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

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Abstract

Comparison of six urease sequences revealed the presence of 10 conserved histidine residues (H96 in the γ subunit, H39 and H41 in β, and H134, H136, H219, H246, H312, H320, and H321 in the α subunit of the *Klebsiella aerogenes* enzyme). Each of these residues in *K. aerogenes* urease was substituted with alanine by site-directed mutagenesis, and the mutant proteins were purified and characterized in order to identify essential histidine residues and assign their roles. The γH96A, βH39A, βH41A, αH312A, and αH321A mutant proteins possess activities and nickel contents similar to wild-type enzyme, suggesting that these residues are not essential for substrate binding, catalysis, or metal binding. In contrast, the αH134A, αH136A, and αH246A proteins exhibit no detectable activity and possess 53%, 6%, and 21% of the nickel content of wild-type enzyme. These results are consistent with αH134, αH136, and αH246 functioning as nickel ligands. The αH219A protein is active and has nickel (~1.9% and ~80%, respectively, when compared to wild-type protein) but exhibits a very high K_m value (1,100 ± 40 mM compared to 2.3 ± 0.2 mM for the wild-type enzyme). These results are compatible with αH219 having some role in facilitating substrate binding. Finally, the αH320A protein (K_m = 8.3 ± 0.2 mM) only displays ~0.003% of the wild-type enzyme activity, despite having a normal nickel content. Unlike the wild-type and αH219A ureases, this mutant protein was not inactivated by diethylpyrocarbonate (DEP), consistent with αH320 being the DEP-reactive general base that facilitates catalysis.

Keywords: histidine; *Klebsiella aerogenes*; nickel ligands; site-directed mutagenesis; urease

Urease (EC 3.5.1.5), found in a variety of plants and a broad range of bacterial species, is a nickel-containing enzyme that catalyzes the hydrolysis of urea to form carbonic acid and two molecules of ammonia (Mobley & Hausinger, 1989). The best studied microbial urease is that from the enteric bacterium, *Klebsiella aerogenes*. The enzyme is comprised of three subunits (M_r = 60,304 [α], 11,695 [β], and 11,086 [γ] [Mulrooney & Hausinger, 1990]) and possesses two nickel ions per $\alpha\beta_2\gamma_2$ catalytic unit (Todd & Hausinger, 1987, 1989). Although *K. aerogenes* urease has been crystallized and the crystals shown to diffract to less than 2 Å (Jabri et al., 1992), the three-dimensional structure of the enzyme has not yet been elucidated. Nevertheless, several structural features of the bacterial urease active site have been characterized, and multiple essential roles for histidine residues have been implicated. For example, the pH dependence of enzyme activity is consistent with the presence of two chemical groups at the active site that participate in catalysis as a general base (pK_a = 6.55) and a general acid (pK_a = 8.85) (Todd & Hausinger, 1987). Chemical modification studies with the histidine-selective reagent DEP were compatible with a histidine residue serving as the general base (Park & Hausinger, 1993). Additional chemical modification

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Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; DEP, diethylpyrocarbonate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IAM, iodoacetamide; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; TAPS, *N*-tris(hydroxylmethyl)methyl-3-aminopropanesulfonic acid; Tricine, *N*-tris(hydroxymethyl)-methylglycine.

and site-directed mutagenesis studies demonstrated the presence of an active-site cysteine residue (C319 in the α subunit) in the *K. aerogenes* enzyme (Todd & Hausinger, 1991b; Martin & Hausinger, 1992). The pH dependence of urease inactivation by disulfide and alkylating reagents (Todd & Hausinger, 1991a), and the shift in pH optimum observed for C319A, C319S, and C319D mutant proteins compared to wild-type enzyme (Martin & Hausinger, 1992) were interpreted in terms of the cysteine residue ionically interacting with a second residue (X), together acting as a proton donor during catalysis. Although the identity of X is unknown, the pH-dependent behavior of urease inactivation by thiol-specific chemical reagents is reminiscent of studies involving papain where a Cys–His ion pair has been characterized (Brocklehurst, 1987). Finally, Lee et al. (1990) compared the chemical reactivity of *K. aerogenes* holoenzyme and apoprotein toward DEP and found that more histidines are accessible to the reagent in the nickel-free protein. The enhanced DEP reactivity of apoprotein is consistent with histidine residues participating as nickel metallocenter ligands in urease.

