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Molecular Biology of Microbial Ureases

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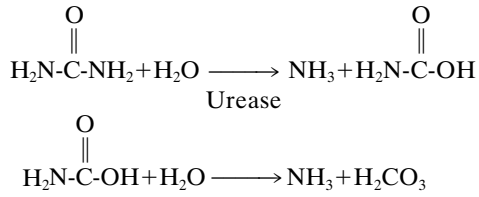
INTRODUCTION

Urease (urea amidohydrolase; EC 3.5.1.5) catalyzes the hydrolysis of urea to yield ammonia and carbamate. The latter compound spontaneously decomposes to yield another molecule of ammonia and carbonic acid:



In solution, the released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms, respectively. The net effect of these reactions is an increase in pH.

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$

$$2NH_3 + 2H_2O \rightleftharpoons 2NH_4^+ + 2OH^-$$

Interestingly, both urea and urease represent landmark molecules in early scientific investigation. Urea was the first organic molecule synthesized (2), and urease from jack bean was the first enzyme crystallized (274) in addition to being the first enzyme shown to contain nickel (64).

In 1989, two of us published a review of the microbial urease literature which assessed the state of knowledge at that time (201). We highlighted the significance of the enzyme, including (i) its capacity to serve as a virulence factor in human and animal infections of the urinary and gastrointestinal tracts, (ii) its role in recycling of nitrogenous wastes in the rumens of domestic livestock, and (iii) its importance in environmental transformations of nitrogenous compounds, including urea-based fertilizers. Furthermore, we described the physiology of microbial ureases, including aspects of their cellular location and regulation. The molecular details of the latter topic, however, were virtually unknown at the time. In addition, we summarized the small body of work that had been published on microbial urease enzymology, focusing on the relationships between the microbial enzymes and the well-characterized nickel-containing urease from jack bean. Finally, we described what was known about the genetics and molecular biology of urease, including the first snapshots of DNA sequence information and the requirement for nonsubunit "accessory genes" that were required for the production of active enzyme.

In the intervening years, there has been remarkable progress in better understanding all aspects of urease. In this review, we have endeavored to summarize these exciting new findings. We now are able to compare numerous urease gene clusters for which the entire nucleotide sequence is known. As well, we are beginning to understand the mechanisms by which urease gene expression is regulated in different species. X-ray crystallographic studies have given us the first glimpse of the three-dimensional structure of this high-molecular-weight, multimeric metalloenzyme. Chemical modification studies, site-directed mutagenesis approaches, and spectroscopic analyses have yielded insights into the structural components at the active site and the catalytic mechanism of the enzyme. The function of the mysterious accessory gene products in nickel metallocenter assembly are beginning to be unraveled. Finally, the detailed roles of the enzyme in various pathogenic processes are being clarified. In particular, because of the newly identified relationship between the highly ureolytic *Helicobacter pylori* and peptic ulceration, much of the recent urease literature has focused on the enzyme produced by this microorganism. Here, we critically assess recent advances in each of these research areas and expand upon their treatment in other reviews (2, 22, 41, 42, 108, 201, 205a).

GENETICS OF UREASE GENE CLUSTERS

In our 1989 review (201), we were able to report only the first glimpses of DNA sequence from bacterial urease gene clusters. Now what we believe to be the complete nucleotide sequences are available for urease gene clusters from five species and additional sequences of urease structural genes and urease-related open reading frames (ORFs) have been reported for other species. Analysis of these data now allows us to identify common features for urease, including gene organization, genes required for apourease synthesis, genes required for insertion of nickel ions into the apoenzyme, optional accessory genes that play specialized roles such as nickel transport, and genes involved in regulation of transcription.

Cloned and Sequenced Genes

The genes that make up complete urease gene clusters have been delineated by nucleotide sequencing of cloned determinants for five bacterial species including *Bacillus* sp. strain TB-90 (172), *H. pylori* (39, 53, 151), *Klebsiella aerogenes* (156, 211), *Proteus mirabilis* (132, 224, 270), and *Yersinia enterocolitica* (58, 264) (Table 1). In addition, sequences of a portion of the genes, usually the structural genes that encode the enzyme subunits, have been reported for the jack bean plant *Canavalia ensiformis* (247) and other bacterial species including *Bacillus pasteurii* (210), the rare ureolytic strains of *Escherichia coli* (plasmid-encoded locus) (68, 69), *Helicobacter felis* (82), *Helicobacter heilmannii* (267), *Klebsiella pneumoniae* (46), *Lactobacillus fermentum* (275), *Proteus vulgaris* (209), *Rhizobium-*

TABLE 1. Cloned and sequenced urease genes

Species	Genes sequenced	Length (kb)[a]	Predicted molecular size of *ure*-encoded polypeptides (kDa)										GenBank accession no.	Reference(s)
			A	B	C	D	E	F	G	H	I	R		
Complete gene cluster sequenced														
Bacillus sp. strain TB-90	*ureABCEFGDHI*	5.86	11.2	12.1	61.5	30.9	17.4	25.4	22.3	25.6	7.9		D14439	172
H. pylori[b,c]	*ureABIEFGH*	6.13	(UreA) 26.5		(UreB) 60.3, 60.5, 61.6	(UreH) 29.6	19.5	28.6	21.7		21.7		M84338, X57132, X17079, M60398	39, 53, 151
K. aerogenes	*ureDABCEFG*	5.15	11.1	11.7	60.3	29.8	17.6	25.2	21.9				M55391, M36068	156, 211
P. mirabilis	*ureRDABCEFG*	6.45	11.0	12.2	61.0	31.0	17.9	23.0	22.4			33.4	M31834, Z18752, Z21940	132, 224, 270
Y. enterocolitica	*ureABCEFGD*	6.07	11.1, 11.3	17.9	61.0, 61.5	36.4	29.5	25.0	24.1				Z18865, L24101	58, 264
Complete sequence for selected genes[d]														
B. pasteurii	*ureABC*		11.1	13.9	61.4								X78411	210
E. coli (plasmid encoded)[e]	*ureRDAB....G*		11.1	12.1		31.5			22.4			34.1	L12007, L03307, L03308	68, 69
H. felis[c]	*ureAB*		(UreA) 26.1		(UreB) 61.7								X69080	82
H. heilmannii[c]	*ureAB*		(UreA) 25.7		(UreB) 61.8								L25079	267
K. pneumoniae	*ureDA*		11.1			30.0							L07039	46
L. fermentum	*ureABC*		11.2	14.1	61.8								D10605	275
P. vulgaris	*ureABC*		11.0	12.1	61.0								X51816	209
R. meliloti	*ureDABC*		11.1	10.8	60.7	24.2							S69145	193, 194
S. xylosus	*ureABC*		11.0	15.4	61.0								X74600	135
U. urealyticum	*ureABC*		11.2	13.6	66.6								X51315	23
C. ensiformis[f]				91.0									M65260	247

[a] Length of complete gene cluster in kilobases.

[b] ORFs designated *ureC* and *ureD* were previously thought to be associated with the *H. pylori* urease gene cluster; these genes are no longer thought to be urease genes and are therefore not listed (see the discussion on genetic organization in the section on genetics of urease gene clusters).

[c] The peptides designated UreA in the three *Helicobacter* species represent fusions of UreA and UreB peptides in other microorganisms, and the peptides designated UreB in this genus and UreH in *H. pylori* are homologous to UreC and UreD in other bacteria.

[d] Urease gene clusters that have been cloned but report no complete sequence for any gene include *Salmonella cubana* (68), two strains of *S. aureus* (134), *S. epidermidis* (103), and *S. saprophyticus* (95).

[e] Additional urease genes are present in *E. coli* between *ureB* and *ureG*, but incomplete sequence information is available.

[f] Sequence information for the jack bean plant urease is included for comparison with bacterial genes. The indicated peptide corresponds to a fusion of UreA, UreB, and UreC.

meliloti (193, 194), *Staphylococcus xylosus* (135), and *Ureaplasma urealyticum* (23). Partial nucleotide sequences of urease genes are available from a number of other bacterial species, including *Bordetella bronchiseptica* (205), *E. coli* (chromosomal locus) (43), and the soybean plant (150, 288). Furthermore, the gene clusters have been cloned from a number of other species including *Morganella morganii* (118), *Providencia stuartii* (68, 203), *Salmonella cubana* (68), two strains of *Staphylococcus aureus* (134), *Staphylococcus epidermidis* (103), and *Staphylococcus saprophyticus* (95). Although these gene clusters have been subjected to genetic analysis, no nucleotide sequence is available for these loci.

Regulatory Genes

For all the urease gene clusters, only one true regulatory gene has been identified and is present only in those gene clusters that are inducible by urea. *ureR* of *P. mirabilis* and its close homolog (also designated *ureR*) in the plasmid-encoded urease gene clusters of *Providencia stuartii* and *E. coli* act as an AraC-like positive activator of gene expression in the presence of urea. The function of these genes and other aspects of regulatory control are discussed in the section on regulation of urease synthesis, below. The *ureR* regulatory gene of *P. mirabilis* (224), *Providencia stuartii*, and *E. coli* (69) does not have a homolog in the urease gene clusters of *H. pylori*, *K. aerogenes*, *Bacillus* sp. strain TB-90, or other bacterial species that have been examined thus far. Two genes, denoted *ureC* and *ureD* but unrelated in sequence to other urease *ureC* and *ureD* genes, are located upstream of the urease structural genes in *H. pylori* (151). Despite their location, there is no evidence that these genes serve a regulatory role, and it is not clear that these genes are components of the urease gene cluster of this microorganism.

Structural Genes

The first published nucleotide sequence of any urease gene, which appeared in 1989, was that of *P. mirabilis* (132). The sequence predicts that the structural subunits of the enzyme are encoded by three contiguous genes, *ureA*, *ureB*, and *ureC* (Fig. 1). When the deduced amino acid sequences of the *P. mirabilis* urease subunits were compared with the amino acid sequence of the single jack bean urease subunit (178, 279), significant amino acid similarity was observed (58% exact matches; 73% exact plus conservative replacements). The 11.0-kDa polypeptide aligns with the N-terminal residues of the plant enzyme, the 12.2-kDa polypeptide lines up with internal residues, and the 61-kDa polypeptide matches with the C-terminal residues, suggesting an evolutionary relationship of the urease genes of jack bean and *P. mirabilis*. Some clues are available to possibly explain the relationship between synthesis of a single subunit for jack bean urease and three subunits for *P. mirabilis*. Sequences which are very similar to the intron splice acceptor consensus sequences were found in the DNA between *ureA* and *ureB* ORFs of *P. mirabilis*, suggesting that the origin of *ureA* and *ureB* may have resulted from an intron splice site that is not functional in procaryotes. Such a scenario is consistent with a eucaryote-to-procaryote gene exchange during the evolution of urease. In addition, examination of the junction between the *ureB* and *ureC* cistrons showed that the two ORFs overlap by a single nucleotide. This feature is compatible with either a eucaryote-to-procaryote or procaryote-to-eucaryote gene exchange and requires only a small modification in nucleotide sequence that results in disruption or fusion of *ureB* and *ureC*.

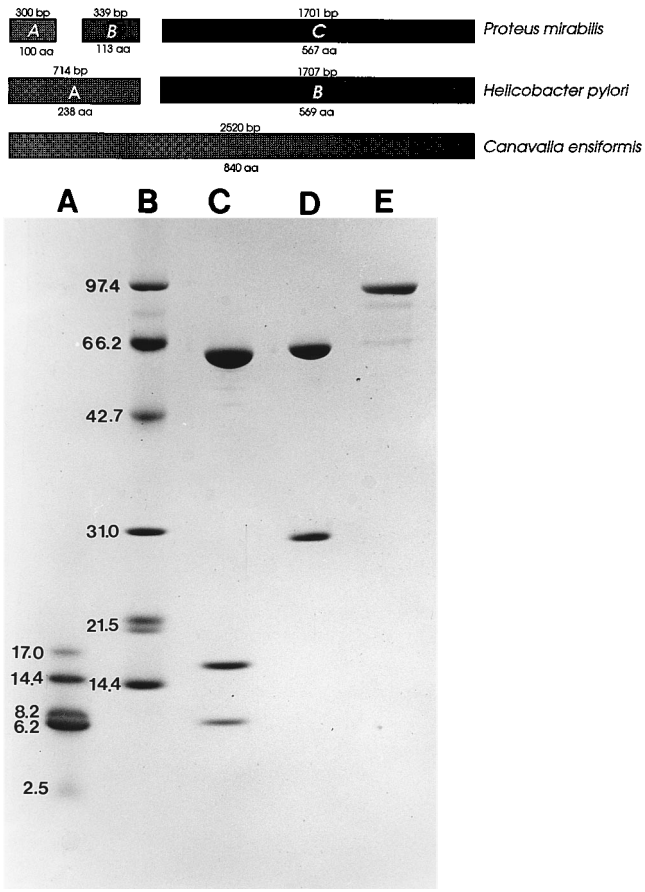


FIG. 1. Structural subunits of urease. Although ureases can have one, two, or three distinct subunits, these enzymes are nevertheless closely related. For example, in the diagram showing a representative three-subunit urease encoded by the *P. mirabilis ureA*, *ureB*, and *ureC* genes (top part of figure), the smallest subunit is homologous to the amino-terminal amino acids (aa) of jack bean (*C. ensiformis*) urease, the medium-size subunit is homologous to internal sequences, and the large subunit is homologous to the carboxy-terminal sequences of the plant enzyme. The same relationship holds true for the two-subunit enzymes of *H. pylori* and other members of this genus. Below the diagram is a polyacrylamide gel of purified ureases composed of one, two, and three distinct subunits. Purified ureases (10 μg of protein), electrophoresed on a 10 to 20% polyacrylamide gradient gel, were stained with Coomassie blue. Lanes: A and B, molecular mass markers in kilodaltons; C, purified *M. morganii* urease; D, purified *H. pylori* urease; E, jack bean urease purified from a partially purified commercial preparation. Reprinted from reference 202 with permission of the publisher.

When urease structural gene sequences appeared for *H. pylori* (39), *P. vulgaris* (209), *K. aerogenes* (211), *U. urealyticum* (23), and others that followed, it became clear that all ureases are related and share common ancestral genes. While the ureases of most bacterial species have been found to possess three distinct subunits, *Helicobacter* species produce ureases with only two distinct subunits. This observation is easily reconciled when it is noted that genetic fusion of the *ureA* and *ureB* genes of non-*Helicobacter* species could result in the appropriate-sized *Helicobacter* urease subunit gene (designated *ureA* in this species). Unfortunately, the present nomenclature is not uniform; e.g., *ureB* in the *Helicobacter* species is equivalent to *ureC* in the organisms possessing a three-subunit enzyme (Fig. 1).

Thus, urease from non-*Helicobacter* bacterial species are composed of three distinct subunits (Fig. 1) encoded by three contiguous genes; ureases from *Helicobacter* species are composed of two distinct subunits encoded by two adjacent genes;

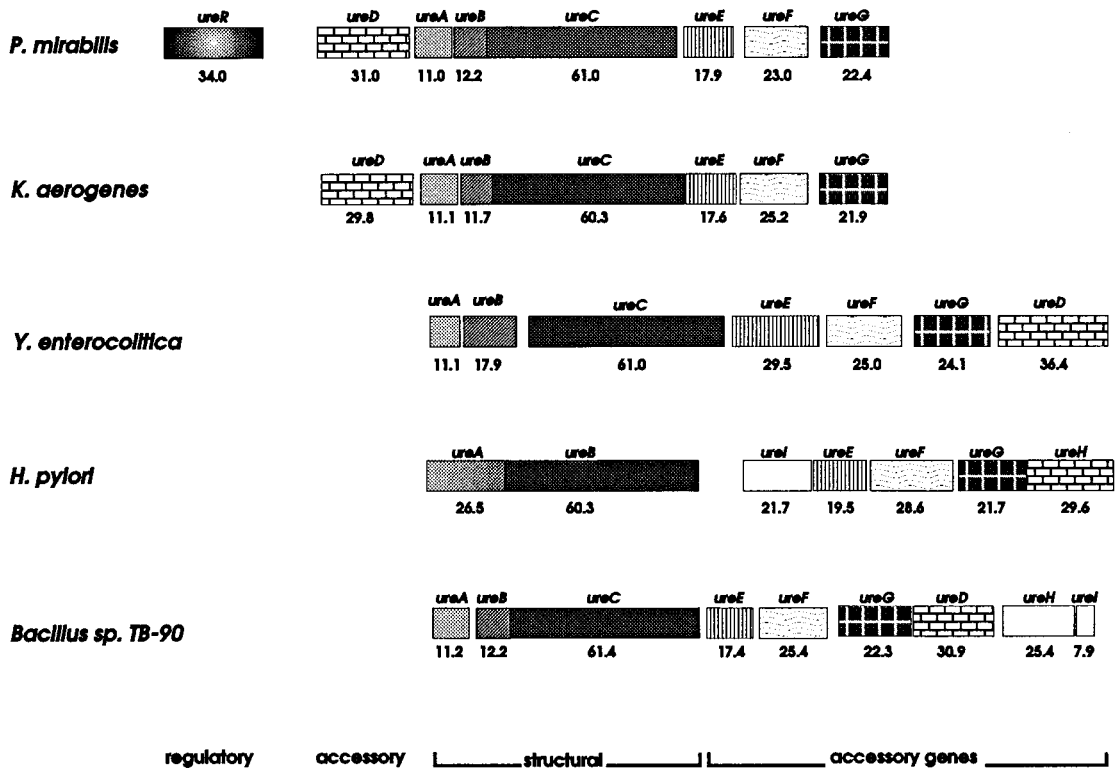


FIG. 2. Genetic organization of urease gene clusters. The genetic organization of urease gene clusters is depicted for the five bacterial species for which the entire nucleotide sequence is known. The relative positions of each gene are assigned as predicted by the nucleotide sequence and rely on the same scale. Gene assignments are shown above the rectangles; the predicted molecular size of the polypeptide encoded by each gene is shown below the rectangles. Patterns and shading within each rectangle are used to identify homologous polypeptides encoded by the urease genes of each of the five species. White rectangles indicate genes for which no homolog is found among other urease gene clusters. *ureR* is identified as a regulatory gene in the urea-inducible urease. Structural genes encode the subunit polypeptides that form the enzyme itself. Accessory genes encode polypeptides that play a role in the assembly of the nickel metallocenter. *ureI* genes of *H. pylori* and *Bacillus* sp. strain TB-90 have no known function. *ureH* of *Bacillus* sp. strain TB-90 appears to be involved with nickel transport.

and the jack bean enzyme is made up of only one distinct subunit encoded by one gene.

Accessory Genes

In addition to the structural genes, urease gene clusters always have accessory genes that are required for synthesis of catalytically active urease when the gene clusters are expressed in a recombinant bacterial host. From studies of *K. aerogenes* and *P. mirabilis* genes and gene products (121, 156, 157, 205a, 211, 234, 237, 269, 270), some of the accessory genes were shown to play a role in activation of the apoenzyme. These genes are now known to be required for assembly of the nickel metallocenter within the active site of the enzyme; they are discussed in some detail below. For most cases, the individual accessory genes are homologous between urease gene clusters from distinct species (Table 1; Fig. 2).

Genetic Organization

The organization of the urease genes of the five species for which the entire nucleotide sequence is known is shown in Fig. 2. At first glance, there appears to be significant diversity with respect to the organization of genes among different microorganisms. However, there are some general themes. First, urea-inducible gene clusters begin with the *ureR* regulatory gene, which is transcribed in the direction opposite to that of the rest of the gene cluster. Second, the structural subunit genes are always aligned the same way, from smallest subunit to largest subunit. Third, accessory genes *ureEFG* are always contiguous. Fourth, *ureD* and its *Helicobacter* homolog *ureH* can either precede structural gene *ureA* or follow *ureG*.

From the data available, the minimal requirements for synthesis of a catalytically active urease appear to include the structural subunit genes and four accessory genes (i.e., seven genes in total for the common three-subunit urease systems). Using *P. mirabilis* or *K. aerogenes* as examples, *ureABC* are required for assembly of the catalytically inactive apoenzyme and *ureDEFG* are required in vivo for assembly of the nickel metallocenter, the completion of which results in active enzyme.

Additional genes such as *ureI* of *H. pylori* or *ureH* of *Bacillus* sp. strain TB-90 play specialized functions that are not required for the expression of active ureases of all species. Two ORFs, designated *ureC* and *ureD*, were originally assigned to the *H. pylori* urease gene cluster (151). The lack of significant function and the lack of homologs in other species prompt us to drop these two putative genes from our current model of the *H. pylori* urease gene cluster.

Transcriptional Organization

Limited information is available on the transcriptional organization of urease gene clusters. For the most part, nucleotide sequence information has been used to identify putative promoter sequences based on homology to canonical RNA polymerase recognition sites. However, these analyses only provide a basis for experimental determinations. In a few cases, North-

ern (RNA) blots have been used to determine mRNA transcript length and *lacZ* fusions to various points within cloned genes have identified the location of regulated promoters (see the section on regulation of urease synthesis, below).

