# Metal Ion Interactions with Urease and UreD-Urease Apoproteins †

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# Metal Ion Interactions with Urease and UreD-Urease Apoproteins†

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ABSTRACT: *Klebsiella aerogenes* urease is a Ni-containing enzyme (two Ni per $\alpha\beta\gamma$ unit) that is purified as an apoprotein from cells grown in Ni-free medium. Partial activation of urease and UreD-urease apoproteins is achieved in vitro by incubation in the presence of Ni(II) and $CO_2$, whereas incubation of these proteins with Ni alone leads to the formation of inactive species [Park, I.-S., & Hausinger, R. P. (1995) *Science 267,* 1156−1158]. Here we determined the kinetics of these inhibitory reactions and demonstrated the presence of two Ni ions per $\alpha\beta\gamma$ unit in the inactive proteins. Although metal-substituted urease has never been purified from Ni-deprived cells, several other metal ions were shown to bind to the urease apoproteins. Divalent Zn, Cu, Co, and Mn all inhibited Ni- and $CO_2$-promoted urease activation at concentrations below that of Ni, whereas Mg and Ca ions did not inhibit this process. Ni-inhibited species recovered their ability to be partially activated after EDTA treatment. In contrast, samples that were exposed to Co or Cu ions were irreversibly inactivated, and EDTA treatment of Zn- or Mn-inhibited samples led to reduced levels of activation competence. Mn-substituted urease, generated from urease apoprotein samples in a Mn- and $CO_2$-dependent manner, was shown to be active, whereas other metal-substituted forms of urease lacked activity. The Mn-protein possessed only 2% of the activity of Ni-activated apoprotein [$\sim$8.0 vs $\sim$400 $\mu$mol min$^{-1}$ (mg of protein)$^{-1}$], but its $K_M$ value was only moderately altered from that of the native enzyme (3.86 $\pm$ 0.15 mM vs 2.3 $\pm$ 0.2 mM). Unlike the Ni-containing enzyme, Mn-urease was inhibited by EDTA. Given the evidence that urease apoprotein binds numerous metal ions, we speculate on possible roles for the UreD, UreF, and UreG accessory proteins in urease activation.

Urease (EC 3.5.1.5), found in many plants, fungi, algae, and bacteria, is a Ni-dependent metalloenzyme that catalyzes the hydrolysis of urea (Hausinger, 1993). The plant enzyme played an important historical role in biochemistry; e.g., jack bean urease was the first enzyme ever crystallized (Sumner, 1926) and the first enzyme demonstrated to contain nickel ion (Dixon *et al*., 1975). Because of the relationships between microbial urease and urinary stone formation, gastric ulceration, and other medical complications, bacterial ureases have been subjected to detailed structural and mechanistic analyses [reviewed by Mobley *et al*. (1995)]. The structure of *Klebsiella aerogenes* urease is known to 2.2 Å resolution and consists of a tightly associated trimer of trimers [$(\alpha\beta\gamma)_3$; $\alpha$, 60.3 kDa; $\beta$, 11.7 kDa; $\gamma$, 11.1 kDa] containing three bi-Ni catalytic sites (Jabri *et al*., 1995). The two nickel ions are located 3.5 Å apart and are bridged by a carbamylated lysine residue (K217-carbamate). Ni-2 possesses distorted trigonal bipyramidal or square pyramidal geometry and is coordinated by H134, H136, K217-carbamate, D360, and a solvent molecule. Ni-1 possesses pseudotetrahedral geometry and is coordinated by H246, H272, K217-carbamate, and weak occupancy by the same solvent molecule that is bound to Ni-2. A model for urease catalysis has been proposed in which urea displaces solvent from the weakly occupied ligand position of Ni-1 and binds to this metal ion in *O*-coordination, with stabilization provided by H219. The solvent molecule coordinated to Ni-2 attacks the urea carbonyl to form a tetrahedral transition state. Release of ammonia from this species results in the formation of carbamate which spontaneously decomposes to form carbon dioxide and a second molecule of ammonia.

Assembly of the urease metallocenter is a surprisingly complex process. In vivo, the products of four accessory genes (*ureD*, *ureE*, *ureF*, and *ureG*) are required to obtain fully active enzyme, but their roles remain unclear [reviewed by Moncrief and Hausinger (1996)]. Purified *K. aerogenes* urease possesses only Ni among the metal ions, and when nickel ions are not provided to the cell only urease apoprotein is obtained (Lee *et al*., 1990). In vitro activation of urease apoprotein has been achieved and found to require both Ni(II) and $CO_2$, with the latter compound provided as bicarbonate (Park & Hausinger, 1995a). With our current knowledge of the urease crystal structure (Jabri *et al*., 1995), the role of the bicarbonate is clear: the activating $CO_2$ molecule reacts with K217 to form a carbamate that serves to ligate both nickel ions in the active site. Despite efforts to optimize the activation conditions, only 12.5% of the apoprotein was found to be activated. UreD ($M_r$ 29.8 kDa) forms a series of three distinct complexes with urease apoprotein that are likely to correspond to the $(\alpha\beta\gamma)_3$ apoprotein associating with one, two, or three molecules of UreD (Park et al., 1994). (UreD is insoluble when synthesized in the absence of the urease peptides and has not been purified or characterized.) A mixture of the three complexes containing an average of 1.6 UreD peptides bound per $(\alpha\beta\gamma)_3$ protein, hereafter termed UreD-urease apoprotein, also is activated by incubation with nickel ions and bicarbonate, and in this case 24% of the

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urease apoprotein was shown to be activated (Park & Hausinger, 1995a). For both the urease and UreD-urease apoproteins, addition of nickel ions prior to provision of bicarbonate prevents the activation from occurring.

The studies described here (i) examine the kinetics and stoichiometry of Ni binding in the inhibitory reactions, (ii) detail the effects of other metal ions on the activation processes, (iii) demonstrate that other metals can bind to urease and UreD-urease apoproteins, (iv) characterize the reversibility of the metal ion binding to inactive, metal ion-bound ureases, and (v) provide evidence that Mn-substituted urease possesses catalytic activity. These results clearly show for the first time that metal-substituted ureases can be formed from urease apoprotein in vitro. On the basis of these findings, we speculate on the in vivo roles of the urease accessory proteins.