This study combines site-directed mutagenesis and enzyme characterization methods to identify several essential histidine residues in *K. aerogenes* urease and to define their roles. Comparison of the urease amino acid sequences from jack bean (Takishima et al., 1988), *Helicobacter pylori* (Clayton et al., 1990; Labigne et al., 1991), *Ureaplasma urealyticum* (Blanchard, 1990), *Proteus vulgaris* (Mörsdorf & Kaltwasser, 1990), *Proteus mirabilis* (Jones & Mobley, 1989), and *K. aerogenes* (Mulrooney & Hausinger, 1990) revealed the presence of 10 conserved histidines: H96 in the γ subunit, H39 and H41 in β, and H134, H136, H219, H246, H312, H320, and H321 in the α subunit of the *K. aerogenes* enzyme. We substituted each of the conserved histidine residues in the *K. aerogenes* enzyme with alanine (a residue that contains a side chain that cannot function as a metallocenter ligand or general base or general acid, cannot participate in hydrogen bond interactions, and is smaller than the wild-type residue so that it will not cause steric disruption of the structure), purified the mutant proteins, and characterized their enzyme activities, nickel contents, and reactivities with DEP and IAM. The results suggest that histidine residues in the α subunit may play key roles with H134, H136, and H246 participating in the ligation of nickel, H219 facilitating the binding of substrate, and H320 acting as a general base in catalysis.

Results

Initial characterization of mutant proteins

Escherichia coli cells containing the *K. aerogenes* urease genes on plasmid pKAU17 or derivative plasmids with His → Ala substitutions were grown under culture conditions that led to high level synthesis of the wild-type and mutant ureases. The wild-type and mutant proteins were highly purified (Fig. 1) and the specific activities, K_m values, and nickel contents were determined (Table 1). The γH96A, βH39A, βH41A, αH312A, and αH321A proteins have K_m values, specific activities, and nickel contents that are similar to the wild-type enzyme, indicating that these histidine residues are not likely to be important for substrate binding, catalysis, or nickel ligation. In contrast, the other five mutant proteins exhibit significant changes in their properties, consistent with important roles for the αH134, αH136, αH219, αH246, and αH320 residues. These mutant proteins fall into three classes, each of which is described separately below.

The αH134A, αH136A, and αH246A proteins are inactive and possess approximately 50% of the normal metal content (αH134A) or the near absence of nickel (αH136A and αH246A). These results are compatible with αH134, αH136, and αH246 functioning as nickel ligands in the enzyme. It remains unclear whether the latter two residues may bridge the two metal atoms at the active site so that

