

## CO<sub>2</sub> Is Required for the Assembly of the Binuclear Metal Center of Phosphotriesterase

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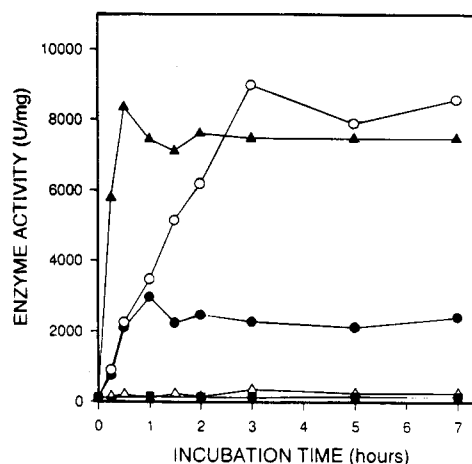
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Phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme that catalyzes the hydrolysis of a broad spectrum of organophosphate triesters, including the pesticides paraoxon and parathion.<sup>1</sup> Although the natural biological substrate for the phosphotriesterase has not yet been identified, the enzyme has received considerable attention because it is also capable of hydrolyzing chemical warfare agents such as soman and sarin.<sup>2</sup> The overall chemical mechanism for the hydrolysis of paraoxon has been well described, but the precise role of the metal center in catalysis is less well understood.<sup>3</sup> The metal center of phosphotriesterase is binuclear, and the two native zinc ions can be substituted with Co<sup>2+</sup>, Ni<sup>2+</sup>, Cd<sup>2+</sup>, or Mn<sup>2+</sup> to yield catalytically active enzyme.<sup>4</sup> EPR experiments with the Mn<sup>2+</sup> reconstituted protein have shown that the two metal ions are antiferromagnetically coupled through a common ligand.<sup>5</sup> The protein and solvent ligands to the metal ions are composed of a mixture of nitrogen and oxygen atoms as determined by <sup>113</sup>Cd NMR spectroscopy.<sup>6</sup> Site-directed mutagenesis experiments have indicated that six of the seven histidine residues in the native phosphotriesterase are at or near the active site.<sup>7,8</sup>

The X-ray crystal structure of the phosphotriesterase has recently been determined by the Holden laboratory at a resolution of 2.0 Å.<sup>9,10</sup> The three-dimensional structure of the holoenzyme, reconstituted with Cd<sup>2+</sup>, indicates the existence of two bridging ligands to the two metal ions, Lys-169 and a solvent water molecule.<sup>10</sup> The other ligands are His-55, His-57, His-201, His-230, and Asp-301 plus two additional solvent water molecules. The most surprising feature of the holoenzyme structure is that the electron density for the side chain of the bridging lysine is best modeled as a carbamate. In this communication, we report the effect of bicarbonate and other reagents on the reconstitution of the binuclear metal center of the apo-phosphotriesterase as well as the enhancement effect exhibited by short-chain carboxylic acids on the activity of two mutants of the wild type enzyme at Lys-169. The carbamate functional group has also been confirmed by NMR spectroscopic analysis of the <sup>13</sup>C-labeled protein.

Phosphotriesterase was purified as described by Omburo et al.<sup>4</sup> The preparation of apoprotein and reactivation conditions were as described by Kuo and Raushel.<sup>7</sup> The reactivation of apo-phosphotriesterase in the presence of Cd<sup>2+</sup> was determined after a 1 h preincubation with either 100 mM potassium



**Figure 1.** The time course for the reconstitution of the binuclear metal center of phosphotriesterase (33  $\mu$ M) when either HCO<sub>3</sub><sup>-</sup> or HCHO was added along with 2 equiv of Cd<sup>2+</sup>. The apoprotein in 50 mM HEPES buffer (pH 7.85) was preincubated with 100 mM KHCO<sub>3</sub> (▲) or 10 mM HCHO (△) at room temperature for 1 h, and then the reaction was initiated with the addition of 2 equiv of Cd<sup>2+</sup>. For the control experiments, the partially degassed (●) or nondegassed (○) apoprotein was activated only with 2 equiv of Cd<sup>2+</sup> without the supplementation of HCO<sub>3</sub><sup>-</sup>. The endogenous bicarbonate concentration was estimated as 0.5 mM. The activity of apoprotein (■) remained the same during the whole time course for the reactivation of the enzyme.

bicarbonate, 10 mM formic acid, 10 mM formaldehyde, or 10 mM glyoxylate. The enhancement of activity for the K169A and K169M mutants was determined in the presence of 10, 50, and 100 mM acetic, propionic, or butyric acid at 25 °C, pH 9.0. The unbound H<sup>13</sup>CO<sub>3</sub><sup>-</sup> was separated from the reconstituted protein by passage through a PD-10 Pharmacia column. The NMR experiments were performed at 125.7 MHz for <sup>13</sup>C. The <sup>13</sup>C spectra were referenced to an external standard of dioxane with an acquisition time of 1.3 s and a 50° pulse width.

