Chemical Rescue of *Klebsiella aerogenes* Urease Variants Lacking the Carbamylated-Lysine Nickel Ligand[†,‡]

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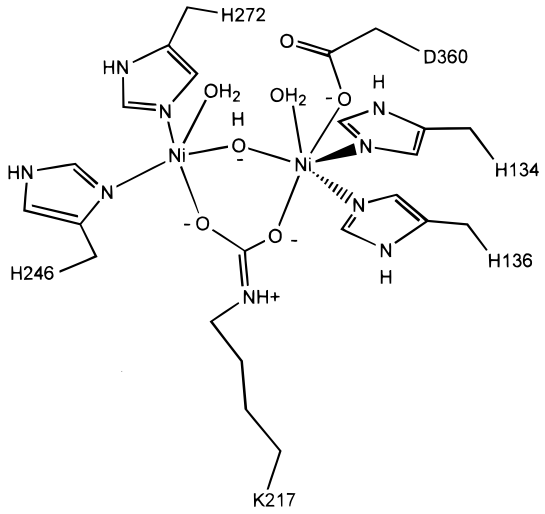
ABSTRACT: *Klebsiella aerogenes* urease possesses a dinuclear metallocenter in which two nickel atoms are bridged by carbamylated Lys217. To assess whether carbamate-specific chemistry is required for urease activity, site-directed mutagenesis and chemical rescue strategies were combined in efforts to place a carboxylate group at the location of this metal ligand. Urease variants with Lys217 replaced by Glu, Cys, and Ala (K217E, K217C/C319A, and K217A proteins) were purified, shown to be activated by incubation with small organic acids plus Ni(II), and structurally characterized. K217C/C319A urease possessed a second change in which Cys319 was replaced by Ala in order to facilitate efforts to chemically modify Cys217; however, this covalent modification approach did not produce active urease. Chemical rescue of the K217E, K217C/C319A, and K217A variants required 2, 2, and 10 h, respectively, to reach maximal activity levels. The highest activity generated [224 μmol of urea degraded·min^{-1}·(mg of protein)$^{-1}$, for K217C/C319A urease incubated with 500 mM formic acid and 10 mM Ni at pH 6.5] corresponded to 56% of that measured for in vitro activation of the wild-type apoprotein. While the K217E apoprotein showed minimal structural perturbations, the K217C/C319A apoprotein showed a disordering of some active site residues, and the K217A apoprotein revealed a repositioning of His219 to allow the formation of a hydrogen bond with Thr169, thus replacing the hydrogen bond between the amino group of Lys217 and Thr169 in the native enzyme. Importantly, these structures allow rationalization of the relative rates and yields of chemical rescue experiments. The crystal structures of chemically rescued K217A and K217C/C319A ureases revealed a return of the active site residues to their wild-type positions. In both cases, noncovalently bound formate was structurally equivalent to the Lys-carbamate as the bridging metallocenter ligand. We conclude that carbamate-specific chemistry is not required for urease catalysis.

Urease, a nickel-containing enzyme that catalyzes the hydrolysis of urea to form ammonia and carbamate, plays important roles in the nitrogen metabolism of plants and microbes and has been implicated as a virulence factor of some human and animal pathogens (*1*). Three-dimensional structures of *Klebsiella aerogenes* urease and several active-site variants have been resolved to 2.0−2.5 Å (*2*−*4*). The active site is located in the $(\alpha/\beta)_8$-barrel domain of the α subunit and contains a dinuclear nickel metallocenter (Chart 1) ligated by His134, His136, His246, His272, and Asp360 and bridged by carbamylated Lys217, whose presence explains the CO_2 dependence for in vitro activation of the apoprotein (*5*, *6*) (the following experiments deal solely with the urease active site, and all residues discussed are found in the α subunit of the enzyme). Carbamates have somewhat different chemical properties than carboxylates, especially in that they include a resonance form with both oxygen atoms carrying formal negative charges (Chart 2). To address whether carbamate-specific chemistry is required for activity, we attempted to replace the K217-carbamate with a carboxylate group. Herein, we describe the purification and

Chart 1



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‡The coordinates for all structures have been deposited in the Brookhaven Protein Data Bank with access codes 1a5k (K217E), 1a5l (K217C), 1a5m (K217A), 1a5n (rescued K217A), and 1a5o (rescued K217C).