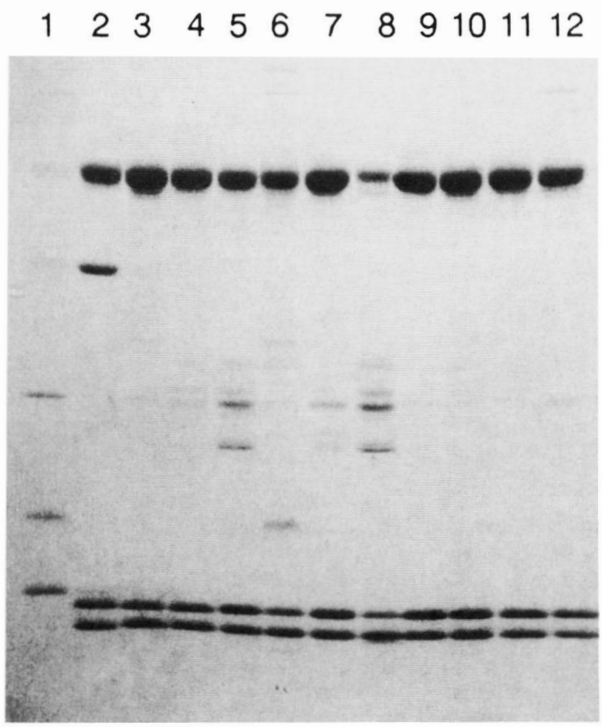

Fig. 1. Denaturing gel electrophoretic analysis of partially purified urease proteins. Samples were run on a 10–15% polyacrylamide gradient gel and stained with Coomassie brilliant blue. The percent purity for each sample was assessed by using an AMBIS gel scanner. Lane 1, molecular weight markers (phosphorylase *b*, M_r 92,500; bovine serum albumin, M_r 66,200; ovalbumin, M_r 45,000; carbonic anhydrase, M_r 31,000; soybean trypsin inhibitor, M_r 21,500; and lysozyme, M_r 14,400). The protein designations and percent purities for the samples are indicated for: lane 2, γH96A (72%); lane 3, βH39A (96%); lane 4, βH41A (90%); lane 5, αH134A (70%); lane 6, αH136A (80%); lane 7, αH219A (93%); lane 8, αH246A (52%); lane 9, αH312A (>98%); lane 10, αH320A (>98%); lane 11, αH321A (98%); and lane 12, wild type (88%).

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

Urease	K_m (mM)	Specific activity		Nickel content	
		U/mg	%	No./ catalytic unit	%
Wild-type	2.3 ± 0.2	1,900	100	2.1	100
γH96A	1.9 ± 0.2	1,700	90	2	95
βH39A	1.5 ± 0.2	1,500	79	2.1	100
βH41A	1.4 ± 0.2	1,300	68	2.5	120
αH134A	–	<0.001	–	1.1	53
αH136A	–	<0.001	–	0.13	6
αH219A	1,100 ± 40[b]	36[b,c]	1.9	1.7	80
αH246A	–	<0.001	–	0.44	21
αH312A	1.6 ± 0.2	1,800	95	2.2	105
αH320A	8.3 ± 0.2[d]	0.051[b,e]	0.0027	2.3	110
αH321A	2.0 ± 0.2	1,700	90	2.4	114

[a] Determined for enzyme purified by DEAE-Sepharose and Mono-Q column chromatographies as illustrated in Figure 1, except where indicated.
[b] Determined by using enzyme purified only with DEAE-Sepharose column chromatography.
[c] Determined by using 1 M urea instead of the standard assay.
[d] Determined for enzyme in cell extracts.
[e] Determined by using pH 6.75 buffer instead of the normal 7.75 buffer.

neither nickel can bind in the absence of these double ligands, or if the increased lability of one active-site nickel atom by loss of a ligand leads to lability of the second metal ion. Similarly, it is unknown whether the 50% nickel content of the αH134A protein represents a case where one nickel is incorporated into each catalytic unit randomly between the two nickel sites or whether one of the two nickel sites is filled while the other remains unoccupied due to the loss of an essential ligand.

The αH219A protein exhibits a very high K_m value for urea coupled with a large decrease in specific activity (although the enzyme activity could not be assayed under saturating conditions, the calculated V_{max} value was about 3% of that found in the wild-type protein). The altered properties of the αH219A protein apparently are not due to changes in nickel content because, at most, 20% of the nickel was lost in this mutant protein compared to wild-type enzyme. Rather, the high K_m value of the αH219A protein suggests that the αH219 residue is somehow important to substrate binding. For example, the histidine residue may facilitate urea binding by forming a hydrogen bond with the substrate. Alternatively, however, the results are compatible with a model in which the imidazole group simply props open the substrate-binding site and maintains its accessibility.

