Substitution of the Urease Active Site Carbamate by Dithiocarbamate and Vanadate†

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ABSTRACT: Urease possesses a dinuclear nickel active site with the metals bridged by a carbamylated lysine residue. In vitro activation of apoprotein (Apo) is achieved by incubation with Ni(II) and bicarbonate as a source of CO_2. Analogues of CO_2 and bicarbonate were examined for their effects on the Apo activation process. While SO_2 had little effect, CS_2 was shown to inhibit Apo activation via its ability to substitute for CO_2 to yield an inactive dithiocarbamate-containing protein. Sulfur-to-Ni charge-transfer transitions arising from this species yielded an electronic absorption band at 324 nm with a shoulder at 382 nm. Borate, sulfate, phosphate, and molybdate had essentially no effect on Apo activation and did not substitute for bicarbonate, while treatment of Apo with Ni(II) plus vanadate led to the production of active urease containing two Ni and one V per active site. Vanadate-dependent activation of Apo resembled the normal activation process in terms of concentration of anion required, optimal pH, and incubation time needed. Furthermore, the UV−visible spectrum, maximal specific activity [386 ± 26 U·(mg of protein)$^{-1}$], K_m (1.83 ± 0.20 mM urea), and pH dependence for the vanadate-containing urease were essentially identical to properties observed for bicarbonate-activated enzyme. Vanadate-activated Apo is proposed to possess a vanadylated lysine that bridges the two Ni ions comprising its metallocenter.

Urease (EC 3.5.1.5), a Ni-containing enzyme isolated from various plants, fungi, algae, and bacteria, catalyzes the hydrolysis of urea to form ammonia and carbamate (reviewed in *1*). Of historical interest, plant urease isolated from jack bean was the first enzyme ever crystallized (*2*) and the first enzyme shown to possess nickel (*3*). More recent studies have focused on bacterial ureases. These enzymes play important roles in nitrogen metabolism and as virulence factors associated with various human and animal pathogens, including those causing urinary stones and gastric ulceration (reviewed in *4*). Crystallographic analysis of *Klebsiella aerogenes* urease reveals a structure comprised of a tightly associated trimer of trimers [$(\alpha\beta\gamma)_3$; α, 60.3 kDa; β, 11.7 kDa; γ, 11.1 kDa] containing three dinuclear catalytic sites (*5*). The two Ni ions, separated by 3.5 Å, are bridged by a carbamylated lysine residue (K217-carbamate) and by a solvent molecule (Wat-500, present at partial occupancy) (*6*). In addition to the bridging ligands, Ni-1 is bound by two histidines (H246 and H272) and a solvent molecule (Wat-501, at partial occupancy), whereas Ni-2 is coordinated by two histidines (H134 and H136), an aspartate (D360), and a solvent molecule (Wat-502). Supported by model compound chemistry (*7*, *8*) and by crystallographic analysis of the acetohydroxamate-inhibited enzyme (*6*), urea is suggested to bind in *O*-coordination to Ni-1 (with stabilization provided by H219), resulting in polarization of the urea carbonyl group. Wat-502 bound to Ni-2 is proposed to attack the carbonyl carbon to form a tetrahedral intermediate which, assisted by H320 acting as a general acid, decomposes to yield the products (*9*).

Assembly of the *K. aerogenes* urease dinuclear metallocenter is a complex process. In vivo synthesis of active urease requires the participation of three accessory proteins (UreD, UreF, and UreG) and is further aided by a fourth (UreE) (*10*). UreE binds Ni and has been suggested to function as a Ni donor to urease apoprotein (Apo[1]) (*11*, *12*). In contrast, the essential auxiliary proteins form complexes with Apo including UreD−Apo (*13*), UreD−UreF-Apo (*14*), and UreD−UreF−UreG-Apo (*15*) species. Despite efforts to purify and characterize these complexes, the detailed roles for the accessory proteins in the in vivo activation process remain unclear (reviewed in *16*). In addition to the cellular activation process, Apo can be activated in vitro simply by incubation with Ni(II) and carbon dioxide (*17*). The activating CO_2, in equilibrium with bicarbonate ion, is incorporated into the protein as the lysine carbamate metallocenter ligand. In the absence of CO_2/bicarbonate, two Ni ions are bound per $\alpha\beta\gamma$ unit, but no activity is generated. Optimized activation conditions involve incubation of Apo with 100 mM bicarbonate and 100 μM $NiCl_2$ at pH 8.3, resulting in a slow increase in urease

