Self-Assembly of the Binuclear Metal Center of Phosphotriesterase†

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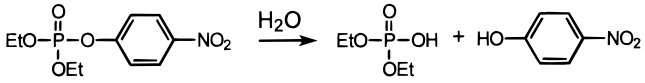
ABSTRACT: The active site of the bacterial phosphotriesterase (PTE) from *Pseudomonas diminuta* contains two divalent metal ions and a carboxylated lysine residue. The native enzyme contains two Zn^{2+} ions, which can be replaced with Co^{2+}, Cd^{2+}, Ni^{2+}, or Mn^{2+} without loss of catalytic activity. Carbon dioxide reacts with the side chain of lysine-169 to form a carbamate functional group within the active site, which then serves as a bridging ligand to the two metal ions. The activation of apo-PTE using variable concentrations of divalent metal ions and bicarbonate was measured in order to establish the mechanism by which the active site of PTE is self-assembled. The time courses for the activation of apo-PTE are pseudo-first-order, and the observed rate constants are directly proportional to the concentration of bicarbonate. In contrast, the apparent rate constants for the activation of apo-PTE decrease as the concentrations of the divalent cations are increased and then become constant at higher concentrations of the divalent metal ions. These results are consistent with a largely ordered kinetic mechanism for the assembly of the binuclear metal center where CO_2/bicarbonate reacts with the apo-PTE prior to the binding of the two metal ions. When apo-PTE is titrated with 0−8 equiv of Co^{2+}, Cd^{2+}, or Zn^{2+}, the concentration of activated enzyme increases linearly until 2 equiv of metal ion is added and then remains constant at elevated levels of the divalent cations. These results are consistent with the synergistic binding of the two metal ions to the active site, and thus the second metal ion binds more tightly to the protein than does the first metal ion. Measurement of the mean dissociation constant indicates that metal binding to the binuclear metal center is strong [$(K_\alpha K_\beta)^{1/2} = 6.0 \times 10^{-11}$ M and $k_{off} = 1.5 \times 10^{-3}$ min^{-1} for Zn^{2+}]. The removal of the carbamate bridge through the mutagenesis of Lys-169 demonstrates that the carbamate bridge is required for both efficient catalysis and overall stability of the metal center.

The bacterial phosphotriesterase (PTE),[1] originally isolated from the soil bacterium *Pseudomonas diminuta*, catalyzes the detoxification of a wide variety of organophosphate nerve agents (*1*, *2*). The reaction catalyzed by this enzyme, illustrated with the insecticide paraoxon, is shown in Scheme 1. Even though organophosphate triesters are not naturally occurring compounds, the enzymatic hydrolysis of these substrates is remarkably efficient. For example, with the native enzyme, the k_{cat} and k_{cat}/K_m values for the hydrolysis of paraoxon are ∼2200 s^{-1} and ∼4 × 10^7 M^{-1} s^{-1}, respectively (*3*). Moreover, solvent viscosity and Brønsted analyses have demonstrated that the catalytic turnover of activated substrates, such as paraoxon, is not limited by the actual chemical cleavage of the P−O bond, but rather is hindered by diffusional steps that occur during the association of substrates and release of products with the enzyme (*3*, *4*).

Biochemical and X-ray structural investigations of PTE have identified the major catalytic components within the active site of this protein (*5*−*9*). The catalytic center is composed of two closely spaced divalent cations that are

Scheme 1



ligated to the protein via direct interactions with His-55, His-57, His-201, His-230, and Asp-301 (*8*). In addition, the two metal ions are bridged by a single solvent molecule and a carboxylated lysine residue (*8*). A representation of the binuclear metal center of PTE is shown in Figure 1. Catalytic activity is retained when the divalent cations are substituted with Zn^{2+}, Co^{2+}, Ni^{2+}, Cd^{2+}, or Mn^{2+} (*5*). The cobalt-substituted PTE has the highest reported value for k_{cat} (7800 s^{-1}) using paraoxon as substrate, followed by nickel (6000 s^{-1}), cadmium (4600 s^{-1}), zinc (2200 s^{-1}), and manganese (1800 s^{-1}). EPR analysis of the Mn/Mn-PTE has demonstrated that the two metal ions are antiferromagnetically coupled to one another (*10*). The structure of the binuclear Ni^{2+} center in urease is architecturally similar to the metal center found within the active site of PTE (*11*). However, PTE does not catalyze the hydrolysis of urea, and urease does not catalyze the hydrolysis of organophosphates. It appears certain that these two proteins are evolutionarily related through a common ancestral precursor and are members of the same superfamily of metal hydrolase proteins (*12*).