## EXPERIMENTAL PROCEDURES

*Protein Purification*. *Escherichia coli* DH5[pKAU22Δ-*ureD1*] (Lee *et al*., 1992), in which the plasmid carries the *K. aerogenes* urease gene cluster with a deletion in *ureD*, and *E. coli* DH5[pKAUD2] (Park *et al*., 1994), which exhibits enhanced expression of the *ureD* gene in the presence of the other urease genes, were grown with rapid agitation in Luria−Bertani medium to stationary phase at 37 °C. Cells were harvested by centrifugation, resuspended in 20 mM phosphate buffer, pH 7.0, containing 1 mM EDTA and 1 mM 2-mercaptoethanol, and disrupted by three passages through a French pressure cell at 18 000 lb/in$^2$. Cell extracts were obtained after centrifugation at 100 000*g* for 60 min at 4 °C. Urease apoprotein was purified from *E. coli* DH5[pKAU22Δ*ureD-1*] cell extracts by using the standard method for purification of the holoprotein (Todd & Hausinger, 1987). UreD-urease apoprotein was purified from the *E. coli* DH5[pKAUD2] cell extracts by following the described method (Park *et al*., 1994) through the Mono Q HR 10/10 step. The proteins were greater than 95% homogeneous on the basis of denaturing polyacrylamide gel analysis.

*Assays*. Urease activities were assayed in 25 mM HEPES buffer, pH 7.75, containing 50 mM urea and 0.5 mM EDTA, unless otherwise indicated. Linear regression analysis of the released ammonia, determined by conversion to indophenol (Weatherburn, 1967), versus time yielded initial rates. One unit (U) of activity is defined as the amount of enzyme that is required to degrade 1 $\mu$mol of urea min$^{-1}$ at 37 °C. Protein concentrations were assessed by using the spectrophotometric Bio-Rad assay, with bovine serum albumin as the standard. Where indicated, calculation of the kinetics constants made use of the method of Wilkinson (1961).

*Activation of Urease Apoprotein Species.* Standard activation conditions for the various urease apoprotein species made use of 100 mM HEPES buffer, pH 8.3, containing 150 mM NaCl, 100 mM bicarbonate, and 100 $\mu$M $NiCl_2$. Changes to this method are indicated where described. Protein was added to a concentration of 5 or 10 $\mu$M urease $\alpha\beta\gamma$ unit. The final concentration of EDTA carried over from the protein addition was $\leq$5 $\mu$M. The activation mixtures were incubated at 37 °C for 2 h, on the basis of the previously established activation kinetics (Park & Hausinger, 1995a), and urease activity was assayed. In the case of $MnCl_2$ replacing $NiCl_2$, the time dependence of activation was determined by removing aliquots at the indicated timepoints.

*Metal Inhibition and Restoration of Activation Competence*. Ni-inhibited urease samples were prepared by incubating urease apoprotein and UreD-urease apoprotein (both at 10 $\mu$M $\alpha\beta\gamma$ unit) for 80 min in activation buffer to which no bicarbonate was added. Urease proteins inhibited by other metal ions were prepared by incubating samples for 80 min at 37 °C in 100 mM HEPES buffer, pH 8.3, containing 150 mM NaCl, 100 mM bicarbonate, and 40 $\mu$M $ZnCl_2$, 40 $\mu$M $CuCl_2$, 100 $\mu$M $CoCl_2$, or 300 $\mu$M $MnCl_2$. Excess metal ions were removed from the samples by using a Centricon-30 to repeatedly concentrate and dilute the protein in 100 mM HEPES buffer, pH 8.3, containing 150 mM NaCl. Restoration of activation competence for the samples was examined by adding EDTA to a concentration of 1 mM and incubating at 25 °C. At the indicated time points, aliquots were removed from the chelation mixtures, EDTA was removed by repeated Centricon-30 concentration and dilution in 150 mM NaCl, 100 mM HEPES, pH 8.3, and the samples were subjected to the standard activation conditions for 2 h.

*Metal Analyses*. Protein samples that were activated or inhibited with $NiCl_2$ were centrifuged to remove any precipitate (e.g., UreD that dissociates from the UreD-urease complexes is highly insoluble) and repeatedly subjected to concentration/dilution cycles using a Centricon-30 filter and 100 mM HEPES buffer, pH 8.3, until the free metal ion concentration was estimated by the dilution factors to be below 1 $\mu$M and the NaCl concentration was less than 10 mM. Protein concentrations were measured after washing. The protein samples were hydrolyzed in 1 N $HNO_3$, dried, dissolved in 50 mM $HNO_3$, and assayed for nickel by using a computer-interfaced Varian SpectraAA-400Z atomic absorption spectrophotometer equipped with an autosampler and a graphite furnace, and Zeeman background correction.

## RESULTS

*Kinetics of Ni-Inhibition of Apoprotein Activation*. Incubation of urease apoprotein with nickel ions in the absence of bicarbonate is known to lead to the production of inactive enzyme (Park & Hausinger, 1995a), but the kinetics of this reaction and the Ni content of the inhibited species had not been examined. To characterize the kinetics of this process, urease and UreD-urease apoproteins were incubated in nitrogen-purged buffer (to remove dissolved $CO_2$) containing 100 $\mu$M $NiCl_2$ for varying lengths of time, aliquots were transferred to activation buffer containing 100 mM bicarbonate and 100 $\mu$M $NiCl_2$, and the urease activity was monitored after 2 h. Representative results from one of three such experiments are illustrated in Figure 1. All experiments yielded the same qualitative findings: loss of activation competence was not instantaneous, and it occurred more rapidly for apoprotein than for UreD-urease apoprotein.