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

Thermal stabilities of mutant ureases

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

pH dependence of mutant urease activities

The pH optimum of the wild-type enzyme (Fig. 2A) at around pH 7.75 is retained in the active mutant proteins γH96A, βH39A, βH41A, αH219A, αH312A, and αH321A, as illustrated for the αH219A protein (Fig. 2B). The results are consistent with the wild-type general base and general acid groups being retained in these mutant proteins. In contrast, the αH320A protein exhibited a significantly shifted pH optimum at around 6.75 (Fig. 2C). Hence, one of the residues serving as a general base or general acid may have been mutated in the αH320A protein or the mutation may have indirectly shifted one of these pK_a values.

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

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

[a] Values are expressed as percentage of that for control samples that were not subjected to high temperature incubation. In each case, cell extracts were incubated in 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM β-mercaptoethanol at the indicated temperatures for up to 2 h, and the activities were measured by using the standard assay conditions, except where indicated.
[b] Activities for the αH219A protein were measured by using a concentration of 100 mM urea.
[c] Assays for the αH320A protein were carried out at pH 6.75.

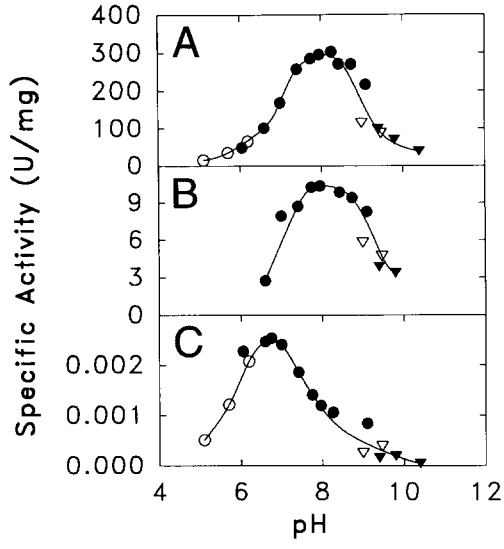

Fig. 2. The pH dependence of urease activity. Analysis using (**A**) cell extracts of *E. coli* DH5[pKAU17], (**B**) purified αH219A enzyme, and (**C**) cell extracts containing the αH320A mutant protein. The reaction mixtures contained urea (50 mM for wild-type enzyme and αH320A protein or 1 M for the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

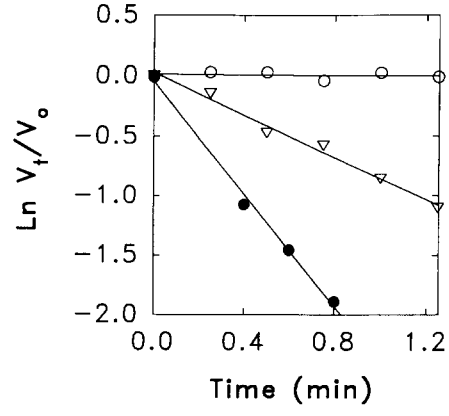

Fig. 3. Kinetics of urease inactivation by DEP. Purified wild-type (●), αH219A (▽), and αH320A (○) proteins were treated with DEP (50 μM, 1 mM, and 1 mM, respectively) in 1 mM EDTA and 50 mM HEPES (pH 7.0) buffer. The natural logarithm of V_t/V_0 (where V_t is the velocity at time t and V_0 is the initial velocity) is shown as a function of time.