The mechanism for the assembly of the binuclear metal center found in PTE is unknown. In the virtually identical

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[1] Abbreviations: PTE, phosphotriesterase; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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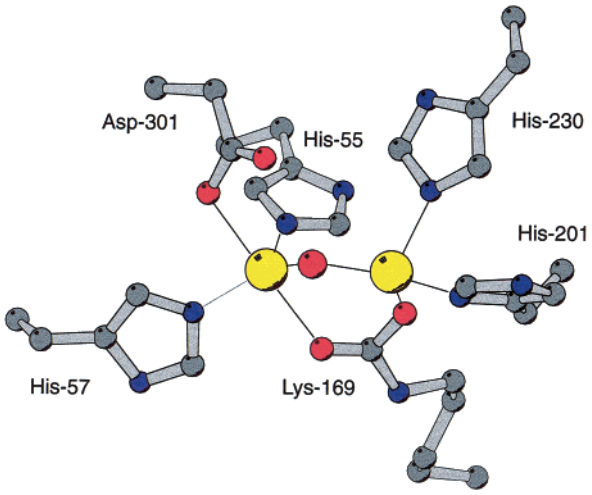


FIGURE 1: X-ray crystallographic structure of the metal center of the phosphotriesterase with two Zn^{2+} ions (*9*).

metal center of urease, there appears to be a host of accessory proteins, some with functions that are not fully determined, which are required for the construction and assembly of the fully activated urease (*13*). However, no such proteins are apparently required for the full reconstitution of the apo-PTE following the addition of divalent cations and bicarbonate (*5*, *7*). The most unusual feature that must occur during the assembly of the binuclear metal center of PTE is the posttranslational modification of Lys-169 with CO_2/HCO_3^-. The formation of the carbamate functional group is thermodynamically unfavorable, but it can be stabilized through interactions with the pair of divalent cations.

In this investigation, we have utilized kinetic and thermodynamic approaches to elucidate the mechanism for the self-assembly of the binuclear metal center of PTE. These studies with the wild-type protein and selected mutants are consistent with a mechanism whereby the assembly process is initiated by the reaction of CO_2 with the ϵ-amino group of Lys-169. This event triggers the subsequent binding of the two divalent cations to complete the formation of the binuclear metal center. The overall rate of the self-assembly process is limited by carbamate formation, and the addition of the two metal ions is highly cooperative.

MATERIALS AND METHODS

Materials. The mutant K169A and the wild-type PTE were prepared and purified as previously described (*5*, *6*). All of the other chemicals used for this investigation were commercially available and used without further purification. The catalytic activity of PTE was routinely determined by measuring the hydrolysis of 1.0 mM paraoxon at pH 9.0 (100 mM CHES) at 400 nm and using $\epsilon = 1.7 \times 10^4$ M^{-1} cm^{-1} for the *p*-nitrophenolate product.

Activation of Apo-enzyme. Divalent metal ions (0−8 mol equiv) and potassium bicarbonate (10−500 mM) were added to the apo-enzyme (wild type or K169A mutant) at pH 8.5 (50 mM HEPES) and then incubated at 25 °C for various periods of time. Aliquots were periodically removed from the enzyme solution, and the catalytic activity was determined as a function of incubation time. The changes in specific activity were fitted to eq 1 where v is the specific catalytic activity of PTE at time t, V_m is the final specific activity, and k is the observed pseudo-first-order rate constant for the activation process.

$$v = V_m(1 - e^{-kt}) \quad (1)$$

Dissociation Constants for Divalent Cations. The dissociation constants for the metal ions from the holo-enzyme were determined using the method of Kiefer et al. (*14*). The apo-enzyme was dialyzed against a large excess of a solution containing known amounts of dipicolinate and a divalent cation. The concentration range of dipicolinate was varied from 1 to 3.0 mM, and the total divalent cation concentration was varied from 30 to 480 μM. The concentration of free metal ion was determined from the known stability constants for the dipicolinate−metal ion complexes (*15*). For the $M^{2+}L_2$ complexes, the stability constants for Zn^{2+}, Co^{2+}, and Cd^{2+} are $10^{-13.0}$, $10^{-12.5}$, and $10^{-10.0}$ M^2, respectively (*15*). After 20 h of dialysis, the free metal ion and dipicolinate were removed from the protein solution by passing the enzyme through a PD-10 gel filtration column (Pharmacia). The catalytic activity of the enzyme was measured, and then the ratio of activated enzyme to total protein was determined. The data were fitted to eq 2 to determine the product of the apparent dissociation constants (K_α and K_β) for the binding of the two metal ions to apo-PTE. This equation assumes that the binding of the second metal ion to the protein is significantly tighter than the binding of the first metal ion.

$$[M_\alpha M_\beta{-}PTE]/[E]_t = [M_f]^2/([M_f]^2 + K_\alpha K_\beta) \quad (2)$$

Disassembly of the Binuclear Metal Center. The fully activated wild-type enzyme was mixed with EDTA (10−50 mM), and the decrease in the catalytic activity was followed as a function of time. The data were fitted to the eq 3, where v is the activity of the enzyme at time t, V_m is the initial enzymatic activity, and k is the observed pseudo-first-order rate constant for the dissociation of metal ion from PTE. The apparent dissociation rate constants were determined at various concentrations of EDTA, and then the data were extrapolated to a concentration of zero EDTA in order to determine the rate constant, k_{off}, for the dissociation of the first metal ion from $M_\alpha M_\beta$−PTE.

$$v = V_m(e^{-kt}) \quad (3)$$

The rate of dissociation of metal ions from the K169A mutant enzyme was too rapid to determine by this method. Therefore, the loss in catalytic activity was determined continuously by adding paraoxon and EDTA simultaneously to the Zn/Zn−K169A enzyme while monitoring the change in absorbance at 400 nm. The assay solution contained 1.0 mM paraoxon, 100 mM CHES (pH 9.0), and varying amounts (0.75−10 mM) of EDTA in the presence or absence of 100 mM propionic acid. The data were fitted to eq 4 where A_{obs} is the observed absorbance at time t, A_t is the total absorbance change, and k is the observed rate constant for the loss of catalytic activity.