*Stoichiometry of Ni Binding*. When urease and UreD-urease apoproteins were subjected to normal activation conditions [yielding specific activity values of 442 and 818 U (mg of protein)$^{-1}$], separated from excess nickel ions, and subjected to atomic absorption spectroscopic analysis, 1.74 $\pm$ 0.17 and 1.86 $\pm$ 0.19 Ni per $\alpha\beta\gamma$ unit were found (triplicates of single samples). When the two apoproteins were incubated with the identical conditions without added bicarbonate, separated from excess nickel ions, and analyzed

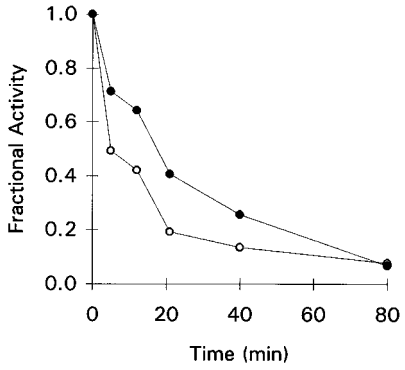


FIGURE 1: Kinetics of inhibition of urease apoprotein activation by nickel ions. Urease apoprotein (○) or UreD-urease apoprotein (●), both at 10 $\mu$M concentration of $\alpha\beta\gamma$ unit, was incubated in nitrogen-purged activation buffer with no added bicarbonate and containing 100 $\mu$M $NiCl_2$. At the indicated time points, aliquots were transferred to normal activation conditions. After 2 h of incubation, the urease activity was assayed and the data were plotted as fractional activity. Ni- and bicarbonate-dependent activation in the absence of the Ni-inhibition treatment resulted in a specific activities of 320 ± 37 and 712 ± 57 U (mg of protein)$^{-1}$ for the urease and UreD-urease apoprotein preparations used in this experiment, respectively.

by atomic absorption spectroscopy, 2.13 ± 0.21 and 2.87 ± 0.29 Ni per $\alpha\beta\gamma$ unit were found. The high levels of nickel ion bound to UreD-urease apoprotein in the absence of bicarbonate may reflect some metal ion binding to UreD that was not completely dissociated from urease. In summary, the Ni-treated apoproteins, like fully active enzyme [2500 U (mg of protein)$^{-1}$; Todd & Hausinger, 1989], bind approximately two nickel ions per $\alpha\beta\gamma$ unit.

*Metal Ion Inhibition of Apoprotein Activation.* The effects of other metal ions on the Ni- and bicarbonate-dependent activation of urease and UreD-urease apoproteins were examined. As shown in Figure 2, divalent zinc, copper, and cobalt reduced the extent of activation of the apoproteins by 50% when present at concentrations of ~10 $\mu$M, i.e., 10-fold less than the nickel ion concentrations. Slightly higher concentrations of manganese ions were required to achieve 50% inhibition of the two samples, and the behavior of Mn(II) at high concentrations was distinct from the other metals. Even in the presence of 3 mM manganese ion, partial activation of urease was observed. For each of these four metal ions inhibition was more pronounced in the urease apoproteins (panel A) than in the UreD-urease apoproteins (panel B). In contrast to these inhibitory metal ions, magnesium and calcium ions exhibited no significant inhibition of urease apoprotein activation, even at 10 mM concentrations.

The number of zinc ions associated with inhibition of the activation processes for urease and UreD-urease apoproteins was determined graphically as shown in Figure 3. The urease apoprotein (panel A) and UreD-urease apoprotein (panel B) plots show the effect on final activity of incubating the proteins with selected concentrations of zinc ions in the activation mixture when using protein concentrations ranging from 1 to 10 $\mu$M of $\alpha\beta\gamma$ unit. In the absence of added zinc ions, the data for both plots fall on straight lines that pass through the origins, as one would expect. The data for samples incubated with zinc ions were nonlinear and appeared to approach lines that were parallel to the non-inhibited lines, consistent with the typical behavior of a tight-binding inhibitor as assessed by this type of Ackerman–

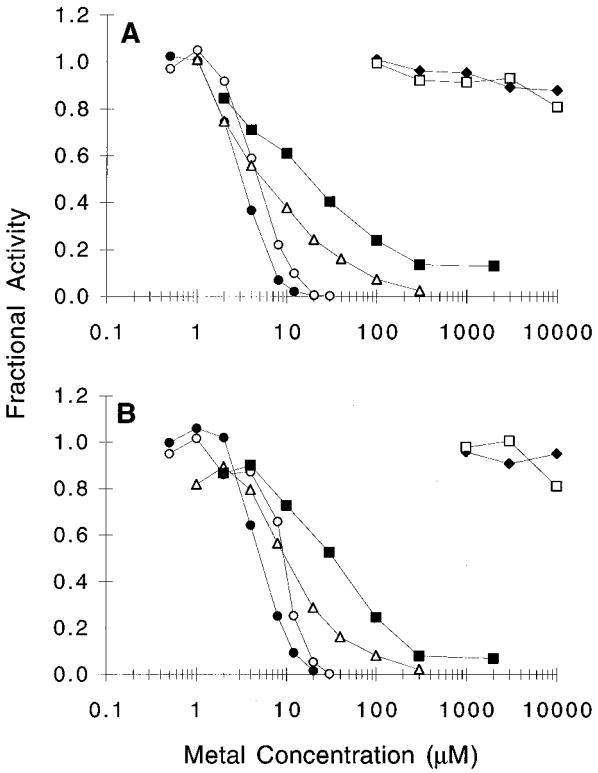


FIGURE 2: Concentration dependence of various metal ions for inhibiting activation of urease and UreD-urease apoproteins. Urease (panel A) and UreD-urease (panel B) apoproteins (5 $\mu$M $\alpha\beta\gamma$ unit) were incubated under standard activation conditions with the inclusion of the indicated concentrations of metal salts: $ZnCl_2$ (●), $CuCl_2$ (○), $CoCl_2$ (△), $MnCl_2$ (■), $MgCl_2$ (◆), and $CaCl_2$ (□). After 2 h, samples were assayed for urease activity and the results were plotted as fractional activity. In control experiments, the urease and UreD-urease apoproteins were activated to yield specific activities of 372 ± 6 and 652 ± 8 U (mg of protein)$^{-1}$, respectively.