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[1] Abbreviations: Apo, urease apoprotein; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; V-urease, Apo that is activated by vanadate and Ni(II); CO_2-urease, Apo activated by bicarbonate and Ni(II); CS_2-urease, Apo treated with CS_2 and Ni(II).

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activity (requiring more than 60 min for completion) to reach approximately 400 μmol of urea degraded·min^{-1}·(mg of protein)$^{-1}$ or ~15% of fully active enzyme (*18*). Analogous studies carried out using the UreD−Apo complex yield activities of approximately 800 μmol of urea·min^{-1}·(mg protein)$^{-1}$, resulting in about 30% active enzyme.

In this paper, we report the effect of compounds analogous to CO_2 or bicarbonate on urease activation. We demonstrate that carbon disulfide substitutes for CO_2, but yields an inactive urease in which the Ni ions are coordinated by a dithiocarbamate. Further, we show that vanadate can be used to generate active urease from Apo in the presence of Ni ions and describe the properties of the vanadate-activated urease.

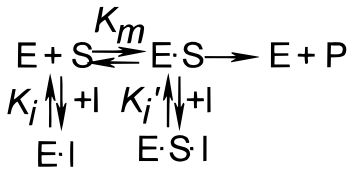
EXPERIMENTAL PROCEDURES

Protein Purification. Apo and UreD−Apo were purified from *Escherichia coli* DH5[pKAU22*ureD-1*] and *E. coli* DH5[pKAUD2], respectively, by methods that have been described previously (*18*). The former plasmid carries the *K. aerogenes* urease gene cluster with a deletion in *ureD*, and the latter plasmid exhibits enhanced expression of *ureD* in the presence of the other urease genes. The purified proteins were greater than 95% homogeneous on the basis of denaturing polyacrylamide gel electrophoresis analysis (*19*).

Assays. Urease activity typically was assayed in 25 mM HEPES buffer, pH 7.75, containing 50 mM urea and 1 mM EDTA at 37 °C. For analysis of the pH dependence of activity, the buffer and pH values were varied as indicated. Linear regression analysis of the released ammonia, determined by conversion to indophenol (*20*), versus time yielded initial rates. One unit of activity is defined as the amount of enzyme that is required to degrade 1 μmol of urea·min^{-1}. Protein concentrations were assayed by using the spectrophotometric Bio-Rad assay, with bovine serum albumin as the standard.

Activation of Apo Species. The standard Apo and UreD−Apo activation procedure involved 2 h incubation of protein sample in 100 mM HEPES buffer, pH 8.25, containing 150 mM NaCl, 100 mM bicarbonate, and 100 M $NiCl_2$ at 37 °C (*18*). Variations to this method (detailed in the text and figure legends) included changes to the buffer concentration, elimination of NaCl, addition of CO_2 or bicarbonate analogues to the activation buffer, substitution of these analogues for CO_2/bicarbonate (while using buffers and analogue solutions that were purged with nitrogen gas to eliminate any dissolved CO_2), and extensive examination of the vanadate-dependent activation process. Specifically, Apo activation by vanadate (prepared from Na_3VO_4) plus Ni(II) was assessed using alternative buffers and pH values, varied vanadate and Ni(II) concentrations, and a range of incubation times.