Inactivation of mutant ureases by DEP

The properties of the αH219A and αH320A mutant proteins were consistent with these two histidine residues playing important roles in the enzyme other than metallocenter ligation. Because chemical modification studies of urease using DEP had provided evidence for an essential histidine residue acting as a general base in the native enzyme (Park & Hausinger, 1993), the DEP reactivities of these two mutant proteins were assessed. Although the wild-type enzyme was rapidly inactivated by 50 μM DEP (Fig. 3), both the αH219A and αH320A proteins appeared to retain full enzyme activity after the same treatment (data not shown). At 1 mM DEP, however, the H219A protein was inactivated in a pseudo-first-order process, whereas the αH320A protein continued to be resistant to inactivation by DEP. Further characterization of DEP inactivation of the αH219A protein demonstrated that the rate was pH dependent (Fig. 4) with the pattern for inactivation of the αH219A ($pK_a = 6.8$) and wild-type ($pK_a = 6.5$) proteins being nearly identical. These data are inconsistent with αH219 serving as the general base for catalysis in the wild-type enzyme. Rather, the reduced level of DEP reactivity observed for the αH219A protein may be due to the same features that account for the high K_m value of this enzyme. The structure of DEP shares some similarity to that of urea, hence, the binding of this reagent to the active site correspondingly may be reduced in affinity. Alternatively, if αH219 is important for maintaining access to the catalytic site the reduced reactivity in the mutant protein may derive from partial closure of this region. In contrast to the DEP reactivity of the αH219A protein, the resistance to inactivation by DEP displayed by the αH320A protein is compatible with αH320 serving as the target of DEP in the native enzyme, i.e., the general base that facilitates catalysis.

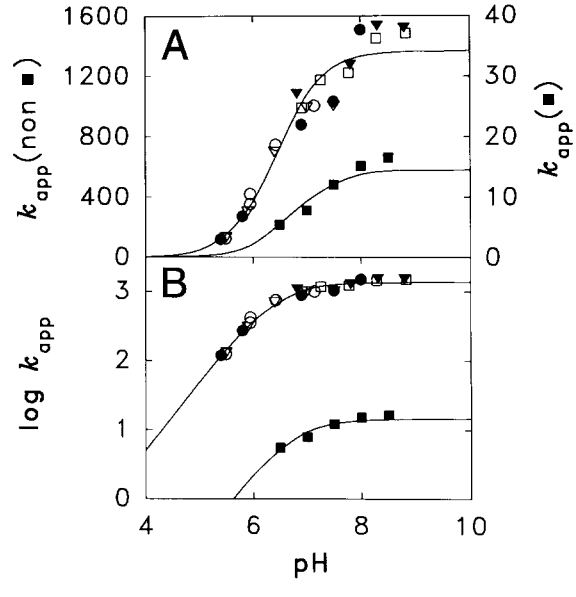

Fig. 4. The pH dependence of DEP inactivation of wild-type and αH219A ureases. **A:** Apparent second-order rate constants ($M^{-1}\ s^{-1}$) for urease inactivation with DEP (left axis for wild-type enzyme and right axis for the αH219A enzyme) are plotted as a function of pH. **B:** The same data plotted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

Inactivation of mutant proteins by IAM

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

Discussion

Dixon et al. (1980) proposed an elegant model for the hydrolysis of urea by the bi-nickel active site of jack bean (*Canavalia ensiformis*) urease that serves as an excellent framework for discussion of *K. aerogenes* urease site-directed mutagenesis. In their model, one nickel ion is

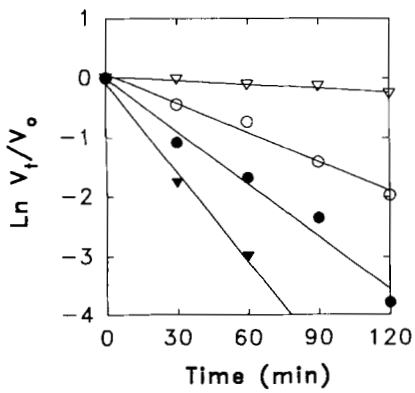

Fig. 5. Kinetics of urease inactivation by IAM. Purified wild-type (●), αH219A (▽), and αH320A (○) proteins were treated with 40 mM IAM and purified αH219A (▼) protein was treated with 400 mM IAM in 1 mM EDTA and 50 mM HEPES (pH 7.75) buffer. The natural logarithm of V_t/V_0 (where V_t is the velocity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

