thiol protection.

Urease inactivation by DTNB- In contrast to the simple, second-order reaction observed between urease and six of the reagents examined, the pseudo-first-order rates of inactivation were not proportional to DTNB concentration. Rather, the data were fit to Scheme 2, involving a preliminary DTNB binding step prior to disulfide exchange leading to inactivation. This type of saturation behavior is not uncommon (9); indeed similar kinetics

could occur for the other reagents, but the saturating concentrations are higher than the range examined. The pH dependence of urease inactivation by DTNB is consistent with a modified Scheme 3 which includes reversible formation of a complex between enzyme and modifying reagent. Binding is unaffected by the ionization status of the essential thiol, whereas the DTNB inactivation step behaves similarly to k_{app} in urease reactions with IAM or DTDP.

The effect of urea on the rate of urease modification by DTNB can be accounted for, in part, by a modification of Scheme 1 to yield



(Scheme 4)

As shown by Cardemil (1987), the rate of inactivation in this case is

$$k_{app} = \frac{[R]\left[K_L\, k_1\, K_i' + k_2\,[L]\,K_i\right]}{K_i'\,K_L\,(K_i + [R]) + K_i\,[L]\,(K_i' + [R])} \qquad (13)$$

The observed parallel lines in the $1/k_{app}$ versus 1/[DTNB] plot (Figure 6) require that

$$\frac{k_1}{k_2} = \frac{K_i}{K_i'} \qquad (14)$$

so that the binding affinity increases (K_i' decreases) as the rate k_2 decreases with increasing concentration of ligand. We do not have an explanation for this fortuitous relationship.

Spectroscopic quantitation of urease thiols- Using subunit molecular weights and cysteine contents derived from recently published sequence data (16) and the $\alpha_2\beta_4\gamma_4$ subunit stoichiometry derived form integration of bands in Coomassie Blue stained SDS gels (25), the holoenzyme is predicted to possess 20 thiols. Modification of denatured enzyme by DTDP showed that urease possessed 25.5 ± 0.3 mol thiol/mol enzyme based on Lowry protein determination. These results are consistent with an absence of disulfides in the native protein. Spectroscopic analysis of jack bean urease using DTNB combined with the amino acid composition indicated that 15 thiols and one disulfide were present per subunit (M_r = 96,600; 20), whereas protein sequencing later demonstrated the presence of only 15 cysteines per subunit (M_r = 90,770; 24).

We have previously shown that *K. aerogenes* urease possesses two active sites per native molecule (26), hence, we can conclude from the spectroscopic analysis

that 4 thiols per catalytic unit react with DTDP at pH 7.75. Modification of at least one of the these thiols is associated with urease inactivation. In the presence of PPD, a tight-binding competitive inhibitor of urease, only 3 DTDP reacted per catalytic unit, and the enzyme was active after PPD removal. These results are consistent with the presence of a single essential thiol per active site. AHA, a slow binding inhibitor, did not provide comparable protection of the essential thiol. Studies at pH 6.1 using the competitive inhibitor phosphate also demonstrated that inactivation was associated with the modification of a single thiol per catalytic unit.

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IDENTIFICATION OF THE ESSENTIAL CYSTEINE RESIDUE IN *KLEBSIELLA AEROGENES* UREASE

ABSTRACT

During reaction with ^{14}C-iodoacetamide at pH 6.3, radioactivity was incorporated primarily into a single *K. aerogenes* urease peptide concomitant with activity loss. This peptide was protected from modification at pH 6.3 by inclusion of phosphate, a competitive inhibitor of urease, which also protected the enzyme from inactivation. At pH 8.5, several peptides were alkylated; however, modification of one peptide, identical to that modified at pH 6.3, paralleled activity loss. The N-terminal amino acid sequence and composition of the peptide containing the essential thiol was determined. Previous enzyme inactivation studies of *K. aerogenes* urease could not distinguish whether one or two essential thiols were present per active site (20); we conclude that there is a single essential thiol present and identify this residue as Cys_{319} in the large subunit of the heteropolymeric enzyme.

INTRODUCTION

Urease (urea amidohydrolase, EC 3.5.1.5) is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate; carbamate spontaneously hydrolyzes to H_2CO_3 and a second molecule of ammonia. The best characterized urease is the homohexameric protein from *Canavalia ensiformis* (jack bean) which contains 2 nickel per subunit (M_r = 90,770) (reviewed by Andrews et al., 2). The function of urease in this and other plants is unknown (21). In contrast, bacterial ureases play important roles in nitrogen metabolism and have been implicated as virulence factors in various human and animal diseases (reviewed by Mobley and Hausinger, 9). The most extensively studied bacterial urease is that from the Gram-negative enteric bacterium, *Klebsiella aerogenes* (currently *K. pneumoniae*). This enzyme possesses three different subunits [M_r = 60,304 (α), 11,695 (β), and 11,086 (γ)], in an apparent $\alpha_2\beta_4\gamma_4$ stoichiometry, and has two bi-nickel active sites (18, 19). The DNA-sequence of the *K. aerogenes* urease operon (11) revealed that each bacterial subunit possesses significant homology to portions of the plant urease sequence, consistent with gene fusion in the later case. As described below, both of these enzymes possess an essential cysteine residue;

however the bacterial and plant enzyme thiols differ in their chemical properties (13; 20).

Labelling studies of jack bean urease indicated that 4 cysteines/subunit react rapidly with a disulfide reagent, DTDP, with no loss of activity, whereas modification of a fifth cysteine was correlated to activity loss (13). The essential thiol exhibited a pH dependence of reactivity similar to that of a free thiol, with a pK_a = 9.1. Takishima et al. (16) followed up on this work by modifying the non-essential enzyme thiols with N-ethylmaleimide, then derivatizing the essential thiol with the chromophore N-(4-dimethylaminodinitrophenyl) maleimide. They demonstrated the specific labelling of a single cysteine residue, Cys_{592}. In contrast, Sakaguchi et al. (15) partially modified jack bean urease with diazonium-1 H-tetrazole, then added ^{14}C-labelled reagent to derivatize the more slowly reacting thiols. They showed that label was incorporated into 2 cyanogen bromide fragments; one fragment was further studied and contained Cys_{207}. More recently, jack bean urease was modified using N-iodoacetyl-N'(5-sulfo-1-naphthyl)ethylene diamine and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (17): three thiols were modified with no loss of activity, whereas alkylation of 2 additional thiols (Cys_{207} and Cys_{592}) was concomitant with activity loss. Complete modification of Cys_{207} with the latter reagent resulted in an activity decrease of only 50%; thus the authors concluded that Cys_{592} (but not Cys_{207}) is essential for activity.

No active site labelling studies have been reported for any bacterial urease; however kinetic analysis of *K. aerogenes* urease revealed a pseudo-first-order loss of activity in the presence of disulfide reagents or alkylating reagents (20). The addition of substrate or inhibitors altered the rates of inactivation, consistent with localization of the essential thiol proximal to the active site. Competitive inhibitors (PPD at pH 7.5, and phosphate at pH 6.1) were shown to protect one thiol per active site from modification by DTDP. The apparent second-order rate constant for activity loss as a function of pH (using either IAM or DTDP) differed notably from the behavior of the plant enzyme in being constant over the range of pH 5 to pH 8. The results are consistent either with the essential thiol interacting with a nearby ionizable amino acid or with the presence of two essential cysteines per active unit (20). In the latter case, inactivation at low pH would involve modification of a cysteine which is distinct from that modified at high pH; both cysteine residues would be required for activity.