$$A_{obs} = A_t(1 - e^{-kt}) \quad (4)$$

Inhibition by Hydrogen Sulfide. Sodium bisulfide was utilized as an inhibitor of the phosphotriesterase-catalyzed reaction. At elevated concentrations, this compound induced

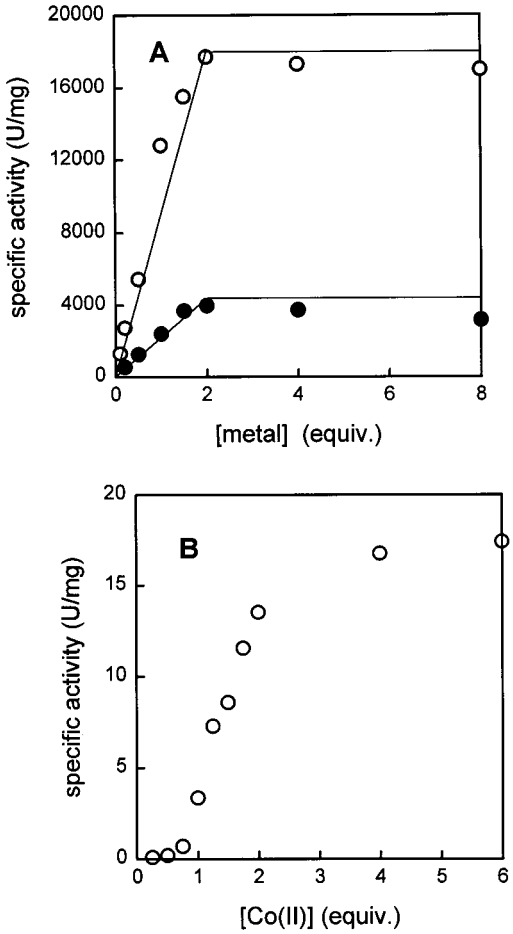


FIGURE 2: (A) Activation of apo-PTE with variable amounts of Zn^{2+} (●) and Co^{2+} (○). The solid lines are drawn to indicate a theoretical titration for the synergistic binding of two metal ions to a binuclear metal center. (B) Activation of the apo-form of the K169A mutant enzyme with variable amounts of Co^{2+}. The concentration of the enzyme is 100 μM. Additional details are provided in the text.

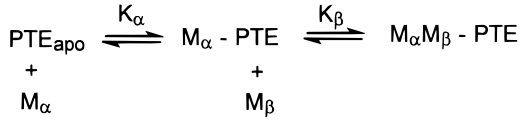
substrate inhibition by paraoxon. The bisulfide concentration was varied from 0 to 2.0 mM at concentrations of paraoxon ranging from 0.02 to 2.0 mM. The data were fitted to eq 5 where A is the concentration of paraoxon, I is the concentration of bisulfide, K_a is the Michaelis constant for paraoxon, and K_{ii} and K_{is} are the intercept and slope inhibition constants, respectively, for bisulfide. The value of K_I is the upper limit for the product of two dissociation constants [the dissociation constant of paraoxon from the E−bisulfide−paraoxon complex (K_i) and the dissociation constant for bisulfide from the E−bisulfide complex (K_{Ia})].

$$v = V_m A/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii}) + A^2 I/K_I] \quad (5)$$

RESULTS AND DISCUSSION

Reconstitution of the Binuclear Metal Center. Full catalytic activity can be recovered when the apo-form of phosphotriesterase is incubated with an excess of divalent cations and bicarbonate at pH 8.5. Shown in Figure 2A is the overall effect of adding variable amounts of Zn^{2+} and Co^{2+} to the apo-form of PTE. The final catalytic activity was measured after an incubation period of 24 h at 4 °C, using paraoxon as the test substrate. The Co/Co-substituted enzyme has a value for k_{cat} that is ~4-fold greater than the Zn/Zn-substituted enzyme (*5*). For both divalent cations, there is a linear increase in the specific activity until the metal:protein ratio is equal to ~2. With the wild-type enzyme, there is no further increase in specific activity up to 8 equiv of metal:protein ratio. The sharp break in the reconstitution profile indicates that the binuclear metal center can be self-assembled in high yield without any additional requirements for other cofactors or auxiliary proteins. Moreover, these results also indicate that the thermodynamic dissociation constant for the disassembly of the metal cluster is significantly below the concentration of apo-protein (25 μM) used in these studies. Otherwise, more than 2 equiv of metal ion would be required to fully saturate the active site.