Inhibition Kinetics. Samples of activated urease were kinetically characterized for inhibition by vanadate. Initial velocities were determined for each of five inhibitor concentrations (10, 20, 30, 40, and 50 mM) and four urea concentrations (5, 10, 20, and 50 mM) in the presence of 1 mM EDTA and 25 mM HEPES buffer (pH 7.75) at 37 °C. The data appeared to fit the following model describing mixed inhibition, and values for K_i and K_i' were calculated by standard methods (*21*).



Spectroscopic Methods. Electronic spectra of activated urease were collected after extensive dialysis by using a Beckman DU7500 spectrophotometer. Nickel content was examined by using a Varian SpectraAA-400Z atomic absorption spectrophotometer equipped with an autosampler, a graphite furnace, and Zeeman background correction, as previously described (*18*). Quantitations of vanadium and nickel were obtained by the University of Georgia Chemical Analysis Laboratory using inductively-coupled plasma emission spectroscopy.

RESULTS

Interaction of Apo with Analogues of CO_2 and Bicarbonate. Carbon dioxide, supplied as bicarbonate, is incorporated into Apo during in vitro activation of urease by Ni ions (*17, 18*). Analogues of CO_2 and bicarbonate were examined for their ability to substitute for CO_2 or to inhibit the CO_2-dependent process. As shown in Table 1, incubation of Apo with 100 μM Ni(II) in the presence of 100 mM bicarbonate resulted in the formation of active enzyme with a specific activity of approximately 400 units·mg^{-1}, while incubation of Apo plus Ni(II) in the absence of bicarbonate failed to activate the enzyme. Among the series of compounds examined, inclusion of 50 mM CS_2 or thiocyanate in the bicarbonate-containing activation mixtures led to significant reductions in urease activity, suggesting that these compounds somehow inhibit the activation process. In the absence of bicarbonate, inclusion of 50 mM SO_2, borate, sulfate, phosphate, or molybdate with Apo and Ni(II) gave rise to trace levels of activity, but these values may be derived from contaminating bicarbonate that was introduced with the analogue solutions. In contrast, clearly significant levels of activity were observed for Apo incubated with just vanadate and Ni(II). Further analyses focused on the interactions of CS_2 and vanadate with Apo.

Interaction of Apo with CS_2. To assess whether CS_2 interacted reversibly or irreversibly with Apo, the protein sample that was treated with Ni(II) and CS_2 was subjected to buffer exchange and reexamined for activation competence using the standard activation conditions. No urease activity was generated during this process, indicating that CS_2 somehow irreversibly inhibited the protein. The effect of CS_2 on Apo activation was consistent with a direct substitution of CS_2 for CO_2, resulting in a dithiocarbamate form of the protein (CS_2-urease) rather than the normal carbamylated species (CO_2-urease). Such a situation would be expected to alter the electronic spectrum arising from the protein. CS_2-urease was dialyzed to remove non-protein-bound complexes of Ni(II) and CS_2, and the UV−visible spectrum was obtained (Figure 1). The protein exhibited a band at 324 nm (ϵ = 3.6 mM^{-1}·cm^{-1}, measured on the basis of $\alpha\beta\gamma$ units) and a shoulder at 382 nm. By contrast, the spectrum of native enzyme lacks these peaks and has an extinction coefficient of less than 1 at 324 nm.

Interaction of Apo with Vanadate. Activation of Apo with vanadate and Ni(II) depended on the concentrations of both

Table 1: Effect of Compounds Resembling CO_2 or Bicarbonate on Urease Apoprotein Activation[a]

compound added	specific activity (units· mg^{-1}) with 100 mM HCO_3	specific activity (units· mg^{-1}) without HCO_3[b]
none	402 ± 2	0 ± 0
CS_2	99 ± 33	0 ± 0
SO_2/HSO_3^-	333 ± 19	4 ± 2
$B(OH)_3$	380 ± 20	9 ± 3
SO_4^{2-}	374 ± 39	10 ± 2
PO_4^{3-}	407 ± 36	18 ± 1
SCN^-	262 ± 23	0 ± 0
VO_4^{3-}	386 ± 26	130 ± 33
MoO_4^{2-}	404 ± 32	6 ± 2