For *P. mirabilis*, a urea-regulated, *ureR*-dependent promoter was found immediately upstream of *ureD* (121). This same promoter apparently controls expression of *ureA*, since *ureA* is regulated in a similar manner but no independent urea-regulated promoter occurs immediately upstream of *ureA*. Similarly, the region immediately upstream of *ureF* has limited ability to drive transcription. Northern blot analysis revealed transcript lengths of 2.8 and 3.6 kb for the *P. mirabilis ure* region, the longer of which could represent *ureDABCDE* (226). Possible *rho*-independent transcriptional terminators have been identified following *ureD*, *ureC*, *ureF*, and *ureG. ureR* is transcribed in a direction opposite the remaining genes and is preceded by a consensus σ^{70} promoter sequence (224). D'Orazio and Collins (69) have provided evidence that *ureR* may be transcribed separately but also under the control of UreR. Furthermore, for the plasmid-encoded urease of *Providencia stuartii* (203), *Salmonella cubana*, and *E. coli* (68), the transcriptional organization appears to be similar to that of *P. mirabilis.*

In *Klebsiella* species, a nitrogen-regulated promoter has been identified upstream of *ureD* (46) and a *rho*-independent transcriptional terminator signal was noted after *ureG* (211). For *Y. enterocolitica*, a putative σ^{70} site was identified upstream of *ureA* and a *rho*-independent terminator sequence was noted immediately downstream of *ureD* (58). Similarly, for *Bacillus* sp. strain TB-90, putative σ^{70} promoter sequences were found upstream of *ureA* and *ureG* and two possible terminators were found at the end of the gene cluster, downstream of *ureI* (172). While no experimental determination of transcriptional organization has been published for *H. pylori*, it has been suggested that *ureAB* represents one mRNA transcript while *ureIEFGH* are encoded by a second transcript (53).

Because of the uncertainty about transcriptional organization of urease gene clusters, it is perhaps premature to confer the term "operon" upon any of the urease clusters described thus far. Clearly, additional studies are required to determine the transcriptional organization of these genes.

Expression of Recombinant Ureases

For the cloning of most urease genes, successful isolations of the relevant gene clusters were based on screening for urease-positive colonies on a urea test medium. The degree to which these heterologous genes were expressed in *E. coli* varies widely, from complete lack of expression of *U. urealyticum* genes, presumably because of the use of UGA as a codon specifying Trp (23), to very weak activity of *M. morganii* urease (118) to overexpression of *H. pylori* (117), *P. mirabilis* (271), and *K. aerogenes* genes (213).

With the exception of the plasmid-encoded ureases, each species appears to contain only one chromosomal copy of urease genes. In most instances, the urease activity produced by *E. coli* containing multicopy urease-encoding plasmids does not exceed the activity of the wild-type species.

A systematic study of urease expression in *E. coli* was undertaken for the *H. pylori* genes (39, 53, 117, 151) and revealed some of the factors that govern expression of active recombinant enzyme. Most of the findings would probably be applicable to the urease genes cloned from other species. The intact but inactive apoenzyme can be produced from clones containing only *ureA* and *ureB* (115). The apoenzyme produced from these clones is indistinguishable from native enzyme except that it lacks nickel and thus has no detectable activity (115). Larger clones containing additional genes are capable of synthesizing active urease. Enzymatic activity, however, was shown to remain very weak in these recombinants compared with that in clinical isolates of *H. pylori* (53, 115). Conditions under which near-wild-type urease activity is achieved were developed, as follows. *E. coli* SE5000 was cotransformed with plasmid pHP808 encoding the entire *H. pylori* urease gene cluster and plasmid pHP902 encoding only the structural genes. When *E. coli* SE5000(pHP808/pHP902) is grown in minimal medium containing no amino acids, with $NiCl_2$ added to 0.75 μM and with the *ureA* and *ureB* genes overexpressed in *trans* to the complete urease gene cluster, these cells express urease activity up to 87 μmol of urea per min per mg of protein (87 U/mg of protein). This level approaches that of the wild-type *H. pylori* strain (100 U/mg of protein), from which the genes were cloned. Poor catalytic activity of recombinant clones grown in Luria broth or M9 medium containing 0.5% Casamino Acids was shown to be due to chelation of nickel ions by medium components, particularly histidine and cysteine. In cultures containing these amino acids, $^{63}Ni^{2+}$ is prevented from being transported into the cell and is not incorporated into urease protein. As a consequence, M9 minimal medium cultures containing histidine or cysteine produce only 0.05 and 0.9%, respectively, of active urease produced by control cultures containing no added amino acids. Therefore, expression of active recombinant *H. pylori* urease is increased when nickel transport is not inhibited and when sufficient synthesis of urease subunits UreA and UreB is achieved.

Since *H. pylori* obviously does not live under the conditions described above but still has very high levels of urease expression, it was reasoned that additional genes were required to accumulate the metal ion under conditions where complex macromolecules and potential chelating agents are present. A gene encoding an apparent high-affinity nickel transport protein, *nixA*, was cloned (200). *E. coli* containing separate clones of the *H. pylori* urease gene cluster and *H. pylori nixA* is capable of producing urease with very high specific activity, independent of the growth conditions. NixA is a 32-kDa membrane protein that mediates the high-affinity transport of nickel ions into *E. coli* (K_T = 11 nM; V_{max} = 1,750 pmol of Ni^{2+} per min per 10^8 bacterial cells). While *nixA* is not associated with the urease gene cluster of *H. pylori*, a homolog, *ureH*, which encodes a protein that shares significant amino acid sequence identity with NixA is encoded as a part of a thermophilic *Bacillus* urease gene cluster (172). HupN of *Bradyrhizobium japonicum* (91) and HoxN of *Alcaligenes eutrophus* (76) are homologs required for activity of hydrogenases, another nickel-dependent metalloenzyme.

REGULATION OF UREASE SYNTHESIS

Whereas urease is synthesized constitutively in some organisms, notably *Bacillus pasteuri*, *Sporosarcina ureae*, *Morganella morganii*, and the chromosomally encoded ureases of some *E. coli* isolates, ureases are also synthesized in response to environmental conditions via positive control of transcription of the relevant genes. In one mode of regulation by environmental conditions, urease expression is regulated by the global nitrogen control system. The best-characterized organisms in this category belong to the genus *Klebsiella*. In the presence of poor nitrogen sources, synthesis of urease is activated. This control is dependent on the nitrogen regulatory system (NTR) and ultimately the action of the positive regulator NAC (nitrogen assimilation control) at the level of urease transcription. In a second mode, urease expression in organisms such as *P.*

mirabilis and those which bear plasmid-encoded ureases (*Providencia stuartii*, *Salmonella cubana*, and some *E. coli* strains) is induced by the presence of the substrate urea. In *P. mirabilis* and *E. coli*, induction requires a gene encoded by the *ure* locus (*ureR*) which is a member of the AraC family of positive activators. In addition, urease may be developmentally regulated in *Proteus* species in which swarm cells have higher levels of urease and of urease transcript. Finally, the urease of *Streptococcus salivarus* is reported to be regulated by pH. Each of these mechanisms is described in more detail below.

Constitutive Expression

Synthesis of *M. morganii* urease appears to be constitutive. In examining the effect of the urease inhibitor acetohydroxamic acid on induction of bacterial ureases, Rosenstein et al. (249) observed no induction by urea in four strains of *M. morganii*. Senior (260) examined an additional four strains and found those to be constitutive as well. The effects of other environmental conditions on urease expression, however, have not been rigorously tested.

A limited number of *E. coli* strains are urease positive, and the phenotype is unstable in a significant fraction of these isolates (294). Collins and Falkow (44) examined two urease-positive *E. coli* strains and found that urease was chromosomally encoded in one isolate. The constitutive locus was closer in homology to those of *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Serratia* species (44) than that of *Proteus* species. Limited sequence data (45) suggest that the gene organization of this locus is similar to that in *Klebsiella* species as well.

Finally, urease is produced constitutively in some soil bacteria (201), including *Bacillus pasteurii* (208) and *Sporosarcina ureae* (138), and in the cyanobacterium *Anabaena variabilis* (96).

Nitrogen Regulation

Nitrogen control of urease synthesis occurs in a number of bacterial species. In *K. aerogenes* (90), urease is not synthesized when the cells are grown in the presence of high-quality nitrogen sources such as ammonia. In contrast, urease synthesis is activated under conditions of nitrogen starvation, as when cultures are grown in minimal medium containing a poor nitrogen source such as proline, arginine, or histidine. Nitrogen regulation of the cloned *Klebsiella* urease genes occurs in a number of host backgrounds (e.g., *Escherichia*, *Salmonella*, and *Klebsiella* spp.) in which low enzyme levels are present during growth on nitrogen-rich medium such as Luria broth or defined medium containing ammonia and Casamino Acids, but nitrogen limitation results in derepression of urease activity (213).

A key element in control of nitrogen-regulated genes in *Klebsiella* species is the two-component regulatory system composed of the response regulator NR_I (NTRC) and the modulator (kinase) NR_{II} (NTRB) (173). Phosphorylation of NR_I by NR_{II} in response to nitrogen deprivation activates transcription of promoters dependent on an alternative sigma factor, σ^{54} (NTRA). Consistent with the control of urease by NTR, *K. pneumoniae* strains with mutations in *ntrA* or *ntrC* are defective in synthesis of urease (46). The nitrogen-regulated promoter of the urease locus was localized by these authors to a 312-bp fragment encompassing the start of the *ureD* ORF. Sequences upstream of the *ureD* translational start contained both possible σ^{54}- and σ^{70}-binding sites, but none were identified for NR_I interaction. Expression of *lacZ* fusions to this region was dependent on an additional component, NAC (46).

NAC is required for expression of several nitrogen-controlled genes, including urease and those required for histidine utilization (*hut*) (15, 164). Synthesis of NAC, a member of the LysR family of positive regulators, is controlled by the action of the NTR system. This serves the function of coupling the σ^{70} promoters of some nitrogen-controlled genes to the NTR system. NAC is itself expressed from a σ^{54} promoter which responds to NTRC (14). The LysR family includes a number of DNA-binding regulatory proteins. Members of this family have a predicted helix-turn-helix motif near the amino terminus and regions of similarity across the remaining residues, suggesting a common ancestry (114). Mutants defective in NAC vary in their expression of different nitrogen-regulated genes, and urease expression is activated at low levels of NAC relative to that required for induction of *hut* or repression of *gdh* (257, 258). NAC has been shown to bind closely upstream of *ureD* on the basis of its ability to shift the electrophoretic migration position of a DNA fragment containing this region and on the basis of DNase I protection experiments (98a).

Nitrogen regulation of urease gene expression has been reported or proposed to occur in a number of other ureolytic organisms. Several genes including urease have been shown to be regulated by nitrogen control in *Pseudomonas aeruginosa* (125), and mutants with mutations in *rpoN* (σ^{54}) are defective in synthesis of urease (289). In *B. subtilis*, levels of both asparaginase and urease are increased 20- to 25-fold in cells grown in medium containing poor nitrogen sources (5). Furthermore, ammonia repression of urease was evident in *R. melioti* (195).

The case for nitrogen regulation of *Helicobacter* urease is less clear. Cussac et al. (53) cloned and expressed the *H. pylori* urease genes in *E. coli* and found a higher relative activity in *E. coli* extracts under nitrogen-limiting conditions. In addition, sequences located 310 bp upstream of both *ureD* and *ureA* are similar to the σ^{54}-binding site (151). However, exogenous nickel is required for full expression of recombinant urease since amino acids, primarily histidine and cysteine, chelate available nickel in the medium (117). Thus, the role of nitrogen regulation in various media cannot be determined in the absence of nickel. In this context, Hu and Mobley (117) found no effect of nitrogen-limiting conditions on synthesis of urease subunits in maxicells containing cloned *H. pylori ure* genes.

Induction by Urea

In contrast to *Klebsiella* species and the other microorganisms discussed above, nitrogen control plays no role in regulation of the *P. mirabilis* urease gene cluster (224). Rather, urease of *Proteus* and *Providencia* species is induced by the substrate urea (249, 250) to levels 5- to 25-fold over those in uninduced cultures (130, 198). For the cloned genes in a heterologous *E. coli* host, proper regulation requires the presence of closely linked sequences since a number of *Bal* 31 deletion derivatives demonstrate altered regulation (131). Urea induction of the urease structural genes was confirmed by construction of a *ureA-lacZ* fusion, which also demonstrated that induction was specific for urea. No induction by closely related structural analogs was observed (225).

Induction of urease is mediated by the regulatory protein UreR, which is encoded by a gene about 400 bp upstream of *ureD* (224). A number of observations suggest that UreR is a positive regulator of urease expression. Although levels of expression vary with the replicon carrying the recombinant gene, deletions extending into the UreR ORF result in a loss of urease activity (224). In addition, the predicted sequence of UreR contains a helix-turn-helix motif and an associated AraC family signature and is similar to a number of DNA-binding regulatory proteins (224), including AppY (6), EnvY (162), and RhaR (283). The AraC family includes a number of viru-

lence factor regulators, and members are characterized by a carboxy-terminal DNA-binding domain and are predicted to have an effector-binding region in the amino terminus (93).

Careful genetic analysis of the *P. mirabilis ure* region in an *E. coli* host has extended these observations (120, 121). Precisely localized in-frame insertions constructed in the *ureR* gene in vitro result in a urease-negative phenotype. In addition, studies of a series of nested deletions of the *ureR* gene linked to *ureD-lacZ* fusions have demonstrated that UreR is a positive activator of expression from a putative promoter upstream of *ureD*. A plasmid containing the *ureR* gene will confer proper regulation in *trans* on a *ureD-lacZ* fusion as well, but similar fusions to small fragments containing 229 bp upstream of *ureA* or the region extending 216 bp upstream of *ureF* are not activated, suggesting that independent promoters do not occur in front of these ORFs.

Plasmid-encoded ureases occur in several members of the *Enterobacteriaceae*. The plasmid-encoded ureases of *Providencia stuartii* (68, 100, 197, 203, 212) and *Salmonella cubana* (68) are induced by urea. Also, Collins and Falkow (44) identified a strain of *E. coli* containing a plasmid which encoded an inducible urease. This was confirmed in a second report (69) and shown to possess a regulatory system similar to that in *P. mirabilis*. A homologous *ureR* was identified upstream of *ureD*, and a *ureD-lacZ* fusion was regulated by *ureR* and urea. *ureR* was also found to be involved in regulation of its own expression (69, 70). Urea-inducible promoters were also found to precede *ureR* itself as well as *ureG* (70).

Developmental Control

Some question surrounds the regulation of urease in *Proteus* cells during swarming. Falkinham and Hoffman (80) reported that urease was constitutive for swarming *Proteus* cells, with negligible activity in nonswarmer cells. However, Jin and Murray (126) found that urease activity is present in both cell types and can be induced to high levels by urea in both cases. Allison et al. (1) determined that differentiation into swarm cells results in substantial increases in urease activity. In addition, mutants defective in swarming have reduced urease levels in some cases, and the level of *ureC* mRNA is increased in swarm cells.

Regulation by pH

Urease plays a role in protection from acidic environment in some oral bacteria. *Streptococcus salivarius* urease levels are regulated by pH (262), for example, and the mechanism appears to occur through regulation of the rate of synthesis of urease (263).

BIOCHEMISTRY OF UREASE

The following sections describe several aspects of urease biochemistry, including critical summaries of urease enzymology, protein structural properties, active-site structure and catalytic mechanism, and properties and possible functions of urease-related accessory proteins that facilitate metallocenter assembly.

Urease Enzymology

In our earlier review of microbial ureases (201), we tabulated the reported kinetic parameters of the enzyme from numerous species, described the range of compounds that are known to inhibit the enzyme, and discussed several other aspects of urease enzymology. In the following sections, we summarize the multiple urease assays that are available, detail urease kinetic properties with a focus on recently described enzymes, provide an in-depth review of urease inhibition by three key classes of inhibitory compounds, and comment on the cellular location of these proteins.

Urease assays. A number of assays are available for quantitating urease activity and analyzing its kinetic behavior. The advantages and disadvantages of several methods to measure urease were described previously (201) and will not be repeated here, but it is important to emphasize that the different assays are distinct in their sensitivities, ease of use, and susceptibility to interference. Ammonia released during the reaction can be reacted with phenol-hypochlorite (300) or with Nessler's reagent (Sigma ammonia color reagent) to allow colorimetric determination of activity. Alternatively, ammonia released from urease action can be utilized by an NADH-dependent glutamate dehydrogenase so that the activity of the coupled system is easily monitored spectrophotometrically (139). In addition, ammonium ion-selective electrodes are available for monitoring ammonia release (104, 140, 206). By using [^{14}C]urea, bicarbonate released from the reaction can be trapped and monitored by scintillation counting (185). $^{14}CO_2$ released from this substrate and $^{13}CO_2$ (assayed by mass spectrometry) released from [^{13}C]urea have been used in breath tests to noninvasively detect the presence of *H. pylori* urease in the human gastric mucosa (13, 99) (see the section on molecular pathogenesis, below). Since the urease reaction results in an increase in pH, several pH-dependent methods to assay the enzyme have been developed. These include inclusion of pH-sensitive dyes for use in spectrophotometic methods (104, 253), utilization of a pH stat (21), and analysis of changes observed simply by using a pH electrode (18). In addition to the above assays, several methods have been adapted or devised to detect urease activity in native gels (25, 85, 182, 261).

Kinetic behavior of urease. Ureases exhibit simple Michaelis-Menten-type kinetic behavior. No evidence for substrate inhibition or allosteric behavior has been detected. Since most ureases are intracellular (see below), urease activities are generally measured by using lysed or permeabilized cells to reduce complications arising from urea or ammonia transport across the cytoplasmic membrane. Total urease activity is often found to increase during purification. This effect is probably due to inhibition of the enzyme in cruder preparations by unidentified cellular components. As an extreme example, some preparations of *Sporosarcina ureae* urease exhibit a dramatic increase in total activity after purification consistent with the presence of a tight-binding inhibitor interacting with the enzyme throughout the isolation procedure (184).

In general, the K_m values determined for urease in cell extracts closely match those measured for highly purified samples; thus, purified enzyme is not required for determination of this value. A limited correlation may exist between the value of this kinetic constant and the ecological niche of the host organism. For example, an enzyme with one of the lowest K_m values (0.17 mM) is that from *H. pylori* (116), which inhabits the gastric mucosal lining, where low concentrations of urea are supplied from the serum (1.7 to 3.4 mM in urea). Such a low kinetic constant allows this urease to function under close to saturation conditions despite the low substrate concentration. Similarly, enzyme isolated from the ruminal microorganism *Selenomonas ruminantium* requires a low K_m value to work efficiently in its environment and was found to have a K_m of 2.2 mM (106). In contrast, microorganisms that are exposed to large amounts of urea such as are found in the urinary tract or in the soil typically possess ureases with larger K_m values, as exemplified by the 13, 60, and 40 to 130 mM observed for the

enzymes from *P. mirabilis* (28), *Sporosarcina ureae* (184), and *B. pasteurii* (152), respectively.

The highest specific activity reported for urease is that isolated from *U. urealyticum*, with reported values ranging from 33,530 to 180,000 μmol of urea degraded per min (U) per mg (242, 254, 272, 281). In contrast, as previously pointed out (201), other purified bacterial ureases generally possess specific activities of 1,000 to 5,500 U/mg. Recently published examples of this trend include the isolated *H. pylori*, *M. morganii*, *Helicobacter mustelae*, *S. xylosus*, and *S. saprophyticus* ureases, which possess specific activities of 1,100 to 3,189 U/mg (72, 78, 116), 2,130 U/mg (118), 1,560 U/mg (73), 1,573 U/mg (33), and 1,979 U/mg (255), respectively, whereas a slightly higher value of 9,300 U/mg is reported for the *Sporosarcina ureae* enzyme (184). Ureases from two filamentous cyanobacteria have recently been purified and found to possess specific activities of 200 to 350 U/mg (124). In addition, a distinct class of acid ureases, with optimal activity at pH 2 to 4.5, appear to possess uniformly depressed specific activities. For example, the enzymes purified from selected strains of *Lactobacillus reuteri*, *Lactobacillus fermentum*, and *Streptococcus mitior* exhibit activities of 350 U/mg (136), 304 U/mg (137), and 458 U/mg (306), respectively. As described above, sequences of all urease gene clusters are highly related, including those for *U. urealyticum* and *L. fermentum*, and the factors governing the disparate activities in these different ureases remain elusive.

With the exception of a small group of acid ureases (136, 137, 306), microbial ureases possess an optimum pH of near neutrality and are often irreversibly denatured by exposure to pH values below 5. Detailed kinetic analysis of the neutral pH enzymes isolated from *K. aerogenes* and *P. mirabilis* reveal that the urea K_m values are nearly independent of pH whereas V_{max} values are strongly dependent on pH. The results are consistent with the presence of a general base ($pK_a \approx 6.5$) and a general acid ($pK_a \approx 9$) that function in catalysis by these enzymes (28, 284). The acid ureases also exhibit bell-shaped dependences on pH and are likely to possess a general base and a general acid with pK_a values that are shifted to lower pH values than found in the neutral-pH enzymes. An important point to be made with regard to examination of the pH optima for these enzymes is that urease is inhibited by several common buffers, including phosphate (285), Tris (16), and boric acid (28).

Urease inhibitors. A reasonable approach to the control of urease-related pathogenesis of bacterial infections or excessive rates of ureolysis in soil is to utilize potent and highly specific inhibitors of this enzyme. Several classes of urease inhibitors are known, and some have been examined for their pharmacological and agricultural value, as reviewed previously (201, 214, 251). In addition to their potential value in medicine and agriculture, the study of urease inhibitors can provide insight into selected aspects of the enzyme mechanism and active-site structure. This discussion of urease inhibitors will be limited to three topics: kinetic analysis of hydroxamic acid inhibition of urease, the kinetics and possible mechanism of inhibition by phosphoramide compounds, and the interaction of urease with thiols.