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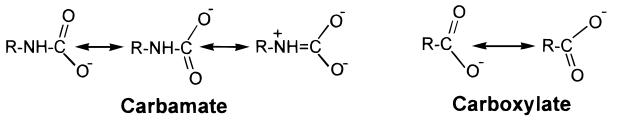
Chart 2



characterization of K217E,[1] K217C/C319A (for convenience this is subsequently referred to as K217C urease), and K217A variant ureases (in which Lys217 was replaced by Glu, Cys, and Ala, respectively). To simplify the interpretation of the results from chemical modification studies, the K217C variant possessed a second change in which Cys319 (a nonessential residue located in the active site) was changed to Ala; C319A urease possesses 50% of the wild-type activity and is resistant to inactivation by thiol-specific chemical reagents (*4*, *7*). The K217E, K217C, and K217A variant proteins lacked urease activity as initially purified or in the presence of added Ni(II); however, the activity was rescued for each protein by the provision of Ni(II) plus small organic acids. The chemical rescue process was kinetically characterized, and the three apoproteins as well as the K217A and K217C variants rescued by formate were structurally characterized by X-ray crystallography. The results obtained for urease are compared with those reported for two other known carbamate-dependent enzymes, ribulosebisphosphate carboxylase/oxygenase (RUBISCO) (*8*) and phosphotriesterase (PTE) (*9*).

EXPERIMENTAL PROCEDURES

Mutagenesis and Protein Purification. Plasmid derivatives were obtained by subcloning a 1.1-kb *Bam*HI-*Sal*I fragment of pKAU17 (*10*) into M13 mp18 and mutagenizing (*11*) with the following oligonucleotides: TTGGCCTGGAGATCCAT, TTGGCCTGGCGATCCAT, and GTTATTGGCCTGTGCATCCATGAGGAC (the underlined bases represent sequence changes). A 560-bp *Hpa*I-*Bam*HI fragment from pC319A (*7*) was substituted into the phage carrying the K217C mutation to change the Cys319 codon to Ala. The 1.1-kb *Mlu*I-*Bam*HI fragments were subcloned into pKAU17 to obtain plasmids encoding the K217E, K217C, and K217A variants. These regions were completely sequenced to confirm that no other mutations had been introduced. A 0.7-kb *Bsa*BI-*Mlu*I fragment from the pKAU17 derivative encoding K217A protein was substituted into plasmid pC319A to generate a doubly mutated gene encoding K217A/C319A urease. The 1.1-kb *Mlu*I-*Bam*HI fragment subsequently was subcloned into pKAU17. Variant ureases were purified from *Escherichia coli* DH5α cells carrying the pKAU17 derivatives and grown on LB medium containing 1 mM $NiCl_2$, as described (*12*). Urease apoprotein was isolated by previously described methods (*6*).

Apoprotein Activation and Assay for Urease. Purified proteins (1 mg·ml^{-1}) were incubated overnight at 37 °C with the indicated concentrations of $NiCl_2$ in 100 mM MES buffer (pH 6.5) containing 500 mM organic acid or in 100 mM EPPS buffer (pH 8.5) containing 500 mM bicarbonate and 500 mM methylamine. Activated samples were assayed at 37 °C in 50 mM HEPES buffer (pH 7.75) containing 1 mM EDTA and 750 mM urea. The released ammonia was measured after its conversion to indophenol (*13*). One unit of urease activity (U) is defined as the ability to degrade 1 μmol of urea·min^{-1}. Protein concentrations were determined by using the BioRad protein assay.

Chemical Modification. K217C urease (1 mg·mL^{-1}) was incubated overnight at 37 °C in the presence of 4-chloro- and 4-bromobutyric acid, 3-chloro- and 3-bromopropionic acid, 3-bromopyruvate, or iodoacetic acid (50 mM final concentrations). In addition, the protein was incubated with 50 mM dithiodipropionic acid under similar conditions in an effort to generate the Cys217-thiopropionate mixed disulfide. In all cases, the mixtures were dialyzed against 100 volumes of buffer to remove excess reagents, incubated in the presence of 1 mM $NiCl_2$ overnight at 37 °C, and assayed. In no case was urease activity detected. Alternatively, protein samples were incubated with 100 mM *N*-(β-iodoethyl)trifluoroacetamide overnight at 37 °C in 100 mM HEPES, pH 8.6, buffer containing 150 mM NaCl, 100 mM bicarbonate, and 1 mM $NiCl_2$, or with 5 mM reagent for 3 h at 50 °C followed by the removal of excess reagent and activation by using the standard conditions (*6*). The aminoethylation procedure, which should form the lysine analogue *S*-(2-aminoethyl)cysteine, failed to generate a urease derivative that could be activated.

Crystallographic Methods. Crystals of the K217E, K217C, and K217A variant ureases were grown as described for the wild-type enzyme (*14*). The crystals of the chemically rescued K217A and K217C ureases were obtained by soaking crystals of the apoproteins for 22 days in artificial mother liquor (pH 7.2) containing 500 mM formate and 1.5 mM $NiCl_2$. All data were collected as described previously (*2*−*4*) from single crystals at room temperature with an ADSC multiwire area detector system and processed using Scalepack (*15*), with the exception of the data from the K217E urease which was collected from two crystals. Data collection and refinement statistics are in Table 1. The initial models for the K217E, K217C, and K217A apoproteins and for the rescued K217C protein were constructed from the refined coordinates of wild-type urease (*2*, *4*, Protein Data Bank entry 1FWJ). The initial model for the rescued K217A enzyme was built using the refined coordinates of the K217A apoprotein. Difference electron density maps ($2F_o - F_c$ and $F_o - F_c$) were used to determine the position and extent of structural changes. The initial difference maps for the rescued enzymes were calculated after removing the water molecules from the active sites so that the water positions would not obscure the electron density for the formate and nickel ions. The models were manually adjusted using the program CHAIN (*16*) and were refined against all data between 10 Å and the nominal resolution limit using the conventional positional and restrained individual *B*-factor refinement protocols in X-PLOR (*17*). Additionally, the occupancies of the nickel ions in the rescued enzymes were refined using the occupancy refinement protocol in X-PLOR (after the *B* factors for the nickel ions were set to 17 Å^2, which is approximately the value observed in wild-type