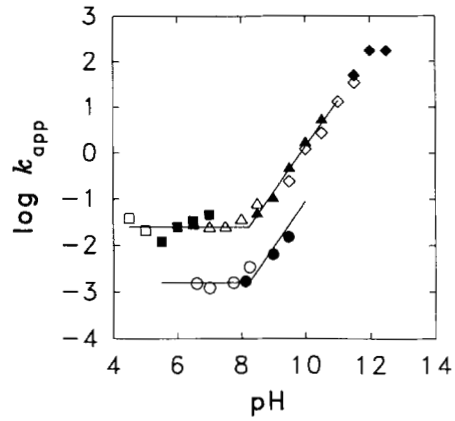

Fig. 6. The pH dependence of IAM inactivation of wild-type and αH219A ureases. Apparent second-order rate constants (M^{-1} s^{-1}) for urease inactivation with IAM (20 mM for wild-type enzyme and 120 mM for the αH219A enzyme) are plotted as a function of pH. The reactions were carried out either in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

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

In studies with *K. aerogenes* urease, known to be ~60% identical in sequence to the jack bean enzyme (Mulrooney & Hausinger, 1990), the Dixon model has undergone further elaboration. The chemical reactivity of the general base ($pK_a \cong 6.5$) that appears to facilitate catalysis (Todd & Hausinger, 1987) was shown to be compatible with that of a histidyl group (Park & Hausinger, 1993). The thiol group that participates in proton donation was identified (Todd & Hausinger, 1991b; Martin & Hausinger, 1992) and shown to function as an ion pair with another resi-

due (X) that could reasonably be accounted for by a histidine group (Todd & Hausinger, 1991a). Finally, the enhanced DEP reactivity of apoprotein over holoenzyme (Lee et al., 1990) is consistent with at least partial metallocenter ligation by histidyl residues. The multiple potential roles for histidine groups in the protein led to the experiments described above.

Our results are consistent with the participation of three histidyl residues (αH134, αH136, and αH246) in nickel coordination, one histidine residue (αH320) serving as the general base in catalysis, and one residue (αH219) somehow facilitating substrate binding. The latter residue may stabilize urea binding by hydrogen-bond formation (similar to the ionic stabilization of bound urea by a postulated carboxyl group in the model of Dixon et al. [1980]), or perhaps αH219 may act by maintaining accessibility to the catalytic site. No evidence was obtained for the presence of a conserved histidine acting as residue X in a Cys–X pair that functions as a general acid. Further efforts toward elucidating the three-dimensional structure of *K. aerogenes* urease by X-ray crystallographic methods (cf. Jabri et al., 1992) will establish the validity of several of the roles for histidine residues that have been proposed here.

Materials and methods

Materials

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

Site-directed mutagenesis

For generation of γH96A, βH39A, and βH41A, a 1.4-kb *Sac*I–*Sma*I fragment of the pKAU17 (Mulrooney et al., 1989) was subcloned into M13 mp18 and mutagenized by the method of Kunkel et al. (1987). For αH134A, αH136A, αH219A, αH246A, αH312A, αH320A, and αH321A, a 1.1-kb *Bam*H1–*Sal*I fragment of the same plasmid was used. Uracil-containing single-stranded template DNA was prepared from *E. coli* CJ236 (*dut*1 *ung*1 *thi*-1 *rel*A1/pCJ105[*cam*^{r} F']). Mutagenized phage were isolated in *E. coli* MV1193 (Δ[*lacI-pro*AB] *rpsL thi endA spcB*15 *hsdR*4 Δ[*srl-rec*A]306::Tn10[*tet*^{r}] F'[*traD*36 *pro*AB$^+$ *lacI*^{q}*lacZ*ΔM15]). The following oligonucleotides were synthesized by using an Applied Biosystems Model 394 DNA synthesizer at the Michigan State University Macromolecular Structural Facility: AATCGGGTTGG CAACGGTGAC, GAAATGGTAGGCCGAACCGAC, CTCGGCGAAAGCGTAGTGCG, CTGAAGATCGCT GAGGACTGG, GGTCGCCCTGGCCAGCGACACC, GGATCGATACCGCTATTCACTG, ACCCATATTGC CTGGATCTGT, CCATCGATGAAGCTCTCGATATG, ATGGTCTGCGCCCATCTGGAC, and GTCTGCCAC GCTCTGGACCCG. These primers were used to alter the 10 conserved histidine codons to encode alanine at each position. Site-directed mutants were identified by DNA sequencing and subcloned back into pKAU17 on a 1.1-kb *Sac*I–*Mlu*I fragment for γH96A, βH39A, and βH41A and a 0.8-kb *Mlu*I–*Bsm*I fragment for the other mutations. These regions were completely sequenced by using Sequenase 2.0 (United States Biochemicals) and the single-strand DNA sequencing method of Sanger et al. (1977) to ensure that no other mutations had been introduced into M13. In one case, sequence analysis demonstrated that a recombination event had occurred, and an alternate clone that possessed the desired muation was chosen for further studies. After subcloning, the mutated sequences were again confirmed by double-strand DNA sequencing methods (Sambrook et al., 1989).