The reactivation of the apo-phosphotriesterase is directly related to the rate of reconstitution of the binuclear metal center. The influence on modification of Lys-169 on the rate of reconstitution of the binuclear metal center of apo-phosphotriesterase was probed by incubation with various modifying agents prior to metal addition. Figure 1 shows the rate of reconstitution of the binuclear metal center in the presence of potassium bicarbonate and formaldehyde. Full reconstitution of the metal center in the absence of these reagents is typically observed in 3 h under the described experimental conditions. Preincubation with 100 mM bicarbonate prior to reconstitution with Cd<sup>2+</sup> enhanced the rate of reactivation by a factor of about 6. No enhancement was observed in the rate of reactivation in the presence of formic acid (data not shown). The ultimate catalytic activity was reduced by 75, 80, and 100% when the reconstitution experiment was carried out in partially degassed buffer, 10 mM glyoxylate (data not shown), and 10 mM formaldehyde, respectively.

When the bridging Lys-169 is mutated to either methionine (K169M) or alanine (K169A), the catalytic activity is drastically reduced. However, a significant fraction of the wild type activity can be restored upon the addition of low molecular weight carboxylic acids. The activity of K169A was enhanced 13-, 25-, and 8-fold in the presence of 100 mM acetic, propionic, and butyric acid, respectively. Similarly, the activity of K169M was enhanced 5- and 2-fold in the presence of 100 mM acetic and propionic acid, respectively. No enhancement was obtained with butyric acid.

The structure of the carbamate was confirmed by NMR spectroscopy of the <sup>13</sup>C-labeled enzyme. Figure 2 shows a

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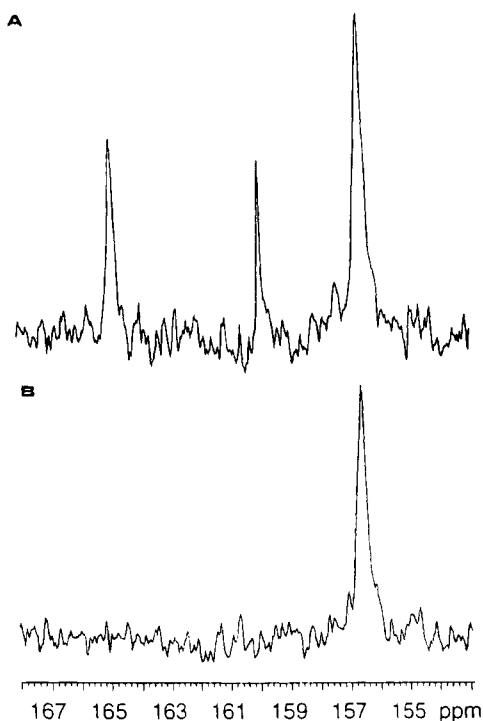
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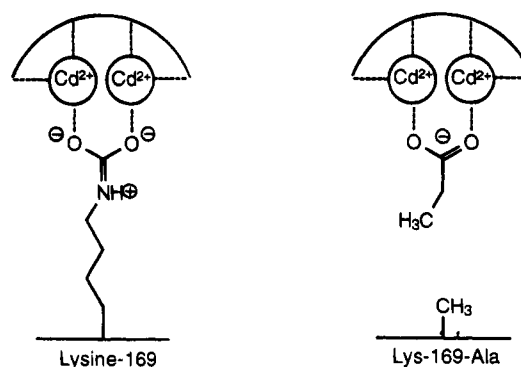
**Figure 2.** The  $^{13}\text{C}$  NMR spectra of 0.7 mM phosphotriesterase in 50 mM phosphate buffer, pH 7.5, (A) upon reconstitution with  $\text{H}^{13}\text{CO}_3^-$  and  $^{113}\text{CdCl}_2$  and (B) upon reconstitution with  $\text{H}^{12}\text{CO}_3^-$  and  $^{112}\text{CdSO}_4$ . Additional details are given in the text.