This manuscript describes our efforts to identify the essential thiol(s) of *K. aerogenes* urease and to discriminate between the one- and two-cysteine models.

EXPERIMENTAL PROCEDURES

Enzyme Purification- *K. aerogenes* urease was purified to a specific activity >2500 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ from strain CG253 carrying the recombinant plasmid pKAU19 (12) by using procedures previously described (19). The third and final step of the purification, Mono-Q (Pharmacia) anion exchange chromatography, made use of DTT-containing buffers (1 mM) to ensure the absence of mixed-disulfides between urease and β-ME. Enzyme was stored at 0°C in DTT-containing buffers until used. Protein was assayed by the method of Lowry et al. (8) using bovine serum albumin as the standard and confirmed using the extinction coefficient $\varepsilon^{1\%}_{280\ nm}$ = 8.5 (18). The molecular weight of urease was calculated to be 211.8 μg/nmol (11).

Uniform labelling of cysteine residues in urease- Purified urease was desalted by ultrafiltration using a Centricon-30 microconcentrator (5 X 1:10 dilution) to remove thiols used to stabilize the enzyme during storage, then dried using a speed-vac concentrator (Savant). 15 mg urease was denatured in 6 M Gn·HCl, 10 mM EDTA, 0.1 M Tris·HCl, pH 8.0, reduced by incubation with DTT (500 nmole) for 60 min at 50°C, and carboxamidomethylated with ^{14}C-IAM (2.5 μmole @ 0.185 GBq/mmol, Amersham) for 30 min at 25°C. The modification reaction was quenched by adding excess β-ME.

Specific labelling of the cysteine residues in native urease- *K. aerogenes* urease (20-50 μM) was desalted into 1 mM EDTA, 80 mM buffer (MES, pH 6.3, HEPES, pH 7.75, or TAPS, pH 8.5) and allowed to react with 5 mM ^{14}C-IAM at 37°C until < 5% of the activity remained, as determined by assaying aliquots using a standard assay procedure (19). Reactions at pH 6.3 were done both with and without 20 mM phosphate, a pH-dependent competitive inhibitor of urease (19), to protect the essential cysteine from modification. Some reactions at pH 7.75 included the competitive inhibitors AHA (25 mM) or PPD (25 μM). During the reactions, aliquots were quenched by addition of excess β-ME, then desalted on a Superose-12 gel filtration column (1 X 30 cm, Pharmacia), equilibrated with 1 mM EDTA, 20 mM phosphate, pH 6.5, containing 1 mM β-ME. Radioactively labelled urease samples were denatured in 6 M Gn·HCl, reduced with DTT, and the remaining cysteines were modified with unlabeled IAM using procedures analogous to those described above.

Standardized protocol for peptide mapping- The α subunit of urease was separated from the β and γ subunits by Superose-12 gel filtration chromatography (1.0 X 30 cm) in 3 M Gn·HCl, 1 mM EDTA, 0.1 M Tris·HCl, pH 8.0. Fractions containing the α subunit were pooled, concentrated, and adjusted to 1 M Gn·HCl. $CaCl_2$ was added to 1.0 mM, then

samples were digested with 4% TPCK-treated trypsin (Type XIII, Sigma) for 24-40 hours at 37°C (2% added initially, another 2% added after 8-12 hours digest). Tryptic peptides were separated by using reverse phase chromatography (Pro-RPC, 0.5 X 10.0 cm, Pharmacia), with a multistep gradient of 0-50% IPN containing 0.05% TFA. Two peptides which did not bind to the Pro-RPC could be separated on a Pep-RPC column (Pharmacia) with a 0-100% acetonitrile gradient, containing 0.1% TFA.

Characterization of the specifically labelled peptide- Sequence analysis of the specifically-labelled tryptic peptide was done by using an Applied Biosystems Model 477 protein sequencer followed by HPLC analysis of phenylisothiocyanate derivatives. Amino acid composition was determined by HPLC analysis after converting the hydrolyzed sample to the phenylisothiocyanate derivatives.

RESULTS AND DISCUSSION

Uniform labelling of urease cysteines and development of a peptide mapping protocol- Denatured *K. aerogenes* urease was uniformly labelled with ^{14}C-IAM at pH 8.0. The α subunit (M_r = 60,304) was separated from the β and γ subunits (M_r = 11,695 and 11,086, respectively) by gel-filtration chromatography (Figure 1), which also removed excess

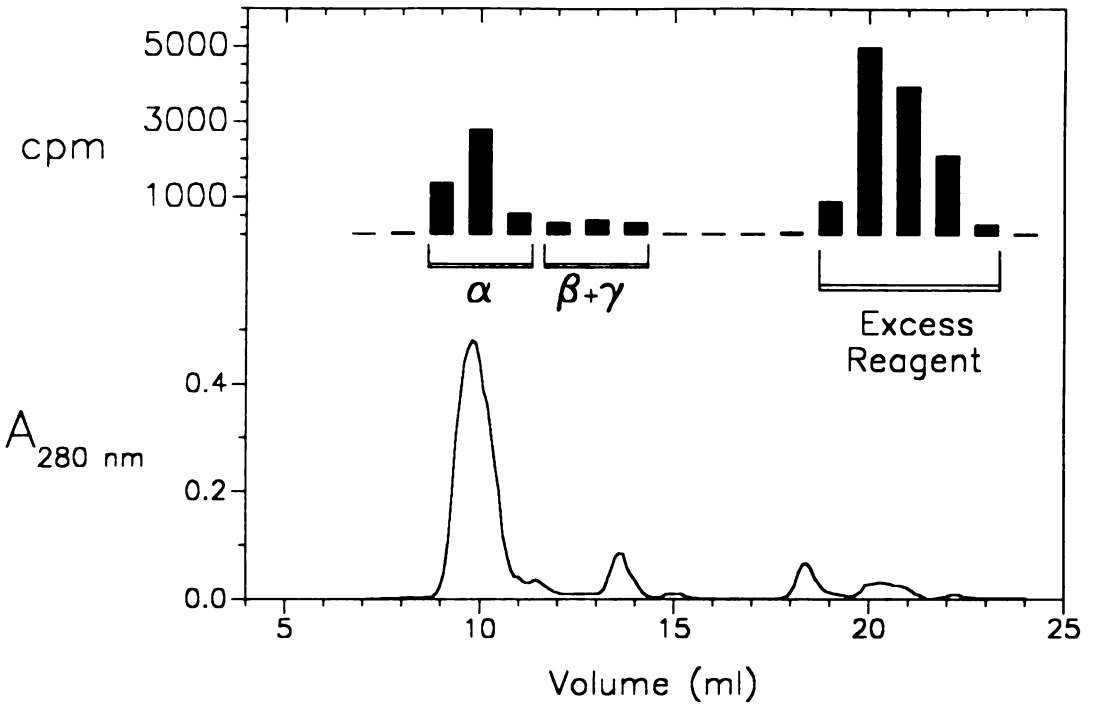

Figure 1. **Subunit distribution of urease thiols.** Urease was denatured, reduced, and uniformly modified with ^{14}C-IAM. Alkylated protein was applied to a Superose-12 column (1.0 X 30 cm, Pharmacia) equilibrated with 3.0 M Gn·HCl, 10 mM EDTA, 0.1 M Tris·HCl, pH 8.0. Cpm was determined in 5% of each 1.0 ml fraction, and absorbance was monitored at 280 nm. Brackets indicate fractions included in calculations of the relative amount of label in each subunit.

reagent. The ratio of ^{14}C in the α subunit to that in the sum of the β and γ subunits was 4.8:1. The β and γ subunits could be separated by reverse phase chromatography (data not shown), and all of the radioactivity was found in the β subunit; thus the ratio of thiols in the α, β, and γ subunits was 4.8:1:0. The genes encoding these proteins (11) indicate an 8:1:0 ratio of thiols in the α, β, and γ subunits. Hence, there are twice as many β as α subunits in the native enzyme, consistent with the proposed $\alpha_2\beta_4\gamma_4$ subunit structure (18).