Scheme 2



The linear increase in catalytic activity with an increasing ratio of divalent cation to protein is indicative of a highly cooperative assembly process. Thus, the dissociation constant for the binding of the second metal ion to the cluster is substantially smaller than the binding of the first metal ion to the apo-form of the protein. This conclusion can be supported with the simple model that appears in Scheme 2. It is assumed in this model that only protein with a fully assembled binuclear metal center ($M_\alpha M_\beta$−PTE) is catalytically active. When apo-protein is titrated with 0−2 equiv of divalent cation, the metal ions distribute among the three enzyme forms (there is no need in this simple example to distinguish between the two possible mononuclear forms: M_α−PTE and M_β−PTE). At one extreme, if the binding of the first metal ion is significantly tighter than the binding of the second metal ion, then no catalytic activity would be observed until the first site is completely occupied. After the first equivalent of metal ion is added, then there will be a linear increase in specific activity until the second full equivalent of metal ion is added. At the other extreme, there will be a linear increase in catalytic activity in the titration from 0 to 2 equiv of metal ion since, in a highly synergistic model, the divalent cations will effectively be adding in pairs. The concentration of enzyme with a single metal ion bound to the protein would be insignificant at any point in the titration. If, however, the dissociation constants were identical, then the titration curve would be intermediate in form when compared to either of the two extreme cases. At a metal:protein ratio of 1:1, the specific catalytic activity would be 33% of the maximum value since the distribution of apo-PTE:M_α−PTE:$M_\alpha M_\beta$−PTE would be 1:1:1. Shown in Figure 3 are theoretical plots using variable ratios for the two dissociation constants. The assembly of the wild-type binuclear metal center of PTE conforms to the highly cooperative model where the divalent cations are effectively adding in pairs.

Reconstitution of the apo-enzyme form of the K169A mutant was conducted in order to explore the role of the bridging carbamate functional group in the assembly of the binuclear metal center. The relationship for the specific catalytic activity as a function of the ratio of added Co^{2+} to the apo-protein is shown in Figure 2B. In this titration, there is clearly a lag in the appearance of catalytic activity. The

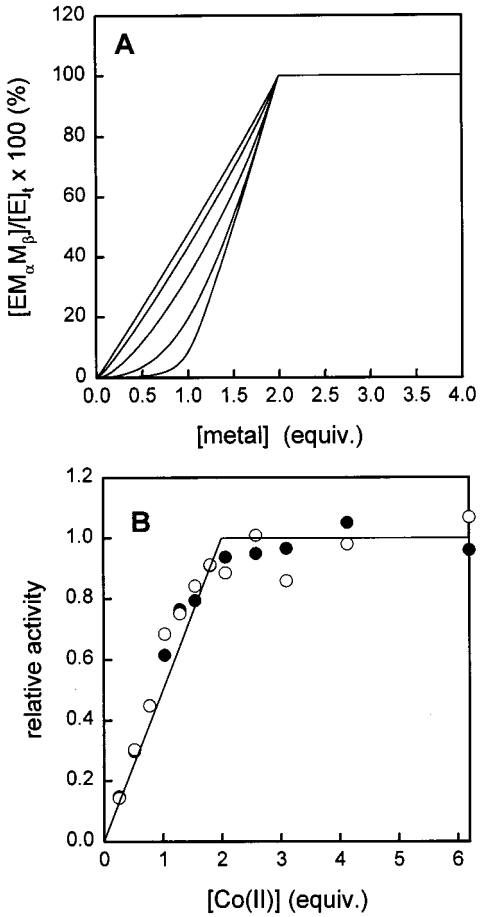


FIGURE 3: (A) Theoretical plots for the titration of PTE based on the model for the self-assembly of the metal center shown in Scheme 2, with variable ratios for the two dissociation constants. The values for K_α/K_β from top to bottom are 100, 10, 1, 0.1, and 0.01, respectively. (B) Titration profile for apo-PTE with Co^{2+} using demeton-S (●) or paraoxon (○) as the test substrate. The solid lines are drawn to indicate a theoretical titration for the synergistic binding of two metal ions to a binuclear metal center. Additional details are provided in the text.

titration is distinctly dissimilar to that observed for the wild-type enzyme, and thus the metal ions are not cooperatively adding in pairs. Comparison with the theoretical plot presented in Figure 3A indicates that the dissociation constant for the first metal ion to bind to the apo-K169A is ~10-fold lower than the dissociation constant for the second metal ion. These results indicate that the carbamate bridge plays a significant role in the promotion and assembly of the binuclear metal center. The overall value of k_{cat} for the K169A mutant is 10 s^{-1} when the protein is activated by Co^{2+}, and thus the loss of the carboxylated lysine that serves to bridge the two divalent cation results in a net reduction in k_{cat} by a factor of ~800.

It has been reported by diSioudi et al. (*16*) that an active site containing a single metal ion is all that is required to activate PTE for the hydrolysis of demeton-S (I) whereas two metal ions are required for catalytic activity with paraoxon (II). Since we have repeatedly demonstrated that the apo-protein of wild-type PTE adds two metals in a highly cooperative fashion, it is difficult to understand how the wild-type protein with a single metal ion could be prepared and stabilized. Moreover, at ambient temperature, the assembly and disassembly processes are kinetically sluggish. Thus, it is not obvious how protein, prepared with 1 equiv of a