[a] Urease apoprotein solutions (0.40 mg·mL^{-1}) were incubated in 100 mM HEPES buffer (pH 8.25) for 2 h at 37 °C containing 100 μM Ni ion and 50 mM of the compounds indicated in the presence or absence of 100 mM bicarbonate. [b] For removing dissolved CO_2 in the HEPES buffer, the solution was maintained under N_2.

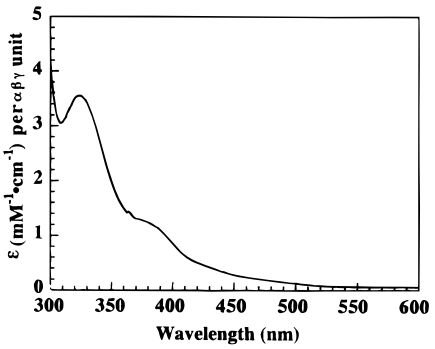


FIGURE 1: Electronic absorption spectrum of CS_2-urease. Apo (0.40 mg·mL^{-1}) was treated with 50 mM CS_2 and 1 mM $NiCl_2$ in 100 mM HEPES buffer (pH 8.25) containing 150 mM NaCl for 2 h at 37 °C under N_2. Excess CS_2 and Ni(II) were removed by washing with 100 mM HEPES buffer, pH 8.3, and CS_2-urease was concentrated to 4.0 mg·mL^{-1} by Centriprep-30.

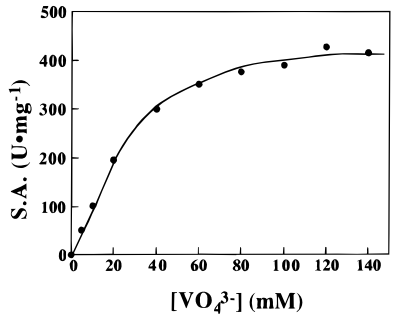


FIGURE 2: Vanadate ion concentration dependence for urease activation. Apo (0.16 mg·mL^{-1}) was incubated for 2 h at 37 °C in 250 mM HEPES buffer (pH 8.25) containing 150 mM NaCl, 1 mM $NiCl_2$, and the indicated concentrations of vanadate under a nitrogen atmosphere, and aliquots were assayed for urease activity.

vanadate[2] (Figure 2) and Ni(II) (Figure 3). All solutions were maintained under a nitrogen atmosphere to eliminate contamination from CO_2. Maximal levels of activity reached ~400 units·(mg of protein)$^{-1}$ with half-maximal activation occurring at ~20 mM vanadate or ~0.3 mM $NiCl_2$. Concentrations of Ni exceeding 1 mM resulted in a reduction

[2] Vanadate concentration refers to the total concentration of vanadate regardless of speciation. In the activation mixture, vandate will be present in the monomeric form as well as the dimeric, tetrameric, and decameric species. For example, at pH 9, a solution containing 40 mM total vanadate will consist of ~25% monomer, 20% dimer, 35% tetramer, and 10% decamer (*22*).

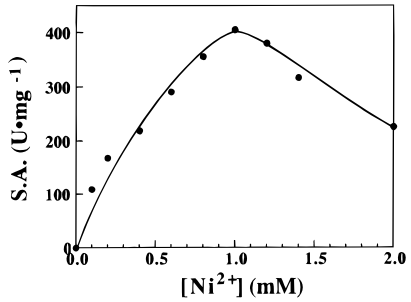


FIGURE 3: Nickel ion concentration dependence of urease activation. Apo (0.17 mg·mL^{-1}) was incubated for 2 h at 37°C in 250 mM HEPES buffer (pH 8.25) containing 150 mM NaCl, 100 mM vanadate, and the indicated concentrations of $NiCl_2$ under a nitrogen atmosphere, and aliquots were assayed for urease activity.