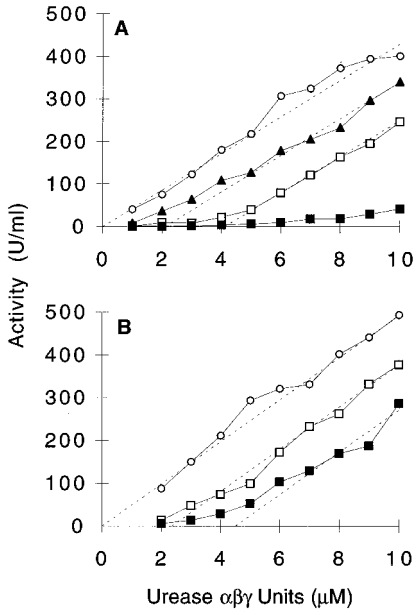


FIGURE 3: Quantitation of Zn-dependent inhibition of urease apoprotein activation. The indicated concentrations of urease apoprotein (panel A) and UreD-urease apoprotein (panel B) were incubated in activation buffer containing 100 $\mu$M $NiCl_2$ and 0 (○), 2.5 (▲), 5 (□), or 10 $\mu$M (■) $ZnCl_2$. The activities were determined after 2 h. Linear regression analysis of the 0 $\mu$M data was used to calculate the slope for lines that were fitted to each data set.

Potter plot (Williams & Morrison, 1979). In the urease apoprotein case, the line for 2.5 $\mu$M zinc ions intercepted

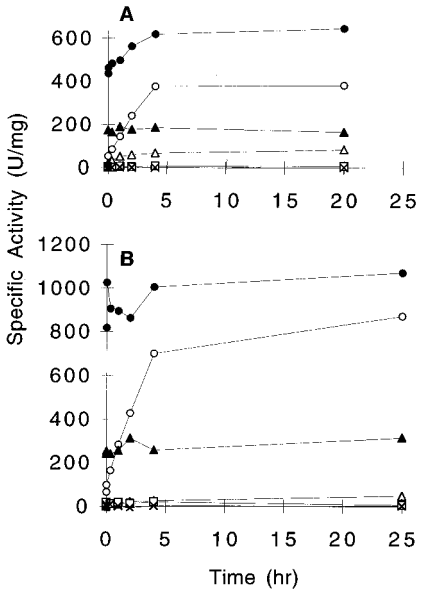


FIGURE 4: Restoration of activation competence to metal ion-inhibited urease apoproteins. Urease apoprotein (panel A) or UreD-urease apoprotein (panel B) samples (10 $\mu$M $\alpha\beta\gamma$ unit) were subjected to a variety of conditions, washed to remove any free metal ions that were present, and treated with EDTA (1 mM). At the indicated time points during incubation at 25 °C, aliquots were subjected to the standard activation conditions for 2 h and urease activity was assayed. The initial conditions included use of the standard activation buffer (●), the standard activation buffer lacking bicarbonate (○), or the standard activation buffer lacking nickel ions but containing 40 $\mu$M $ZnCl_2$ (△), 40 $\mu$M $CuCl_2$ (□), 100 $\mu$M $CoCl_2$ (×), or 300 $\mu$M $MnCl_2$ (▲).

the *X*-axis at about 2 $\mu$M $\alpha\beta\gamma$ unit and the line for 5 $\mu$M zinc ions intercepted at about 4 $\mu$M $\alpha\beta\gamma$ unit. Thus, the inhibition of urease apoprotein activation appeared to be associated with binding of approximately one zinc ion per $\alpha\beta\gamma$ unit. By contrast, the UreD-urease apoprotein data were more consistent with binding of two zinc ions per $\alpha\beta\gamma$ unit in order to inhibit the activation. The requirement for higher levels of zinc ion to inhibit activation of UreD-urease apoprotein over that needed for urease apoprotein inhibition may arise from the zinc binding to UreD.

*Restoration of Activation Competence from Metal-Inhibited Ureases*. We examined whether EDTA treatment could restore activation competence to metal-inhibited urease and UreD-urease apoprotein samples. These data are shown in Figure 4 and compared with Ni- and bicarbonate-activated samples that were EDTA-treated in a similar manner.

The closed circles in Figure 4 illustrate the behavior of samples of urease apoprotein (panel A) and UreD-urease apoprotein (panel B) that had been activated by the normal process [resulting in activities of 442 and 818 U (mg of protein)$^{-1}$, respectively, at the $t = 0$ points in these plots], subjected to 1 mM EDTA treatment for the indicated amount of time, and activated a second time. EDTA is known to be incapable of removing nickel ions from active urease; however, the results in Figure 4 suggest that some portion of non-productively bound nickel ions associated with inactive urease holoproteins could be removed by the chelator. The increase in activation competence for both samples appeared to be nearly complete after about 4 h incubation with EDTA. For both the activated urease and activated UreD-urease samples, the chelation/activation cycle increased the enzyme activity by an amount that correlates to further activation of 10%−15% of the inactive $\alpha\beta\gamma$ units, i.e., (645 − 442)/(2500 − 442) and (1071 − 818)/(2500 − 818), where 2500 U (mg of protein)$^{-1}$ is the nominal specific activity of fully active enzyme (Todd & Hausinger, 1989).

As shown by the open circles in Figure 4, Ni-inhibited ureases, prepared from urease apoprotein (panel A) or UreD-urease apoprotein (panel B) that had been treated with nickel ions in the absence of bicarbonate, regained the ability to be activated after prolonged treatment with 1 mM EDTA. In agreement with the data in Figure 1, the samples possessed only low levels of activity prior to EDTA treatment [12.8 and 66 U (mg of protein)$^{-1}$ at $t = 0$ in these plots]. Incubation of these samples with EDTA restored normal levels of activation competence; i.e., after 4 h incubation with the metal chelator, the Ni-inhibited urease apoprotein and Ni-inhibited UreD-urease apoprotein samples could be activated by using standard conditions to yield specific activity values of 372 and 872 U (mg of protein)$^{-1}$, respectively. The kinetics of the restoration processes were nearly identical for the two samples, with ∼50% activation competence developed after approximately 2 h of incubation with EDTA. These kinetics for regain in activation competence resemble those observed for EDTA treatment of activated samples, as described above.