Purified carboxamidomethylated-urease α subunit was highly insoluble unless maintained in the presence of a denaturant such as 1 M Gn·HCl. A standard protocol for peptide mapping was developed by digesting the subunit dissolved in this chaotropic agent with TPCK-treated trypsin for various lengths of time and analyzing the peptides by Pro-RPC chromatography (Figure 2). Optimum resolution of cysteine-containing peptides (determined by monitoring absorbance at 214 nm and by analysis of fractions for cysteine-containing peptides using liquid scintillation counting) was only achieved after 30-40 hours of digestion, at 37°C. Six major cysteine-containing peaks were identified as flow-thru (FT), and peptides A, B, C, D, and E (Figure 2). The flow-thru fraction could be further resolved into two additional peptides, F and G, on the more hydrophobic column, Pep-RPC (data not shown). This protocol yielded consistent peptide maps in which the 7 peaks accounted for

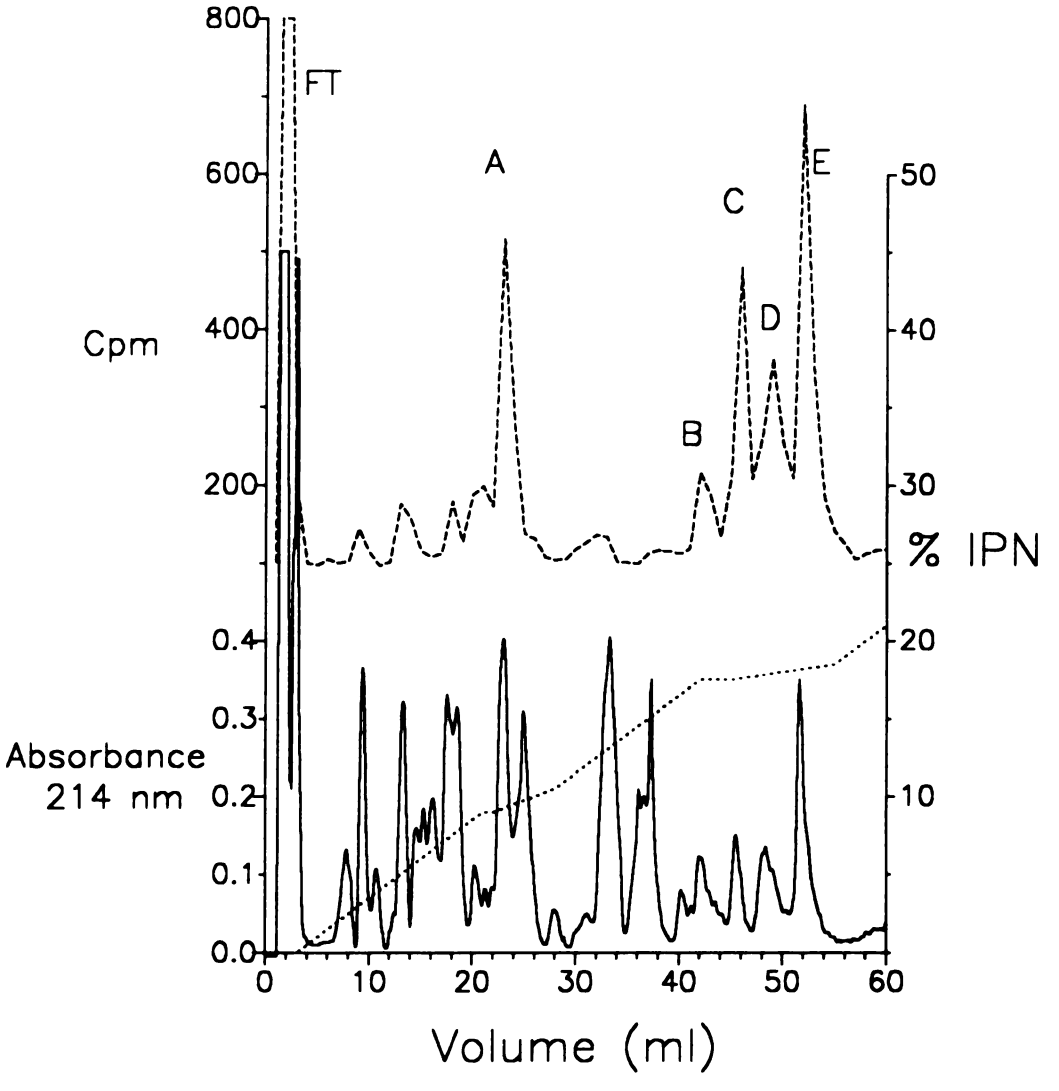

Figure 2. **Pro-RPC analysis of uniformly-labelled trypsin-digested urease α-subunit.** Purified urease α-subunit was digested with TPCK-treated trypsin (as described in the text) and applied to a Pro-RPC column. The column was eluted using the indicated gradient of IPN/0.05 % TFA (dotted line) while monitoring the absorbance at 214 nm (solid line). Fractions (1 ml) were collected and radioactivity was determined in 0.1 ml aliquots by scintillation counting (dashed line).

at least 75 % of the applied label in uniformly-labelled samples, and clearly resolved the specifically labelled peptides in the experiments described below.