[1] Abbreviations: K217E, K217C/C319A (or K217C), and K217A proteins are *Klebsiella aerogenes* ureases with Lys217 replaced by Glu, Cys, and Ala, respectively, and with the K217C protein having a second change in which Cys319 is replaced with Ala; EDTA, ethylenediaminetetraacetic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; PTE, phosphotriesterase; RUBISCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

Table 1: Crystallographic Data Collection and Refinement[a]

	K217E	K217C	K217A	rescued K217A	rescued K217C
resolution (Å)	2.2	2.2	2.0	2.4	2.5
unique reflections	37683	38169	48752	31434	27738
completeness (%)	92 (74)	93 (69)	90 (59)	100 (100)	100 (100)
redundancy	5.1 (2.2)	2.0 (1.3)	1.8 (1.4)	4.0 (2.7)	4.1 (2.8)
R_{meas} (*31*)	0.105 (0.443)	0.096 (0.420)	0.092 (0.440)	0.115 (0.416)	0.134 (0.474)
R_{mrgd-F} (*31*)	0.124 (0.392)	0.129 (0.367)	0.136 (0.382)	0.131 (0.328)	0.143 (0.374)
R_{cryst}[b]	0.178	0.186	0.194	0.167	0.181

[a] Values in parentheses correspond to data in the highest resolution bin as reported by Scalepack. [b] $R_{cryst} = \sum|F_{obs} - F_{calc}|/\sum|F_{obs}|$, where F is the structure factor.