Zn-inhibited species (Figure 4, open triangles) were partially restored to an activation competent state by EDTA treatment; however, the levels of activity generated by incubation with 1 mM EDTA for the Zn-inhibited urease apoprotein (panel A) and UreD-urease apoprotein (panel B) were only 22% and 5.6% of those obtained with the corresponding Ni-inhibited samples. Zn-inhibited UreD-urease apoprotein formed in the absence of bicarbonate also was examined for its ability to regain activity by EDTA treatment (data not shown). The protein (5 $\mu$M $\alpha\beta\gamma$ unit) was incubated with 100 $\mu$M $ZnCl_2$ in activation buffer lacking bicarbonate and $NiCl_2$ for 80 min, adjusted to 1 mM EDTA, concentrated, washed with HEPES buffer, and activated under standard conditions for 2 h. In contrast to the value of 49 U (mg of protein)$^{-1}$ observed after prolonged efforts to restore activation competence to UreD-urease apoprotein that was treated with zinc ion and bicarbonate, the brief EDTA treatment of bicarbonate-free, Zn-inhibited protein yielded enzyme with a specific activity of 280 U (mg of protein)$^{-1}$ after activation. Thus, restoration of activation competence was more facile in samples treated with zinc ion in buffer lacking bicarbonate.

Treatment of urease apoprotein (panel A) or UreD-urease apoprotein (panel B) with copper or cobalt ions led to the formation of species that were unable to be restored to activation competent states by incubation with 1 mM EDTA (Figure 4, open squares and X symbols). No precautions were taken to exclude oxygen from the inhibition, EDTA treatment, or activation buffers, and we cannot rule out the possibility of oxidative denaturation taking place; however, the presence of a substantial loss of activation competence by zinc ions, where oxidative denaturation is less likely to occur, argues against this possibility.

The behavior of the Mn-inactivated samples of urease was distinct from all other metal ions tested (Figure 4, closed triangles). For both the Mn-treated urease apoprotein (panel A) and UreD-urease apoprotein (panel B), Ni-dependent activation had little effect on the initial activites [∼20 and ∼10 U (mg of protein)$^{-1}$, respectively] if the samples were

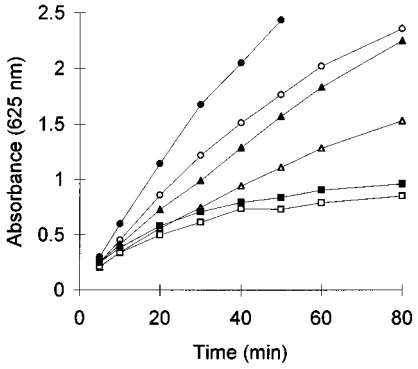


FIGURE 5: Chelator-dependent activity of Mn-containing urease. Urease apoprotein (open symbols) and UreD-urease apoprotein (closed symbols), 2.5 $\mu$M $\alpha\beta\gamma$ unit for each, were incubated in activation buffer lacking $NiCl_2$ and containing 100 $\mu$M $MnCl_2$ for 30 min. Aliquots of 100 $\mu$L were transferred to 2 mL of assay buffer lacking EDTA (triangles), to assay buffer lacking EDTA and containing 100 $\mu$M $MnCl_2$ (circles), or to assay buffer containing 1 mM EDTA (squares). Aliquots (20 $\mu$L) of the reaction mixtures were withdrawn at the indicated time points, and the ammonia produced as a function of time was quantitated by color development.

not exposed to a metal chelator. Treatment of Mn-inhibited samples with 1 mM EDTA resulted in almost immediate restoration of activation competence to a portion of the samples so that activities of 174 $\pm$ 10 and 268 $\pm$ 32 U (mg of protein)$^{-1}$ were generated. Further incubation with EDTA did not significantly affect the level of activation that could be restored. These results suggest that at least a portion of the manganese ions may be loosely bound to the urease active site.

*Demonstration of an Active Mn-Containing Urease.* Following up on the observation that high concentrations of manganese ions failed to completely inhibit urease apoprotein activation (Figure 2), we examined whether Mn-substituted enzyme might possess urease activity. As shown in Figure 5, Mn-treated urease and UreD-urease apoproteins were active. It is important to note that, unlike the case for the Ni-containing enzyme, activity was slowly lost in the presence of 1 mM EDTA. Furthermore, the rate of the enzymatic reaction was enhanced by inclusion of 100 $\mu$M $MnCl_2$ in the assay buffer. These results are consistent with manganese ions being weakly bound to the enzyme, compatible with the results observed in experiments related to restoration of activation competence (Figure 4). The effects of EDTA and $MnCl_2$ in the assay buffer were not observed during the first 4 min of the assay (data not shown); hence, this time range was chosen for detailed kinetic characterization. The Mn-enzymes generated from urease and UreD-urease apoproteins appeared to be identical and exhibited typical Michaelis−Menten type kinetics with $K_M$ = 3.86 $\pm$ 0.15 mM and $V_{max}$ = 8.0 $\pm$ 0.1 U mg$^{-1}$. This $K_M$ is only 1.6-fold the value for the Ni-containing enzyme (2.3 mM). The specific activity of the Mn-containing enzyme is approximately 2% of that for Ni-activated apoprotein or 0.3% of that for the wild type enzyme.

The Mn-dependent activation processes for urease and UreD-urease apoproteins were examined in more detail. The bicarbonate-dependence of activation was identical for the two samples and exhibited saturation at approximately 100 mM (data not shown), in agreement with the bicarbonate-dependence for Ni-promoted activation (Park & Hausinger, 1995a). As shown in Figure 6, the concentration of

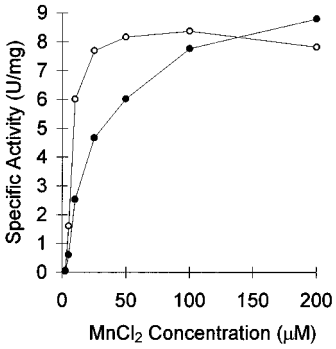


FIGURE 6: Mn-dependence of urease apoprotein activation. Urease apoprotein (○) and UreD-urease apoprotein (●), each at 5 $\mu$M concentration of $\alpha\beta\gamma$ unit, were incubated in activation buffer lacking nickel ion and containing the indicated concentrations of $MnCl_2$ for 30 min, and aliquots were assayed for urease activity in buffer containing 100 $\mu$M $MnCl_2$.