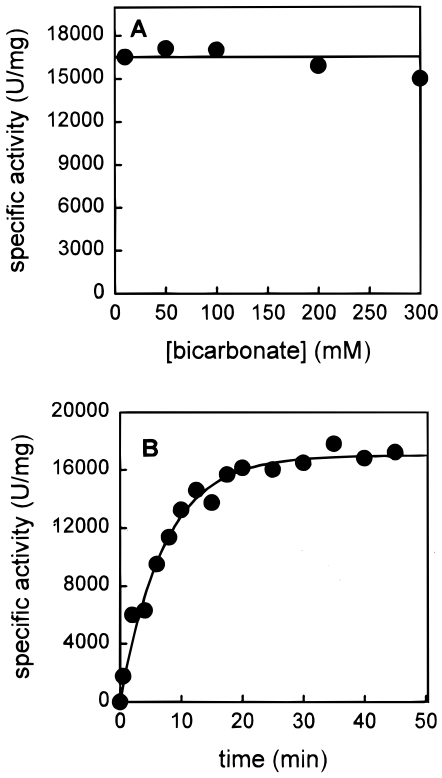
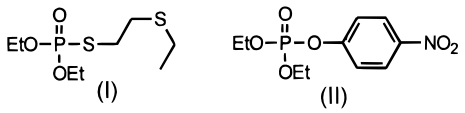


FIGURE 4: (A) Equilibrium activity of PTE samples when the apo-form of PTE is incubated with 100 equiv of Co^{2+} and variable amounts of bicarbonate. (B) Time course of PTE activation with 100 equiv of Co^{2+} and 100 mM bicarbonate. The data were fitted to eq 1. The values for V_m and k were found to be 1.7×10^4 units/mg and 0.14 min^{-1}, respectively. Additional details are provided in the text.

Scheme 3



divalent cation, could spontaneously equilibrate between a completely mononuclear site and a binuclear site (with 50% occupancy) depending on which substrate is used to monitor the catalytic activity. The thermodynamic titration curve of apo-PTE with Co^{2+} as the divalent cation using demeton-S as the assay substrate is shown in Figure 3B. Relative to paraoxon, the enzymatic hydrolysis of demeton-S is quite slow, but the titration curve and assembly mechanism for the binuclear metal center are the same. Therefore, a binuclear metal center is required with the wild-type enzyme to hydrolyze either paraoxon or demeton-S (Scheme 3).

Effect of Bicarbonate. It has previously been shown that the assembly of the binuclear metal center within PTE is accelerated by the addition of bicarbonate (*7*). A plot of the final specific activity when the apo-form of PTE is incubated with 100 equiv of Co^{2+} and variable amounts of bicarbonate is shown in Figure 4A. There is no significant effect on the final specific activity since even the low levels of endogenous bicarbonate in the buffer solution are sufficient to fully assemble the binuclear metal center of PTE. Although there is no effect on the final catalytic activity, there is, however, a substantial effect on the rate of formation of the binuclear metal center when the concentration of bicarbonate is varied. When apo-PTE is incubated with 100 equiv of Co^{2+} and

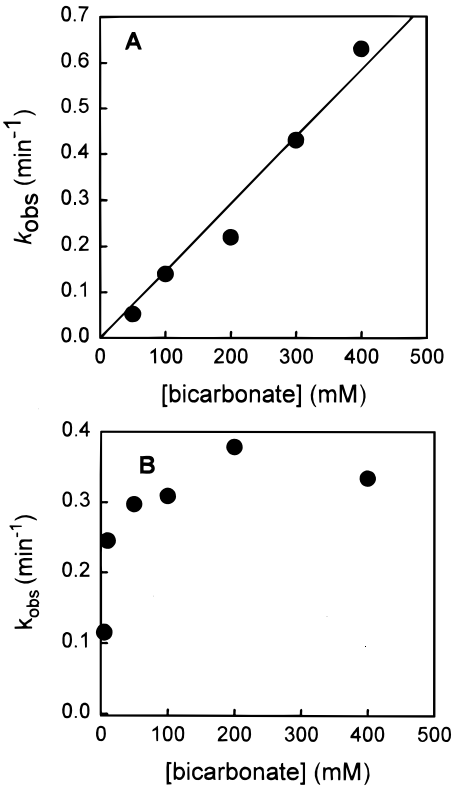
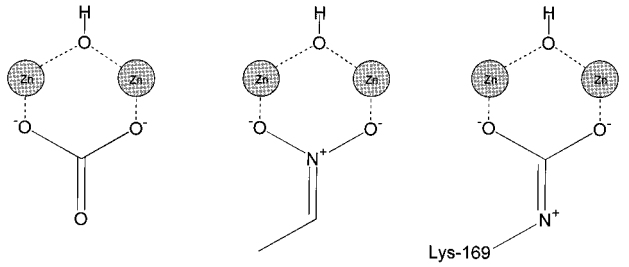


FIGURE 5: (A) Rate of activation of apo-PTE with 100 equiv of Co^{2+} and variable amounts of bicarbonate. (B) Rate of activation of apo-K169A with 8 equiv of Co^{2+} and variable amounts of bicarbonate. Additional details are provided in the text.

Scheme 4



100 mM bicarbonate, the regain of catalytic activity is first-order (Figure 4B) with a rate constant of 0.14 min^{-1}. If the observed first-order rate constants are plotted as a function of the initial bicarbonate concentration, then a second-order rate constant of 1.5 ± 0.1 M^{-1} min^{-1} is obtained at an initial Co^{2+} concentration of 2.5 mM (Figure 5A). The actual substrate for the activation of the wild-type enzyme is presumed to be carbon dioxide rather than bicarbonate.