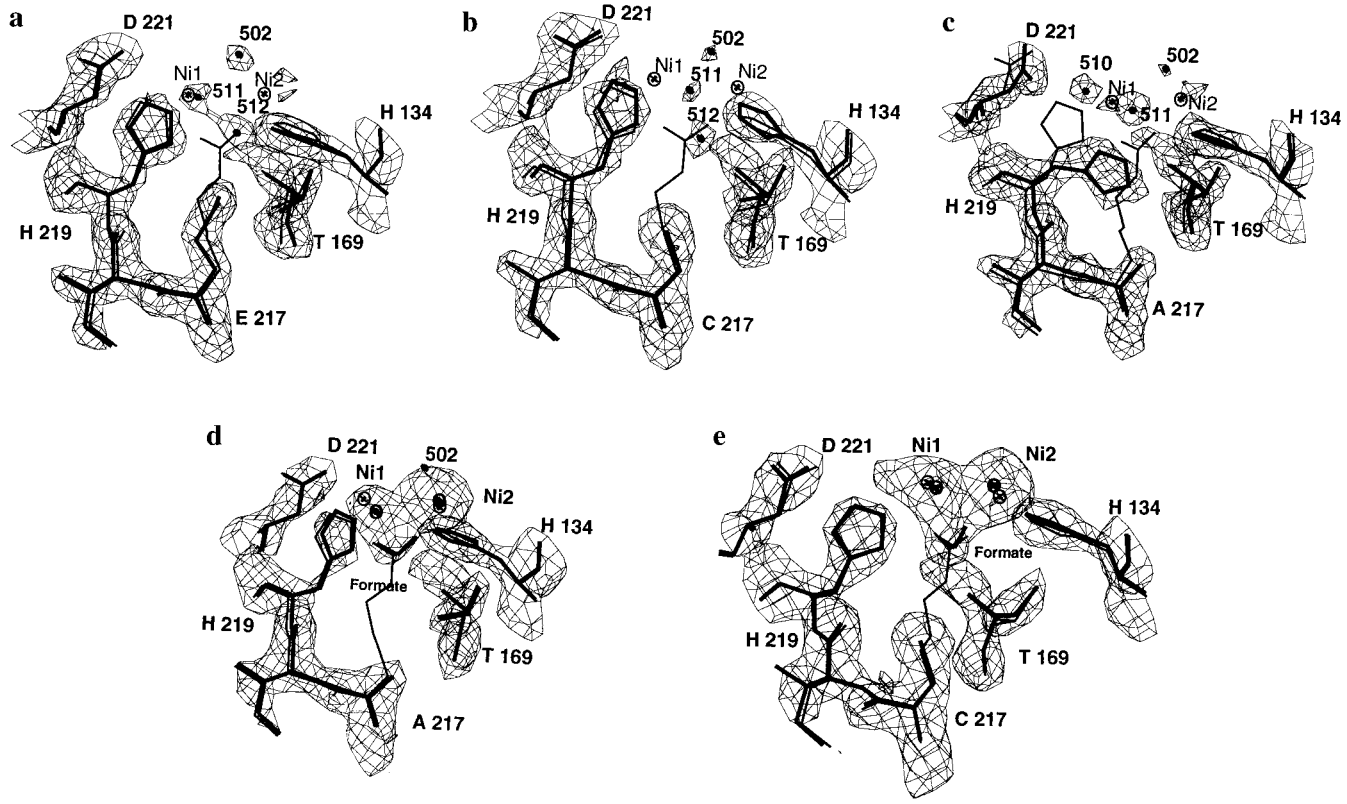


FIGURE 1: Active-site crystal structures of the K217E, K217C, K217A, and chemically rescued K217A and K217C ureases. The variant structures are shown (thick line) along with their $2F_o - F_c$ electron density (at a contour level of 1.5 ρ_{rms}) and the wild-type urease structure (thin line) for the K217E (A), K217C (B), and K217A (C) apoproteins and for the rescued K217A (D) and K217C (E) enzymes. The positions of nickel ions in the wild-type enzyme and rescued proteins are indicated by circles. For clarity, the active site water molecules are not included for the wild-type structure. These figures were prepared with the program CHAIN (*16*).

urease). In the K217C urease, the occupancies of the two alternate conformations of His272 were refined in X-PLOR after setting the *B* factors for the side-chain atoms to 15 Å^2, which is approximately the value observed for the main-chain atoms of this residue in the variant.

RESULTS

Direct Replacement of the Lys217-Carbamate by Glu. The K217E urease variant was designed to directly replace the carbamylated lysine with an acidic amino acid residue. This protein had no bound nickel ions, and the 2.2-Å resolution crystal structure of K217E urease (Figure 1A) revealed that the carboxylate side chain of Glu217 was not long enough to reach to the metal binding site and act as a nickel ligand. In place of the nickel ions, three water molecules bind in the active site: Wat-502 (which is equivalent to a site present in wild-type holourease (*4*)), Wat-511, and Wat-512. There were no significant changes in the positions of the active site residues.

Efforts to Replace the Lys217-Carbamate by Chemical Modification with Carboxylated Reagents. The K217C protein was meant to be used in chemical modification experiments. This variant also has Cys319 replaced by Ala to circumvent enzyme inactivation by thiol-selective reagents, since C319A urease has been shown to be resistant to alkylating agents and disulfides, and retains 50% of the wild-type activity (*4*, *7*). All efforts to extend the cysteine side chain in this variant by chemically modifying Cys217 (see Experimental Procedures) were unsuccessful, consistent with Cys217 being in a position which is unable to react with these reagents.

The crystal structure of the K217C variant (Figure 1B) confirms that this protein does not bind nickel ions. In place of the metal ions, three water molecules (Wat-502, Wat-511, Wat-512) bind to the active site in nearly the same positions found in the K217E structure. The addition of the bulky S_γ atom of Cys217 forces the surrounding residues to shift from their wild-type positions (Figure 2). The side

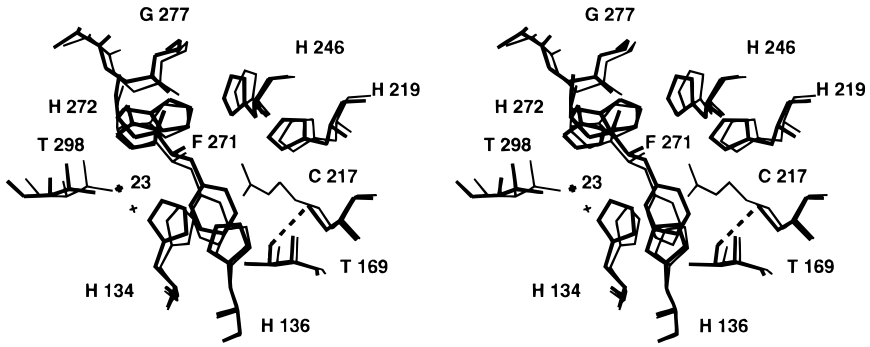


FIGURE 2: Structural changes caused by the K217C mutation. The structure of the residues surrounding residue 217 are shown for the K217C variant (thick line) and wild-type urease (thin line). The dashed line indicates the contact (at a distance of 3.3 Å) between the side chains of Cys217 and Thr169. The shift of this threonine forces several other residues to shift from their wild-type positions.