Hydroxamic acids are well-known inhibitors of urease, as first demonstrated for the jack bean enzyme in 1962 (146). Acetohydroxamic acid is the most widely exploited example of this inhibitor class; however, numerous other hydroxamic acid derivatives have been synthesized and shown to be very effective inhibitors of the plant and bacterial enzymes (66, 92, 105, 147, 215, 221, 229). These compounds include various *n*-aliphatic hydroxamic acids, *o*- or *p*-substituted benzohydroxamic acids, *trans*-cinnamoyl hydroxamic acid, and other derivatives.

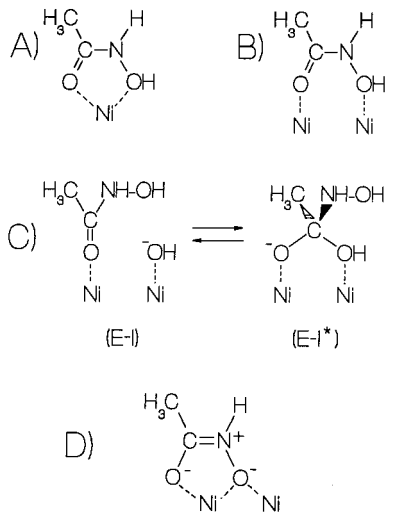


FIG. 3. Structural hypotheses for the interaction of the urease bi-nickel active site with the slowly binding inhibitor, acetohydroxamic acid. Four distinct structural models for the acetohydroxamic acid-bound form of urease include a bidentate complex in which the carbonyl and hydroxyl oxygen atoms of the inhibitor bind to same nickel ion (22) (A), a complex in which the carbonyl and hydroxyl oxygen atoms are bound separately to each nickel ion (B), an initial species in which the carbonyl oxygen is bound to one nickel ion and a hydroxyl group is coordinated to the second nickel ion, followed by the generation of a nickel-bridging species possessing a tetrahedral geometry at the inhibitor carbon atom (285) (C), and a bidentate complex in which the carbonyl oxygen binds to one nickel ion and the hydroxyl group bridges the two metal ions (273) (D). The two structural species in the third model have been suggested to be related to the kinetically distinguishable E-I and E-I* forms of the inhibited enzyme and may mimic structures present during urea ureolysis. It is also possible, however, that two-step binding may be associated with the other three models shown.

By using acetohydroxamic acid as the benchmark inhibitor and *K. aerogenes* urease as the canonical enzyme, the mechanism of inhibition was shown to be that of a slow, tight-binding competitive inhibitor (285). Thus, the inhibitor competes with substrate to bind to the enzyme as an E-I complex that is slowly transformed to an E-I* complex. The latter complex is very stable, with a dissociation constant of $\sim 9 \times 10^{-5}\ s^{-1}$, resulting in an overall inhibition constant of approximately 2.6 μM (285). It is important to note that non-steady-state kinetic measurements must be used for proper analysis of this inhibitor class. Reports of noncompetitive inhibition for these compounds (see, e.g., references 94, 250, and 293) are probably in error. Hydroxamic acids are known to be good metal chelators, and binding of acetohydroxamic acid to jack bean urease results in a UV-visible spectral perturbation (66) and alteration of the magnetic susceptibility of the metallocenter (35). From these results, at least four structural hypotheses can be put forward to describe the interaction of these compounds with the binuclear active site of urease. In one proposal (22), hydroxamic acids are suggested to form a bidentate complex with one of the nickel ions at the active site of the enzyme (Fig. 3A). Second, the bound species may be envisioned as bridging the two nickel ions (Fig. 3B). Third, the E-I and E-I* complexes for acetohydroxamic acid interaction with *K. aerogenes* enzyme have been suggested (285) to represent an initial monodentate complex and a species that bridges the two nickel ions at the active site, respectively, as illustrated in Fig. 3C. The latter species could represent a reasonable model for a transition state complex thought to be present during urea hydrolysis. A final possible structural hypothesis for the acetohydroxamate-inhibited enzyme involves a bidentate-bound species in which

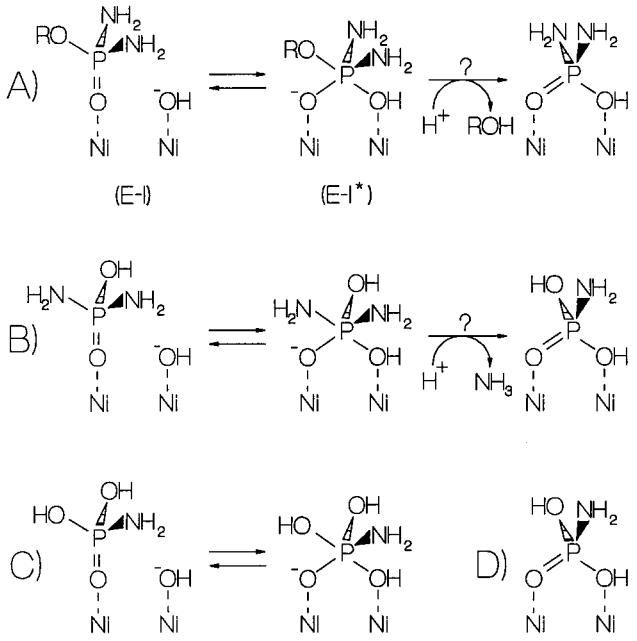


FIG. 4. Postulated interactions of phosphoroamide compounds with the urease bi-nickel active site. (A) Phenylphosphorodiamidate (where R is a benzene ring) and related compounds are kinetically characterized as slowly binding urease inhibitors that form distinct E-I and E-I* species (285). By analogy to the acetohydroxamic acid structures shown in Fig. 3C, the urease-arylphosphorodiamidate species could reasonably possess the phosphoryl oxygen-bound and bridging structures indicated. In addition to their action as inhibitors, these compounds have been proposed to be slow substrates of the enzyme (3, 80a, 183), and hydrolysis of the latter complex to form diamidophosphate is illustrated. (B) Diamidophosphate may interact with urease via similar structural species and, similarly, is proposed to be hydrolyzed to form phosphoroamide. (C) Phosphoroamide could reasonably bind in analogous monodentate and bridging modes. No evidence for hydrolysis of this compound has been reported, but the trigonal bipyramidyl intermediate shown in panel C could eliminate hydroxide to produce the bridging species shown in panel D.

the carbonyl oxygen binds to one nickel ion while the hydroxyl group bridges the two metal ions (Fig. 3D). A model complex with this type of coordination has recently been characterized (273). Further structural studies are required to distinguish between these proposals.

Many phosphoroamide compounds are even more potent inhibitors of urease than the hydroxamic acids. Effective inhibitors range from simple compounds such as phosphoramidate and diamidophosphate to substituted phenylphosphorodiamidates and a range of *N*-acyl phosphoric triamides (3, 65, 80a, 147, 148, 183, 196). Analogous to the acetohydroxamic acid inhibition mechanism, these compounds are kinetically characterized as slowly binding competitive inhibitors (3, 80a, 183, 285) and non-steady-state kinetic methods must be used for valid analysis. As a specific example, kinetic analysis of phenylphosphorodiamidate interaction with *K. aerogenes* urease is consistent with formation of E-I and E-I* complexes with a dissociation constant of 4.7×10^{-5} s^{-1} and an overall inhibition constant of 94 pM (285). These authors proposed that in parallel to the proposed mechanism for acetohydroxamic inhibition, the E-I state represents the inhibitor bound in a unidentate mode to one nickel ion and the E-I* species involves the inhibitor bridging the two nickel ions, as illustrated in Fig. 4A, where R is the aromatic ring. Again, the proposed E-I* structure is thought to represent a possible transition state model for urea hydrolysis in which the substrate would form a tetrahedral intermediate rather than the hypothetical trigonal bipyramidyl intermediate illustrated for the inhibitor. Unlike the case of hydroxamic acids, it would not be surprising if the bound phenylphosphorodiamidate were in fact hydrolyzed by the enzyme, and this possibility is included as the simple in-line displacement shown in Fig. 4A. Very slow substrates are known to yield identical kinetics to that observed. Although no evidence has been reported to directly demonstrate the presence of hydrolysis products from phosphoroamides, the results from three studies are consistent with such a mechanism. In one study (80a), the second-order rates of inhibition for a series of substituted phenyl phosphorodiamidates were found to correlate with the phenol pK_a values, consistent with a hydrolytic inhibition mechanism. In two additional studies (3, 183), a series of phosphoroamides all yielded the same apparent dissociation rate as if identical kinetic species were present. It was suggested that the larger compounds are hydrolyzed by urease to form diamidophosphate and that the identical dissociation rates for the different compounds actually represent dissociation of the same compound, i.e., diamidophosphate. Diamidophosphate is itself also thought to be a substrate as well as an inhibitor of urease (3), and hypothetical interactions of this compound with the urease active site are represented in Fig. 4B. Finally, speculative structures for the phosphoroamide-bound species are shown in Fig. 4C and D (22). Again, further studies are needed to better define the structure of the inhibited urease species.

Thiol compounds are not potent inhibitors of urease; however, they have been very useful for mechanistic studies and for better characterization of the enzyme metallocenter. With *K. aerogenes* urease, kinetic analyses for a range of thiol compounds reveal simple competitive inhibition in which the presence of other charged groups has a significant effect on the inhibition constant: cysteamine, containing a cationic β-amino group, exhibits the highest affinity for urease ($K_i \approx 5$ μM), whereas thiolates containing anionic carboxyl groups are uniformly poor inhibitors (285). Additionally, pH dependence studies demonstrate that the actual inhibitor is the thiolate anion. The presence of thiol leads to an alteration of the UV-visible spectrum for both jack bean urease (63) and *K. aerogenes* urease (285), in which the spectroscopic perturbation is consistent with the development of a thiolate→Ni(II) charge-transfer complex (20). In both cases, the spectrally observed K_D matches the K_i determined kinetically for this competitive inhibitor. Since thiols compete with urea for the active site and since thiol compounds appear to bind to nickel, these results strongly support the notion that urea binds to the enzyme metallocenter. Further discussion of the interaction of thiols with the enzyme metallocenter is found in the section on active-site studies of urease, below.

Enzyme location. With the possible exception of the enzyme from *H. pylori*, described separately below, microbial ureases appear to be cytoplasmic proteins. This statement contradicts a body of early work (see, e.g., reference 246), as well as some more recent electron-microscopic histochemical studies involving a ruminal *Staphylococcus* sp. and *P. mirabilis* (186, 187). The histochemical studies involved a technique in which bacteria are incubated with urea, urease-generated ammonia is reacted with tetraphenyl boron, the complexed ammonia is exchanged for silver ions, and the electron-dense material is visualized in thin sections. The apparent localization of urease to the membrane and periplasm in these species may reflect the inability of tetraphenyl boron to enter the microorganism. Ammonia that is generated intracellularly may simply exit the cell and react with the compound extracellularly. It is important to note that this method detects ammonia rather than urease itself. In contrast, antibodies developed against urease

have been used to localize the enzyme to the cytoplasm of *K. aerogenes* (213) and *U. urealyticum* (217). Furthermore, cell fractionation studies generally demonstrate that urease partitions with the cytoplasmic fraction (reviewed in reference 201).

The urease from *H. pylori* is an apparent exception to the general rule that these proteins are cytoplasmic enzymes. From 53 to 80% of the intact-cell urease activity is recovered by simply washing the cells with deionized, distilled water (72). Furthermore, approximately 90% of the whole-cell activity is recovered by extraction with the detergent *n*-octylglucoside at a concentration of 1% (78). Importantly, the water extraction process preserves the viability of up to 80% of the cells. Because of complications related to urea or ammonia transport across the cytoplasmic membrane, it is unlikely that the whole-cell urease assays detect all of the urease associated with the microorganism; nevertheless, it is clear that a significant amount of *H. pylori* urease is loosely associated with the bacterium. An extracellular localization of this enzyme is consistent with the strong immunological response mounted against urease in individuals infected with this microorganism. Results from immunogold localization studies further buttress this conclusion by indicating a cell surface location (110). Additional electron-microscopic localization studies found no urease activity in one strain of *H. pylori*, cell membrane-associated urease activity in seven strains, and cytoplasmic urease activity in eight strains (26); however, the results of these studies can be questioned because the investigators used the histochemical method, for which limitations have been described above. Because the protein sequences predicted from DNA sequencing of the *H. pylori* urease genes (39, 151) do not indicate the presence of a signal sequence, the mechanism of its export to the cell surface is of interest if the *H. pylori* urease is indeed an extracellular enzyme. Because an inordinate number of purified *H. pylori* proteins have been localized to the cell surface and because "cell surface" proteins have later been determined to reside intracellularly, it may still be premature to assign the natural location of *H. pylori* urease to the exterior of the cell.

General Protein Structure of Ureases

Tabulations of the apparent subunit sizes and stoichiometries for ureases representing a wide range of organisms are available (108, 201); however, much of this work is superseded by recent advances in the field. Here, we compare the known primary structures for numerous ureases and explore aspects of the quaternary structure of the enzyme, including very recent structural results from X-ray crystallographic analysis.

Primary structures. Although ureases may be composed of one, two, or three distinct types of subunits (Fig. 1), the proteins are all closely related, as detailed in Fig. 5. Figure 5 compares the sequences of the three subunits (UreA, UreB, and UreC) of *K. aerogenes* urease (211) with other three-subunit protein sequences derived from DNA sequence analysis (e.g., *P. mirabilis* [132], *P. vulgaris* [209], *U. urealyticum* [23], *L. fermentum* [275], two strains of *Y. enterocolitica* [58, 264], *S. xylosus* [135], *R. meliloti* [194], the thermophilic *Bacillus* sp. strain TB-90 [172], and *B. pasteurii* [210]), with the two-subunit ureases found in two strains of *H. pylori* (39, 151), *H. felis* (82), and *H. heilmannii* (267), and with the single-subunit enzyme from jack bean (247, 279). Of interest, the spacing between conserved regions encoding the subunits differs in the different gene clusters, and the *R. meliloti* urease genes are interrupted by other genes (194).

Comparison of these sequences by various computer algorithms has provided only limited phylogenetic information. Although not designed for determination of phylogeny, Bestfit alignments (61) provided some potentially interesting results. The UreC sequence from *K. aerogenes* is 72, 72, and 73% identical to the corresponding proteins from the enteric gram-negative organisms *P. mirabilis*, *P. vulgaris*, and *R. meliloti*, respectively; however, alignments with two strains of another gram-negative enteric organism, *Y. enterocolitica*, reveal identities of only 57 and 59%. The values for *Y. enterocolitica* are even lower than those observed when *K. aerogenes* UreC is compared with UreC of the gram-positive *Bacillus* sp. strain TB-90 (71%) or with the corresponding region of the plant urease (61%). A more appropriate phylogenetic analysis of the UreA + UreB + UreC sequences (including only the regions that are shared by all proteins) was carried out with the program PAUP (phylogenetic analysis using parsimony) (277). Three distinct unrooted trees of equal weight were observed, indicating the difficulty in defining the phylogeny of these protein sequences. Bootstrap analysis demonstrated that the urease sequences for the four *Helicobacter* species always group together, as expected from Fig. 5. Also as expected, the protein sequences from the two *Y. enterocolitica* ureases always group together. Furthermore, the protein sequences from the two *Proteus* species group together 100% of the time, these group with *K. aerogenes* protein sequence 91% of the time, and these three group with the *R. meliloti* protein sequence 92% of the time. Beyond these features, additional phylogenetic information was not justified in being assigned. Nevertheless, this conservative interpretation of the data reveals an important feature. There is no clear phylogenetic division in the sequences between the gram-negative and gram-positive bacteria or between the bacteria and the plant. These results raise questions concerning the origin of the genes encoding the urease subunits and the mechanisms of their dispersal.

The high sequence similarity observed among the proteins described above indicates that all ureases are variants of the same enzyme and are likely to possess the same general three-dimensional structure and catalytic residues at the active site. This assertion is strengthened by the finding that for many inhibitors, the inhibition kinetics are generally similar when the bacterial and plant enzymes are compared. Thus, further discussion of the quaternary structure of urease and studies involving the enzyme active site will, when appropriate, include results obtained with the jack bean enzyme.

Quaternary structures. Most bacterial ureases are heteropolymeric enzymes that possess a native M_r of 200,000 to 250,000 (e.g., the proteins recently isolated from *L. reuteri* [136], *Streptococcus mitior* [137], *L. fermentum* [306], two filamentous cyanobacteria [124], and other microorganisms [reviewed in reference 201]); however, exceptions to this general rule are known (e.g., the heteropolymeric proteins from *S. xylosus* [33], *S. saprophyticus* [255], *M. morganii* [118], and several other species [201] are larger). In particular, the *H. pylori* enzyme consistently has been reported to be significantly larger, with an M_r of 380,000 to 600,000 (72, 78, 116, 291). For comparison, jack bean urease is known by equilibrium ultracentrifugation studies to have an M_r of ~590,000 and to be composed of a homohexamer of its single subunit (66). In the presence of glycol, glycerol, and 1,2-propanediol, the native plant enzyme readily dissociates into half-units that retain activity (25, 47).

The stoichiometry of the subunits in bacterial ureases has been the subject of considerable investigation. Separation of individual subunits by denaturing polyacrylamide gel electrophoresis and integration of the staining intensities for the multiple bands reveal near-integer values for the subunit stoichi-

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UreA   1                                                  48
K.a.   MELTPREKDK LLLFTAALVA ERRL..ARGL KLNYPESVAL ISAFIMEGAR
Y.e.-2 MQLTPREVEK LMIYTLSDVA FKRK..ARGL KLNYPEAVSI ITETAMEGAR
Y.e.-1 MQLTPREVEK LMIYTLSDVA FKRKPKARGL KLNYPEAVSI ITVTAMEGAR
H.p.-2 MKLTPKELDK LMLHYAGELA KKRK..EKGI KLNYVEAVAL ISAHIMEEAR
H.p.-1 MKLTPKELDK LMLHYAGELA KKRK..EKGI KLNYVEAVRL ISAHIMEEAR
H.f.   MKLTPKELDK LMLHYAGRLA EEAL..ARGV KLNYTEAVAL ISGRVMEKAR
H.h.   MKLTPKELDK LMLHYAGELA KQRK..AKGI KLNYTEAVAL ISAHVMEEAR
P.m.   MELTPREKDK LLLFTAGLVA ERRL..AKGL KLNYPERVAL ISCAIMEGAR
P.v.   MELTPREKDK LLLFTAGLVA ERRL..AKGL KLNYPESVAL ISCAIMEGAR
R.m.   MNLTPREKDK LLISMAAMVA RRRL..ERGV KLNHPEAIAL ITDFVVEGAR
Bac.   MKLTSREMEK LMIVVAADLA RRRK..ERGL KLNYPEAVAM ITYEVLEGAR
B.p.   MHLNPAEKEK LQIFLASELL LRRK..ARGL KLNYPEAVAI ITSFIMEGAR
S.x.   MHFTQREQDK LMLVIAADLA RRRQ..QRGL KLNYPEAVAI ISFELLEGAR
C.e.   MKLSPREVEK LGLHNAGYLA QKRL..ARGV RLNYTEAVAL IASQIMEYAR
L.f.   MRLTKREQEK MMISLAGMIA EKRK..DRGL KLNQPEAVAL ITSRLLEGAR
U.u.   MNLSLREIQK LLVTVAADVA RRRL..ARGL KLNYSERVAL ITDHVVEGAR

UreA   49                                                 97
K.a.   DG.KSVASLM EEGRHVLTRE QVMEGVPEMI PDIQVEATFP DGSKLVTVHN
Y.e.-2 DG.KSVEDVM KEASKVLTKD DVMDGVADLI PNVQVEAIFT DGSRLVTVHD
Y.e.-1 DG.KSVEDVM KEASKVLTKD DVMDGVADLI PNVQVEAIFT DGSRLVTVHD
H.p.-2 AGKKTAAELM QEGRTLLKPD DVMDGVASMI HEVGIEAMFP DGTKLVTVHT
H.p.-1 RGKKTAAELM QEGRTLLKPD DVMDGVASMI HEVGIEAMFP DGTKLVTVHT
H.f.   DGNKSVADLM QEGRTWLKKE NVMDGVASMI HEVGIEANFP DGTKLVTIHT
H.h.   AGKKSVADLM QDGRTLLKAD DVMPGVAHMI HEVGIEAGFP DGTKLVTIHT
P.m.   EG.KTVAQLM SEGRTVLTAE QVMEGVPEMI KDVQVECTFP DGTKLVSIHS
P.v.   EG.KTVAQLM SEGRAVLTAE QVMEGIPEMI KDIQVECTFP DGTKLVSIHD
R.m.   DG.RSVAELM EAGAHVLTRD QVMEGIAEMI HDIQVEATFP DGTKLVTVHE
Bac.   DG.KTVAQLM QYGATILTKE DVMEGVAEMI PDIQIEATFP DGTKLVTVHD
B.p.   DG.KTVAMLM EEGKHVLTRD DVMEGVPEMI DDIQAEATFP DGTKLVTVHN
S.x.   DG.KTVAELM SYGKQILGED DVMEGVADML TEMEIEATFP DGTKLITVHH
C.e.   DGEKTVAQLM CLGQHLLGRR QVLPAVPHLL NAVQVEATFP DGTKLVTVHD
L.f.   DG.KTVGELM NEGATWLTKD DVMEGIPEMI PMIQVEATFP DGTKLITVTD
U.u.   DG.KLVADLM QSAREVLRVD QVMEGVDTMV GIIQVEVTFP DGTKLVSVHD