Specific labelling of the essential cysteine at pH 6.3- The reactivity of the essential thiol of *K. aerogenes* urease exhibited a pK_a of 12 and decreased below this value until about pH 8; below pH 8 the essential thiol showed a constant rate of modification (20). In contrast, the reactivity of most thiols towards modifying reagents decreases with decreasing pH below the thiol pK_a. We attempted to exploit this low pH reactivity as a method to specifically label the essential thiol. In addition, since the competitive inhibitor phosphate was shown to completely protect urease from inactivation by both disulfide-forming reagents and alkylating agents (20), we sought to identify the essential cysteine modified at pH 6.3 by comparing the peptides labelled by ^{14}C-IAM in the presence and absence of phosphate. After 25 hours incubation with ^{14}C-IAM in the absence of phosphate, a total of 7.7 thiols (6 in the α subunits, 1.7 in the β-subunits) were modified with complete loss of activity; in the presence of phosphate, 4 thiols in the α subunits were protected from modification (Figure 3). Phosphate did not protect any cysteine in the β subunit, demonstrating that the essential cysteine is in the α subunit. The finding that only 2 β-subunit thiols are modified in inactivated enzyme may indicate that the 4 β-subunits are

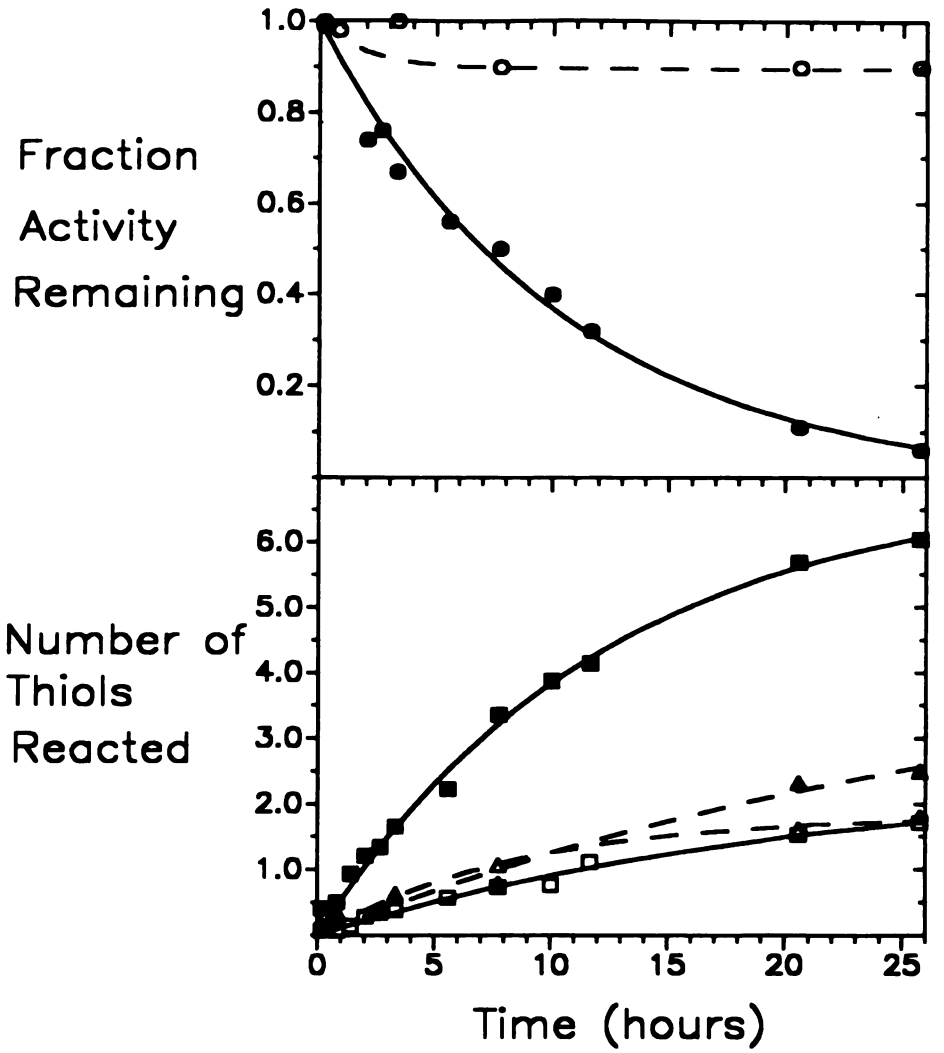

Figure 3. **Effect of phosphate on the modification of urease thiols by IAM.** Urease was inactivated with 5 mM ^{14}C-IAM in the presence (open symbols) and absence (closed symbols) of 20 mM phosphate, in 1 mM EDTA, 80 mM MES, pH 6.3. **A)** The remaining activity is shown as a function of time. **B)** The number of α-subunit (squares) and β-subunit (triangles) thiols reacted was assessed as described in the text.

present in 2 distinct conformations: the thiols in two of these subunits are accessible to solvent, whereas the other 2 β-subunit thiols are sequestered.

Purified α-subunit was digested with trypsin, and subjected to the Pro-RPC peptide mapping protocol. In the absence of phosphate, the label was incorporated most specifically into peptide D, and the extent of peptide modification was reasonably well correlated to activity loss. Furthermore, phosphate provided significant protection of this same cysteine-containing peptide (Figure 4). Other cysteine-containing peptides were protected to a lesser extent, probably because phosphate stabilizes the enzyme from low-pH induced denaturation.

Specific labelling of the essential cysteine at high pH- At higher pH values, competitive inhibitors of urease did not protect the essential thiol from alkylation by IAM (Figure 5). This result was surprising because we had previously shown that the slow-tight binding inhibitor PPD (K_i = 95 pM), but not AHA (K_i = 2.6 μM), protected 1 mole of thiol per mole of active site from modification by DTDP (20). PPD may be able to sterically exclude DTDP from the active site, whereas the smaller, uncharged alkylating reagent, IAM, is still able to react with the essential thiol.

Because the competitive inhibitor protection approach was untenable for identification of the essential cysteine

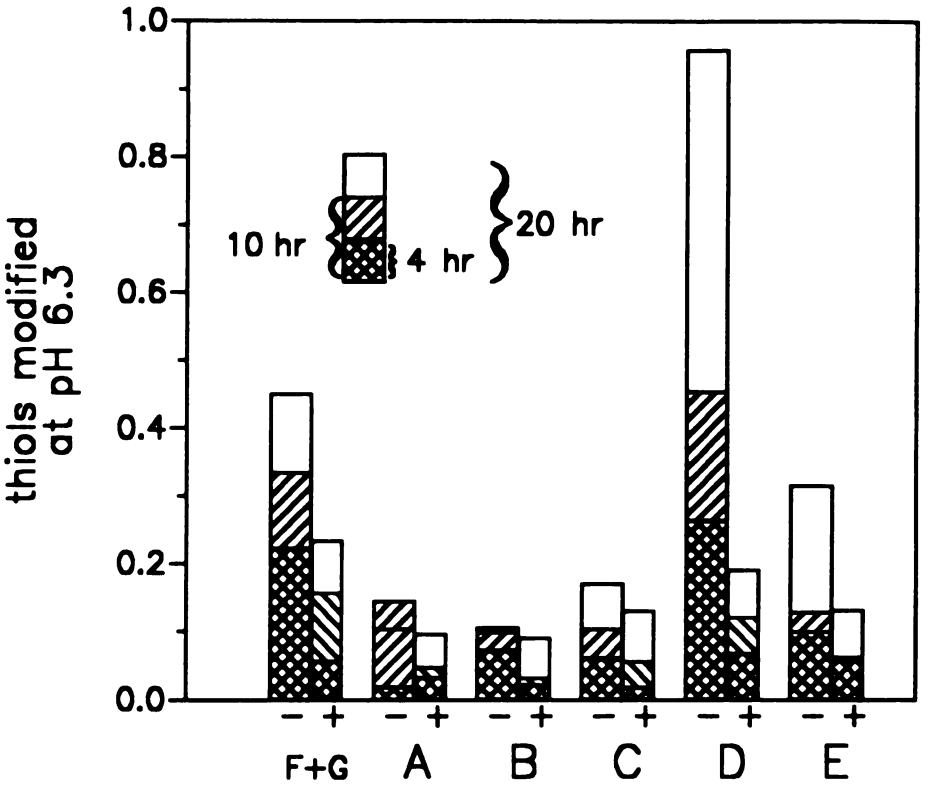

Figure 4. **Peptide distribution of urease thiols modified at low pH.** The α-subunit of urease, modified at pH 6.3, was purified and digested with trypsin. Pro-RPC chromatography was used to determine the amount of labelled thiol in each peptide in the presence (+) and absence (-) of phosphate.