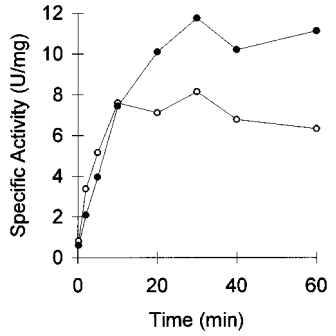


FIGURE 7: Kinetics of Mn activation of urease apoprotein. Urease apoprotein (○) and UreD-urease apoprotein (●), each at 5 $\mu$M concentration of $\alpha\beta\gamma$ unit, were incubated in activation buffer containing 100 $\mu$M $MnCl_2$ for the indicated time periods, and aliquots were assayed for urease activity in buffer containing 100 $\mu$M $MnCl_2$.

manganese ion required for activation of urease apoprotein was lower than that needed for activation of UreD-urease apoprotein; i.e., half-maximal concentrations for activation were <10 $\mu$M $MnCl_2$ and ~25 $\mu$M $MnCl_2$, respectively. These values are the same order of magnitude as those observed for Ni-dependent activation of urease apoprotein (~20 $\mu$M $NiCl_2$) and UreD-urease apoprotein (~25 $\mu$M $NiCl_2$) (Park & Hausinger, 1995a). Using near-saturating concentrations of bicarbonate and $MnCl_2$, the activation kinetics of urease and UreD-urease apoproteins were determined. As shown in Figure 7, activation was not instantaneous; rather, approximately 10 min was required for the urease apoprotein and about 20 min for the UreD-urease apoprotein to reach their final levels of activation. In both cases, Mn-dependent activation was more rapid than the previously described Ni-dependent activation of these proteins which required over 60 min to reach final levels of activation (Park & Hausinger, 1995a).

## DISCUSSION

A general scheme that integrates results related to urease/metal ion interactions is illustrated in Figure 8. Although complex, this model is the most simple one that fits the data presented above. In this figure, P represents the urease apoprotein, P-$CO_2$ denotes the carbamylated protein, and the other states represent various Ni-containing or metal-substituted urease species. Below, we discuss separately the interactions of urease with nickel ions and with other metal

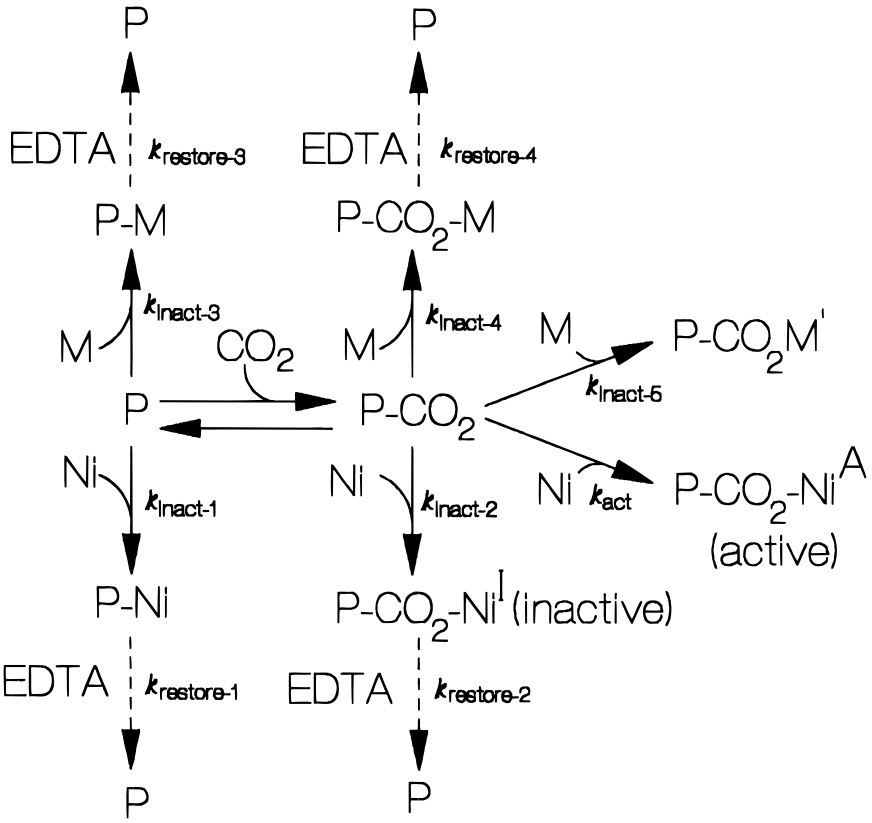


FIGURE 8: Model for the interaction of metal ions with urease apoprotein. The non-carbamylated urease apoprotein is shown as P and the carbamylated protein is indicated by P-$CO_2$. Active enzyme (P-$CO_2$-$Ni^A$) is generated upon productive binding of nickel ions to P-$CO_2$, associated with a rate constant $k_{act}$. Inactive Ni-containing species (P-Ni and P-$CO_2$-$Ni^I$) are formed by non-productive binding of nickel ions to P and P-$CO_2$, with inactivation rate constants $k_{inact-1}$ and $k_{inact-2}$. Other selected metal ions also bind to P to form P-M, with a rate constant $k_{inact-3}$, and bind to P-$CO_2$ to give two distinct P-$CO_2$-M species, with rate constants $k_{inact-4}$ and $k_{inact-5}$, respectively. EDTA treatment removes metal ions from four of the non-productive species with rate constants $k_{restore-1}$ through $k_{restore-4}$, but this chelator is unable to remove to remove nickel ions from active enzyme or other metal ions from the P-$CO_2$-M′ species. The P-Ni and P-$CO_2$-$Ni^A$ species contain 2 Ni per $\alpha\beta\gamma$ unit, whereas the metal contents of the other species remain to be established.

ions in terms of this working hypothesis. Modifications to this scheme are then described to account for the results obtained when using UreD-urease apoprotein, and these results are discussed in relation to the possible role of the UreD protein. Finally, on the basis of the ability of urease to form numerous inactive complexes with metal ions, we speculate on possible roles for other accessory proteins.