UreA/B 98                                  1          12
K.a.   PII*                                MIPGEYH VKP.....GQ
Y.e.-2 PIK*MSTKTN STKATSEKTD SLKTNRGTKS SAGYSDQNIP LGGCILADTP
Y.e.-1 PIK*MSTKTN STKATSEKTD SLKTNRGTKS SAGYSEQNTP LGGCILADTP
H.p.-2 PIEA..... .......... .......... NGKLVPGELF LKN.....ED
H.p.-1 PIEA..... .......... .......... NGKLVPGELF LKN.....ED
H.f.   PVED..... .......... .......... NGKLAPGEVF LKN.....ED
H.h.   PVEAG.... .......... .......... SDKLAPGEVI LKN.....ED
P.m.   PIV*                              MIPGEIR VNA...ALGD
P.v.   PIV*                              MIPGEIR VNQ...ALGD
R.m.   PIR*                              MIPGE.. IIA...AAGE
Bac.   PIR*                              MIPGEYV LKK.....EP
B.p.   PIS*                        VSN NNYIVPGEYR V.....AEGE
S.x.   PIV*                              MKPGEII VKR.....TE
C.e.   PISRENGELQ EALFGSLLPV PSLDKFAETK EDNRIPGEIL CED.....EC
L.f.   PIR*                              MVPGEYK LQP.....DK
U.u.   PIYK*               MI ICQDQSNQFT PGKLVPGAIN FAE.....GE

UreB   13                                                 53
K.a.   IALNTGRATC RVVVENHGDR PIQVGSHYHF AEVN.PALKF D........R
Y.e.-2 ITFNENKPVT KVKVRNTGDR PIQVGSHFHF FEAN.RALEF D........R
Y.e.-1 ITFNENKPVT KVKVRNTGDR PIQVGSHFHF FEAN.RALEF D........R
H.p.-2 ITINEGKKAV SVKVKNVGDR PVQIGSHFHF FEVN.RCLDF D........R
H.p.-1 ITINEGKKAV SVKVPPVGDR PVQIGSHFHF FEVN.RCLDF D........R
H.f.   ITINAGKEAI SLKVKNKGDR PVQVGSHFHF FEVN.KLLDF D........R
H.h.   ITLNAGKHAV QLKVKNKGDR PVQVGSHFHF FEVD.KLLDF D........R
P.m.   IELNAGRETK TIQVANHGDR PVQVGSHYHF YEVN.EALRF A........R
P.v.   IELNAGRETK IIQVANHGDR PVQVGSHYHV YEVN.EALKF E........R
R.m.   IELNAGLETV SIEVANSGDR PVQVGSHYHF AETN.PGLIF D........R
Bac.   ILCNQNKQTI KIRVLNRGDR PVQVGSHFHF FEVN.QSLQF H........R
B.p.   IEINAGREKT TIRVSNTGDR PIQVGSHIHF VEVN.KELLF D........R
S.x.   IEVNRGHNAT ILDVKNTGDR PIQVGSHYHF FEAN.PALQF E........R
C.e.   LTLNIGRKAV ILKVTSKGDR PIQVGSHYHF IEVN.PYLTF D........R
L.f.   VPYNVGYDDI SLKVKNVGDR PVQVGSEYHF YEANEQGLQF D........R
U.u.   IVMNEGREAK VISIKNTGDR PIQVGSHFHL FETNSALVFF DEKGNEDKER

UreB   54                                                 102
K.a.   QQAAGYRLNI PAGTAVRFEP GQKREV.ELV AFAGHRAVFG FRGEVMGPLE
Y.e.-2 AAAYGKRLNI SSTTAIRFEP GDETEV.PLI PFGGKQTLYG FNNLVDGWTG
Y.e.-1 AAAYGKRLNI SSTTAIRFEP GDETEV.PLI PFGGKQTLYG FNNLVDGWTG
H.p.-2 EKTFGKRLDI ASGTAVRFEP GEEKSV.ELI DIGGNRRIFG FNALVDRQAD
H.p.-1 EKTFGKRLDI ASGTAVRFEP GEEKSV.ELI DIGGNRRIFG FNALVDRQAD
H.f.   AKSPCKRLDI ASGTAVRFEP GEEKSV.ELI DIGGNKRIYG FNSLVDRQAD
H.h.   EKAYGKRLDI ASGTAVRFEP GEEKTV.ELI DIGGNKRIYG FNALVDRQAD
P.m.   KETLGFRLNI PAGMAVRFEP GQSRTVDELV AFAGKREIYG FHGKVMGKLE
P.v.   EETLGFRLNI PAGMAVRFEP GQCRTV.ELV AFDGKREIYG FHGKVMGKLE
R.m.   DAARGKRLDI PAGTAVRFEP GQTRQV.TLI PLSGKREVFG FRQQVMGKL*
Bac.   EKAFGMRLNI PAGTAVRFEP GDAKEV.EII PFSGERKVYG LNNVTNGSVE
B.p.   AEGIGRRLNI PSGTAARFEP GEEMEV.ELT ELGGNREVFG ISDLTNGSVD
S.x.   EKAYGKRLDI PAGAAVRFEP GDEKEV.QLV EYSGKRRIFG FHGEVNGPID
C.e.   RKAYGMRLNI AAGTAVRFEP GDCKSV.TLV SIEGNKVIRG GNAIADGPVN
L.f.   SQAWGKHLDI PAGTAIRLEA GEERTV.KLI DFGGKRRVFG FNNKVNGWLD
U.u.   KVAYGRRFDI PSGTAIRFEP GDKKEV.SVI DLVGTRWSLR CKRLS*

UreB/C 103                                      1          13
K.a.   VNDE*                                   MSN ISRQAYADMF
Y.e.-2 EGVVPNSERP DKLEAIRRAA ERGFKSSK*         MPQ ISRQEYAGLF
Y.e.-1 EGVVPNSERP DKLEAIRRAA ERGFKSSK*         MPQ ISRQEYAGLF
H.p.-2 NESKKIALHR AKERGFHGAK SDDNYVKTIK E*     MKK ISRKEYVSMY
H.p.-1 NESKKIALHR AKERGFHGAK SDDNYVKTIK E*     MKK ISRKEYASMY
H.f.   ADGKKLGLKR AKEKGFGSVN CGCE.ATKDK Q*     MKK ISRKEYVSMY
H.h.   HDGKKLALKR AKEKHFGTIN CGCD.NK*          MKK ISRKEYVSMY
P.m.   SEKK*                                   MKT ISRQAYADMF
P.v.   SENK*                                   MKT ISRQAYADMF
R.m.                                           MSYR MSRAAYANMF
Bac.   MGKRK*                                  MSFS MSRKQYADMF
B.p.   NKELILQRAK ELGYKGVE*                      VK INRQQYAESY
S.x.   EARVYKAEDD DSATEIIAEE NKVSENANKE SGYNR*MSFK MTQSQYTSLY
C.e.   ETNLEAAMHA VRSKGFGHEE EKDASEGFTK EDPNCPFNTF IHRKEYANKY
L.f.   VDKKADTYNG YPLEKSYPEE K*                 MSFD MDHEQFASFY
U.u.                                           MFK ISRKNYSDLY

UreC   14                                                 62
K.a.   GPTVGDKVRL ADTELWIEVE DDLTTYGEEV KFGGGKVIRD GMGQGQML.A
Y.e.-2 GPTTGDKIRL GDTNLFIEIE KDLRGYGEES VYGGGKSLRD GMGANNHLTR
Y.e.-1 GPTTGDKIRL GDTNLFMQIE KDLRGYGEES VYGGGKSLRD GMGANNHLTR
H.p.-2 GPTTGDKVRL GDTDLIAEVE HDYTIYGEEL KFGGGKTLRE GMSQSNNPSK
H.p.-1 GPTTGDKVRL GDTDLIAEVE HDYTIYGEEL KFGGGKTLRE GMSQSNNPSK
H.f.   GPTTGDRVRL GDTDLILEVE HDCTTYGEEI KFGGGKTIRD GMSQTNSPSS
H.h.   GPTTGDKVRL GDTDLILEVE HDCTTYGEEI KFGGGKTIRD GMGQTNSPSS
P.m.   GPTTGDRLRL ADTELFLEIE KDFTTYGEEV KFGGGKVIRD GMGQSQVV.S
P.v.   GPTTGDRLRL ADTELFLEIE QDFTTYGEEV KFGGGKVIRD GMGQSQVV.S
R.m.   GPTVGDKVRL ADTELFIEVE KDFTTHGEEV KFGGGKVIRD GMGQSQVTRE
Bac.   GPTVGDAIRL ADSELFIEIE KDYTTYGDEV KFGGGKVIRD GMGQHPLATS
B.p.   GPTVGDRVRL ADTDL.GEVE KDYYYLGDEV NFGGGKVLRE GMGENGTYTR
S.x.   GPTVGDSVRL GDTNLFARVE KDYATYGDEA AFGGGKSIRD GMAQNPNVTR
C.e.   GPTTGDKIRL GDTNLLAEIE KDYALYGDEC VFGGGKVIRD GMGQSCGHPP
L.f.   GPTTGDSVRL GDTDLFAKIE KDLTVHGQES LFGGGKVLRD GMGVSATETR
U.u.   GITTGDSVRL GDTNLWVKVE KDLTTYGEES VFGGGKTLRE GMGMNSTMKL

UreC   63                                                105
K.a.   AD....C.V. DLVLTNALIV DHWGIVKAD. IGVKDGRIFA IGKAGNPDIQ
Y.e.-2 DN...GV.LD LVITNVTIVD ARLGVIKAD. VGIRDGKIAG IGKSGNPGVM
Y.e.-1 DN...GGYLE FSHNQRHYCC SPFRVIKAD. VGIRDGKIVG IGKSGNPGVM
H.p.-2 EE.......L DLIITNALIV DYTGIYKAD. IGIKDGKIAG IGKGGNKDMQ
H.p.-1 EE.......L DLIITNALIV DYTGIYKAD. IGIKDGKIAG IGKGGNKDTQ
H.f.   YE.......L DLVLTNALIV DYTGIYKAD. IGIKDGKIAG IGKAGNKDMQ
H.h.   HE.......L DLVITNALIV DYTGIYKAD. IGIKNGKIHG IGKAGNKDLQ
P.m.   AE....C.V. DVLITNAIIL DYWGIVKAD. IGIKDGRIVG IGKAGNPDVQ
P.v.   AE....C.V. DVLITNAIII DHWGIVKAD. IGIKDGRITG IGKAGNPDVQ
R.m.   GG....A.V. DTVITNALIL DHWGIVKAD. IGLKDGRIAA IGKAGNPDMQ
Bac.   DE....C.V. DLVLTNAIIV DYTGIYKAD. IGIKDGMIAS IGKAGNPLLM
B.p.   TE....N.VL DLLLTNALIL DYTGIYKAD. IGVKDGYIVG IGKGGNPDIM
S.x.   DD...KQ.VA DLVITNALIL DYDKIVKAD. IGVKNGYIMK IGKAGNPDIM
C.e.   A.....I.SL DTVITNAVII DYTGIIKAD. IGIKDGLIAS IGKAGNPDIM
L.f.   AE...NPMVA DLIISDAIII DWTGIYKAD. IGIRDGKILA IGKGGNPDVM
U.u.   DDKLGNAEVM DLVITNALIL DYTGIYKADP IGIKNGKIAS IGKSGNPHLT

UreC   106                                   * *         153
K.a.   PNVT..IPIG AATEVIAAEG KIVTAGGIDT HIHWICPQQA EEALVSGVTT
Y.e.-2 DGVTPGMVVG VSTDAISGEH LILTAAGIDS HIHLISPQQA YHALSNGVAT
Y.e.-1 DGVTPGLVVG VSTDAISGEH LILTAAGIDT HIHLISPQQA YHALSNGVAT
H.p.-2 DGVKNNLSVG PATEALAGEG LIVTAGGIDT HIHFISPQQI PTAFASGVTT
H.p.-1 DGVKNNLSVG PATEALAGEG LIVTAGGIDT HIHFISPQQI PTAFASGVTT
H.f.   DGVDNNLCVG PATEALAAEG LIVTAGGIDT HIHFISPQQI PTAFASGVTT
H.h.   DGVCNRLCVG PATEALAAEG LIVTAGGIDT HIHFISPQQI PTAFASGITT
P.m.   PNVD..IVIG PGTEVVAGEG KIVTAGGIDT HIHFICPQQA QEGLVSGVTT
P.v.   PNVD..IVIG PGTEVVAGEG KIITAGGVDT HIHFICPQQA EEGLISGVTT
R.m.   PGVT..IIVG PGTEVIAGEG KIVTAGGMDS HIHFICPQQI EEALMSGLTC
Bac.   DGVD..MVIG AATEVIAAEG MIVTAGGIDA HIHFICPQQI ETALASGVTT
B.p.   DGVTPNMIVG TATEVIAAEG KIVTAGGIDT HVHFINPDQV DVALANGITT
S.x.   DNVD..IIIG ATTDIISAEG KIVTAGGIDT HVHFVNPEQS QVALESGITT
C.e.   NGVFSNMIIG ANTEVIAGEG LIVTAGAIDC HVHYICPQLV YEAISSGITT
L.f.   DNVD..FIVG ASTEAISGEG MIVTAGGIDL HVHYITPSIA QAALDNGITT
U.u.   DGVD..MVVG ISTEVSAGEG KIYTAGGLDT HVHRLEPEIV PVALDGGITT

UreC   154                                                203
K.a.   MVGGGTGPAA GTHATTCTPG PWYISRMLQA ADSLPVNIGL LGKGNVSQPD
Y.e.-2 FPGGGIGPTD GTNGTTVTPG PWNIRQMLRS VEGLPVNVGI LGKGNSYGRG
Y.e.-1 FPGGGIGPTD GTNGTTVTPG PWNIRQMLRS VEGLPVNVGI LGKGNSYGRG
H.p.-2 MIGGGTGPAD GTNATTITPG RRNLKWMLRA AEEYSMNLGF LAKGNASNDA
H.p.-1 MIGGGTGPAD GTNATTITPG RRNLKFMLRA AEEYSMNFGF LAKGNASNDA
H.f.   MIGGGTGPAD GTNATTITPG RANLKSMLRA AEEYAMNLGF LAKGNVSYEP
H.h.   MIGGGTGPAD GTDATTITPG RWNLKEMLRA SEEYAMNLGY LGKGNVSFEP
P.m.   FIGGGTGPVA GTNATTVTPG IWNMYRMLEA VDELPINVGL FGKGCVSQPE
P.v.   FIGGGTGPVA GTNATTVTPG IWNMHRMLEA VDELPINVGL FGKGCVSQPE
R.m.   MLGGGTGPAH GTLATTCTPG PWHIARMIEA ADAFPMNLAF AGKGNASLPG
Bac.   MIGGGTGPAT GTNATTCTPG PWNIHRMLQA AEEFPINLGF LGKGNCSDEA
B.p.   LFGGGTGPAE GSKATTVTPG PWNIEKMLKS TEGLPINVGI LGKGHGSSIA
S.x.   HIGGGTGASE GAKATTVTPG PWHLHRMLLA AESLPLNIGF TGKGQAVNHT
C.e.   LVGGGTGPAA GTRATTCTPS PTQMRLMLQS TDYLPLNFGF TGKGSSSKPD
L.f.   LFGGGTGPAN GTRSATCNPG AWYTHRMLQA VDNAPINYGL MAKGSGSRPE
U.u.   VITGGTGMND GTKATTVSPG NSELIC.LQA ADGLPINAGF LAKGQG.MED

UreC   204          * S                          *     253
K.a.   ALREQVAAGV IGLKIHEDWG ATPAAIDCAL TVADEMDIQV ALHSDTLNES
Y.e.-2 PLLEQAIAGV VGYKVHEDWG ATANALRHSL RMADEMDIQV SVHTDSLNEC
Y.e.-1 PLLEQAIAGV VGYKVHEDWG ATANALRHSL RMADEMDIQV SVHTDSLNEC
H.p.-2 SLADQIEAGA IGFKIHEDWG TTPSAINHAL DVADKYDVQV AIHTDTLNEA
H.p.-1 SLADQIEAGA IGLKIHEDWG TTPSAINHAL DVADKYDVQV AIHTDTLNEA
H.f.   SLRDQIEAGA IGFKIHEDWG STPAAIHHCL NVADEYDVQV AIHTDTLNEA
H.h.   ALIDQLEAGA IGFKIHEDWG STPSAINHAL NIADKYDVQV AIHTDTLNEA
P.m.   AIREQITAGA IGLKIHEDWG ATPMAIHNCL NVADEMDVQV AIHSDTLNEG
P.v.   AIREQIEAGA IGLKIHEDWG ATPMAIHNCL NVADEMDVQV AIHSDTLNEG
R.m.   ALVEMVLGGA TSLKLHEDWG TTPAAIDCCL SVADEYDVQV MIHTDTLNES
Bac.   PLKEQIEAGA VGLKLHEDWG STAAAIDTCL KVADRYDVQV AIHTDTLNEG
B.p.   PIMEQIDAGA AGLKIHEDWG ATPASIDRSL TVADEADVQV AIHSDTLNEA
S.x.   ALVEQIHAGA IGLKVHEDWG ATPSALDHAL QVADDYDVQI ALHADTLNEA
C.e.   ELHEIIKAGA MGLKLHEDWG STPAAIDNCL TIAEHHDIQI NIHTDTLNEA
L.f.   LIGEQIEAGC AAIKTHEDWG ATAAGIENSI AAANKYDVQV AVHTDSLNEG
U.u.   PIFEQIVAGA CGLKIHEDWG ANRNAIDLAL TVAEKTDVAV AIHTDTLNEA

UreC   254          *                                303
K.a.   GFVEDTLAAI GGRTIHTFHT EGAGGGHAPD IITACAHPNI LPSSTNPTLP
Y.e.-2 GYVEDTIDAF EGRTIHTFHT EGAGGGHAPD IIRVASQPNV LPSSTNPTLP
Y.e.-1 GYVEDTIDAF EGRTIHTFHT EGAGGGHAPD IIRVASQPNV LPSSTNPTLP
H.p.-2 GCVEDTMAAI AGRTMHTFHT EGAGGGHAPD IIKVAGEHNI LPASTNPTIP
H.p.-1 GCVEDTMAAI AGRTMHTYHT EGAGGGHAPD IIKVAGEHNI LPASTNPTIP
H.f.   GCVEDTLEAI AGRTIHTFHT EGAGGGHAPD VIKMAGEFNI LPASTNPTIP
H.h.   GCVEDTLEAI AGRTIHTFHT EGAGGGHAPD VIKMAGEFNI LPASTNPTIP
P.m.   GFYEETVKAI AGRVIHVFHT EGAGGGHAPD VIKSVGEPNI LPASTNPTMP
P.v.   GFYEETVKAI AGRVIHVFHT EGAGGGHAPD VIKSVGEPNI LPASTNPTMP
R.m.   GFVEDTIAAI KGRTIHAYHT EGAGGGHAPD IIRICGQPNV IPSSTNPTRP
Bac.   GFVEDTLKAI DGRVIHTYHT EGAGGGHAPD IIKAAGFPNI LPSSTNPTRP
B.p.   GFLEDTVRAI NGRVIHSFHV EGAGGGHAPD IMAMAGHPNV LPSSTNPTRP
S.x.   GFMEETMAAV KDRVLHMYHT EGAGGGHAPD LIKSAAYSNI LPSSTNPTLP
C.e.   GFVEHSIAAF KGRTIHTYHS EGAGGGHAPD IIKVCGIKNV LPSSTNPTRP
L.f.   GFVENTINAF NGNTVHTFHT EGAGGGHAPD IMVVAGQDNV LPSSTTPTNP
U.u.   GFVEHTIAAM KGRTIQLTIQ KVLVVDMLQI FLESVKYAHI LPASTNPTIP
                          (HAYHT EGAGGGHAPD I)
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UreC   304              B                                    353
K.a.   YTLNTIDEHL DMLMVCHHLD PDIAEDVAFA ESRIRRETIA AEDVLHDLGA
Y.e.-2 YGVNSQAELF DMIMVCHNLN PNVPADVSFA ESRVRPETIA AENVLHDMGV
Y.e.-1 YGVNSQAELF DMIMVCHNLN PNVPADVSFA ESRVRPETIA AENVLHDMGV
H.p.-2 FTVNTEAEHM DMLMVCHHLD KSIKEDVQFA DSRIRPQTIA AEDTLHDMGI
H.p.-1 FTVNTEAEHM DMLMVCHHLD KSIKEDVQFA DSRIRPQTIA AEDTLHDMGI
H.f.   FTKNTEAEHM DMLMVCHHLD KSIKEDVQFA DSRIRPQTIA AEDQLHDMGI
H.h.   FTKNTEAEHM DMLM.CHHLD KNIKEDVEFA DSRIRPQTIA AEDKLHDMGI
P.m.   YTINTVDEHL DMLMVCHHLD PSIPEDVAFA ESRIRRETIA AEDILHDMGA
P.v.   YTINTVDEHL DMLMVCHHLD PSIPEDVAFA ESRIRRETIA AEDILHDMGA
R.m.   YTVNTLAEHL DMLMVCHHLS PTIPEDIAFA ESRIRKETIA AEDILHDIGA
Bac.   YTINTLEEHL DMLMVCHHLD ANIPEDIAFA DSRIRKETIA AEDVLHDLGV
B.p.   FTVNTIDEHL DMLMVCHHLK QNIPEDVAFA DSRIRPETIA AEDILHDLGI
S.x.   YTVNTIDEHL DMVMITHHLN ASIPEDIAFA DSRIRKETIA AEDVLQDIGV
C.e.   LTSNTIDEHL DMLMVCHHLD REIPEDLAFA HSRIRKKTIA AEDVLNDIGA
L.f.   YCKNTLDELF YMTMVCHNLN PKIPDDVAFA ESRIRKQTEA AEDVLQDMGA
U.u.   YTVNTIAEHL DMLMVCHHLN PKVPEDVAFA DSRIRSQTIA AEDLLHDMGA