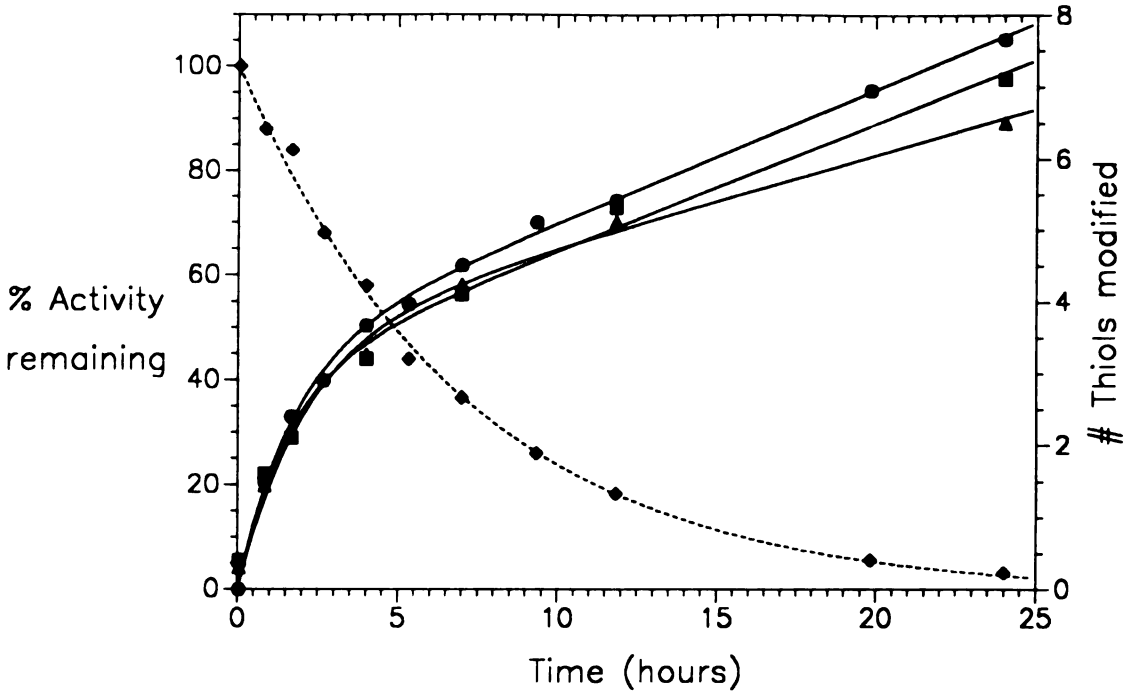

Figure 5. **Effect of competitive inhibitors on urease thiol modification at pH 7.75.** Urease was inactivated at pH 7.75 in 10 mM EDTA, 100 mM HEPES, pH 7.75 using ^{14}C-IAM, in the absence of inhibitors (●), and in the presence of 25 mM AHA (■) or 25 μM PPD (▲). Aliquots were quenched with an excess of β-ME, desalted using Superose-12 gel-filtration chromatography, and the incorporated radioactivity was measured to determine the number of thiols modified. The time-dependent decrease in activity is that for urease inactivated in the absence of inhibitors (◆). Activity of the inhibited samples was not determined because the rate of inhibitor dissociation to yield active enzyme is slow ($T_{0.5}$ ~ 2.5 hr, Todd and Hausinger, 1989).

residue at higher pH values, the reactivity of individual cysteine-containing peptides was examined in uninhibited samples as a function of remaining activity. The pattern of subunit labelling at pH 8.5 is shown in Figure 6a. 2-3 thiols were modified with essentially no loss of activity; activity loss occurred upon modification of an additional 5-6 thiols per holo-enzyme, with 1-2 thiols modified in the β subunits, and 4 thiols modified in the α subunits. Tryptic peptide analysis of α-subunit purified from urease modified at pH 8.5 revealed that activity loss paralleled the labelling of only peptide D (Figure 6b).

As in the pH 6.3 study, only half of the β-subunits were alkylated at pH 8.5, perhaps indicating that the holo-enzyme has two distinct populations of β-subunits. (We assume that the same two thiols are reactive at each pH). The low pH/inhibitor protection analysis demonstrated that the β-subunit thiol is not essential for catalysis; however, modification of this thiol (at pH 8.5) closely followed activity loss in the absence of inhibitors. The observed similarity in reaction rates between the β-subunit thiol and the essential thiol in *K. aerogenes* urease may relate to the anomalous jack bean urease active site labeling studies of Sakaguchi et al., (15). The bacterial β-subunit is homologous to residues 132-237 in the jack bean urease sequence (11, 17) and the only cysteine in this region of the plant urease is Cys_{207}; Sakaguchi et al. (15) may have