*Nickel Ion Interactions with Urease Apoprotein.* We propose that nickel ions bind to urease apoprotein to yield three distinct species: the active, carbamylated enzyme (P-$CO_2$-$Ni^A$), an inactive non-carbamylated enzyme (P-Ni), and an inactive carbamylated species (P-$CO_2$-$Ni^I$). In studies where urease apoprotein is exposed to nickel ion in the absence of bicarbonate (see Figure 1) a Ni-inhibited species (P-Ni) is generated with a pseudo-first-order rate constant, $k_{inact-1}$, of approximately 0.07 $min^{-1}$ ($t_{1/2} \approx 10$ min) in the presence of 100 $\mu$M $NiCl_2$. Atomic absorption analysis results are consistent with this species containing approximately two nickel ions per $\alpha\beta\gamma$ unit. The nickel ions can be removed from the P-Ni species by EDTA treatment (Figure 4A) to restore the ability of the protein to be activated. This regaining of activation competence occurs with a pseudo-first-order rate constant, $k_{restore-1}$, of approximately 0.006 $min^{-1}$ ($t_{1/2} \approx 2$ h) in 1 mM EDTA. The product of this reaction is likely to be P because activation of the Ni-inhibited, EDTA-treated sample yielded enzyme that was identical in activity to that obtained by activation of fresh apoprotein. Addition of nickel ions to apoprotein in the presence of bicarbonate gives rise to active urease with a pseudo-first-order rate constant, $k_{act}$, of roughly 0.046 $min^{-1}$ ($t_{1/2} \approx 15$ min) when using 100 $\mu$M $NiCl_2$ (Park & Hausinger, 1995a); however, we propose that an inactive P-$CO_2$-$Ni^I$ form of the protein is formed in addition to active enzyme. Two types of experimental evidence support this hypothesis. First, the $k_{act}$ value is approximately $^2/_3$ that of $k_{inact-1}$; if these were the only two reactions occurring one would expect that $\sim^2/_5$ of the apoprotein should be activated, but this does not occur. Rather, only 10%−15% of urease apoprotein is typically converted into active enzyme under activation conditions that include saturating levels of bicarbonate. Secondly, $^{14}C$-labeling studies with [$^{14}C$]$NaHCO_3$ (Park & Hausinger, 1995a) reveal the incorporation of 0.48 bicarbonate carbon atoms per $\alpha\beta\gamma$ unit, despite the activation of less than 10% of the protein in that experiment. The $^{14}C$-incorporation numbers generated during the radiolabeling experiment are likely to be underestimated due to the presence of contaminating unlabeled bicarbonate in the buffer solutions. Because the metal content of Ni-activated urease apoprotein is slightly less than two Ni per $\alpha\beta\gamma$ unit, the inactive carbamylated species may contain one or two nickel ions. After a single round of activation, the inactive P-$CO_2$-$Ni^I$ species can be restored to a state of activation competence by treatment with EDTA (Figure 4A) and active enzyme can be generated from 10% to 15% of the apoprotein formed. The rate of the restoration process, $k_{restore-2}$, appears to be identical to that for the non-carbamylated protein. In contrast

to the P-Ni and P-$CO_2$-$Ni^I$ species, nickel ion is not removed from the active enzyme by EDTA treatment.

*Other Metal Ion Interactions with Urease Apoprotein.* The results shown in Figures 2−7 represent the first clear demonstration that urease apoprotein can bind a variety of other metal ions. The only published indication that metal-substituted ureases can form is found in work by Zerner (1991), where jack bean urease was dialyzed against buffers containing divalent zinc or cobalt for nearly 2 months and the metal content changed from 2 Ni per subunit to 0.83 Zn + 0.89 Ni per subunit and 0.66 Co + 1.17 Ni per subunit, respectively. Our data are consistent with the rapid generation of three metal-substituted urease species (P-M, P-$CO_2$-M, and P-$CO_2$-M′) from urease apoprotein. The metal contents of these species are not established, but it is reasonable to speculate that they reflect the metal contents of the nickel-containing species.

Zinc ions inhibit urease apoprotein activation. Under normal activating conditions where the $NiCl_2$ concentration is 100 $\mu$M, ∼3 $\mu$M $ZnCl_2$ reduces the extent of activation by half (Figure 2A). Our kinetic analysis of urease interaction with $ZnCl_2$ appears to fit the upper half of the scheme for urease/metal ion interactions shown in Figure 8. Zinc ions bind to the non-carbamylated apoprotein to yield an inhibited species that rapidly recovers activation competence when exposed to EDTA. In contrast, only ∼20% of the Zn-inhibited protein prepared in the presence of bicarbonate is restored to an activation competent state by EDTA treatment, and this restoration is a much slower process (Figure 4A). These results support the proposal for a P-M state (associated with $k_{\text{restore-3}}$) and two P-$CO_2$-M species (one associated with $k_{\text{restore-4}}$ and one with the metal ion "locked in"). The basis for the differences in the latter two species may parallel those found in the Ni-activated protein where the metal in active enzyme is inaccessible to the chelator.

Copper and cobalt ions also are highly inhibitory to urease activation (Figure 2A). The inability to restore activation competence to the Cu- or Co-inhibited proteins (Figure 4A) may indicate tht P-$CO_2$-M′ is formed to the exclusion of P-$CO_2$-M, or the stability of the P-$CO_2$-M species may be sufficiently great that $k_{\text{restore-4}}$ is not observed.

Manganese ion interactions with urease apoprotein are of special interest. Mn-substituted urease is active and possesses ∼2% of the activity observed in Ni-activated enzyme. This is the first demonstration of an active metal-substituted urease. In contrast to the wild-type enzyme, activity is lost by incubation of Mn-urease in the presence of EDTA (Figure 5). EDTA treatment of Mn-urease also leads to an increase in activation competence of the protein, yielding a final activity of 174 ± 10 U (mg of protein)$^{-1}$ (Figure 4A). These results are compatible with the formation of approximately equal amounts of P-$CO_2$-M and P-$CO_2$-M′ species when protein is incubated with manganese ion and bicarbonate. In contrast to the situation for Ni-activated protein, the active Mn-urease appears to be the P-$CO_2$-M species that dissociates during EDTA treatment, while the P-$CO_2$-M′ species is inactive. It is presently unclear why manganese ion is able to confer partial activity to urease apoprotein.