chain of Thr169 is forced to shift away from Cys217 by 0.5 Å so that its O_γ atom is 3.3 Å away from the Cys217 S_γ atom. The shift of this threonine leads to a series of other structural changes: the side chain of His134 (a nickel ligand in wild-type urease) is forced to shift 1 Å away from the shifted Thr169, and this shift is propagated through Wat-23 to the main chain of His272 (also a nickel ligand in the wild-type enzyme). The side chain of His272 is present in two alternate conformations: the first has a χ_1 torsion angle of 24° (which puts the side chain in approximately the wild-type position) and has an occupancy of 0.3, while the alternate conformation has a χ_1 torsion angle of −57° and has an occupancy of 0.7. The shifted positions of His134 and His272 cause the neighboring residues, Phe271, Thr298, His246, and His219, to shift by 0.6−1.0 Å. The His272 shift also appears to cause an increase in the disorder of a nearby loop containing residues 274−283, whose main chain *B* factors increase from 8−13 Å^2 to 30−60 Å^2. The active site flap (residues 312−336) also becomes so disordered in the K217C variant that residues 309−338 were not included in this model. In contrast, the residues immediately surrounding Cys217 appear to be well-ordered, and a solvent-accessible surface calculated with a 1.3-Å probe radius indicates that Cys217 is completely buried by these surrounding residues.

Replacement of the Lys217-Carbamate by Chemical Rescue of the K217A Protein. The K217A variant urease was created in order to test whether exogenously added organic acids could replace the K217-carbamate to rescue urease activity. As shown in Table 2, formate was especially effective in restoring activity to this protein, but some activity was also formed in the presence of acetate, bicarbonate, or bicarbonate plus methylamine. In contrast, other organic acids such as propionate, oxalate, α-ketoglutarate, and glyoxylate were unable to activate the K217A protein. Activation of K217A urease by organic acids and Ni(II) was a slow process that required approximately 10 h to reach completion (Figure 3). Very high levels of organic acids were needed to affect activation; for example, maximal activity was achieved in the presence of approximately 500 mM formate (Figure 4). The requirement for such high concentrations of formate during activation raised concerns that excess formate may carry over into the assay mixture and serve as an inhibitor; however, insignificant inhibition was observed at the formate levels present after dilution into the assay mixture (<2.5 mM) when compared to the inhibition constant (K_i ~4 mM) for this mixed inhibitor of wild-type enzyme. The nickel ion concentration required to observe the greatest activity levels was also very high (Figure 5). For bicarbonate-containing buffers, the examination of high concentrations of Ni was precluded by the precipitation of the Ni salt.

Once formed, the K217A-formate-nickel metallocenter was stable to multiple rounds of dilution and reconcentration in EDTA-containing phosphate buffer (pH 7.5) lacking the

Table 2: Activation of K217 Urease Variants by Chemical Rescue[a]

organic acid and other additives	[Ni(II)] (mM)	specific activity (U·(mg of protein)$^{-1}$)[b]		
		K217A	K217E	K217C
none	0.0	<0.05	<0.05	<0.05
bicarbonate	1.0	0.30 ± 0.04	<0.05	94 ± 6[c]
bicarbonate/CH_3NH_2	1.0	0.8 ± 0.1	<0.05	71 ± 3[c]
formate	1.0	3.4 ± 0.2	1.2 ± 0.2	26.8 ± 0.2
	5.0	9.9 ± 0.3	5.2 ± 0.4	145 ± 3
	10.0	21.4 ± 0.9	10.7 ± 0.7	224 ± 14
acetate	1.0	0.17 ± 0.01	<0.05	<0.05
	5.0	1.4 ± 0.2[c]	<0.05	<0.05
	10.0	2.0 ± 0.3[c]	<0.05	<0.05

[a] Proteins were incubated with 500 mM concentrations of the indicated short-chain organic acids and the concentrations of Ni(II) as shown. The results represent the average and standard deviations for three determinations unless otherwise indicated. [b] For comparison, 2-h incubation of wild-type apoprotein in the presence of 100 μM Ni(II) and 100 mM bicarbonate results in the formation of active enzyme with a specific activity of 400 U·(mg of protein)$^{-1}$ (*6*). [c] Average of two determinations.

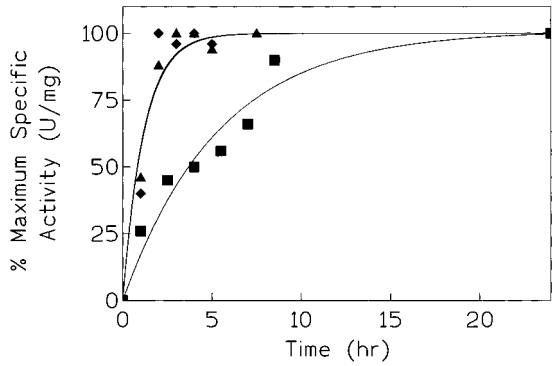


FIGURE 3: Chemical rescue time dependencies for the K217A, K217E, and K217C proteins. The K217A (■), K217E (◆), and K217C (▲) apoproteins (1 mg·ml^{-1}) were incubated for the indicated time periods at 37 °C in the presence of 500 mM formate and 1 mM $NiCl_2$ in 100 mM HEPES buffer (pH 6.5). The specific activities were determined in assay buffer containing 750 mM urea. The data were plotted as percent maximal activity and fit to a first-order activation process.