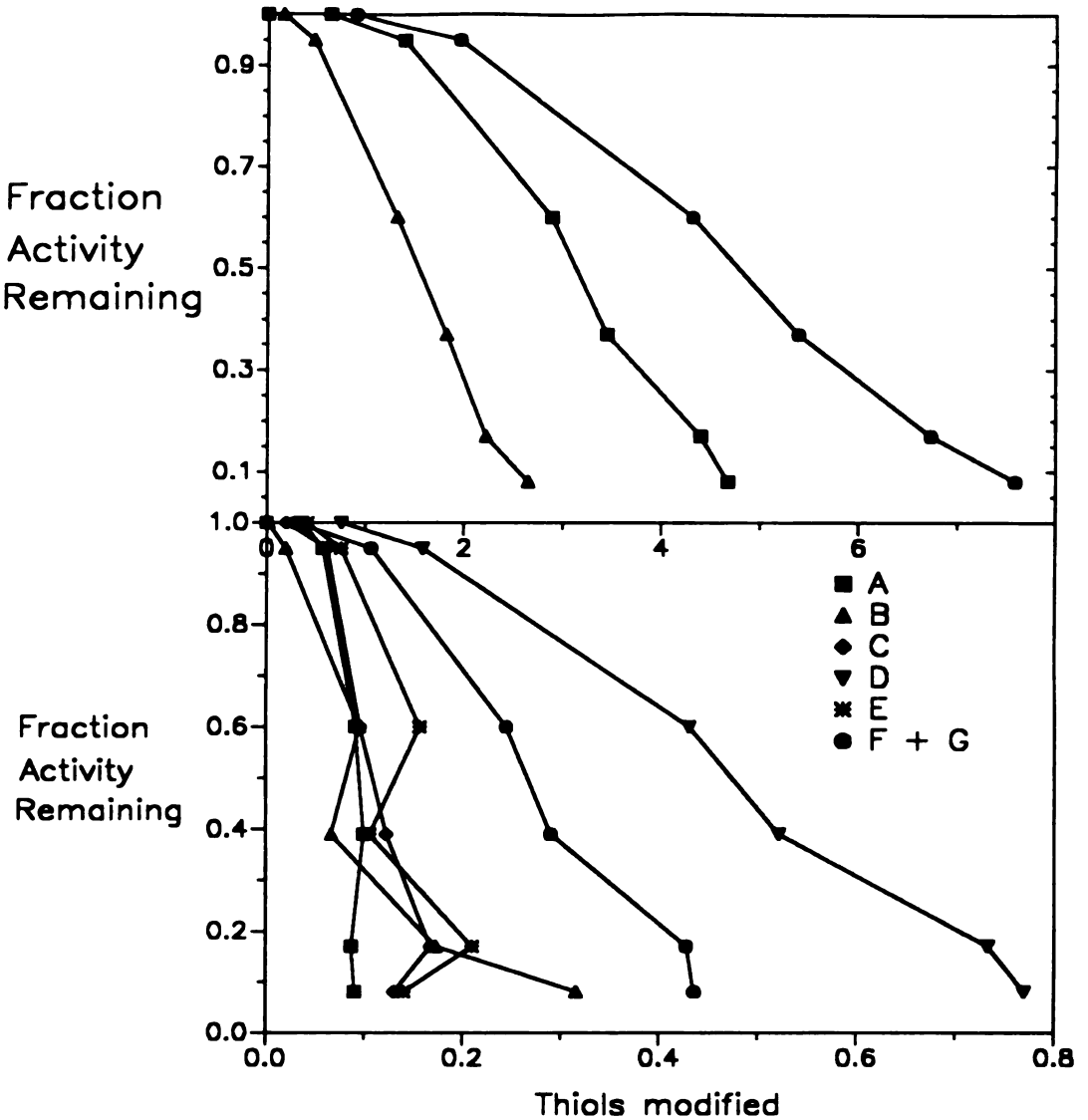

Figure 6. **Peptide distribution of urease thiols modified at high pH.** Urease was inactivated with 5 mM ^{14}C-IAM at pH 8.5. **A)** The activity remaining as a function of the total number of thiols modified (●) was determined. In addition, the distribution of label in the α (■) and β (▲) subunits was measured. **B)** Activity is shown as a function of the amount of label incorporated into the α-subunit tryptic peptides.

misidentified Cys_{207} as the essential cysteine if this residue reacts similarly to that in the bacterial β-subunit.

In contrast to jack bean studies reporting the rapid modification of several thiols followed by slower reaction with the essential thiol (13, 1516, 17), we saw no cysteine-containing peptide in the α-subunit labelled faster than peptide D. Rather, our peptide mapping protocol demonstrated that a small amount of each cysteine-containing peptide was labelled with no loss of activity, perhaps due to the presence of a small (<10%) fraction of inactive protein.

Identification of the cysteine in peptide D- N-terminal sequence analysis of peptide D, purified from uniformly labelled urease, revealed the sequence TLNTI. Comparison of this sequence to that predicted from DNA sequence analysis (11) allows us to conclude that peptide D begins at residue 305 of the α-subunit. This peptide is produced by cleavage after Tyr_{304}, indicating that after 30 hours digestion, significant Ψ-tryptic cleavage had occurred. Ψ-trypsin is an autolytic product of trypsin which possesses chymotryptic-like activity, although at a much slower rate (1). Peptide D was subjected to amino-acid analysis (Table 1). The composition is consistent with peptide D extending from position 304 to the tryptic cleavage site at Arg_{336}. The only cysteine in this peptide is Cys_{319}. We conclude that *K. aerogenes* urease possesses a

Table 1

Amino Acid Composition of the Essential Cysteine-Containing Peptide

Amino Acid	Predicted	Experimental
Asx	6	4.9
Glx	3	3.7
Ser	1	1.3
Gly	0	2.7
His	3	2.4
Arg	1	1.1
Thr	2	2.3
Ala	3	3.4
Pro	1	1.4
Cys[1]	1	0.8
Tyr	1	0.5
Val	2	2.1
Met	2	0.8
Val	0	0.0
Ile	2	2.1
Leu	4	4.1
Phe	1	1.1
Lys	0	0.3
Trp	0	nd[2]

[1] Quantitated as carboxymethyl cysteine (although modification reactions were carried out using IAM, the carboxamidomethyl derivative is hydrolyzed to the carboxymethyl derivative during acid hydrolysis in 6 N HCl).

[2] not detected

single essential cysteine residue, and identify this residue as Cys_{319}.

Comparison of K. aerogenes urease sequence to other ureases- Ureases from *K. aerogenes* (11), jack bean (17), *Helicobacter pylori* (4, 6), *Ureaplasma urealyticum* (3), *Proteus mirabilis* (5), and *Proteus vulgaris* (10) have been sequenced. Only one cysteine (Cys_{319} by *K. aerogenes* numbering or Cys_{592} by jack bean numbering) is conserved among all species. As shown in Figure 7, the sequence immediately surrounding the essential cysteine, is identical at 22 of 29 amino acids. This region also contains three conserved histidine residues at 312, 320 and 321; histidines have been suggested to participate in binding the active site nickel ions (14, 7). Additionally, one of these histidines could interact with the essential thiol to enhance its reactivity at low pH values (20). Although data from both the bacterial and plant urease studies are consistent in identifying the essential cysteine, its precise role in catalysis remains unknown.

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                ** ***** * *  **  ** *** ******      **  ** **
Jb 560-VCGIKNVLPS STNPTRPLTS NTIDEHLDML MVCHHLDREI PEDLAFAHSR-610
Hp 289-VAGEHNILPA STNPTIPFTV NTEAEHMDML MVCHHLDKSI KEDVQFADSR-339
Uu 293-SVKYAHILPA STNPTIPYTV NTIAEHLDML MVCHHLNPKV PEDVAFADSR-343
Pv 287-SVGEPNILPA STNPTMPYTI NTVDEHLDML MVCHHLDPSI PEDVAFAESR-337
Pm 287-SVGEPNILPA STNPTMPYTI NTVDEHLDML MVCHHLDPSI PEDVAFADSR-337
Ka 287-ACAHPNILPS STNPTLPYTL NTIDEHLDML MVCHHLDPDI AEDVAFAESR-337
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Figure 7. **Comparison of urease sequences surrounding the essential cysteine residue.** The two letter abbreviations stand for: Jb-jack bean (Takishima et al., 1988); Hp-*Helicobacter pylori* (Labigne et al., 1991); Uu-*Ureaplasma urealyticum* (A. Blanchard, 1990); Pv-*Proteus vulgaris* (Mörsdorf & Kaltwasser, 1990); Pm-*Proteus mirabilis* (Jones & Mobley, 1989); Ka-*Klebsiella aerogenes* (Mulrooney & Hausinger, 1990). The indicated amino acid positions refer to the α-subunits of the bacterial ureases. Residues which are identical among all sequences are marked by an asterisk; the conserved cysteine and the three conserved histidines are highlighted in bold.

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SUMMARY and FUTURE DIRECTIONS

Upon initiation of this project little was known about microbial ureases. Most research on this medically and agriculturally important enzyme was done by scientists studying mixed microbial populations, whole cells, or impure proteins. The few known characteristics of microbial ureases were similar to the extensively studied plant enzyme, thus some researchers exploited the plant enzyme to draw conclusions about bacterial ureases! This chapter summarizes the purification and characterization of urease from *K. aerogenes,* describes active site studies using competitive inhibitors and amino acid modifying reagents, and exposes interesting questions arising from this work.