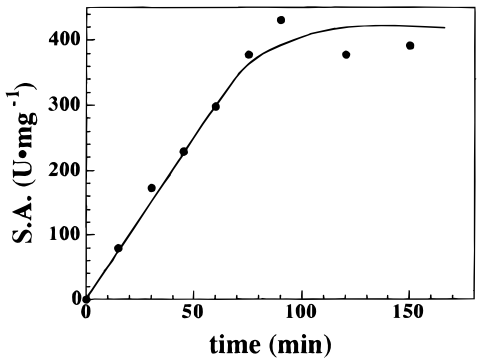


FIGURE 4: Time dependence of urease activation by vanadate and Ni. Apo (0.40 mg·mL^{-1}) was incubated at 37 °C in 250 mM HEPES buffer (pH 8.25) containing 150 mM NaCl, 1 mM $NiCl_2$, and 100 mM vanadate under a nitrogen atmosphere. At the indicated time points, aliquots were removed from the incubation mixture and assayed for urease activity.

of activity. As illustrated in Figure 4, the Ni- and vanadate-dependent activation of Apo is a slow process that requires about 100 min to reach completion. Activation of Apo required the simultaneous presence of Ni and vanadate. For example, no activity was generated when Apo was incubated with 1 mM Ni(II) for 2 h at 37 °C in HEPES buffer (pH 8.3), washed with Ni-free buffer, and subsequently incubated with a buffered solution containing 100 mM vanadate. Similarly, active urease was not formed by incubating Apo first with vanadate, washing, and subsequently treating with Ni(II) in HEPES buffered solutions. The pH dependence of Apo activation by vanadate and Ni exhibits a maximum of approximately 8.4−9 (Figure 5).

Characterization of V-Urease. Apo that had been activated by vanadate plus Ni (V-urease) was shown to possess 1.87 ± 0.02 mol of Ni per $\alpha\beta\gamma$ unit (triplicate determination) based on atomic absorption spectroscopic analysis or 2.05 mol of Ni and 1.05 mol of V per $\alpha\beta\gamma$ unit based on plasma emission spectroscopic analysis. The metals appeared to be tightly bound as demonstrated by the absence of activity loss when V-urease samples were incubated at 4 °C for 12 h in buffer containing 10 mM EDTA. Furthermore, an additional round of activation of V-urease failed to alter the enzyme activity.

Several properties of V-urease were compared to the CO_2-urease species. For example, the UV−visible spectrum (not shown) and the effects of pH on the activities for the two proteins (Figure 6) were nearly identical. Similarly, the K_m values obtained for the two forms of activated enzyme were very similar (Table 2). Finally, only small changes were

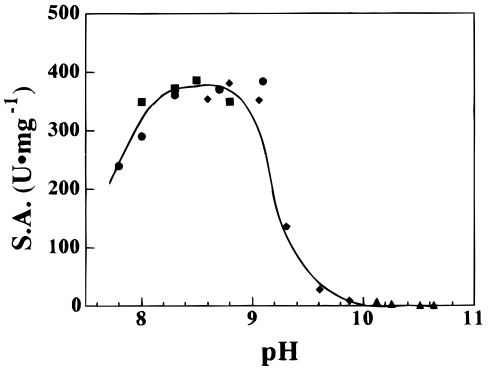


FIGURE 5: pH dependence of urease activation by vanadate and Ni. Apo (0.13 mg·mL^{-1}) was incubated for 2 h at 37 °C in 250 mM HEPES (●), CHES (◆), CAPS (▲), or EPPS (■) buffers containing 150 mM NaCl, 1 mM $NiCl_2$, and 140 mM vanadate under a nitrogen atmosphere, and urease activity was assayed.