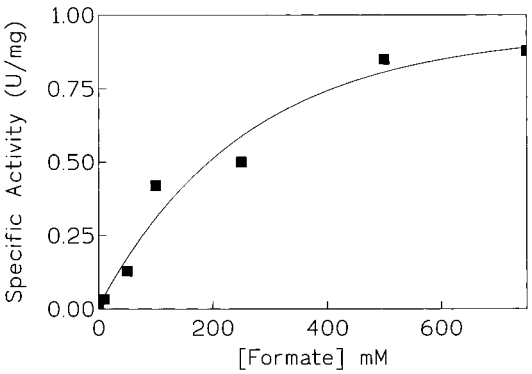


FIGURE 4: Formic acid concentration dependence of chemical rescue for K217A urease. Apoprotein (1 mg·ml^{-1}) was incubated in the presence of the indicated concentrations of formate and 1 mM $NiCl_2$ in 100 mM HEPES buffer (pH 6.5) overnight at 37 °C and assayed for urease activity in the presence of 50 mM urea.

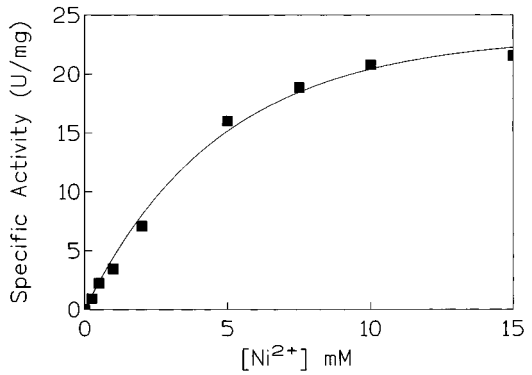


FIGURE 5: Nickel ion concentration dependence for the chemical rescue of K217A urease. Apoprotein (1 mg·ml^{-1}) was incubated in the presence of the indicated concentrations of Ni(II) and 500 mM formate in 100 mM MES buffer (pH 6.5) overnight at 37 °C and assayed for urease activity in the presence of 750 mM urea.

organic acid and nickel ions. The K_m for formate-activated K217A urease (220 ± 50 mM urea) was greatly increased over that observed for the wild-type enzyme [2.4 mM (*18*)]. In contrast, the pH dependence of the activity for the formate-activated protein was similar to that for the wild-type enzyme and exhibited an optimum at pH 7.5−8.

Crystal Structures of K217A and Chemically Rescued K217A Protein. The crystal structure of K217A urease (Figure 1C) verified that this protein does not possess bound nickel ions and revealed a rearrangement of the side chain of His219, which rotates by 84° around the $C_\alpha-C_\beta$ bond (χ_1) to form a hydrogen bond with the side chain of Thr169 (with a distance of 2.7 Å between the N_ϵ atom of His219 and the O_γ atom of Thr169). This interaction replaces that formed by the amino group of Lys217 with Thr169 in the wild-type enzyme and partially fills in the cavity left by the missing lysine side chain. This change is accompanied by shifts in the side chains of Asp221 (Figure 1C) and His320 (not shown), and the active site flap (which includes the latter residue) becomes more ordered than in wild-type urease. The active site contains three water molecules: Wat-502 and Wat-511 are found in the other K217 variants, and Wat-510 is a position unique to the K217A protein which fills part of the cavity left near the wild-type position of His219.

The structure of K217A urease chemically rescued by the addition of formate and nickel ions showed that formate binds in the same position as the carbamate group in the wild-type protein (Figure 1D). Two nickel ions also bind to the active site, demonstrating that formate can functionally replace the carbamylated Lys in its role as a bridging ligand to the dinuclear center. However, Ni1 and Ni2 are 0.8 and 0.3 Å from their wild-type positions, leading to a Ni−Ni distance of 3.0 Å in the variant compared to 3.6 Å in the wild-type enzyme. The two nickel ion positions also have very different electron density strengths. We have modeled this difference by refining occupancies rather than *B* factors under the premise that an active, EDTA stable metallocenter will have a mobility similar to that of the wild-type metal center. The occupancy of Ni2 refined to 0.9, but that of Ni1 refined to only 0.4. The low occupancy of Ni1 may contribute to the lower activity and higher K_m of the rescued K217A enzyme relative to the activated wild-type urease. The side chain of His219 returns to its wild-type position in the rescued K217A protein which leaves open the cavity occupied by the carbon chain of Lys217 in the wild-type enzyme. The electron density gives no evidence for any preferred solvation sites (ordered water molecules) in this cavity. At this resolution, only one of the water molecules that act as nickel ligands in the wild-type urease (Wat-502) is visible in the electron density. The active site flap becomes disordered in this rescued variant, so residues 316−330 were not included in the model.