In retrospect, it is not too surprising that other metal ions bind to urease apoprotein. Adenosine deaminase and phosphotriesterase are known to possess structures with folding patterns that are similar to the urease catalytic domain, and these proteins retain many features of the urease active site environment (Jabri *et al*., 1995). Adenosine deaminase possesses residues comparable in position to the urease H134, H136, H246, and D360 Ni-ligands, and these amino acids function as ligands to the single, requisite zinc ion in that protein (Wilson *et al*., 1991). Thus, it may be expected that non-carbamylated urease could reasonably bind one or more zinc ions. The apparent ability of a single zinc ion to inhibit urease apoprotein activation (Figure 3A) may be related to this behavior. Phosphotriesterase possesses residues corresponding to all of the urease Ni-ligands, including a carbamylated lysine residue that bridges the binuclear metal center of this enzyme (Benning *et al*., 1995). Phosphotriesterase is known to be active with divalent zinc, cadmium, nickel, cobalt, and manganese ions, and will bind selected other metals such as copper to form inactive species (Chae *et al*., 1995). Hence, it is not unreasonable to expect that urease, with an active site of similar structure, could also accommodate a range of metal ions as we have now demonstrated. These findings now raise two questions: Why does only nickel (and to a small extent manganese) ion confer activity to the urease protein? How does the cell ensure that only nickel ions are incorporated? The answer to the first question remains obscure, but it is important to note that the metal ions in several of these metal-substituted forms of urease may serve as useful spectroscopic probes to further characterize the enzyme mechanism, including features related to this question. With regard to the second question, an obvious possibility is that the urease accessory proteins must play a key role in the specificity of metal ion incorporation.

*Metal Ion Interactions with UreD-Urease Apoprotein*. Comparison of the activation properties for the urease and UreD-urease apoproteins allows us a means to examine the role of UreD in metallocenter assembly. In general, the interactions of nickel and other metal ions with UreD-urease apoprotein follows the same scheme as shown for the apoprotein (Figure 8) except that metal ion binding is accompanied by UreD dissociation. In the absence of bicarbonate, nickel ions bind to UreD-urease apoprotein to form an inhibited species (Figure 1) with a pseudo-first-order rate constant ($k_{\text{inact-1}}$) of approximately 0.035 min$^{-1}$ ($t_{1/2}$ ≈ 20 min) at 100 $\mu$M $NiCl_2$, or approximately half of that observed for Ni-inhibition of the urease apoprotein. In the presence of saturating levels of bicarbonate, UreD-urease apoprotein is activated with a pseudo-first-order rate constant ($k_{\text{act}}$ ≈ 0.028 min$^{-1}$, $t_{1/2}$ ≈ 25 min; Park & Hausinger, 1995a) that is ∼60% of that observed for the apoprotein. Again, two Ni-containing carbamylated species are posited in order to account for the observed activation of 25%−30% of the protein under these conditions and to explain the results from prior $^{14}$C-bicarbonate radiolabeling studies (Park & Hausinger, 1995a). Atomic absorption analysis results are compatible with the P-Ni species containing 2 Ni per $\alpha\beta\gamma$ unit, as known for the active enzyme (Jabri *et al*., 1995). Both the Ni-inhibited species are restored to an activation competent state by treatment with EDTA (Figure 4B). The kinetics of these activation processes appear to be identical to those seen with Ni-inhibited urease apoprotein. Curiously, the P-Ni species generated from UreD-urease apoprotein can be activated, after EDTA treatment, to a level of 30%−35%, rather than only 10%−15% as seen with apoprotein alone. In contrast, the second round of activation generates 15% additional active enzyme from the P-$CO_2$-$Ni^I$ pool, as

expected if this becomes apoprotein in the presence of EDTA. The behavior of UreD-urease apoprotein with other metal ions again parallels the urease apoprotein results. Slightly higher concentrations of Zn, Cu, Co, and Mn salts are required to inhibit activation by 50% compared to the urease apoprotein (Figure 2). Mn-urease generated from UreD-urease apoprotein is active, but the formation of this species requires longer times and higher nickel ion concentrations than are needed for apoprotein (Figures 6 and 7). In summary, the presence of UreD appears to reduce the rate of apoprotein interaction with nickel and other metal ions in all of its reactions. If the $k_{inact}$ rate constants were reduced to a greater extent than the reduction in rate of $k_{act}$, the net effect would be an increase in the amount of enzyme activated (~30%) compared to that for urease apoprotein (~15%). It remains unclear whether these small changes are related to the physiological role of UreD.

*Roles of Urease Accessory Proteins.* Our primary interest in urease metallocenter assembly focuses on how nickel ions are correctly incorporated into the protein. The studies reported here demonstrate that nickel ions can be misincorporated into urease and that the apoprotein binds numerous other metal ions, some with higher affinity than seen for nickel. These results highlight the need for additional factors in order to activate urease in vivo. These additional factors clearly include the UreD, UreF, and UreG accessory proteins that are required for generating active urease in the cell [reviewed by Moncrief and Hausinger (1996)]. We previously demonstrated the presence of a cellular UreD-UreF-UreG-urease apoprotein complex and proposed that this complex functions in urease activation (Park & Hausinger, 1995b). In that work, we speculated that the accessory proteins may function in provision of $CO_2$ or nickel ion. On the basis of results reported here demonstrating that numerous metal ions bind to urease apoprotein, we suggest two additional potential roles for the accessory proteins. These proteins may function to impart specificity on urease apoprotein for binding nickel ion or to reverse the binding of inappropriate metal ions or of non-productively bound nickel ion. Further studies are needed to test these hypotheses and to further assess the validity of the working model for urease-metal ion interactions shown in Figure 8.

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