The rate of activation of the apo-K169A mutant also depends on the initial concentration of bicarbonate in the reconstitution solution, while the ultimate specific activity is unaffected by the addition of bicarbonate over that found adventitiously in the buffer solution. However, unlike the wild-type enzyme, the increase in the rate of activation is saturated when the concentration of bicarbonate is higher than 50 mM (Figure 5B). The activation by added bicarbonate during the assembly of the binuclear metal center within the K169A mutant suggests that bicarbonate (or carbonate) itself is able to act as a weak bridge between the two metal ions as illustrated in Scheme 4. When incubated with 8 equiv of Co(II) and 50 mM bicarbonate, the apo-form of the

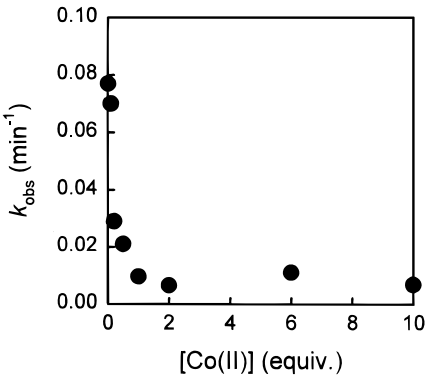


FIGURE 6: Observed pseudo-first-order rate constant for the activation of apo-PTE, as a function of the initial concentration of Co^{2+}. Additional details are provided in the text.

K169A mutant is activated with a value for $k_{obs} = 3.0 \times 10^{-1}$ min^{-1}, while under the same conditions the observed rate constant of activation for the wild-type enzyme is 6.0×10^{-2} min^{-1}. Presumably, the activation of the K169A mutant is significantly faster than the activation of the wild-type enzyme because the unstable carboxylated lysine residue does not need to form prior to the addition of the metal ions to the apo-protein.

It has previously been reported that small ligands such as acetic acid, propionic acid, or ethylamine (in the presence of high concentrations of bicarbonate) can rescue the activity of Lys-169 mutants (*17*). Specifically, propionic acid can be used to activate the assembly of the binuclear metal center within the K169A mutant (*7*). The rate of activation is linearly dependent upon the concentration of propionic acid in the activation medium, much like the bicarbonate-dependent activation of the wild-type enzyme (data not shown). However, unlike the wild-type enzyme, the ultimate specific activity ($k_{cat} = 4700$ s^{-1} at 200 mM propionic acid) is dependent on the amount of propionic acid added to the activation solution because of the relatively weak binding of propionic acid to the K169A mutant. Interestingly, nitroethane can also enhance the catalytic activity of the K169A mutant enzyme ($k_{cat} = 700$ s^{-1} at 100 mM nitroethane), suggesting that the nitro group can function as a bridging ligand. It is likely that the activated form of the bridging ligand is the deprotonated nitroethane in the aci-configuration as depicted in Scheme 4. A representation of the bridging ligand in the wild-type enzyme is also presented in this scheme.

Variation of Divalent Cation. With an excess of Co^{2+}, there is a linear increase in the rate of assembly of the binuclear metal center when bicarbonate is varied up to a concentration of at least 400 mM (Figure 5A). When the bicarbonate concentration is held constant and the [Co^{2+}] is varied, the pseudo-first-order rate constant for the formation of active enzyme diminishes as the Co^{2+} concentration increases. At excess Co^{2+}, the rate constant for the formation of active enzyme is constant and linearly dependent on the concentration of added bicarbonate. A plot of the observed pseudo-first-order rate constant as a function of the initial Co^{2+} concentration is presented in Figure 6. At high levels of divalent cation, the observed first-order rate constant has a value of 0.008 ± 0.002 min^{-1} while the extrapolated value at zero Co^{2+} is 0.08 ± 0.01 min^{-1} when the total bicarbonate concentration is ~1 mM.

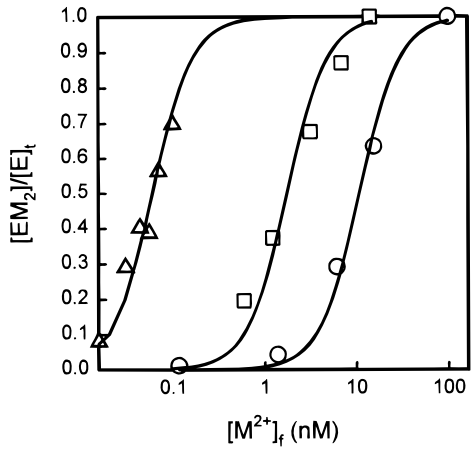


FIGURE 7: Measurement of metal dissociation constants of Zn^{2+} (△), Co^{2+} (□), and Cd^{2+} (○) with PTE. Additional details are provided in the text.