Enzyme purification

Ureases were purified from *E. coli* DH5 carrying pKAU17 or the site-directed mutants of pKAU17 by procedures described previously (Todd & Hausinger, 1989), except that cells were grown in LB medium containing 1 mM $NiCl_2$. As noted by Lee et al. (1992) and Martin and Hausinger (1992), the wild-type enzyme isolated from cells grown under these conditions does not exhibit the maximum specific activity observed for enzyme isolated from *K. aerogenes* [pKAU19] cells (2,500 units mg^{-1}; Todd & Hausinger [1989]). This decreased activity does not result from a difference in nickel content but rather may be related to problems arising from the high levels of urease biosynthesis or to unknown host-dependent effects (Lee et al., 1992). Because urease synthesis in cells containing the mutated plasmids is similar to that in cells producing wild-type enzyme, it is reasonable to directly compare their relative specific activities and nickel contents. Purification of inactive mutant proteins (αH134A, αH136A, αH219A, αH246A, and αH320A) was monitored for urease-containing fractions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using 10–15% polyacrylamide gradient gels and the buffers described by Laemmli (1970). Sample purities were determined by using a gel scanner (AMBIS Inc.), and the measured values were used for correction of enzyme activities and nickel contents.

Assay of enzyme activity

The urease activities for wild-type and mutant proteins except αH219A and αH320A were assayed in 25 mM

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

Nickel quantitation

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

Thermal stability

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

Inactivation by DEP

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

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

Inactivation by IAM

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

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References

Blanchard, A. (1990). *Ureaplasma urealyticum* urease genes; use of a UGA tryptophan codon. *Mol. Microbiol. 4*, 669–679.

Brocklehurst, K. (1987). Acyl group transfer—Cysteine proteinases. In *Enzyme Mechanisms* (Page, M.I. & Williams, A., Eds.), pp. 140–158. Royal Society of Chemistry, Burlington House, London.

Clayton, C.L., Pallen, M.J., Kleanthous, J., Wren, B.W., & Tabaqchali, S. (1990). Nucleotide sequence of two genes from *Helicobacter pylori* encoding for urease subunits. *Nucleic Acids Res. 18*, 362.

Cousineau, J. & Meighen, E. (1976). Chemical modification of bacterial luciferase with ethoxyformic anhydride: Evidence for an essential histidyl residue. *Biochemistry 15*, 4992–5000.

Dixon, N.E., Riddles, P.W., Gazzola, C., Blakeley, R.L., & Zerner, B. (1980). Jack bean urease (EC 3.5.1.5). V. On the mechanism of action of urease on urea, formamide, acetamide, *N*-methylurea, and related compounds. *Can. J. Biochem. 58*, 1335–1344.

Jabri, E., Lee, M.H., Hausinger, R.P., & Karplus, P.A. (1992). Preliminary crystallographic studies of urease from jack bean and from *Klebsiella aerogenes* urease. *J. Mol. Biol. 227*, 934–937.

Jones, B.D. & Mobley, H.L.T. (1989). *Proteus mirabilis* urease: Nucleotide sequence determination and comparison with jack bean urease. *J. Bacteriol. 171*, 6414–6422.