UreC   354   *                                             403
K.a.   FSLTSSDSQA MGRVGEVILR TWQVAHRMKV QRGALAEETG DNDNFRVKRY
Y.e.-2 ISMFSSDSQA MGRVGENWLR VMQTANAMKA SRGKLPEDAP GNDNFRVLRY
Y.e.-1 ISMFSSDSQA MGRVGENWLR VMQTANAMKA SRGKLPEDAP GNDNFRVVRY
H.p.-2 FSITSSDSQA MGRVGEVITR TWQTADKNKK EFGRLKEEKG DNDNFRIKRY
H.p.-1 FSITSSDSQA MGRVGEVITR TWQTADKNKK EFGRLKEEKG DNDNFRIKRY
H.f.   FSITSSDSQA MGRVGEVITR TWQTADKNKK EFGRLKEEKG DNDNFRIKRY
H.h.   FSITSSDSQA MGRVGEVITR TWQTADKNKK EFGRLPEEKG DNDNFRIKRY
P.m.   ISVMSSDSQA MGRVGEVILR TWQCAHKMKL QRGTLAGDSA DNDNNRIKRY
P.v.   ISVMSSDSQA MGRVGEVVMR TWQCAHKMKL QRGSLAGDTA ENDNNRIKRY
R.m.   FSIISSDSQA MGRVGEVAIR TWQTADKMKR QRGRLKEETG DNDNFRVKRY
Bac.   FSMISSDSQA MGRVGEVIIR TWQTADKMKK QRGKLQEDNG VGDNFRVKRY
B.p.   ISMMSTDALA MGRAGEMVLR TWQTADKMKK QRGPLAEEKN GSDNFRLKRY
S.x.   FSMVSSDSQA MGRVGEVITR TWQVAHRMKE QRGSLDGDSE YNDNNRIKRY
C.e.   ISIISSDSQA MGRVGEVISR TWQTADPMKA QTGPLKCDSS DNDNFRIRRY
L.f.   LSMMTSDAQA MGRVGEVAMR CWQLASKMKK VRGPLDGDSK YDDNNRIKRY
U.u.   ISIMSSDTLA MERIGEVVTR SWQMAHKMKA QFGALKGDSE FNDKQPCKRY

UreC   404                                                 453
K.a.   IAKYTINPAL THGIAHEVGS IEVGKLADLV VWSPAFFGVK PATVIKGGMI
Y.e.-2 VAKITINPAI AQGVSHVIGS VEVGKMADLV LWDPRFFGAK PKMVIKGGMI
Y.e.-1 VAKITINPAI AQGVSHVIGS VEVGKMADLV LWVPRFFGAK PKMVIKGGMI
H.p.-2 LSKYTINPAI AHGISEYVGS VEVGKVADLV LWSPAFFGVK PNMIIKGGFI
H.p.-1 LSKYTINPAI AHGISEYVGS VEVGKVADLV LWSPAFFGVK PNMIIKGGFI
H.f.   ISKYTINPGI AHGISDYVGS VEVGKYADLV LWSPAFFGIK PNMIIKGGFI
H.h.   ISKYTINPAI THGISEYVGS VEVGKYADLV LWSPAFFGIK PNMIIKGGFI
P.m.   IAKYTINPAL AHGIAHTVGS IEKGKLADIV LWDPAFFGVK PALIIKGGMV
P.v.   IAKYTINPAL AHGIAHEVGS IEKGKLADIV LWDPAFFGVK PALIMKGGMV
R.m.   IAKYTINPAI AHGLSHEIGS LEVGKRADLV LWNPAFFGVK PDMVLLGGTI
Bac.   IAKYTINPAI AHGIADYVGS VEVGKLADLV VWNPAFFGVK PELVLKGGMI
B.p.   VSKYTINPAI AQGMAHEVGS IEEGKFADLV LWEPKFFGVK ADRVIKGGII
S.x.   IAKYTINPAI THGISDYVGS IDEGKLADII MWEPAFFAVK PDVIVKGGLI
C.e.   IAKYTINPAI ANGFSQYVGS VEVGKLADLV MWKPSFFGTK PEMVIKGGMV
L.f.   VAKYTINPAI CNGISDYVGS IEVGKYADLN IWDPKYFGTK PDMVIKNGMI
U.u.   VAKYTINPAI AHGIDSYVGS IEVGKLADIV AWEPKFFGAK PYYVVKMGVI

UreC   454                                                 503
K.a.   AIAPMGDINA SIPTPQPVHY RPMFGALGSA RHHCRLTFLS QAAAANGVAE
Y.e.-2 NWAAMGDPNA SLPTPQPVFY RPMFGAMGKT MQDTCVTFVS QAALDDGVKE
Y.e.-1 NWAAMGDPNA SLPTPQPVFY RPMFGAMGKT MQDTCVTFVS QAALDDGVKE
H.p.-2 ALSQMGDANA SIPTPQPVYY REMFAHHGKA KYDANITFVS QAAYDKGIKE
H.p.-1 ALSQMGDANA SIPTPQPVYY REMFAHHGKA KYDANITFVS QAAYDKGIKE
H.f.   ALSQMGDANA SIPTPQPVYY REMFGHHGKN KFDTNITFVS QAAYKAGIKE
H.h.   ALSQMGDANA SIPTPQPVYY REMFGHHGKA KFDTNITFVS QVAYENGIKH
P.m.   RYAPMGDINA AIPTPQPVHY RPMYACLGKA KYQTSMIFMS KAGIEAGVPE
P.v.   AYAPMGDINA AIPTPQPVHY RPMYACLGKA KYQTSMIFMS KAGIDAGVPE
R.m.   AAAPMGDPNA SIPTPQPVHY RPMFGAYGRS RTNSSVTFVS PASLDAGLAG
Bac.   AYSTMGDPNA SIPTPQPVLY RPMFAAKGDA KYQTSITFVS KAAYEKGIHE
B.p.   AYAQIGDPSA SIPTPQPVMG RRMYGTVGDL IHDTNITFMS KSSIQQGVPA
S.x.   NPAINGDANG SIPTSEPLKY RKMYGQLGGN MQGTSMTFVS TTAYENDIGK
C.e.   AWADIGDPNA SIPTPEPVKM RPMYGTLGKA GGALSIAFVS KAALDQRVNV
L.f.   TYGIAGDPSS SLPTPEPVLE RFLYGAEGRA VNHTCVTYVS QYAYDHGIKE
U.u.   ARCVAGDPNA SIPTCEPVIM RDQFGTYGRS LTSTSVSFVS KIGLENGIKE

UreC   504                                                 551
K.a.   RLNLRSAIAV VKGCR.TVQK ADMVHNSLQP N.ITVDAQTY EVRVDGELIT
Y.e.-2 KAGLDRQVIA VKNCR.TISK HDLVRNDQTP N.IEVDPETF AVKVDGVHAT
Y.e.-1 KAGLDRQVIA VKNCR.TISK HDLVRNDQTP N.IEVDPETF AVKVDGVHAT
H.p.-2 ELGLERQVLP VKNCR.NITK KDMQFNDTTA H.IEVNPETY HVFVDGKEVT
H.p.-1 ELGLERQVLP VKNCR.NITK KDMQFNDTTA H.IEVNSETY HVFVDGKEVT
H.f.   ELGLDRAAPP VKNCR.NITK KDLKFNDVTA H.IDVNPETY KVKVDGKEVT
H.h.   ELGLQRVVLP VKNCR.NITK KDLKFNDVTA H.IEVNPETY KVKVDGNEVT
P.m.   KLGLKSLIGR VEGCR.HITK ASMIHNNYVP H.IELDPQTY IVKADGVPLV
P.v.   KLGLQSLIGR VEGCR.KVTK ASMIHNSYVP H.IELEPQTY IVKADGVPLV
R.m.   RLGVAKELVA VQNTRGGIGK ASMIHNSLTP H.IEVDPETY EVRADGELLT
Bac.   QLGLKKKVKP VHGIR.KLTK KDLILNDKTP K.IDVDPQTY EVKVDGQLVT
B.p.   KLGLKRRIGT VKNCR.NIGK KDMKWNDVTT D.IDINPETY EVKVDGEVLT
S.x.   LLGLKRKLRP VHNIR.KLTK ADMKNNSATP K.IDVDPQTY EVFVDGEKIT
C.e.   LYGLNKRVEA VSNVR.KLTK LDMKLNDALP E.ITVDPESY TVKADGKLLC
L.f.   QLGLNKTILP VHNTR.SLTK ANMKLNNYTP KTIEIDPQTY DVKIDGKLIT
U.u.   EYKLEKELLP VKNCR.SINK KSMKWNSATP N.LEVDPQTF DAAVDYNDLE