Chemical Rescue of K217E and K217C Urease Variants. As shown in Table 2, formate was also effective at restoring activity to K217E and K217C variants. The K_m value of formate-rescued K217C urease (260 ± 60 mM) was similar to that for the rescued K217A variant. In contrast to the K217A protein, activation of the K217E and K217C proteins was complete by about 2 h (Figure 3). For the K217C protein, bicarbonate was more effective than formate when activating with 1 mM Ni(II); higher metal ion concentrations could not be compared due to the formation of a nickel/bicarbonate precipitate. No activity was detected during efforts to rescue K217E or K217C proteins with acetate or larger compounds. Because formate proved to be effective for the activation of each urease variant, it was also examined for its ability to activate the wild-type apoprotein. Incubation of native apoprotein with 500 mM formate and 10 mM Ni(II) led to the development of urease activity to a value of ~3 U·(mg of protein)$^{-1}$, demonstrating that chemical rescue can also be achieved in protein containing Lys217. The formate-dependent activation rate for the wild-type protein was similar to that for formate activation of K217E or K217C proteins or for bicarbonate-dependent activation of wild-type apoprotein (data not shown).

The crystal structure of chemically rescued K217C was obtained (Figure 1E) and shown to possess two nickel ions and a bound formate acting as a bridging ligand. As in the rescued K217A protein, the formate is bound in nearly the same position occupied by the carbamate in wild-type urease. In contrast to the K217A variant, Ni1 and Ni2 are 0.4 and 0.5 Å, respectively, from their wild-type positions in this variant, leading to a Ni−Ni distance of 2.9 Å. The electron densities for the nickel ions are also more equal in this variant, and the refined occupancies are 0.8 for Ni1 and 1.1 for Ni2. As in the rescued K217A variant, the active site residues shift back to positions near those observed in the wild-type enzyme, with the exception of Thr169 which is forced to remain near its shifted position by its close contact

with Cys217. The side chains of His134 and His272 return to their wild-type positions and act as nickel ligands. The side chain of His136, a Ni2 ligand, has shifted by 0.4 Å from its wild-type position, consistent with the shift of the nickel ion. The loop containing residues 274−283 also becomes more ordered, as it is in the wild-type enzyme. However, the majority of residues in the active site flap remain disordered, and residues 316−335 were not included in the model for this variant.

To assess whether the Cys319 to Ala change in K217C urease may have affected its activation competence, an analogous second site change was introduced into the K217A protein. Thus, a plasmid encoding K217A/C319A urease was constructed, and the doubly variant protein was purified and characterized for its activation properties. Rather than enhancing the activation level over that observed for the K217A protein, the doubly variant urease was activated to a specific activity of only 2.5 U·(mg of protein)$^{-1}$ using the conditions that gave the highest specific activity for the singly variant enzyme (21.4 U·mg^{-1}).

DISCUSSION

Chemical Rescue of Urease. Each of the K217E, K217C, K217A, and wild-type urease apoproteins were capable of being chemically rescued by small organic acids and nickel ions, and the structures of K217A- and K217C-formate-nickel complexes reveal that the metallocenters in these proteins are authentic reproductions of the wild-type metallocenter. An important result of the structural information is that it allows us to rationalize the time courses and selectivities of urease activation. The K217A protein is more slowly activated than the other apoproteins because the rearranged His219 side chain is in a stabilized position which sterically blocks formate binding. In contrast, the activation of the other two variants is more rapid because the K217E protein is still perfectly preorganized for nickel ion binding, and in the K217C protein, although there are structural rearrangements required for activation, the formate binding site is not blocked. We suggest that the K217E protein is activated only to a small extent by formate and not at all by bicarbonate due to charge repulsion between the ions and the glutamate side chain. The K217E and K217C proteins are not activated by acetate because they cannot accommodate the larger ion for steric reasons (e.g., the acetate methyl carbon would be within 2.6 Å of the S_γ atom of Cys217). There is space for an acetate ion to bind to the K217A urease, and we speculate that the acetate-rescued variant has lower activity than the formate-rescued variant because interactions made by the acetate methyl group cause it to bind in a less productive orientation. Such additional interactions may also account for the fact that propionate does not activate the K217A variant at all, although the cavity appears large enough to accommodate the larger ion. The K217C urease is activated to a greater extent than the K217A urease because the cysteine side chain partially fills the cavity left by the loss of Lys217, which may lead to better packing interactions in the active site of the rescued K217C protein relative to the rescued K217A protein. This improved packing may lead to higher activity by increasing the occupancy of Ni1, which is required for catalysis. Finally, we propose that the lack of reactivity of K217C urease with covalent rescue reagents is due to the inaccessibility of the S_γ atom in a well-ordered region of the protein.