Kunkel, T.A., Roberts, J.D., & Zakour, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol. 154*, 367–382.

Labigne, A., Cussac, V., & Courcovx, P. (1991). Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *J. Bacteriol. 173*, 1920–1931.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature 227*, 680–685.

Lee, M.H., Mulrooney, S.B., & Hausinger, R.P. (1990). Purification, characterization, and in vivo reconstitution of *Klebsiella aerogenes* urease apoenzyme. *J. Bacteriol. 172*, 4427–4431.

Lee, M.H., Mulrooney, S.B., Renner, M.J., Markowicz, Y., & Hausinger, R.P. (1992). *Klebsiella aerogenes* urease gene cluster: Sequence of *ureD* and demonstration that four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are involved in nickel metallocenter biosynthesis. *J. Bacteriol. 174*, 4324–4330.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193*, 265–275.

Martin, P.R. & Hausinger, R.P. (1992). Site-directed mutagenesis of the active site cysteine in *Klebsiella aerogenes* urease. *J. Biol. Chem. 267*, 20024–20027.

Miles, E.W. (1977). Modification of histidinyl residues in proteins by diethylpyrocarbonate. *Methods Enzymol. 47*, 431–442.

Mobley, H.L.T. & Hausinger, R.P. (1989). Microbial urease: Significance, regulation, and molecular characterization. *Microbiol. Rev. 53*, 85–108.

Mörsdorf, G. & Kaltwasser, H. (1990). Cloning of the genes encoding urease from *Proteus vulgaris* and sequencing of the structural genes. *FEMS Microbiol. Lett. 66*, 67–74.

Mulrooney, S.B. & Hausinger, R.P. (1990). Sequence of the *Klebsiella aerogenes* urease genes and evidence for accessory proteins facilitating nickel incorporation. *J. Bacteriol. 172*, 5837–5843.

Mulrooney, S.B., Pankratz, H.S., & Hausinger, R.P. (1989). Regulation of gene expression and cellular localization of cloned *Klebsiella aerogenes* (*K. pneuminae*) urease. *J. Gen. Microbiol. 135*, 1769–1776.

Park, I.-S. & Hausinger, R.P. (1993). Diethylpyrocarbonate reactivity of *Klebsiella aerogenes* urease: Effect of pH and active site ligands on rate of enzyme inactivation. *J. Protein Chem. 12*, 51–56.

Sakaguchi, K., Mitsui, K., Kubashi, K., & Hase, J. (1983). Photo-oxidation of jack bean urease in the presence of methylene blue. *J. Biochem. 93*, 681–686.

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Sanger, F., Mickle, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA 80*, 3963–3965.

Takishima, K., Suga, T., & Mamiya, G. (1988). The structure of jack bean urease. The complete amino acid sequence, limited proteolysis and reactive cysteine residues. *Eur. J. Biochem. 175*, 151–165.

Todd, M.J. & Hausinger, R.P. (1987). Purification and characterization of the nickel-containing multicomponent urease from *Klebsiella aerogenes*. *J. Biol. Chem. 262*, 5963–5967.

Todd, M.J. & Hausinger, R.P. (1989). Competitive inhibitors of *Klebsiella aerogenes* urease: Mechanisms of interaction with the nickel active site. *J. Biol. Chem. 264*, 15835–15842.

Todd, M.J. & Hausinger, R.P. (1991a). Reactivity of the essential thiol of *Klebsiella aerogenes* urease. *J. Biol. Chem. 266*, 10260–10267.

Todd, M.J. & Hausinger, R.P. (1991b). Identification of the essential cysteine residue in *Klebsiella aerogenes* urease. *J. Biol. Chem. 266*, 24327–24331.

Weatherburn, M.W. (1967). Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem. 39*, 971–974.

Wilkinson, G.N. (1961). Statistical estimations in enzyme kinetics. *Biochem. J. 80*, 324–332.