UreC   552          567
K.a.   SEPADVLPMA QRYFLF*
Y.e.-2 CEPIDTAAMN QRYFFG*
Y.e.-1 CEPIDTAAMN QRYFFG*
H.p.-2 SKPANKVSLA QLFSIF*
H.p.-1 LNQPIK*
H.f.   SKAADELSLA QLYNLF*
H.h.   SHAADKLSLA QLYNLF*
P.m.   CEPATELPMA QRYFLF*
P.v.   CEPATELPMA QRYFLF*
R.m.   CEPATVLPMA QRYFLF*
Bac.   CEPAEIVPMA QRYFLF*
B.p.   CEPVKELPMA QRYFLF*
S.x.   SEPATELPLT QRYFLF*
C.e.   VSEATTVPLS RNYFLF*
L.f.   CDAAPTLPLT QRYYLY*
U.u.   NWLEQPAAEL AKKLKKTANG KYVLACRTSN RSSISTKILL ILILELFWFSNSISNYI*
       (AEPLTEAPLA QRYFLF*)
```

ometries, but these ratios vary for the enzymes from different sources. For example, the α/β/γ (UreC/UreB/UreA) ratio for three-subunit enzymes from *Providencia stuartii* and *K. aerogenes* is nearly 1:2:2 (212, 284), that from *L. reuteri*, *L. fermentum*, and *Streptococcus mitior* is 1:2:1 (136, 137, 306), that from *Sporosarcina ureae* is 1:1:2 (184), and that from *S. saprophyticus* and *U. urealyticum* is 1:1:1 (255, 281). Integration of the staining intensity for the bands arising from the two-subunit enzyme of *H. pylori* and other *Helicobacter* species is consistent with a 1:1 stoichiometry (72, 73, 116, 291). Despite the calculations showing differences in subunit stoichiometries among the three-subunit enzymes, the nonequivalent ratios are probably artifactual. As described below, the crystallographically determined structure of *K. aerogenes* urease clearly demonstrates the presence of a 1:1:1 subunit ratio (122). On the basis of the extensive sequence similarities among these proteins (Fig. 5), it is likely that all ureases possess equal numbers of each of their distinct subunit polypeptides.

Electron-microscopic images of negatively stained jack bean urease were interpreted to suggest that the enzyme is composed of two cyclic trimers that are paired in an eclipsed position (86). Analogous electron-microscopic images of the *H. pylori* enzyme (8, 9, 110) similarly suggest a threefold rotational symmetry, whereas analysis of ureases from *S. saprophyticus* and *S. xylosus* show symmetric particles divided in two by a dark-staining line (255). Overall, the contrast between subunits in all of these samples is too low to clearly establish the packing arrangement. A reasonable interpretation of these data is that most bacterial ureases possess a structure similar to the jack bean homotrimer [i.e., the bacterial enzymes possess an $(\alpha\beta\gamma)_3$ structure] whereas *H. pylori* and perhaps a few other prokaryotic ureases are related in three-dimensional structure [i.e., an $(\alpha\beta)_6$ or $(\alpha\beta\gamma)_6$ structure] to the jack bean homohexamer.

Crystal structures. Crystallographic studies of urease have a long history with exciting recent developments. Although jack bean urease was the first enzyme ever crystallized (274), the crystal structure of this protein has never been solved. Recently, crystals of the plant enzyme were reproduced, found to be in space group $F4_132$, and shown to diffract to 3.5 Å (1 Å = 0.1 nm) when a synchrotron source was used (123). At the same time, crystals of *K. aerogenes* urease were described, shown to belong to space group $I2_13$, and found to diffract to better than 2-Å resolution when a laboratory X-ray source was used (123). As illustrated by the space-filling model in Fig. 6, the crystal structure has now been solved to 2-Å resolution

FIG. 5. Comparison of urease sequences. Ureases for which the complete primary structures are known from DNA or protein sequence analysis were compared by using the PILEUP program (61) with additional minor adjustments. The subunit designation and the numbering are according to the three-subunit *K. aerogenes* (*K.a.*) urease sequence (211). Other sequences shown include the protein subunits from two strains of *Y. enterocolitica* (*Y.e.*-1 and *Y.e.*-2 [58, 264]), two strains of *H. pylori* (*H.p.*-1 and *H.p.*-2 [39, 151]), *H. felis* (*H.f.* [82]), *H. heilmannii* (*H.h.* [267]), *P. mirabilis* (*P.m.* [132]), *P. vulgaris* (*P.v.* [209]), *R. meliloti* (*R.m.* [194]), the thermophilic *Bacillus* sp. strain TB-90 (*Bac.* [172]), *B. pasteurii* (*B.p.* [210]), *S. xylosus* (*S.x.* [135]), jack bean (*C. ensiformis*, *C.e.* [247, 279]), *L. fermentum* (*L.f.* [275]), and *U. urealyticum* (*U.u.* [23]). The jack bean enzyme possesses a single subunit, the *Helicobacter* species all possess two-subunit ureases, and the other proteins contain three subunits. The *U. urealyticum* sequence possesses two italicized regions with alternative sequence from different reading frames indicated in parentheses on the subsequent lines. The first region is known to include a sequencing error (see the footnote in reference 122), and the second region (at the carboxyl terminus) is also suspicious, because the alternative sequence provides a much better match. Ligands to the *K. aerogenes* nickel metallocenter are shown above the sequence (*). Also indicated is the likely general base (B) required in catalysis and a residue that appears to be involved in substrate binding (S).

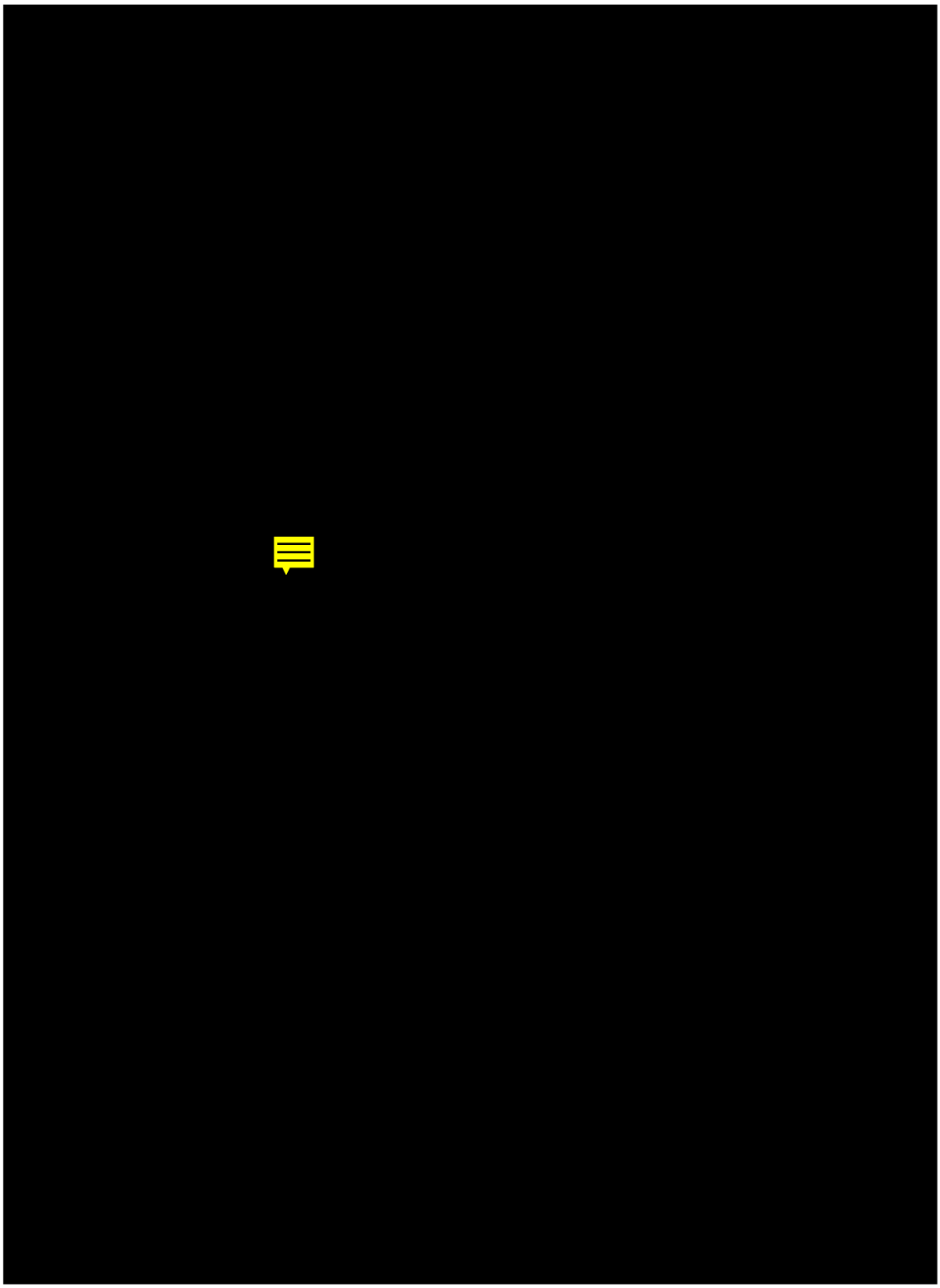

FIG. 6. Space-filling model of the urease crystal structure. The structure of the *K. aerogenes* urease $(\alpha\beta\gamma)_3$ trimer is shown as viewed down the threefold axis (top) and perpendicular to the threefold axis (bottom). One $\alpha\beta\gamma$ unit is shown in white, another is shown in blue, and the third is colored according to the individual subunits: α (UreC, red), β (UreB, orange), and γ (UreA, yellow). Figure courtesy of Evelyn Jabri and P. Andrew Karplus at Cornell University.

(122). The protein is a trimer of trimers [i.e., $(\alpha\beta\gamma)_3$] possessing three active sites per enzyme. The amino and carboxy termini for each of the *K. aerogenes* urease subunits are accessible; thus, additional residues, as are found in urease sequences from other microorganisms, can be accommodated without disturbing the structure. In particular, there is adequate room for inclusion of a connecting linker between the UreA and UreB subunits, as is present in the *H. pylori* and jack

bean enzymes, as well as between the UreB and UreC subunits, as exists in the jack bean enzyme. Further details of the active-site structure and catalytic mechanism, as revealed by crystallographic analysis, are summarized below.

Active-Site Studies of Urease

In 1989, our review was able to provide only the barest hints of information regarding the urease active site (201). More recently, the results from a variety of biophysical, spectroscopic, chemical modification, and site-directed modification studies were summarized to provide a more detailed view of the urease catalytic center (108). Below, we briefly reiterate the results of these studies and extend this discussion to include more-current work, including that from X-ray crystallographic analysis. In light of these results, we describe a potential mechanism of urease.

Presence of nickel in urease. All purified ureases that have been analyzed for metal content have been shown to possess nickel, and the presence of urease activity in ureolytic organisms uniformly exhibits a dependence on nickel in the growth medium (reviewed in references 107 and 108). The two-nickel-ions-per-subunit content of jack bean urease (62, 64) forms the benchmark with which all other ureases are compared. Atomic absorption spectroscopic measurement of nickel coupled with the use of a slow, tight-binding inhibitor to quantitate the number of active sites was used to demonstrate the presence of two nickel ions per active site in *K. aerogenes* urease (285). Recent X-ray crystallographic structure analysis confirms the presence of a binuclear active site in this protein (122). As detailed above, urease sequences make up a highly related family of proteins; hence, it is likely that all ureases contain a metallocenter containing two nickel ions. It must be pointed out, however, that selected microorganisms (e.g., *Arthrobacter oxydans* [256], *B. pasteurii* [34], *Brevibacterium ammoniagenes* [219], *H. pylori* [109, 291], *S. xylosus* [33], and *S. saprophyticus* [255]) are reported to have lower nickel contents that, in some cases, are near those expected for proteins possessing a mononickel center. Although it is possible that ureases fall into two classes with distinct nickel-containing active sites, it seems more likely that the anomalous low levels of nickel in these isolated examples arise from incomplete loading of the protein with the metal ion.

Magnetic and spectroscopic properties of the urease metallocenter. Essentially all biophysical and spectroscopic characterization studies of the bacterial urease metallocenter have been done with protein isolated from *K. aerogenes*. UV-visible spectroscopy indicates that thiols (competitive inhibitors of the enzyme) bind to the nickel metallocenter (285). Whereas the native enzyme has only a very weak UV-visible spectrum, the addition of thiol compounds leads to spectroscopically detectable thiolate anion $\rightarrow$ Ni(II) charge transfer transitions (322, 374, and 432 nm for β-mercaptoethanol). Saturation magnetization measurements of the native protein can be fitted by assuming that all nickel ions are magnetically isolated and composed of a population of high-spin ($S = 1$) and low-spin ($S = 2$) species, with the diamagnetic contribution increasing with increasing pH (57). These results differ from the conclusions derived from magnetic susceptibility measurements of jack bean urease that suggest the presence of a ferromagnetically coupled bi-nickel center (35). The differences were shown not to arise from inherent differences in the bacterial and plant enzymes; rather, they are likely to be due to inappropriate assumptions and invalid fitting methods used in the plant enzyme study as detailed in reference 57. These magnetic techniques show that both the bacterial and plant enzymes become diamagnetic in the presence of thiols. X-ray absorption spectroscopic analysis of the bacterial enzyme reveals an edge spectrum that is most consistent with five-coordinate geometry, while extended X-ray absorption fine-structure analysis suggests the presence of two or three histidyl imidazole groups per nickel ion (297). Whereas the spectra indicate that metal ions in native enzyme possess all N or O ligands, the spectrum of the β-mercaptoethanol-bound form exhibits clear Ni-S as well as Ni-Ni scattering interactions. The Ni-Ni distance is estimated to be 3.26 Å by this technique. Variable-temperature magnetic circular dichroism spectroscopic results clearly demonstrate the presence of antiferromagnetic coupling between the nickel ions in the β-mercaptoethanol-bound form of the enzyme (128). Thiolate-induced antiferromagnetic coupling of the two nickel ions in the plant enzyme has also been described previously (84). These data are consistent with the model shown in Fig. 7, where a thiol inhibitor bridges the two nickel ions at the active site.

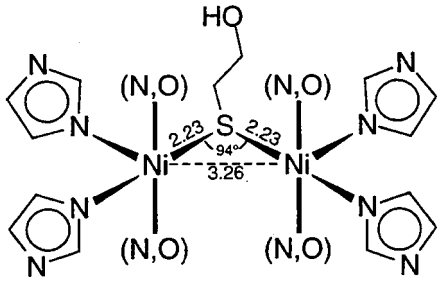

FIG. 7. Model of the urease bi-nickel center based on biophysical and spectroscopic analyses. The combination of X-ray absorption (297) and variable-temperature magnetic circular dichroism (84) spectroscopic data, along with results from saturation magnetization measurements (57), were used to generate a structural model of the metallocenter. In this model, the nickel ions are separated by about 3.5 Å, each metal ion is coordinated by two histidyl residues and approximately three additional N or O donors to provide a net coordination number of about five. Externally added thiols (indicated as β-mercaptoethanol) can displace a ligand to result in bridging of the two nickels. Reproduced from reference 297 with permission of the publisher.

Ligands to the urease metallocenter. In addition to the spectroscopic analyses mentioned above, three further studies relate to identification of the nickel ligands in urease: a site-directed mutagenesis experiment (236), an investigation into apoprotein activation conditions (238), and structural determination of the protein by X-ray crystallographic analysis (122).

Comparison of six urease sequences available in early 1993 revealed conservation of 10 histidine residues (His-96 in UreA, His-39 and His-41 in UreB, and His-134, His-136, His-219, His-246, His-312, His-320, and His-321 in UreC [numbering according to the *K. aerogenes* enzyme]). Each of these residues was substituted with alanine by site-directed mutagenesis, and the mutant proteins were purified and characterized (236). The UreC-H134A, UreC-H136A, and UreC-H246A mutant proteins exhibit no detectable activity and possess 53, 6, and 21%, respectively, of the nickel content of wild-type protein. The authors speculated that these three residues function in metallocenter ligation. Histidine residues comparable to His-134, His-136, and His-246 are conserved in all sequences that have become available since this study, whereas His-96 in UreA, His-39 in UreB, and His-312 and His-321 in UreC are not conserved in more recently derived sequences (Fig. 5). Three additional residues, His-269, His-272, and His-280, were not examined by site-directed mutagenesis approaches because they were not conserved in the published *U. urealyticum* sequence (23). It now appears that a sequencing error led to an

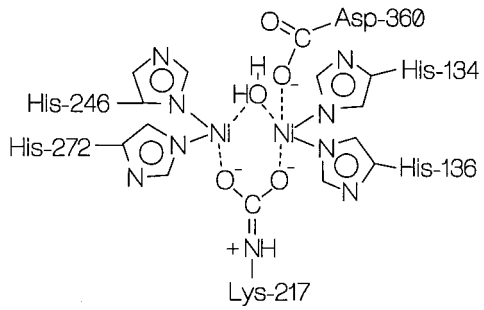

FIG. 8. Structure of the urease bi-nickel center determined from crystallographic analysis. The ligands to the urease metallocenter include the indicated UreC residues (122). Lys-217 is carbamylated, allowing it to bridge the two metal ions. A water molecule is associated mainly with the nickel ion that is coordinated by Asp-360, His-134, and His-136 but exhibits partial occupancy, perhaps as a bridge, in the coordination sphere of the other metal ion. One metal ion is five-coordinate and the other is four-coordinate with low occupancy at one position. His-219 and His-320 are in the immediate environment of the metallocenter. The former residue appears to facilitate substrate binding, whereas the latter residue is thought to serve as the general base in catalysis.

inadvertent frameshift in this sequence (see the footnote in reference 122), and these residues are in fact present in all urease sequences (Fig. 5).

Partial activation of purified *K. aerogenes* urease apoprotein has recently been demonstrated (238). The distinct feature of this process, described in more detail in the section on urease metallocenter assembly (below), is that carbon dioxide is required. The authors speculated that an amino acid side chain, such as a lysine residue, reversibly reacts with carbon dioxide during the activation process. They proposed that the resulting carbamate acts as a metallocenter ligand.

X-ray crystallographic analysis of *K. aerogenes* urease supports the imidazolate ligand assignments that were based on site-directed mutagenesis studies (i.e., His-134, His-136, and His-246) and further indicates that His-272 also is a ligand to the binuclear center (122). The same study additionally demonstrates the validity of the carbamate hypothesis by fitting a modified lysine, with density that is appropriate for a lysine carbamate, at position 217 and allowing it to bridge the two nickel ions in the metallocenter. Lys-217 is conserved in all ureases that have been sequenced (Fig. 5). Finally, the crystal structure has provided insight into two other features of the urease metallocenter ligands. First, density that may arise from a water molecule is associated with one nickel ion and may partially bridge the two nickel ions on the face opposite that of the lysine carbamate. Secondly, Asp-360, known to be conserved in all ureases (Fig. 5), functions as a ligand to one nickel ion in urease (122). Thus, only slight modification of the spectroscopically derived model for the urease metallocenter (Fig. 7) is needed to fit the crystallographically determined structure, as illustrated in Fig. 8.

Of special interest to inorganic chemists, a reasonable model complex of the urease active site has been synthesized (295). In this complex, the two nickel ions are correctly positioned 3.5 Å apart, they appropriately have two nitrogenous ligands per metal ion, and they are bridged by a monodentate carboxylate and two bidentate carboxylate groups such that one of latter ligands could mimic the carbonate bridge to the two nickel ions in urease. Moreover, the model complex coordinates a urea molecule via its carbonyl oxygen atom. As described below, this type of coordination has implications regarding the mechanism of catalysis by the enzyme.

Other active-site residues. As mentioned in the section on urease enzymology (above), studies examining the pH dependence of ureolysis indicate that ureases generally appear to possess a general base and a general acid that function in catalysis. A variety of approaches, summarized in this section, have been used in efforts to identify these and other catalytically important residues.

UreC–His-320 (*K. aerogenes* numbering) is likely to be the general base involved in urea hydrolysis. Bacterial urease is known to be rapidly inactivated by treatment with diethylpyrocarbonate, a reagent that reacts with histidine and several other amino acids, where the rate and extent of this inactivation are reduced by addition of substrate or inhibitors (235). Furthermore, the pH dependence of this inactivation event is consistent with the presence of a reactive residue possessing a pK_a of 6.5, identical to that of the general base in catalysis. Replacement of His-320 with alanine by site-directed mutagenesis results in the synthesis of a protein that possesses very low but detectable activity (0.0027% of the wild-type activity) and contains the standard nickel content (236). In contrast to the behavior of the wild-type enzyme, the activity of the mutant protein is not affected by diethylpyrocarbonate treatment. These results are compatible with His-320 being the general base that participates in catalysis. Replacement of His-320 with leucine in the enzyme from *P. mirabilis* results in an enzyme that lacks activity and also possesses reduced amounts of nickel (271). Results of X-ray crystallographic studies of the *K. aerogenes* enzyme (122) are consistent with the hypothesis that this residue serves as a general base by placing His-320 near, but not serving as a ligand to, the bi-nickel center. The fact that elimination of this putative general base greatly reduces but does not eliminate the activity of the enzyme rules out an alternative role for this residue as a catalytic nucleophile. His-320 is conserved in all ureases that have been sequenced to date (Fig. 5).

Jack bean urease possesses a cysteine residue that initially was proposed to serve as a general acid in catalysis (67, 228, 279), but studies with the bacterial enzyme have called this proposal into question. *K. aerogenes* urease is inactivated by alkylating and disulfide reagents, and the enzyme is protected by the presence of substrate and inhibitors (286). These results demonstrate that chemical modification of a cysteine residue near the active site can eliminate activity. The pH dependence of the inactivation reaction is interpreted in terms of a model involving two ionizable residues that act together to yield macroscopic pK_a values of ~12 and less than 5. Peptide-mapping studies combined with kinetic inactivation approaches were used to identify the residue associated with activity as UreC–Cys-319 (287), identical in position to the "essential" cysteine in jack bean urease. However, site-directed mutagenesis of this residue revealed that it is not essential for catalysis (181). For example, the C319A mutant protein possesses 48% of the wild-type activity, although the pH optimum of the mutant protein is shifted to lower pH compared with that of the wild-type enzyme. The analogous C319A mutation in the *P. mirabilis* protein has lower but still detectable activity (271). As further evidence that a cysteine is not required at this position, the DNA sequence of urease genes from *S. xylosus* indicates the presence of threonine at this position for that protein (135). Finally, X-ray crystallographic studies provide no compelling evidence that Cys-319 plays an important role in catalysis but suggest that chemical alterations at this position may block access to the active site and that mutants generated by site-directed methods may display secondary effects arising from perturbation of the general base at the adjacent position, His-320 (122). At this time, the hypothetical general acid that functions in catalysis has not been identified.

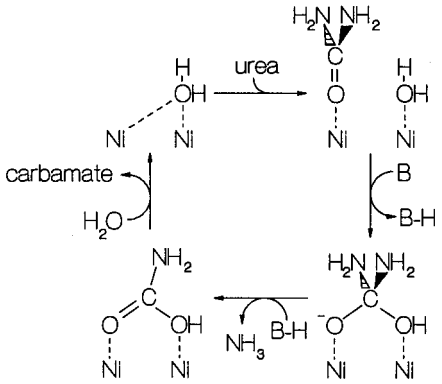

FIG. 9. Mechanistic proposal for urease. The urease active-site structure (Fig. 8) and the proposed structural models for urease-inhibitor complexes (Fig. 3 and 4) are compatible with much of the original mechanistic proposal put forth in 1980 by Dixon et al. (67). Urea is suggested to bind in O coordination to one nickel ion (facilitated by His-219), an active-site base (B, His-320) activates a water molecule bound to the other metal ion, attack by the metal-coordinated hydroxide on the substrate carbon atom results in a tetrahedral intermediate that bridges the two metal sites, a proton is transferred to the intermediate with accompanying ammonia release, and water displaces the carbamate to complete the cycle. The original mechanistic proposal (67) included a general acid role for a cysteine residue; however, this aspect of the former model has been discarded.

An additional residue found at the *K. aerogenes* active site is UreC–His-219. Site-directed modification of this amino acid to form the H219A protein resulted in a dramatic increase in K_m to over 1 M urea (236). These results are consistent with a role in substrate binding, an hypothesis that is compatible with the crystal structure (122).

Urease mechanism. The current model for bacterial urease catalysis (Fig. 9) is a modification and extensive elaboration of the classic urease mechanism proposed by Zerner and colleagues for the plant enzyme (22, 67). In their original mechanism, the Zerner group included an illustration of the active site in which the two nickel ions are separated into two distinct metallocenters, but they suggested that the same basic mechanism could be maintained if the two metals formed a single binuclear center. As detailed above, such a bi-nickel metallocenter is now known to exist. Furthermore, the ligands to the metallocenter in the bacterial enzyme are known and include a bridging lysine carbamate, a partially bridging water molecule, four histidine imidazoles with two bound to each nickel ion, and an aspartic acid residue bound to one nickel ion (122, 236, 238, 297). Supported by model compound chemistry (295), the substrate is suggested to bind in O coordination to one nickel ion, resulting in polarization of the urea carbonyl group. The Zerner model hypothesized the participation of a carboxylate group in stabilizing a particular tautomer of the bound urea, but at this time there is no compelling reason to retain or reject this aspect of their model, and this feature is excluded from Fig. 9 to maintain simplicity. Also not shown is His-219, a residue which may function to further stabilize substrate binding. His-320, acting as a general base (B), activates the water molecule (or hydroxyl group) coordinated to the second nickel ion. Nucleophilic attack on the urea carbonyl by the hydroxyl group gives rise to a tetrahedral intermediate that decomposes to carbamate with elimination of ammonia. The Zerner model had postulated a cysteine serving in this step as a general acid; however, bacterial urease studies have eliminated this residue as part of the mechanism. The required proton donor is shown as the protonated general base in Fig. 9; however, an unidentified general acid must still be considered an alternative possibility. Support for a distinct residue serving in this role arises from the suggested hydrolysis of phosphoroamide compounds described in the section on urease enzymology (above) and shown in Fig. 4. On steric grounds, the same residue would be unlikely to participate as both a general acid and a general base for the in-line hydrolytic mechanism involving a trigonal bipyramidyl intermediate.

Clearly, alternative mechanisms to that shown in Fig. 9 cannot be ruled out at this time. It remains possible that urea binding exhibits N coordination or bidentate coordination to the metallocenter. General base-catalyzed activation of an amino acid side chain and subsequent attack by this residue on the substrate carbonyl, resulting in a covalently bound intermediate, cannot be excluded. It is hoped that this working model of urease catalysis will be the impetus for further mechanistic characterization of this fascinating two-nickel-ion enzyme.

Urease Metallocenter Assembly

A particularly fascinating aspect of urease biochemistry centers on the mechanism of its metallocenter assembly (reviewed in reference 205a). As described below, in vitro activation of purified urease apoprotein recently has been achieved and found to exhibit a surprising requirement for carbon dioxide. Moreover, in vivo activation of urease is a highly complex process that requires the participation of products from numerous accessory genes (enumerated in Table 1). These proteins serve to install the functional metallocenter into the urease apoprotein; however, the detailed roles for the auxiliary proteins are only beginning to be understood.

In vitro activation of urease apoprotein. When ureolytic bacteria, fungi, algae, or plants are grown in medium lacking nickel ions, urease apoprotein is produced (see, e.g., references 12, 167, 213, 244, 245, and 302). Addition of nickel ions to cell extracts from such cultures fails to produce any urease activity. An exception to this statement is the recent demonstration of low levels of activity generated by nickel ion addition to freshly prepared and highly concentrated extracts of *E. coli* cells expressing the *K. aerogenes* urease genes (234). Up to 10% of the urease present in these extracts was shown to be activated in these studies; however, even this rather low level of activation was found to require incubation of extracts at 37°C for over 24 h. Furthermore, additional experiments (234) indicated that the observed activity is probably generated from a UreD-urease apoprotein complex (see below), not from the apoprotein alone. The *K. aerogenes* urease apoprotein purified from cells grown in the absence of nickel ion possesses a quaternary structure identical to that of the holoprotein (155). Moreover, crystallographic analyses demonstrate near identities between the apoprotein and holoprotein structures, except at the metallocenter (122). Addition of nickel ions to purified apoprotein fails to yield active enzyme when the usual purification or assay buffers are used. This finding is not affected by provision of possible protein stabilizers (thiols, glycerol, salt) or inclusion of denaturants (155). However, as described in the next paragraph, a slight modification of the activation procedure results in the rapid generation of significant levels of urease activity from the apoprotein upon nickel addition.