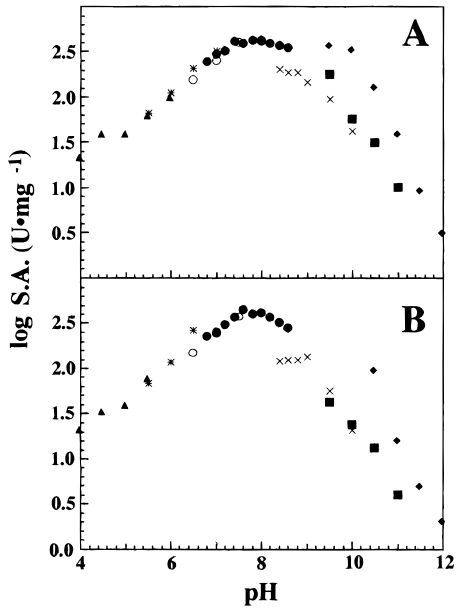


FIGURE 6: pH dependence of activity for V- and CO_2-ureases. Apo (0.20 mg·mL^{-1}) was (A) subjected to the standard bicarbonate-dependent activation procedure or (B) activated at 37 °C for 2 h in 250 mM HEPES buffer (pH 9.0) containing 150 mM NaCl, 1 mM $NiCl_2$, and 100 mM vanadate. Aliquots of the activation mixtures were assayed for urease activity in 200 mM acetate (▲), MES (*), MOPS (○), HEPES (●), CHES (×), CAPS (■), or phosphate (▲) buffers containing 50 mM urea, and the log of the rates were plotted.

Table 2: Kinetic Constants for V-Urease and CO_2-Urease[a]

		vanadate inhibition[b]	
enzyme	K_M (mM)	K_i (mM)	K_i' (mM)
V-urease	1.83 ± 0.20	10.9 ± 0.9	26.6 ± 1.9
CO_2-urease	2.20 ± 0.24	27.3 ± 2.5	52.0 ± 4.5

[a] Urease assay condition: 250 mM HEPES buffer (pH 7.75) containing 1 mM EDTA at 37 °C. [b] Vanadate inhibition constants are based on a mixed inhibition model in which K_i reflects the interaction of vanadate with free enzyme and K_i' indicates vanadate interaction with the enzyme-substrate complex.

seen in the kinetic constants for vanadate inhibition of the V- and CO_2-ureases (Table 2). The vanadate inhibition kinetics were examined to establish that excess vanadate present in the activation mixture did not inhibit the activated enzyme during the assay.

Vanadate Activation of UreD−Apo. Bicarbonate-dependent activation of UreD−Apo is approximately 2-fold more efficient that activation of Apo [i.e., yielding activities of ~800 versus ~400 units·(mg of protein)$^{-1}$] (*18*); hence, it was of interest to compare vanadate activation of UreD−Apo versus Apo. When UreD−Apo (0.40 mg·mL^{-1}) was incubated in HEPES buffer (pH 8.25) containing 100 mM vanadate plus 1 mM $NiCl_2$ for 2 h, the specific activity generated (423 ± 16 units·mg^{-1}) was comparable to that observed for Apo. Thus, the presence of UreD had no significant effect on vanadate-dependent urease activation.

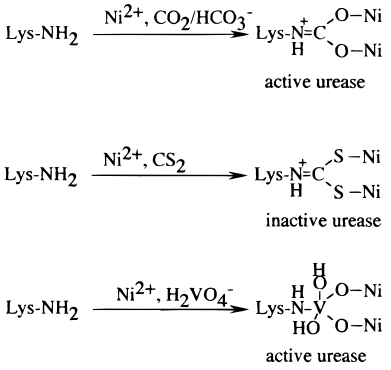


FIGURE 7: Model for the structures of CO_2-, CS_2-, and V-ureases. The structure of the CO_2-activated enzyme is based on the crystallographically determined structure of wild-type enzyme.