The highest level of rescued activity observed (224 U·(mg of protein)$^{-1}$), obtained using the K217C variant, is only 9% of the activity level of native urease [2,500 U·(mg of protein)$^{-1}$ (*18*)], but it corresponds to 56% of the specific activity typically achieved during in vitro activation of wild-type urease apoprotein with Ni(II) and bicarbonate [400 U·(mg of protein)$^{-1}$] (*6*). The K217C variant includes a change at a second position (C319A) which could reasonably affect its ability to be activated; for example, C319A urease has been shown to be superior to the wild-type enzyme in stability and resistance to nickel ion inhibition (*4*). Characterization of the activation behavior of K217A/C319A apoprotein, however, demonstrated that including an additional C319A change in the K217A protein reduced the extent of activation; that is, the doubly variant protein yielded lower activity values that the singly variant K217C protein. Thus, the cysteine to alanine change in K217C protein was unlikely to contribute to its high specific activity after chemical rescue.

The ability to produce any urea-degrading activity is significant given the remarkable stability of this substrate toward hydrolysis. Indeed, the spontaneous hydrolysis of urea has never been observed (*19*). Furthermore, no reports of urea hydrolysis by metal ions or metal complexes have been reported (e.g., refs *20*−*23*), although ethanolysis of urea by a dinuclear Ni complex was shown to be catalytic (*24*). The kinetic and structural results presented here show that urease activity does not require any special chemical properties of the carbamylated lysine found in the natural urease metallocenter. Instead, we conclude that the lysine carbamate simply serves as a dianionic bridging ligand that positions the two metal ions at the proper distance and within the proper constellation of active site residues to allow for catalysis, suggested to occur by a reverse protonation mechanism (*25*). A direct comparison of the k_{cat}/K_m values for rescued K217A or K217C variants with in vitro activated wild-type urease indicates a $\Delta\Delta G$ of 4.6 or 3.2 kcal·mol^{-1}, respectively, in transition-state stabilization. These numbers cannot be directly assigned to the difference between a bridging carboxylate and the carbamate, however, because they include variations in activation efficiency as well as factors associated with the increased disorder of noncovalently held carboxylate. In contrast, however, a comparison of k_{cat} values yields $\Delta\Delta G$ values of 1.8 or 0.36 kcal·mol^{-1} and implies that the electronic differences between the carbamate and carboxylate have only a small effect on the rate-limiting step of urease catalysis.

Relationship to Other Metalloenzymes Possessing Lysine Carbamates. In addition to urease, Lys-carbamates are known to function as metallocenter ligands in two other enzymes: RUBISCO (*8*) and PTE (*9*). The residue that serves as a ligand to the mononuclear magnesium center in RUBISCO (Lys191 in the *Rhodospirillum rubrum* enzyme) was changed to Glu by site-directed modification of the corresponding gene; this change abolished activity (*26*), comparable to the results for K217E urease. Cys substitution of Lys191 in RUBISCO differs from the case of K217C urease in being accessible to chemical reagents; aminoethylation of K191C RUBISCO restored activity to 4−7% of

the wild-type level (*27*). Chemical rescue of this variant RUBISCO was achieved by using aminomethanesulfonic acid; however, the resulting level of activity amounted to only 10^{-5} of that of the wild-type enzyme (*28*), and leaves unresolved whether a carbamate is chemically required for activity in that protein.

A combination of site-directed mutagenesis and chemical rescue studies have also been applied to PTE (*29*). PTE is a distant homologue of urease (*2*, *30*), and its active site possesses an equivalent carbamylated Lys (Lys169) that bridges a dinuclear (normally Zn) metallocenter (*9*). The wild-type PTE metallocenter is, however, EDTA-labile. K169E, K169M, and K169A variant PTE proteins all appear to be slightly active in the absence of added organic acid (the Cd-activated forms of these proteins possess specific activities of 0.3, 0.09, and 7.7 U·(mg of protein)$^{-1}$, respectively, compared to a value of 9200 U·(mg of protein)$^{-1}$ for the wild-type enzyme). The background PTE activity highlights the ease of hydrolysis of the phosphotriester substrate, paraoxon, or diethyl *p*-nitrophenyl phosphate, compared to the difficulty of hydrolyzing urea (*19*, *25*). Four-fold activation of K169E PTE was noted in the presence of formate, whereas larger organic acids were ineffective. The K169M variant was activated best (5-fold) by acetate, while the K169A protein was activated by acetate, propionate, and butyrate (13-, 8-, and 8-fold). Formate activation was not reported for the K169M and K169A PTE proteins. In contrast to the case of urease variants, activation of the PTE apoproteins was immediate and was rapidly reversible by the addition of a chelator, as would be expected on the basis of the properties of wild-type PTE (*29*). Maximal activity was achieved by maintaining the organic acid in the assay buffers, resulting in an activity of 2600 U·(mg of protein)$^{-1}$ for K169A plus acetate and Co(II), corresponding to 30% of that for the Co-containing wild-type enzyme activity (10100 U·(mg of protein)$^{-1}$) (*29*) and clearly indicating that, as for urease, the carbamate in PTE is present for geometric rather than chemical reasons. Whereas no structural characterization of chemically rescued RUBISCO or PTE has been reported, the chemically rescued K217A- and K217C-formate-nickel structures shown in Figure 1, parts D and E demonstrate that formate structurally and functionally replaces the Lys-carbamate in generating an active dinuclear urease metallocenter.

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