portion of the  $^{13}\text{C}$  NMR spectrum of phosphotriesterase in 50 mM phosphate buffer, pH 7.5, upon reconstitution with  $\text{H}^{13}\text{CO}_3^-$  and  $^{113}\text{CdCl}_2$ . The  $^{13}\text{C}$  NMR spectrum obtained with phosphotriesterase reconstituted with unlabeled bicarbonate shows a typical natural abundance protein spectra. Upon reconstitution with  $^{13}\text{C}$ -enriched bicarbonate, two additional resonances are observed at 159.9 and 164.9 ppm. The resonance at 159.9 ppm is due to unbound  $\text{H}^{13}\text{CO}_3^-$  and  $^{13}\text{CO}_3^{2-}$ . This was confirmed by addition of 10 mM  $\text{H}^{13}\text{CO}_3^-$  to the phosphotriesterase previously reconstituted with  $\text{H}^{12}\text{CO}_3^-$ . The new resonance at 164.9 ppm is consistent with a single carboxylated lysine residue. A resonance at the identical location has previously been observed for the  $^{13}\text{CO}_2$ -labeled ribulosebiphosphate carboxylase (Rubisco), which is known to undergo activation by  $\text{CO}_2$  upon formation of a carbamate at an active site lysine.<sup>11</sup> The resonance at 156.5 ppm in both spectra originates from the natural abundance  $^{13}\text{C}$  of the guanidino carbons of arginine and the  $\xi$ -carbons of tyrosine. No spin coupling was observed in the  $^{13}\text{C}$  spectrum between the  $^{113}\text{Cd}^{2+}$  and the  $^{13}\text{C}$  label of the carbamylated lysine. These results indicate that even though the two metals are bridged by a common ligand, the magnetic interaction between the  $\text{Cd}^{2+}$  and the carbamate carbon is not sufficient for observation of nuclear spin coupling under the conditions used to acquire the spectra.

The enhancement of the rate of reactivation in the presence of bicarbonate as well as the reduction in the recovery of catalytic activity in degassed buffer clearly demonstrates that the reconstitution of the binuclear metal center of phosphotriesterase is dependent on either the interaction of  $\text{HCO}_3^-$  or  $\text{CO}_2$  with apoprotein prior to metal binding or the binding of a  $\text{HCO}_3^-/\text{CO}_2$ -metal ion complex. The inhibition of reactivation after incubation with formaldehyde may be explained by interaction of the modifying agent with the apoprotein prior to metal binding since formaldehyde can react chemically with the  $\epsilon$ -amino group of lysine to form a carbinolamine or an imine.

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### Scheme 1



The Lys-169 mutant proteins, K169A and K169M, exhibit 1% and 0.1% of the catalytic activity, respectively, when compared to the wild type protein.<sup>12</sup> The observed activity is increased substantially when short-chain carboxylic acids are added to the assay mixture. K169A had the greatest enhancement in the presence of propionic acid, whereas acetic acid had the greatest effect on K169M. These results are consistent with the binding of the short-chain carboxylic acids in the active site in a geometry that would be analogous to that obtained for a carboxylated lysine. These results complement those of the bicarbonate activation of wild type protein. Noncovalent chemical rescue by alkyl amines for mutants of aspartate aminotransferase has previously been obtained by Toney and Kirsch.<sup>13</sup>

The proposed structure of the carbamate within the binuclear metal center in the phosphotriesterase is illustrated in Scheme 1, where the  $\epsilon$ -amino group of lysine has reacted with  $\text{CO}_2$ . This type of structure has been previously reported for Rubisco and, more recently, urease and is fully consistent with the recently acquired X-ray diffraction data for phosphotriesterase.<sup>10,14,15</sup> Also shown in Scheme 1 is a model for the enhancement of activity of K169A by propionic acid. Phosphotriesterase and Rubisco show complete restoration of activity in the presence of bicarbonate, whereas urease was only partially reactivated. The results from the urease reactivation indicate a need for an accessory gene product, UreD, for activation at low concentrations of  $\text{CO}_2$  *in vitro* and probably *in vivo*.<sup>15</sup> The metabolic function of the phosphotriesterase *in vivo* has yet to be determined, and it is not known if there is an obligatory requirement for an activase protein for this system.

In summary, we have shown that the reconstitution of the binuclear metal center of phosphotriesterase is  $\text{CO}_2$  dependent. We have also demonstrated that it is possible to enhance the activity of phosphotriesterase mutants of Lys-169 by short-chain carboxylic acids. These results are consistent with the carboxylation of the  $\epsilon$ -amino group of a lysine at the active site of phosphotriesterase which facilitates the incorporation of the metal ions in the proper geometry for maximal enzymatic activity.

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