Scheme 5

$$PTE_{apo} \underset{k_2}{\overset{k_1[HCO_3^-]}{\rightleftharpoons}} PTE\text{-}NH\text{-}CO_2^- \xrightarrow{k_3[Co^{2+}]} M_\alpha M_\beta\text{-}PTE$$

The diminution in the observed rate constant for the attainment of catalytic activity with an increasing concentration of the divalent cation is consistent with an assembly process for the wild-type enzyme that proceeds in two stages (*18*). A slower rate-determining step must precede the binding of the two divalent cations to the apo-enzyme. Since the rate of formation of the binuclear metal center is directly proportional to the concentration of added bicarbonate, these results are consistent with the initial formation of the carbamate functional group prior to the binding of the divalent cations to the apo-protein. Using the simplified model that appears in Scheme 5, the observed rate constant at saturating levels of the divalent cations would approximate the value of $k_1[HCO_3^-]$. At lower levels of the divalent cations, the observed rate constant would approximate the sum of the rate constants for the formation and decomposition of the carboxylated lysine residue ($k_1[HCO_3^-] + k_2$). An approximate value for k_2 of 7.2×10^{-2} min^{-1} can thus be obtained by subtraction of the observed rate constant for formation of the binuclear metal center at high and low concentrations of added Co^{2+}(8.0×10^{-2} min^{-1} − 8.0×10^{-3} min^{-1}).

Metal Dissociation Constants. Apparent dissociation constants for the divalent metal ions from the binuclear metal center were determined via competition with 2,6-pyridinedicarboxylic acid (dipicolinic acid) (*14*). The dipicolinic acid−metal ion complex buffers the concentration of free metal ion while serving as a source of metal ion for the assembly of the binuclear metal center. The relationship between the free metal ion concentration and the relative concentration of activated protein is presented in Figure 7 for Zn^{2+}, Co^{2+}, and Cd^{2+}. A fit of the data to eq 2 gives the product of the two dissociation constants ($K_\alpha K_\beta$) for Zn^{2+}, Co^{2+}, and Cd^{2+} as 3.6×10^{-21}, 3.1×10^{-18}, and 1.2×10^{-16} M^2, respectively. The mean dissociation constants, $(K_\alpha K_\beta)^{1/2}$, for Zn^{2+}, Co^{2+}, and Cd^{2+} are thus 6.0×10^{-11}, 1.8×10^{-9}, and 1.1×10^{-8} M, respectively. Zn^{2+} binds tightest to the binuclear metal center of PTE with somewhat weaker binding exhibited by Cd^{2+} and Co^{2+}.

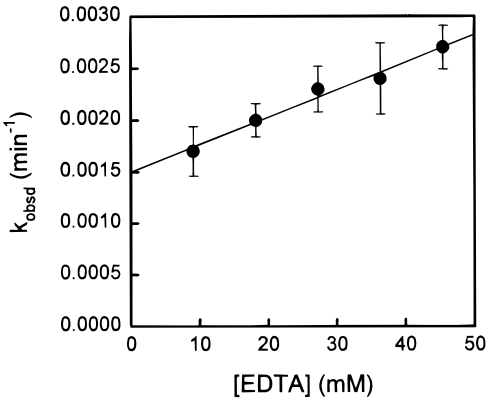


FIGURE 8: Rate of inactivation of Zn/Zn−PTE in the presence of variable amounts of EDTA. Additional details are provided in the text.

Rate of Metal Ion Dissociation. The rate of metal ion release from the binuclear metal center of PTE was measured by sequestering the liberated divalent cation with the chelating agent EDTA. Unlike neutral chelators, such as *o*-phenanthroline, the negatively charged EDTA is thought to be unable to actively remove metal ions from the anionic metal binding site of phosphotriesterase. Instead, it scavenges metal ions that spontaneously dissociate from the enzyme active site. Therefore, the rate of metal ion dissociation can be measured from the time course of inactivation in the presence of EDTA. The small contribution from the EDTA-assisted dissociation pathway can be eliminated by measuring the rate constant for metal dissociation at several different EDTA concentrations. In Figure 8 is shown the effect of added EDTA upon the rate of inactivation of Zn/Zn−PTE. The data were extrapolated to zero EDTA to estimate the rate constant for dissociation of the metal ion from the binuclear metal center. The apparent dissociation rate constants for Zn^{2+}, Cd^{2+}, and Co^{2+} are 1.5×10^{-3}, 6.5×10^{-2}, and 5.7×10^{-2} min^{-1}, respectively. Zn^{2+} dissociates from the enzyme more slowly than does either Cd^{2+} or Co^{2+}.

The K169A mutant enzyme has a significantly faster rate of metal ion dissociation than the wild-type enzyme. In the absence of propionic acid, the rate constant for the loss of catalytic activity (0.67 min^{-1}) is 450-fold faster than the wild-type enzyme under similar conditions. In the presence of propionic acid, the rate constant for the loss of catalytic activity was 0.19 min^{-1}.