Urease purification and characterization- Urease of the Gram negative enteric bacterium, *K. aerogenes,* was purified to homogeneity and was demonstrated to have an $\alpha_2\beta_4\gamma_4$ subunit structure, containing 4 nickel (chapter 2). This was not expected since the well-studied plant enzyme has a homo-hexameric structure containing 12 nickel dispersed among 6 active sites. Using the tight-binding inhibitor PPD we demonstrated that *K. aerogenes* urease has two active sites (Chapter 3); therefore both enzymes have 2

mol nickel/mol active unit. The $\alpha_2\beta_4\gamma_4$ subunit stoichiometry has not been definitively determined since quantitation assumes each subunit has similar binding affinity for dye; however the presence of three distinct subunits was confirmed through the DNA sequencing efforts of Scott Mulrooney. The three subunits share significant homology to the jack bean enzyme and are consistent with gene-fusion in the latter case. Gel filtration chromatography in the presence of salts, glycols, DMSO, reductants, and detergents failed to cause subunit dissociation (Chapter 2). Perhaps moderate concentrations of chaotropic reagents (*e.g.* 2.0 M guanidine·HCl) will be found to disrupt subunit interactions, allowing investigation of the forces determining quaternary structure and identification of the nickel binding subunit(s).

Not only are the subunits held together tightly, but the nickel is also tightly bound. Nickel is released at pH values below 4.5 and examination of the holoenzyme treated at low pH may reveal increased reactivity of the nickel ligands. Low pH treatment may also lead to replacement of one or both of the nickel with other metals, allowing further spectroscopic and biophysical characterization. Nothing, however, is known of the stability of nickel-depleted urease apo-enzyme to low pH; a low-pH treatment could "irreversibly" denature the protein.

Purified urease can migrate as several distinct, active isozymes through native gel electrophoresis. SDS PAGE of

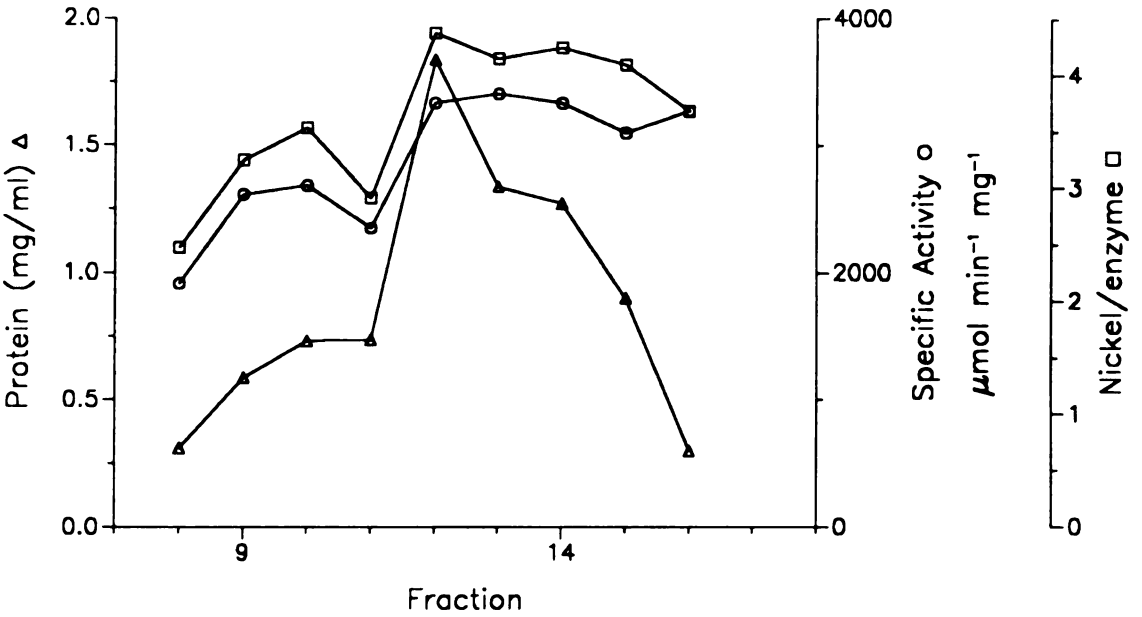

Figure 1. **Separation of urease isozymes on Mono-Q.** Urease was applied to a 1.0 X 10 cm Mono-Q equilibrated with 1 mM EDTA, 20 mM phosphate, pH 6.5, containing 1 mM β-ME. Urease protein was eluted with an increasing gradient of KCl. Protein, activity, and nickel were determined as described in Chapter 2.

each active band showed identical subunit stoichiometry, as assessed by scanning densitometry. These isozymes can also be separated by either ion exchange chromatography or gradient hydrophobic chromatography. The major active form (isozyme B) migrates faster under native gel electrophoresis and elutes later from anion exchange chromatography, consistent with a greater anionic character. One possible explanation for the multiple forms is that the minor form has less than 4 mol nickel/mol enzyme. Figure 1 is a preliminary experiment determining specific activity, protein, and nickel content of urease eluting from Mono-Q anion exchange chromatography. The isozyme which elutes first (A) has only 75% of the nickel (and 75% of the specific activity) of the isozyme which elutes later (B) consistent with A having less than stoichiometric amounts of nickel. While isozyme B is very stable, it will (over time) form some isozyme A; isozyme A looses activity quickly at low pH and has never been observed to form isozyme B. To simplify experiments, all inhibitor and active site studies in chapters 3-5 (and all biophysical characterizations) employed the second isozyme only.

Interpretation of urease structural studies would be easier with knowledge of the urease crystal structure. Methods to crystallize plant ureases have been known for 65 years, yet poor crystal quality and large unit cell have precluded solving the crystal structure. Crystals of the *K. aerogenes* enzyme have been obtained (A. Karplus, in

progress) and structural studies are continuing. Integration of crystal structure with chemical and genetic studies should contribute to understanding the mechanism of urea hydrolysis.

Urease inhibitors- The intelligent design of useful urease inhibitors requires detailed knowledge of enzyme-inhibitor interactions. AHA and PPD were found to be slow binding competitive inhibitors of *K. aerogenes* urease (Chapter 3). A slowly processed substrate is kinetically indistinguishable from a slow-binding inhibitor, therefore assays for PPD hydrolysis should be done. If PPD is hydrolyzed, the rate of hydrolysis may correspond to the rate of formation of enzyme-inhibitor complex, or to the rate of enzyme reactivation. A thorough examination of the pH-dependence for PPD inhibition of urease may help elucidate the mechanism of urea hydrolysis. Preliminary studies using AHA-urease indicated that the rate of reactivation (k_6) increased below pH 6.5, perhaps indicating the enzyme pK_a of 6.55 (required to be deprotonated for urea catalysis) must be deprotonated for the formation of a tight binding transition state complex with AHA or PPD.