The breakthrough in efforts to activate purified urease apoprotein in vitro was achieved by simply adding bicarbonate to the activation buffers (238). For example, addition of apoprotein to buffer containing 100 mM $NaHCO_3$ and 100 μM $NiCl_2$ resulted in activation of over 12% of the protein present. The bicarbonate-dependent activation process is reasonably rapid, being complete in less than 90 min. Because bicarbonate is in equilibrium with carbon dioxide in solution, initial rate studies

were conducted with bicarbonate stock solutions that were adjusted to pH 8.5 or 4.2 (the equilibrium favors greater levels of carbon dioxide at lower pH) to distinguish which of these species is the actual activating factor. The enhanced rate of activation observed when the latter solution was used, coupled with the depressed rate when carbonic anhydrase was included in the solution, demonstrated that the activating factor is carbon dioxide. The kinetics of in vitro activation were found to be consistent with reaction between a protein side chain and carbon dioxide, followed by binding of nickel. The pH dependence of activation reveals that deprotonation of the side chain residue is associated with a pK_a of ≥ 9. The authors speculated that a lysine carbamate, or similar modified residue, is formed on the protein and then serves as a ligand for metal binding (238). The urease crystal structure (122) is entirely compatible with this hypothesis and shows that a modified Lys-217, with electron density that is appropriate for a lysine carbamate, serves as a bridge between the two nickel ions at the active site. In the absence of appropriate levels of carbon dioxide (half-maximal activation is achieved at 0.2% carbon dioxide, compared with atmospheric concentrations of 0.03%), nickel was found to bind to the apoprotein in a nonproductive manner. The nonproductive metal ion-binding process results in formation of a nickel-containing protein that cannot be activated by adding bicarbonate, unless the nickel is first removed by prolonged incubation in the presence of a nickel chelator.

Requirement of accessory genes for in vivo urease activation. Early genetic analyses of many organisms revealed that it is possible to eliminate urease activity by disrupting genes other than those encoding the urease subunits (see, e.g., references 17, 45, 97, 143, 165, 166, 191, 212, and 296). Subsequently, sequence information revealed the presence of numerous nonsubunit genes in urease gene clusters (Table 1; see the section on genetics of urease gene clusters, above). In 1990, it became clear that at least some of these other genes serve to facilitate assembly of a functional metallocenter in the protein. This result came from studies in which inactive urease was purified from a deletion mutant lacking a DNA fragment encoding part or all of the *ureE*, *ureF*, and *ureG* genes (211). The purified protein was found to possess insignificant levels of nickel ions. The urease purification and characterization approach was extended to analyze a series of deletion mutants that lacked the functional genes *ureD*, *ureE*, *ureF*, or *ureG* (156). Disruption of *ureD*, *ureF*, or *ureG* was shown to lead to the formation of inactive urease that, in all cases, lacked nickel ions. In contrast, disruption of *ureE* reduced only the levels of urease activity and correspondingly reduced the nickel content of urease purified from this microorganism. Each of these genes was shown to produce a *trans*-acting factor. These results were interpreted to suggest that the UreD, UreE, UreF, and UreG accessory proteins participate in the functional incorporation of the urease metallocenter. A model illustrating hypothetical roles for the urease accessory proteins is presented in Fig. 10. The evidence related to the functions of these polypeptides is summarized below.

Speculative role for UreD in urease activation. As indicated in Table 1, complete DNA sequence information is available for genes encoding UreD from *Bacillus* sp. strain TB-90 (172), *E. coli* (68), *H. pylori* (unfortunately, the *ureD* homolog is denoted *ureH* in this strain) (53), *K. aerogenes* (156), *K. pneumoniae* (46), *P. mirabilis* (132), *R. meliloti* (193), and *Y. enterocolitica* (58). These sequences are not closely related to each other (205a), they do not exhibit significant homology to other sequences in the GenBank database, and they provide no clue to the protein function. Attempts to characterize the *ureD* gene product have been reported only for the protein from *K. aerogenes* (234). When the *ureD* promoter region was mutagenized to create an improved ribosome-binding site and an ATG initiation codon, high-level synthesis of the protein was achieved. Soluble UreD was found to be associated with urease apoprotein (Fig. 10), and three distinct UreD-urease apoprotein complexes were purified. These complexes appear to correlate to the $(\alpha\beta\gamma)_3$ apoprotein binding one, two, or three molecules of UreD. These complexes were shown to be competent for activation upon addition of nickel ions, with the observed level of activation paralleling the amount of UreD bound (234). Activation of the UreD-urease complexes is accompanied by dissociation of UreD. As found for apoprotein alone, carbon dioxide is required for in vitro activation of the UreD-urease apoprotein complexes (238). A functional UreD is absolutely required for the presence of any urease activity in vivo; however, the role of this protein remains unclear. We have speculated (234, 238) that UreD serves as a urease-specific chaperone protein that facilitates proper assembly of the metallocenter by maintaining a proper protein conformation or perhaps by preventing nonproductive nickel ion binding prior to incorporation of the essential carbon dioxide molecule. Further studies may help to clarify the action of this peptide.

Speculative role for UreE in urease activation. UreE sequences are available from the DNA sequences of the corresponding genes from *Bacillus* sp. strain TB-90 (172), *H. pylori* (53), *K. aerogenes* (211), *P. mirabilis* (132), and *Y. enterocolitica* (58). Only limited homology is observed within this group (205a), and no other proteins in the GenBank database appear to be related to UreE. Three of these proteins are predicted to possess a histidine-rich motif at their carboxyl termini. This feature was utilized to purify the *P. mirabilis* protein by a single-step procedure involving nickel-chelate affinity chromatography (269). As expected for such a histidine-rich peptide, the purified UreE protein from *K. aerogenes* was shown by equilibrium dialysis studies to bind nickel ion. Specifically, UreE binds approximately six nickel ions per dimer with a K_d of $\sim$10 μM (157). X-ray absorption spectroscopic analysis was used to demonstrate that the coordination environment of nickel in the *K. aerogenes* UreE holoprotein primarily involves imidazolate ligands. Crystals of UreE apoprotein were obtained and shown to diffract to less than 3 Å. These crystals fractured upon addition of nickel ions, consistent with the induction of a conformational change upon nickel binding (157). From these studies, it is tempting to speculate that UreE may function to bind cellular nickel ion by its polyhistidine tail and act as the nickel donor during urease activation (Fig. 10). Consistent with a possible nickel donor role, *ureE* deletion mutants exhibit depressed urease activity in minimal media that can be partially restored by adding higher concentrations of the metal ion (269). However, the absence of a polyhistidine tail in the UreE sequences from two species raises questions about whether the protein uniformly serves as a nickel donor. It remains possible that the protein has multiple functions in some microorganisms and a single function in others, that it serves no function in certain species, or that it serves distinct functions in different cells. Overall, the role for UreE has not been established.

Speculative roles for UreF and UreG in urease activation. Sequence information is available for UreF and UreG peptides, via the corresponding DNA sequences, from *Bacillus* sp. strain TB-90 (172), *H. pylori* (53), *K. aerogenes* (211), *P. mirabilis* (132, 270), and *Y. enterocolitica* (58). In addition, the UreG sequence is known from *E. coli* (68). As found for UreD and UreE, the UreF sequences do not exhibit extensive homology with each other (205a) and are not significantly related

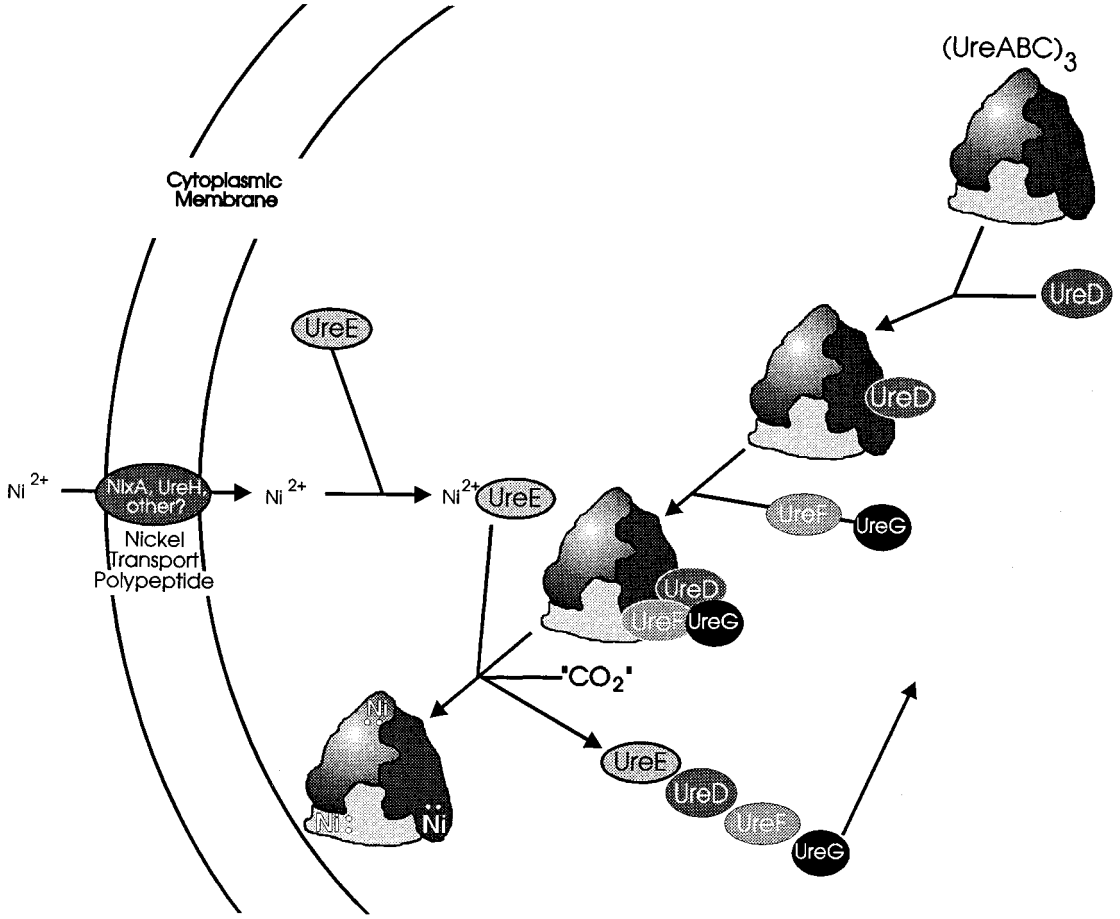

FIG. 10. Model for urease activation by accessory genes. The urease apoenzyme is assembled from three copies of each distinct subunit polypeptide, UreA, UreB, and UreC, resulting in a stoichiometry of $(UreABC)_3$. In vivo activation of the apoprotein requires the presence of nickel ion, carbon dioxide, and numerous urease accessory gene products. This figure represents our current working model in which roles for these auxiliary proteins are proposed. UreD, a possible chaperone-like protein, forms a complex with the apoenzyme. UreF and UreG join the complex, making the apoenzyme competent to accept nickel ions (Ni^{2+}) into the active site in a productive manner. These proteins may function in delivery or production of the requisite carbon dioxide molecule that reacts with apoprotein to become a nickel ligand. Alternatively, UreF and/or UreG may facilitate interaction between the urease apoprotein and the putative nickel donor, UreE. Finally, it is possible that these proteins eliminate non-productively bound metal ions from the active site. Upon activation, UreD, UreE, UreF, and UreG dissociate from the enzyme and are recycled for interaction with the next urease apoenzyme molecule or with other "UreABC" units on the same molecule. The net result is the assembly of three catalytic sites on the enzyme, each containing two nickel ions (white dots). UreE, the nickel-binding protein, acquires its nickel ion with the assistance of NixA (in *H. pylori*) or UreH (in *Bacillus* sp. strain TB-90), which mediate transport of nickel across the cytoplasmic membrane.

to any other proteins accessible in GenBank. In contrast, the UreG sequences are a much more highly conserved group (205a), approaching the homology observed within the structural peptides of urease. Further, the UreG peptides possess a nucleotide-binding motif that is present in a variety of ATP- and GTP-binding proteins. A limited degree of homology between *P. mirabilis* UreG and a 60-kDa chaperonin from a thermophilic bacterium was noted (270), but the significance of this slight similarity is not clear. Of greater interest, the UreG sequences are clearly related to the HypB proteins. For example, the *K. aerogenes* UreG sequence is approximately 25% identical to the *E. coli hypB* gene product (304). This gene is part of the hydrogenase pleiotropic operon that is required for activation of nickel-containing hydrogenases (163). HypB was proposed to have a role in nickel processing based on the ability to complement *hypB* mutations by supplementing the medium with elevated concentrations of nickel ion (299). Purified *E. coli* HypB was shown to be capable of binding and hydrolyzing GTP (176). Coupling this result with the presence of a nucleotide-binding site in UreG raises the possibility that the urease-related protein functions in an energy-dependent step during in vivo urease assembly.

Despite the absolute requirement for a functional UreF and UreG during in vivo activation of urease, it is important to stress that the roles of these peptides remain unknown. Analysis of deletion mutants revealed that neither *ureF* nor *ureG* is required for UreD-urease apoprotein complex formation (237). It is interesting, however, that the chromatographic behavior of the complex is distinct in the *ureF* deletion mutant, suggesting that UreF somehow may alter the UreD-urease complex conformation. Furthermore, recent evidence is consistent with the presence of a series of UreD-UreF-UreG-urease apoprotein complexes, in addition to the UreD-urease apoprotein complexes (Fig. 10), in *E. coli* cells carrying the *K. aerogenes* urease genes (237). These investigators speculate that these larger complexes are required for in vivo activation of urease. Hypothetical roles for UreF and/or UreG in these complexes include the generation or delivery of carbon dioxide to the metallocenter assembly site, facilitation of productive interaction between urease apoprotein and UreE holoprotein,

and release of nickel or other metal ion that is nonproductively bound to urease. Additional studies may further elucidate the properties of this large assembly complex and detail the roles of its components.

Metal transport. There is no published evidence indicating that UreD, UreE, UreF, or UreG participate in nickel transport into the cell. Nevertheless, cellular nickel transport must occur to provide nickel to the urease incorporation machinery. In both *Bacillus* sp. strain TB-90 and *H. pylori*, additional genes that appear to function in metal transport specific for urease assembly have been identified (Fig. 10). It should be pointed out, however, that nickel transport systems may be encoded by loci that are not associated with ureolysis. For example, *E. coli* cells possess the *nik* operon that encodes a five-component system that transports nickel ion when cells are grown under anaerobic conditions (220). This system includes a periplasmic nickel-binding protein and an ATP-dependent integral membrane transporter. The two urease-related systems are described below.

The *ureH* gene is part of the urease gene cluster of *Bacillus* sp. strain TB-90 (172). Deletion of a segment of this gene results in a requirement for higher nickel ion concentrations in the medium to achieve the same urease activities. In addition, the UreH peptide exhibits 23% sequence identity to the HoxN protein of *A. eutrophus*, a high-affinity nickel transport peptide that is required for hydrogenase activity (76). (An additional hydrogenase-related homolog of *ureH* has been identified as the *hupN* gene in *Bradyrhizobium japonicum* [91]; however, the role of the HupN protein is not established.) These results provide compelling circumstantial evidence that UreH functions in nickel transport.

E. coli cells expressing the *H. pylori* urease gene cluster fail to exhibit urease activity in rich medium containing compounds that chelate nickel ion, yet *H. pylori* has a high level of urease activity in such conditions. The *nixA* gene of *H. pylori* was cloned on the basis of its ability to confer high-level urease activity to cells growing in this environment (200) (see the section on genetics of urease gene clusters, above). Although not part of the urease gene cluster, this gene encodes a protein that is related to UreH of *Bacillus* sp. strain TB-90 and to HoxN and HupN of *A. eutrophus* and *Bradyrhizobium japonicum*, respectively. Moreover, cells containing this protein possess an enhanced ability to transport nickel ion (200). In a similar fashion, *E. coli* cells containing a plasmid with the *K. aerogenes* urease gene cluster exhibit significantly enhanced urease activity in the presence of a second plasmid carrying *hoxN* (303). These results are compatible with the presence of unlinked nickel transport-related genes in other microorganisms for which urease clusters have been characterized.

MOLECULAR PATHOGENESIS

Urease activity plays a central role in the pathogenesis elicited by a number of bacterial species. The involvement of this enzyme in urolithiasis (stone formation), catheter encrustation, pyelonephritis, ammonia encephalopathy, hepatic encephalopathy, hepatic coma, and inactivation of complement has been previously reviewed (201). New developments published since that time, however, are summarized below.

Urease as a Virulence Factor in Urinary Tract Infection

P. mirabilis. *P. mirabilis* , a urease-positive bacterium, is not a common cause of urinary tract infection (UTI) in the normal host. Surveys of uncomplicated cystitis or acute pyelonephritis show that *P. mirabilis* causes only a small percentage of cases. Even in patients with recurrent UTI, the incidence of infections caused by this organism is only a few percentage points higher. However, this organism infects much higher proportions of patients with complicated urinary tracts, i.e., those with functional or anatomic abnormalities or with chronic instrumentation. Indeed, nearly half of elderly long-term-catheterized patients are infected with this species (298). In these patients, not only does this bacterium cause cystitis and acute pyelonephritis but also the production of urinary stones, a hallmark of infection with this organism, adds another dimension to the already complicated urinary tract.

Urease as virulence factor. Several investigators (4, 27, 127, 129, 168–170, 216) have used rat or mouse models to compare the severity of pyelonephritis caused by *P. mirabilis*. The effects of urease were studied by injecting acetone-killed organisms that retained urease activity (27); by treating infected animals with oral supplements of acetohydroxamic acid, a specific inhibitor of the enzyme (4, 170, 216); or by inoculation of an ethyl methanesulfonate-generated urease mutant (168). Although all studies supported the supposition that urease played a role in virulence, the use of killed organisms (27) or mutants generated by nonspecific mutagens (168) made interpretation of these data difficult. Additionally, animals in these studies were challenged hematogenously, which establishes kidney infection by a route that is not now believed to mimic the more natural course of infection that develops by the ascending route.

Analysis of virulence with an isogenic urease-negative mutant. To specifically evaluate the contribution of urease to virulence, a mutation in the *ureC* urease structural subunit gene was introduced into a *P. mirabilis* strain by homologous recombination. Virulence was assessed in the CBA mouse model of ascending UTI (129). Twenty mice each were challenged transurethrally with 1×10^9 to 2×10^9 CFU of a *P. mirabilis* strain or its urease-negative derivative. At 48 h, animals were sacrificed and the mean $\log_{10}$ of quantitative cultures of isolates from the urine (parent, 6.23; mutant, 4.19; $P = 0.0014$), bladder (parent, 6.29; mutant, 4.28; $P = 0.0002$), right kidney (parent, 4.11; mutant, 2.43; $P = 0.036$), and left kidney (parent, 4.11; mutant, 1.02; $P = 0.00009$) were all shown to be significantly different.

To assess a longer duration of infection, mice developed significant bacteriuria and struvite renal stones after transurethral challenge with the parent strain (127). The urease-negative mutant had a 50% infectious dose of $>2.7 \times 10^9$ CFU, a value more than 1,000-fold greater than that of the parent strain (2.2×10^6 CFU). The urease-positive parent strain reached significantly higher concentrations and persisted significantly longer in the bladder and kidneys than did the mutant. Indeed, in the kidneys, the concentration of the parent increased while that of the mutant was falling, so that, by 1 week, the concentration of the parent was 10^6 times that of the mutant.

Renal pathology. The *P. mirabilis* urease-positive parent produced significantly more severe renal pathology than the mutant did (127). The initial abnormalities were in and around the pelvis and consisted of acute inflammation and epithelial necrosis. By 1 week, pyelitis was more severe, crystals were seen in the pelvis, and acute pyelonephritis had developed with acute interstitial inflammation, tubular epithelial cell necrosis, and, in some cases, abscesses. By 2 weeks, more animals infected with the parent strain had renal abscesses and radial bands of fibrosis than those infected with the mutant.

Urolithiasis. Infection-induced stones result from urease-mediated urea hydrolysis in urine (87, 101, 102, 158, 171, 188, 204). Generation of ammonia from cleavage of urea, present in

high concentration (0.4 to 0.5 M) in urine, results in an elevation of pH and precipitation of normally soluble polyvalent ions present in urine. The primary compounds formed are struvite ($MgNH_4PO_4 \cdot 6H_2O$) and carbonate apatite [$Ca_{10}(PO_4)_6 \cdot CO_3$].

In the experimental model of UTI with isogenic strains (127, 129), renal pathology in mice infected with the parent strain was accompanied by a tendency to develop urolithiasis. Beginning at 1 week, struvite stones were found frequently in the renal pelvis of mice infected with the parent strain (stones were found in 12 of 39 mice at 1 week and in 8 of 20 mice at 2 weeks), whereas stones were never found in mice infected with the urease-negative mutant ($P < 0.002$). In a separate study, Dumanski et al. (71) determined that *P. mirabilis* capsule specifically contributes to urease-mediated crystal formation. It can be concluded that the urease of *P. mirabilis* is a critical virulence determinant necessary for colonization, urolithiasis, and severe acute pyelonephritis.

S. saprophyticus. *S. saprophyticus*, unlike *P. mirabilis*, is a frequent cause of UTI in young, sexually active women (230). The role of the urease of this species in experimental UTI was investigated by using a nitrosoguanidine-generated urease-negative mutant (95). Rats were inoculated transurethrally with 1.5×10^9 CFU of the parent or mutant strain in a fashion that allowed vesicoureteral reflux (resulting in direct inoculation of the kidneys). Animals were evaluated at 1 week after challenge, and the results revealed a significant role for urease in the colonization and damage to the urinary bladder. A less important role for urease in colonization and damage to the kidneys was observed. Leukocyturia and bacteriuria were significantly more pronounced in the urease-positive parent than in the urease-negative mutant. Bladders of the group challenged with the urease-positive parent strain were enlarged, and the bladder walls were thickened. Bladder stones were found in 3 of 19 animals infected with the parent strain but in 0 of 14 of the animals infected with the urease-negative mutant. On histologic examination, severe destruction of the bladder tissue with loss of the uroepithelium and formation of abscesses was noted in animals infected with the parent strain. Infection with the mutant strain led to only a mild acute cystitis. It was concluded that the urease of *S. saprophyticus* contributed mainly to invasiveness of bladder tissue by the organism and did not play as significant a role in damage to the kidneys.

H. pylori Urease and Gastroduodenal Infection

Since our last major review (201), there has been an explosion of literature on the subject of *H. pylori*, the etiologic agent of gastritis and peptic ulceration (50). Because urease is central to the virulence of this organism, much of that literature focused on this enzyme.

Urease as antigen. Urease is the most prominent protein component of *H. pylori*. Not surprisingly, this protein, described as surface exposed by some investigators (72, 78, 110), serves as a powerful immunogen for this organism (40, 55, 59, 133, 222, 223, 239). Patients with active gastritis due to *H. pylori* show significantly elevated immunoglobulin G and A titers to the urease in serum when compared with preinfection levels. Enzyme-linked immunosorbent assay systems have been developed by using partially purified or purified urease as an antigen to measure these immune responses (for a review, see reference 240). Other outer membrane proteins have been used as well to measure elevated immune responses (240). Such tests are useful for diagnosing acute infection, monitoring eradication of *H. pylori* during antibiotic therapy, and conducting large-scale epidemiological studies.

Avoidance of host defense. Urease is critical for *H. pylori* colonization of the human gastric mucosa. In vitro, the bacterium is quite sensitive to the effect of low pH (111) unless urea is present (180). The initial colonization of the stomach with a pH of 3 or less would be difficult unless the organism could protect itself from exposure to acid. It is postulated that the organism hydrolyzes urea, releasing ammonia, which neutralizes acid and thus enables survival and initial colonization. Urease-negative mutants of *H. pylori* have been generated by nonspecific chemical mutagenesis (24, 259) or by selection of naturally occurring mutants (51) or constructed by allelic exchange of in vitro constructed deletion mutations (81). Representative mutants have demonstrated that urease is necessary for colonization in the gnotobiotic piglet model (74).

Direct toxicity to the host. In addition to the survival benefit of expressing urease, there is evidence that ammonium hydroxide, generated by urea hydrolysis, contributes significantly to histologic damage. It should be emphasized that ammonium ion per se is not toxic but that instead, damage results from the hydroxide ions generated by the equilibration of ammonia with water. To demonstrate the cytotoxic effect of urease, cell cultures of a human gastric adenocarcinoma cell line were seeded with *H. pylori* and supplemented with various concentrations of urea (266). Cell viability was found to be inversely proportional to ammonia concentrations generated by urea hydrolysis. Viability was improved when the urease inhibitor acetohydroxamic acid was added to the culture before the exposure to *H. pylori*. Acetohydroxamic acid slowed the liberation of ammonia and reduced the cytotoxic effect.

Similar effects have been shown on Vero cells overlaid with filtrates of *H. pylori* (11). Cell rounding and loss of viability were observed in cultures to which 30 mM urea had been added. These changes were associated with a rise in pH. Further work with *H. pylori* supernatants used 4 mM urea, duplicating urea concentrations found in the human stomach (305). At this concentration, 10% of the Vero cells showed intracellular vacuolization after 24 h of exposure to *H. pylori* supernatants. Acetohydroxamic acid reduced this effect by 75%. These data suggested that histologic damage may result directly from the localized generation of ammonia due to the hydrolysis of urea.

It has also been postulated that ammonia produced by urea hydrolysis has an additional effect (112). Ammonia may interfere with normal hydrogen ion back-diffusion across gastric mucosa, resulting in cytotoxicity to the underlying epithelium.

Host damage induced by the immune response. Urease activity may also be responsible for damage to the gastric epithelium via its interaction with the immune system. *H. pylori* whole cells can stimulate an oxidative burst in human neutrophils (276). When neutrophils, *H. pylori*, and urea were incubated with rabbit fetal gastric mucosal cells, cytotoxicity was seen, as evidenced by shrunken gastric cells. This cytotoxicity was not observed with urea-free medium, with the addition of acetohydroxamic acid, or by incubation with inhibitors of the oxidative burst. It was suggested that hydrogen peroxide from the oxidative burst oxidizes chlorine ions which react with ammonia liberated by *H. pylori* urease to give the highly toxic product monochloramine. The cytotoxic activity could be mimicked by the addition of monochloramine to the gastric cells.