Effect of COS and CS_2. The two sulfur-containing analogues of carbon dioxide, COS and CS_2, were incubated with the apo-form of PTE in an attempt to assemble the binuclear metal center with a thio- and dithiocarbamate bridge. These efforts were unsuccessful, but a significant degree of inhibition of the catalytic activity of PTE was observed upon addition of COS. The inhibitory properties of COS could be mimicked by the addition of bisulfide to the assay solution. A kinetic analysis of the inhibition by bisulfide indicates that it induces substrate inhibition by paraoxon, as illustrated in Figure 9. The induced substrate inhibition with paraoxon and bisulfide indicates that high concentrations of paraoxon can effectively trap bisulfide on the enzyme. The most likely mechanistic scenario for the inhibitory complex is for bisulfide to replace hydroxide as the bridging ligand to the two divalent cations. The binding of paraoxon prevents the release of the metal-bound bisulfide, and this enzyme−

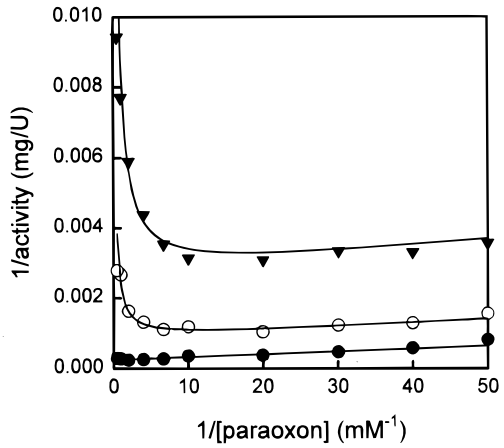
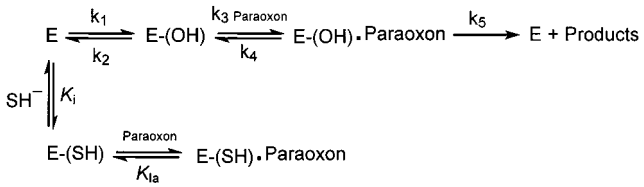


FIGURE 9: Induced substrate inhibition of PTE by paraoxon in the presence of 0 (●), 0.5 (○), and 2.0 mM (▼) NaSH. The data were fitted to eq 5. Additional details are provided in the text.

Scheme 6



substrate complex is unable to consummate an ensuing chemical reaction. A fit of the inhibition data to eq 5 provided values for K_{ii} and K_I of 0.17 ± 0.02 mM and 0.11 ± 0.01 mM^2, respectively. The value for K_{is} was undetermined. The inhibition mechanism proposed for bisulfide is presented in Scheme 6.

Mechanism for Assembly of the Binuclear Metal Center. The binuclear metal center of PTE can be self-assembled in high yield without the need for other accessory proteins or external cofactors. The rate of assembly is linearly dependent on the concentration of added bicarbonate/CO_2 but independent of the divalent cation concentration when supplied in excess of the protein concentration. The kinetic data are consistent with a model that supports the formation of the carbamate functional group with Lys-169 prior to the synergistic binding of the two divalent cations to complete the assembly process. At endogenous levels of bicarbonate/CO_2, the assembly of the binuclear metal center is relatively slow ($t_{1/2}$ > 100 min), and thus there is the distinct possibility that in the native cellular environment additional enzymes or cofactors exist to accelerate the formation of the metal center. One potential mechanism is the activation of the bicarbonate (perhaps via phosphorylation) prior to nucleophilic attack by the ϵ-amino group of lysine-169.

Comparison with Urease. Urease is known to have a binuclear nickel metal center that is strikingly similar to the metal center found within phosphotriesterase (*8*, *11*), and it is thought that these two enzymes are evolutionarily related. Unlike the phosphotriesterase, however, urease can apparently be activated only by Ni^{2+}. Moreover, the activation of this enzyme requires three accessory proteins (UreD, UreF, and UreG) and is facilitated by a fourth (UreE) (*13*). In the absence of these accessory proteins, only ~30% of apo-urease could be activated by Ni^{2+} and CO_2 in vitro. UreE is thought to function as a metallo-chaperone that delivers nickel to the metal center of urease (*19*). The three required accessory proteins form complexes with urease apo-protein, and the quaternary complex UreD−UreF−UreG−apo-urease was suggested to be the key urease activation machinery (*20*). This complex has been isolated, and it was found that it activates apo-urease to near wild-type level in the presence of Ni^{2+} and high concentration of bicarbonate (~100 mM). Notably, the activation of apo-urease in the quaternary complex at a physiologically relevant bicarbonate concentration (~100 μM) is GTP-dependent. It has been suggested that the presence of GTP affects the assembly of the metal center either by a GTP-dependent structural change of the apo-urease complex or through the formation of carboxy phosphate for the carboxylation of the active site lysine residue (*21*).

The activation of apo-urease in vitro, in which the rate of activation is dependent upon the concentration of Ni^{2+} and the overall extent of activation is determined by the CO_2 concentration (*13*), sharply contrasts with that found for the phosphotriesterase. Moreover, metal binding to urease cannot be reversed by the addition of metal chelators. These comparisons suggest that the binding of divalent cations to the phosphotriesterase is more efficient. In addition, nonproductive metal chelation to urease (metal binding to CO_2-free apo-enzyme) leads to inactive enzyme, because the nonproductive interaction can only be reversed in the presence of EDTA. For phosphotriesterase, the dissociation of metal ions is quite fast in the absence of the carbamate bridge, as observed with K169A mutant, and thus nonproductive binding does not inactivate the enzyme. It can be speculated that the comparatively slow and inefficient metal chelation to urease, as well as the irreversible nature of the metal center assembly, are responsible for the different activation kinetics of urease from those of the phosphotriesterase.

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