With the advent of molecular biology and the development of expression vectors producing large quantities of protein, biophysical and spectroscopic studies on the urease nickel center are now possible. Magnetic susceptibility and MCD measurements on urease are consistent with two nickel

per active site being antiferromagnetically coupled in the presence of β-ME. Similar studies using the slow-binding inhibitors PPD and AHA may confirm the proposed bridging interactions of these competitive inhibitors with urease. Additional evidence linking the interactions of AHA and PPD with the nickel center may be deduced if the UV-visible spectrum of *K. aerogenes* urease changes upon binding of these inhibitors. The rate of spectral change may correspond to the rapid equilibration between enzyme and AHA or the slow step leading to our proposed "bridging" intermediate.

Urease active site amino acids- Ureases from all sources examined are susceptible to inactivation by thiol specific reagents. Competitive inhibitors modified the reactivity of an essential thiol in *K. aerogenes* urease and protected one thiol per catalytic unit from modification (Chapter 4). The essential cysteine of the jack bean enzyme is thought to act as a general acid, donating a hydrogen to the ammonia molecule as it leaves. We have demonstrated that at catalytically active pH values, this essential cysteine has appreciable nucleophilic character and that it interacts with another ionizable enzyme group (Chapter 4). The identity of this second ionizable group is not known, however the model of Zerner predicts three additional ionizable groups in close proximity to the active site thiol:

a carboxylate, an un-identified base, and a nickel-bound hydroxide.

Only indirect evidence for a carboxyl group at the active site of urease has been published: comparison of K_i values for various nickel binding inhibitors (Chapter 3) and k_{app} values for several thiol modifying reagents (Chapter 4) were consistent with repulsion of negatively charged molecules by the urease active site. Preliminary studies of *K. aerogenes* urease showed no loss of activity upon exposure to Woodwards Reagent K, at 37°C. However this reagent decomposes rapidly. More thorough analysis using carbodiimides or trialkyloxonium salts are obviously needed. Prior protection of the essential thiol with a reversible modifying reagent (*e.g.* modification by disulfide reagents is reversible) may be necessary.

Other amino acid specific reagents may be used to identify amino acids which participate in catalytic turnover. For example, DEP has been demonstrated to quickly inactivate urease holoenzyme (Mann Hyung Lee), consistent with a histidine being essential for activity; however the effect of active site ligands and the identity of the amino acid modified is not known. Tetranitromethane could be used to modify tyrosine side chains; lysine could be guanidinated using O-methyl isourea or reductively methylated with formate/$NaCNBH_4$. Preliminary experiments using the latter two lysine modifying reagents were hampered because the reagents interfered with the indophenol assay. Figure 2

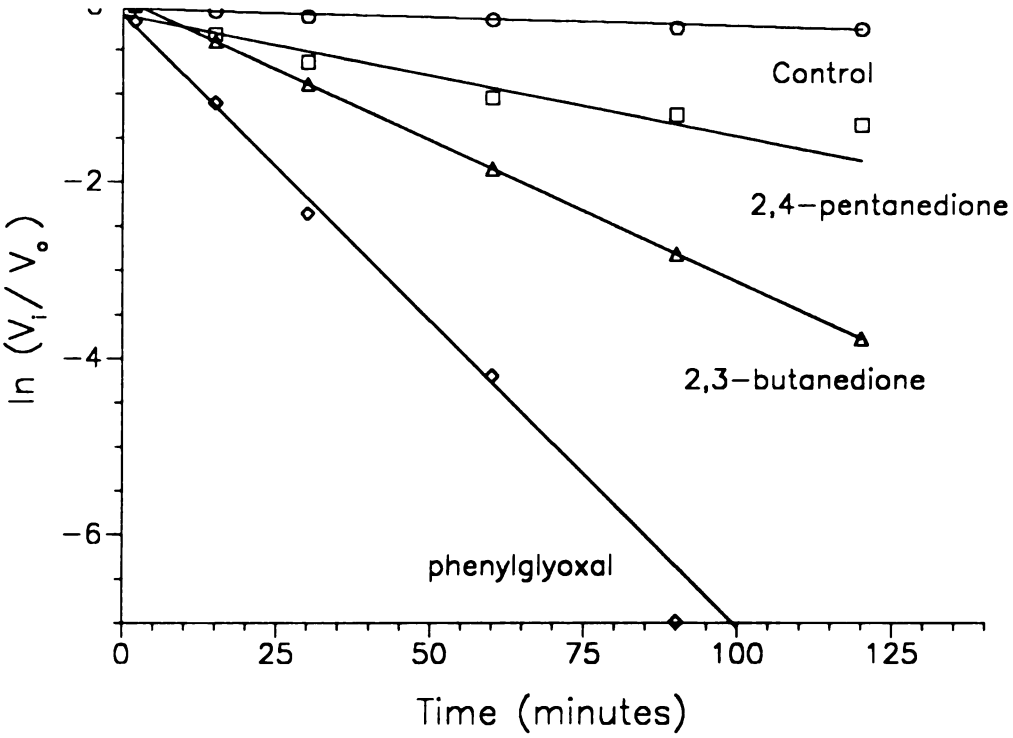

Figure 2. **Pseudo-first order inactivation of urease by arginine modifying reagents.** Urease was incubated at 37°C in 8 mM EDTA, 80 mM HEPES, pH 7.75 alone, or in the presence of 200 mM 2,4-pentane dione, 50 mM 2,3-butane dione, or 100 mM phenylglyoxal. At the indicated intervals, a small aliquot was diluted into the standard assay mixture to determine activity.

demonstrates the pseudo-first order loss of *K. aerogenes* urease activity by three arginine modifying reagents: 2,4-pentanedione, 2,3-butanedione, and phenylglyoxal. A comprehensive analysis of the effects of arginine modifying reagents will include determination of: the apparent second-order rate constants (and the effect of pH on these rate constants), the effects of active site ligands, and the effect of prior modification of the essential thiol. Identification of the essential arginine residue would naturally follow.

Clearly, expanded investigation of essential amino acids could contribute to our understanding of the enzyme mechanism. Once an amino acid is identified (as Cys_{319} was identified as essential for *K. aerogenes* urease activity; Chapter 5), site directed mutagenesis, (varying amino acid size, charge, polarity, or ability to form hydrogen bonds) could distinguish the properties of the amino acid involved in catalysis. Similar site directed mutagenesis studies could be targeted towards proposed nickel ligands. Simultaneous biophysical, spectroscopic, chemical, kinetic, and genetic analysis of the urease active site should generate complementary data, creating a thorough understanding of the catalytic mechanism and leading to the rational design of potent inhibitors for this medically and agriculturally important enzyme.

APPENDIX

SATURATION MAGNETIZATION OF THE NICKEL CENTERS IN UREASE.

E.P. Day, J. Peterson[+], M.J. Todd[*], and R.P. Hausinger[*] University of Minnesota, Minneapolis, MN 55455. [+]University of Alabama, Tuscaloosa, AL 35487. [*]Michigan State University, East Lansing, MI 48824.

The two nickel ions at the active site of urease are believed to be in the Ni(II) oxidation state. We have measured the saturation magnetization of urease isolated from *Klebsiella aerogenes* to determine its spin, spin concentration, average g value, and zero-field splitting. Activity was measured before and after the magnetization data were collected. EPR was used to quantitate Fe(III) impurities and metal analysis to quantitate the nickel. Spin S=1 paramagnetism accounted for roughly half the nickel implying that half the nickel is low spin (S=0). No evidence for exchange coupling between the nickel ions was found.