DISCUSSION

Dithiocarbamate-Containing Urease. Bicarbonate-dependent Apo activation is inhibited by inclusion of CS_2, and Ni(II)/CS_2-treated protein cannot be activated by Ni(II)/CO_2. Furthermore, Ni(II)/CS_2 treatment of Apo results in the generation of an electronic absorption spectrum with a band at 324 nm and a shoulder at 382 nm. This spectrum is reminiscent of that produced by adding thiols to urease (for example, 2-mercaptoethanol addition yields electronic transitions at 322 and 374 nm; *23*), and likely arises from sulfur-to-Ni(II) charge-transfer interactions (*24*). Our results are consistent with the formation of a lysine dithiocarbamate-bridged dinuclear center in Apo treated with CS_2 and Ni(II), as shown in Figure 7. The dithiocarbamate form of the protein is likely to be inactive due to decreased Lewis acidity of the bound nickel ions.

Vanadate-Activated Urease. The evidence reported here for vanadate- and Ni(II)-dependent activation of Apo is the first report of urease activation by a compound other than CO_2/bicarbonate. Vanadate-dependent activation resembles in many ways the standard bicarbonate-dependent activation process. For example, half-maximal Apo activation occurs at a concentration of ~20 mM vanadate[2] versus about 10 mM bicarbonate (*17*). Similarly, the time dependence for activation was comparable for both cases and required about 100 min. In addition, the pH dependencies for the two activation events were in the same range, i.e., pH ~9. Somewhat larger differences were noted in terms of the Ni(II) concentrations required for activation; e.g., half-maximal Apo activation was achieved with approximately 20 μM Ni in the bicarbonate-dependent system, whereas 0.3 mM Ni was required in the vanadate-dependent system. The kinetic

[3] A variant form of urease in which Lys-217 was changed to Ala (M. A. Pearson, R. A. Schaller, L. O. Michel, P. A. Karplus, and R. P. Hausinger, manuscript in preparation) failed to become activated when incubated with vanadate plus Ni(II).

properties and pH dependence on activity of the V-urease also closely resembled the properties of CO_2-urease. The UV−visible spectra and K_m values of both activated samples are nearly identical to those determined for the wild-type enzyme (K_m = 2.4 mM; *23*). Kinetic inhibition constants for vanadate were 2-fold different when comparing the two activated enzymes, suggesting that the substitution of vanadate for CO_2 only slightly perturbs the metallocenter active site. Although vanadate inhibition kinetics were able to be fit to a model for noncompetitive inhibition by the vanadate monomer, it is important to note that vanadate exists as an equilibrium mixture of several species.[2] An alternative interpretation of the kinetic data could include distinct binding affinities for urease by each of the monomer, dimer, tetramer, and decamer forms of vanadate.

The similarities in activation conditions to generate V- and CO_2-ureases and the near identity in properties of these activated species suggest that the two enzyme forms are related in structure. We propose a structure for V-urease in which Lys-217[3] interacts directly with vanadate to form a vanadylated residue that bridges the dinuclear Ni center (Figure 7). Precedence for 5-coordinate vanadate lacking an oxo group and bound to a protein nitrogenous residue is established. For example, crystal structures for both the vanadium-containing chloroperoxidase (*25*) and the vanadate-inhibited rat prostatic acid phosphatase (*26*) reveal the presence of vanadate (V) in trigonal bipyramidal coordination with histidine imidazolates at apical positions.

Conclusions. The studies described above have demonstrated that analogues of CO_2 and bicarbonate can interact with Apo to form inhibited or functionally substituted urease species. It is interesting to note that vanadate is present at high concentrations in certain environments and accumulates to high levels in ascidians (reviewed in *27*). Although it has not been established whether ascidians possess urease activity, it is reasonable to assume that selected microbes associated with bivalve molluscs and tunicates do possess this enzyme. We speculate that vanadate-containing urease may naturally exist in these symbiotic examples or in other special environments.

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