The urease enzyme itself can also cause activation of monocytes and polymorphonuclear leukocytes and recruitment of inflammatory response cells, resulting in indirect damage to the gastric epithelium. Water extracts of *H. pylori*, known to contain urease in high concentration, can activate monocytes

by a lipopolysaccharide-independent pathway (175). In vitro stimulation of human monocytes led to secretion of inflammatory cytokines and reactive oxygen intermediates, all of which may be involved in mediating the inflammatory response in the gastric epithelium. Further investigation has shown that sonicates of *H. pylori* strains could prime and also cause direct activation of the oxidative burst in human polymorphonuclear leukocytes and monocytes (227). Both properties were present in two separate molecular weight size ranges, which did not preclude the UreA subunit of urease. In contrast, it was reported that purified urease could not stimulate natural killer cell activity of isolated granular lymphocytes directly, unlike complete cells of *H. pylori* (280). This finding suggests that such damage caused by urease occurs by its interaction with cells responsible for cellular inflammatory signalling rather than with the cytotoxic cells themselves.

There is also evidence of urease or urease-containing fractions from *H. pylori* acting as chemotactic factors for leukocytes, causing further local inflammation (52, 174). Such chemotactic activity for human monocytes and neutrophils was present in purified urease samples and could be inhibited by specific antibody to the UreB urease subunit. Further, a 20-amino-acid peptide based on the amino terminus of the UreB subunit protein also exhibited similar levels of chemotaxis in a microchamber test system. Immunocytochemical staining showed urease closely associated with the crypt cells in the lamina propria of patients with duodenal ulcers. It is postulated that urease is absorbed into the mucosa, where it attracts leukocytes and causes mucosal inflammation.

Urease, by a variety of mechanisms, is at least partly responsible for the initial recruitment of monocytes and neutrophils and for further activation and stimulation of the immune system to produce the local inflammatory lesion associated with *H. pylori* infection. Further elucidation of the multifactorial role of urease in causing potential damage to the gastric epithelium and its contribution to the variety of disease states associated with the bacterium is awaited.

Urease-negative mutants in animal models of *Helicobacter* infection. The contribution to pathogenesis by the *H. pylori* urease has been assessed by testing the virulence of a urease-negative mutant of an *H. pylori* strain, generated by mutagenesis with nitrosoguanidine, in the gnotobiotic piglet model of gastritis (74). The mutant, which retained only 0.4% of the urease activity of the parent strain, was unable to colonize any of 10 orally challenged piglets as assessed at 3 or 21 days after challenge. The parent strain successfully colonized seven of seven piglets and elicited a lymphofollicular gastritis. No significant histopathologic changes was noted for piglets infected with the mutant strain. Since complementation techniques are not available, it was not possible to determine whether additional defects were present in the nitrosoguanidine-mutated *H. pylori* strain assayed in the piglets. Additional insight, however, was gained in subsequent experiments (75), in which an isogenic urease-negative mutant (*ureG*::Km) was used for challenge. Piglets, treated or not with omeprazole (a proton pump inhibitor) to prevent acid secretion, were challenged with the parent and mutant strains. The parent strain colonized normally in numbers ranging from a mean $\log_{10}$ CFU of 4.4 to 6.9. The urease-negative mutant was unable to colonize the gastric mucosa at normal physiological pH and was recovered in small numbers (mean $\log_{10}$ of <2) from omeprazole-treated, achlorhydric piglets. The results confirmed that urease enzymatic activity and not simply the inactive apourease protein is essential for colonization. Furthermore, protection from low gastric pH appears not to represent the major role of urease in promoting colonization by *H. pylori*. The dramatic results of this experiment were corroborated by the construction of a urease-negative mutant of *H. pylori* by allelic exchange and testing of its virulence by oral administration to nude mice (290). The urease-negative mutant (which had undetectable urease activity) was not recovered from the gastric mucosa of challenged mice, and no pathologic changes were noted. On the other hand, for the animals given the parental strain, gastritis developed and viable bacteria were recovered. These findings are further supported by the inability of an undefined urease-negative mutant to colonize the gastric mucosa of cynomolgus monkeys (278).

Detection of *H. pylori* with Urease

Urease biopsy test. The urease reaction can be exploited in the endoscopy suite by obtaining an endoscopic biopsy specimen and placing it directly in a small volume of Christensen's urea broth or various modifications of this recipe (189). If *H. pylori* cells are present in significant numbers, urea will be hydrolyzed by urease, the pH will rise, and phenol red will turn from light orange to red, usually within 5 min. Since the reaction is due to preformed enzymes, the test can be carried out at 45°C to increase the speed of reaction (199). Various modifications of the test exist, and the reactions can be carried out in a commercially available urea-containing gel. All of the biopsy urease tests have specificities approaching 100% and sensitivities of at least 90% (189). This test has been repeatedly validated in the literature; an exhaustive list of these studies is not provided here.

Urease-positive colonies after culture. *H. pylori* can be cultured from the above-described biopsy specimens on blood-based media including Skirrow's or Dent's medium at 37°C in an anaerobic jar with an activated Campy Pak, which supplies the proper microaerobic environment. After 3 to 5 days of culture, pinpoint colonies can be observed and tested for urease activity. *H. pylori* cultured directly from endoscopic biopsy specimens always gives a strong urease reaction, which, in the presence of positive oxidase and catalase reactions, is diagnostic for this species.

Urea breath test. Although the urease biopsy test reaction is simple, it does require biopsy by endoscopy, an invasive procedure. A noninvasive procedure, the urease breath test, which serves as a sensitive and specific, although qualitative, indicator of infection has been developed. The patient is given an oral dose of labeled urea, either [^{13}C]urea (99) or [^{14}C]urea (13). If the organism is present, urea will be hydrolyzed and $^{13}CO_2$ or $^{14}CO_2$ will be liberated, enter the bloodstream, exchange in the lungs, and be exhaled. Exhaled CO_2 is trapped and quantitated in a mass spectrometer for $^{13}CO_2$ or a scintillation counter for $^{14}CO_2$. Since a mass spectrometer is prohibitively expensive for most institutions, the [^{14}C]urea test used with a liquid scintillation counter may be preferred. Alternatively, samples can be collected in sealed tubes and processed in a central laboratory. While a number of members of the normal anaerobic gut flora are urease positive and potentially could interfere with this test, the data collected thus far indicate that false-positive reactions are rare.

PCR identification. The use of PCR to detect specific DNA sequences has been particularly successful for the identification of slowly growing bacterial pathogens. As urease production is ubiquitous in *H. pylori*, urease gene sequences are a suitable target for identification.

PCR amplification of a 411-bp fragment from the *ureA* gene has been used in identification of *H. pylori* strains (36, 37). The PCR fragment was amplified from boiled supernatants of 50 strains of *H. pylori*. No amplification product was produced

from 60 other strains, including *H. mustelae* and other urease-producing bacterial species. These primers were 100% specific and sensitive and had a limit of detection of 10 to 100 bacterial cells. The primers were also able to amplify the 411-bp fragment from gastric biopsy specimen supernatants (after vortexing of the sample and boiling for 10 min) by using a 35-cycle amplification. These studies were extended by using the same PCR primers but with a more complex lysis of the biopsy material by sodium dodecyl sulfate and proteinase K digestion followed by phenol-chloroform extraction (292). After 35 amplification cycles, 25 of 26 culture positive samples produced the 411-bp amplification product, with 1 of 40 culture-negative samples being PCR positive. The difference in rate of PCR detection of culture-negative samples may have been a reflection of the sensitivity of the culture protocol rather than the PCR assay. PCR amplification of a 365-bp product from an adjacent area of the 5′ end of the *ureA* gene sequence has been reported when a pair of PCR primers, one of which was degenerate, was used (301). This PCR primer pair was also 100% sensitive and specific for *H. pylori*, with a reported limit of detection of 17 bacterial cells. Amplification from gastric juice samples by using these primers resulted in 25 of 26 culture-positive samples producing the expected 365-bp PCR product, with 8 culture-negative patients also being negative by PCR.

PCR amplification from regions of the *ureB* gene have also been used to detect *H. pylori* in paraffin-embedded biopsy samples (301). Two PCR primer pairs were used to amplify a 132-bp product and a 115-bp product. Microtome sections were boiled for 15 min and subjected to two rounds of amplification of 25 cycles each. In four of five samples tested, both *ureB* PCR primer pairs amplified the expected product whereas no product was obtained from the larger *ureA* primer pair. This may reflect the poor quality of DNA obtained from paraffin-embedded samples and the greater efficiency of amplification of smaller PCR products.

Both of the *H. pylori* urease structural genes, *ureA* and *ureB*, have been demonstrated to be useful targets for PCR detection of *H. pylori* from culture supernatants, fresh and paraffin-embedded biopsy tissue, and gastric juice aspirates. These PCR detection systems can be applied to epidemiological and transmission studies, monitoring of treatment regimens, and diagnostic testing of *H. pylori* (19, 79, 248, 268, 307).

PCR typing systems based on urease genes. It has been noted that there is a great deal of diversity among *H. pylori* strains with respect to restriction endonuclease sites within chromosomal DNA (177). Indeed, there was so much diversity that nearly every strain revealed a new pattern. Previous studies have used gross differences in the restriction digest patterns of *H. pylori* chromosomal DNAs to differentiate strains. However, interpretation of these results is difficult because of the complexity of the restriction digest patterns, even with densitometric analysis of one signature area of the pattern.

To simplify comparison, this microdiversity can be examined by using PCR products of specific genes from the *H. pylori* chromosome. The first systems that were developed were based on PCR amplification of urease genes (88, 89). PCR-amplified urease structural subunit genes *ureA* and *ureB*, when digested with appropriate restriction endonucleases, produced different digestion patterns in agarose gels. The substrate for PCR amplification was DNA extracted from *H. pylori* by alkali lysis and phenol-chloroform extraction. The 2.4-kb PCR products, amplified from 22 clinical isolates and subjected to *Hae*III digestion, produced 10 distinct patterns on agarose gels, with two patterns being shared between five and six strains. These patterns allowed easy differentiation between strains. Strains taken from one patient at a 6-month interval revealed the same restriction pattern of the PCR product, suggesting that the same strain was present in both biopsy specimens. Another method amplified the *ureC* gene followed by direct DNA sequencing of the PCR product to distinguish between strains of *H. pylori* (49). Numerous base pair changes were detected, and strains were easily differentiated. Again, strains taken from the same individual over various periods demonstrated the stability of this sequence over time, allowing this method to confirm the presence of the same strain or a different strain.

Seven subsequent reports (10, 38, 60, 119, 161, 207, 231) have confirmed that urease genes (*ureA*, *ureB*, and the former *ureC*, which is no longer recognized as part of the urease gene cluster) can be PCR amplified, subjected to restriction endonuclease digestion, and used to (i) differentiate strains on the basis of patterns on agarose gels, (ii) identify the presence of multiple strains in a single biopsy specimen, (iii) check for reinfection with the same strain following eradication therapy, and (iv) identify similar strains among family members. It is probable that for *H. pylori*, any PCR-amplified fragment will allow this type of differentiation. Indeed, this diversity is observed for a 48-kDa stress protein (*htrA*) and a 26-kDa antigen-encoding gene (38) or by ribotyping (161).

Y. enterocolitica Urease and Reactive Arthritis

Reactive arthritis can develop in patients shortly after development of enteric infection with *Y. enterocolitica*. Those with certain human leukocyte antigen (HLA) types, particularly those positive for HLA-B27, are more prone to development of this syndrome (141). A 19-kDa cationic intracellular polypeptide, isolated from *Y. enterocolitica* O:3, was able to induce experimental reactive arthritis in preimmunized mice (190). By use of N-terminal sequencing, degenerate oligonucleotide synthesis, and PCR, the gene for this polypeptide was isolated and found to encode the UreB subunit of urease. Indeed, clones isolated from a cosmid library of *Y. enterocolitica* chromosomal DNA carried the entire urease gene cluster, which expressed urease activity in *E. coli*. The identity of these genes was confirmed by nucleotide sequencing (58, 264). The UreB subunit was subsequently demonstrated to bear epitopes recognized by *Yersinia*-specific synovial fluid $CD4^+$ T cells in two patients with *Yersinia*-induced reactive arthritis (243). On the basis of the observation that other epitopes cross-react with other enteric organisms that do not cause reactive arthritis, it was suggested that other *Yersinia* macromolecules may also be required for the development of this syndrome. This was later confirmed by Skurnik et al. (264), who also implicated the YadA protein in this process.

U. urealyticum in Urinary Tract Infection and Respiratory Disease

U. urealyticum, a bacterium that lacks a traditional cell wall, produces abundant urease, which is clearly central to the pathogenesis of this species. Construction of urease-negative mutants, however, has not been forthcoming because of the difficulties associated with genetic manipulation of this species. Also, because of the absolute requirement for urea hydrolysis for generation of ATP (265), such a mutation would probably be lethal and therefore no mutant could be isolated. ATP synthesis has been proposed to be driven by an ammonia chemical potential, derived from cytoplasmic urease-mediated ureolysis. This bioenergetic mechanism appears to be unique to the *Ureaplasma* genus and has not been described for other bacterial species.

The pathogenesis ascribed to the *U. urealyticum* urease is associated principally with urolithiasis in patients infected with

this organism (113). In a study of 24 patients with infection-induced stones requiring surgery for removal, *U. urealyticum* was cultured from 7 patients and was the only urease-positive species in 4 individuals. A canine model of upper UTI has been used to demonstrate that while *U. urealyticum* could not survive in canine urine for an extended period, the organism could persist in the kidneys of obstructed animals for at least 21 days (149). A rat model of urinary tract infection has also been developed (7).

U. urealyticum has also been implicated in respiratory disease. This organism was the single most common isolate cultured from endotracheal aspirate from 200 low-birth-weight infants (30). As an animal model, newborn C3H/HeN mice were used to study *Ureaplasma* respiratory infection (252). The organism was found to produce an acute, self-limiting, interstitial pneumonia in the newborn mice; 14-day-old mice were less susceptible to infection. Again, because no mutants were available, it is not possible to assess the specific role of urease in this syndrome.

As a demonstration of the high specific activity of *U. urealyticum* urease, suspensions of these bacteria killed mice 5 min after intravenous injection (160). This toxicity was inhibited by flurofamide, a potent urease inhibitor, demonstrating that urease was the toxic factor. Although such intravenous injection probably does not mimic the course of any natural infection, this study underscores the potential toxicity associated with urease-mediated serum urea hydrolysis.

Bordetella bronchiseptica and Respiratory Disease in Animals

Bordetella bronchiseptica, a urease-positive organism, is the etiologic agent of respiratory diseases in laboratory and domestic animals (kennel cough in dogs and atrophic rhinitis and pneumonia in piglets) (98). To examine the role of urease in such infections, an unmarked *ureC* deletion mutant was constructed in a wild-type *Bordetella bronchiseptica* strain (205). The mutant and wild-type strains were used in coinfection studies to assess colonization of the guinea pig respiratory and digestive tracts. The urease-negative strains outcompeted the urease-positive parent strain, demonstrating that urease is not essential for *Bordetella bronchiseptica* colonization of the guinea pig respiratory and digestive tracts.

Urease in Vaccines

The concept of vaccination with urease has been with us for some time. J. B. Sumner, who earned a Nobel prize in 1946 for his earlier work (274) involving the first crystallization of an enzyme (jack bean urease), demonstrated that this enzyme could be used as an antigen (144, 145) capable of stimulating a strong immunoglobulin response. Sera collected from rabbits immunized with the purified enzyme were shown to inhibit urease activity in a titratable fashion. Subsequent reports (56, 159, 282) of the results of studies in which the plant enzyme was used as a vaccine relied, in theory, on the inhibition of catalytic activity to explain the mechanism of protection. However, no such direct inhibition of enzyme activity by polyclonal antisera has since been described for bacterial ureases. A single report (218), however, describes the synergistic inhibition of *H. pylori* urease activity by two monoclonal antibodies raised against the purified enzyme.

More-recent studies employed jack bean urease as a vaccine to prevent urea hydrolysis by bacterial ureases. In each study, the presumed mechanism of protection was inhibition of bacterial urease activity and thus the prevention of ammonium hydroxide accumulation. Rats vaccinated with jack bean urease were protected from necrosis induced by subcutaneous injection of *M. morganii* (formerly *P. morganii*), a ureolytic uropathogenic enteric species (159). Jack bean urease immunization was also shown to promote increased growth of farm animals including young chickens, hens, lambs, and pigs (241). In a small clinical trial, vaccination with increasing doses of jack bean urease alleviated the symptoms of hepatic encephalopathy due to ammonia toxicity in a limited number of patients (56).

Recently, the concept of using urease as a vaccine has been most sharply focused at prevention of gastritis, peptic ulcer disease, and possibly gastric cancer by protection against infection with *H. pylori*. Because urease is the most prominent antigenic component of the organism and elicits a powerful immunoglobulin response in infected individuals, a number of research groups have reasoned that this enzyme would be a logical agent for a vaccine. The concept is confounded by the fact that a vigorous antiurease response already exists in infected persons and that this response is not sufficient to clear *H. pylori* from the host. The feasibility of vaccine development, however, is driven by the premise that it is easier to prevent an infection caused by a relatively few organisms than it is to clear an established infection in which the gastric mucosa is colonized at a high density. In addition, new techniques of oral immunization coupled with powerful adjuvants may provide a secretory and serum immunoglobulin response that is sufficient to both prevent and perhaps even clear a chronic *H. pylori* infection.

Following the report of the nucleotide sequence of *ureA* and *ureB* (39), it was noted that the predicted amino acid sequence of the *H. pylori* urease shared >50% similarity with that of the jack bean urease. Pallen and Clayton (232) suggested that oral immunization or simple ingestion of the plant urease may serve as the basis for vaccination. This possibility was addressed experimentally by Chen et al. (32), who orally immunized mice with jack bean urease and a cholera toxin adjuvant. Mice were found not to be protected by oral challenge with *H. felis* (a close relative of *H. pylori* that infects mice), thus refuting this interesting hypothesis. It is, of course, more logical to use the urease that is produced by the organism for which the vaccine is being developed. Jack bean urease, however, has been used as a substitute for the bacterial enzymes primarily because of the ready commercial availability of the purified plant enzyme. It is worthy of mention that concanavalin A, a T-cell mitogen, is also produced by the jack bean. Indeed, urease was first called "canavalin" (after the jack bean genus, *Canavalia*), and thus "concanavalin A" originally meant "not urease."

Recently, the feasibility was tested that oral vaccination with *Helicobacter* species (which produce urease as their most abundant antigen) would protect mice against infection with *H. felis*. Czinn et al. (54) reported protection of germ-free mice from infection with *H. felis* by active or passive immunoglobulin A immunization with monoclonal antibodies directed against *H. pylori*. Chen et al. (31) reported that mice, immunized with a *H. felis* sonicate and cholera toxin adjuvant, were protected from colonization by an oral challenge of *H. felis*. This group later demonstrated that the nontoxic cholera toxin B subunit, when used as an adjuvant for the sonicate, also conferred significant protection (153). While some of the protective effect observed in these experiments was undoubtably due to antiurease response, these issues were specifically addressed by two groups using similar strategies, again with the mouse model and an *H. felis* challenge. Michetti et al. (192) found moderate but significant protection when purified urease was used for immunization. However, when urease structural subunits UreA and UreB, purified as histidine-tagged fusion proteins on a Ni^{2+}-nitrilotriacetic acid column, were used for immunization, mice were protected from colonization of the gastric mucosa by *H. felis*. UreB, which harbors the enzyme active site, gave earlier

and more complete protection from the challenge. In later studies (48), oral immunization with UreB was used therapeutically to effect eradication. Likewise, in independent efforts, Ferrero et al. (83) used *H. pylori* and *H. felis* UreA and UreB, purified as maltose-binding protein translational fusions, to immunize mice with cholera toxin as the adjuvant. UreB provided more significant protection than UreA, and the *H. felis*-derived subunits conferred better protection than the *H. pylori* urease subunits. Also, Pappo et al. (233) and Lee et al. (154) used recombinant apourease (catalytically inactive enzyme encoded by clones carrying only *ureAB*) for immunization and also showed significant protection of mice against *H. felis* challenge. In the latter studies, protection was correlated with high secretory immunoglobulin A titer (77, 154, 233). These studies provided strong evidence that oral immunization with urease in the presence of adjuvant is a feasible strategy for development of a vaccine for prevention of *H. pylori* infection in humans. The recent development of a mouse model for the direct study of *H. pylori* rather than the related species *H. felis* will undoubtedly speed the development of vaccines (179). Identification of an adjuvant that is suitable for use in humans will be a necessary next step.

Urease genes have also been used to mark a vaccine strain. *P. mirabilis* urease genes (132) were used as a nonantibiotic genetic marker to tag a recombinant *Vibrio cholerae* live oral attenuated vaccine strain (142). The construct thus possesses a cryptic marker that allows differentiation between wild-type and vaccine strains. Interestingly, the urease expressed in *V. cholerae* was not catalytically active when the organism was grown in culture medium not supplemented with $NiCl_2$. This suggested that *V. cholerae* does not possess a high-affinity nickel transport system that is necessary to provide nickel ions for the active site of the metalloenzyme.

CONCLUSIONS

The progress in the last 6 years has markedly increased our understanding of the genetic organization of urease gene clusters, the regulation of expression of these genes, the biochemistry of ureolysis including nickel metallocenter assembly and the three-dimensional structure of the enzyme, and the molecular pathogenesis of urease including the role of urease in UTI, peptic ulcer disease, and reactive arthritis.

Although the progress has been astounding, specific areas will benefit from intensive analysis. While nucleotide sequences are available for five entire gene clusters, the transcriptional organization is rather poorly understood. True promoters that have been unambiguously identified are few, and mRNA transcript lengths have not been reported. The mechanisms of gene regulation are beginning to be analyzed; however, interaction of specific DNA sequences with regulatory proteins has not been forthcoming. More comprehensive protein structural studies, when combined with further experiments to detail the catalytic mechanism and the interactions with active-site directed inhibitors, may allow the rational design of novel inhibitors for agricultural and pharmacological use. It has been well established that accessory proteins are required for nickel activation, but only a few studies have analyzed the specific interaction of these proteins with the urease apoprotein. Further, urease has been identified as a critical virulence determinant in development of UTI, acute pyelonephritis, and peptic ulcer disease, but the specific mechanisms of pathogenesis have been simplified and are still quite unclear. Judging from the volume of work that has been undertaken in the past 6 years, we anticipate that many of these research questions soon will be addressed.

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