Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 5, No. 24, pp. 3067-3072, 1995

0960-894X/95 $9.50+0.00

0960-894X(95)00539-0

PHOSPHORUS-31 NMR RELAXATION STUDIES OF DIETHYL P-METHOXYPHENYL PHOSPHATE BOUND TO PHOSPHOTRIESTERASE

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Abstract: The effect of Mn^{2+}/Mn^{2+}, Mn^{2+}/Zn^{2+} and Mn^{2+}/Cd^{2+} reconstituted phosphotriesterase on the ^{31}P spin lattice ($1/T_1$) relaxation rate of diethyl *p*-methoxyphenyl phosphate has been investigated. In the presence of Mn^{2+}/Mn^{2+} phosphotriesterase, the spin lattice relaxation rate of the phosphorus atom is enhanced giving an upper limit for the phosphorus-metal root mean-sixth average distance of 4.2 Å. These results demonstrate for the first time that substrates for phosphotriesterase bind in close proximity to the binuclear metal center.

Introduction

Phosphotriesterase from *Pseudomonas diminuta* is a zinc metalloenzyme that catalyzes the hydrolysis of a broad spectrum of organophosphate triesters including organophosphate insecticides and chemical warfare agents.[1,2] Phosphotriesterase is remarkably efficient in catalyzing the hydrolysis of organophosphate triesters even though they are not natural occurring compounds. The best substrate found to date is paraoxon which has a turnover number for hydrolysis of 10^4 s^{-1}.[2] The overall chemical mechanism for the hydrolysis of paraoxon is thought to proceed via the direct attack of an activated water molecule on the phosphorus center of paraoxon resulting in a net inversion of configuration.[3] It is presumed the metal center functions to activate the substrate and water molecule for hydrolytic attack but the exact role of the metal center in catalysis has yet to be defined.

The metal center of phosphotriesterase is binuclear and the native zinc metals can be replaced with a variety of divalent metals yielding catalytically active enzyme.[4] EPR experiments with Mn^{2+}/Mn^{2+} reconstituted protein have shown the two metals are antiferromagnetically coupled by a common ligand.[5,6] Kinetic and ^{113}Cd NMR investigations have shown that the binuclear metal center consists of non-identical sites, M_α and M_β. M_α has been proposed to be the primary catalytic site, whereas the function of M_β is unclear.[7,8] The crystal structure of the cadmium substituted phosphotriesterase has been determined recently.[9,10] The two metal ions are 3.7 Å apart and bridged by a solvent water molecule and a carbamate formed from Lys-169. To date, however, there has been no direct experimental evidence to indicate the substrate is bound at the active site in close proximity to either of the metal atoms.

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One of the few methods available to estimate distances in protein-ligand interactions is the measurement of nuclear magnetic relaxation rates of ligand atoms in the presence of paramagnetic probes, such as protein bound metal ions.[11] To this end, we have investigated the effect of phosphotriesterase reconstituted with paramagnetic ions on the ^{31}P spin lattice ($1/T_1$) relaxation rate of the phosphorus center in diethyl *p*-methoxyphenyl phosphate.

Experimental Procedures

Materials. Phosphotriesterase was purified and reconstituted with Mn^{2+}/Mn^{2+}, Zn^{2+}/Zn^{2+}, Mn^{2+}/Cd^{2+} and Mn^{2+}/Zn^{2+} as previously described.[4,5] Diethyl *p*-methoxyphenyl phosphate was synthesized as described by Hong and Raushel.[12] The metal content of the Mn^{2+}/Mn^{2+} and Zn^{2+}/Zn^{2} reconstituted phosphotriesterases were determined by atomic absorption.[5]

Nuclear Magnetic Resonance. Spin- lattice ($1/ T_1$) relaxation rates for the phosphorus center in diethyl *p*-methoxyphenyl phosphate were measured by the inversion recovery method.[13,14] The $1/T_1$ measurements were made at 81 MHz on a Varian XL 200 spectrometer and at 162 MHz on a Varian XL 400 spectrometer. All samples contained: 2 mM diethyl *p*-methoxyphenyl phosphate, 50 mM HEPES pH 8.5, 10 % D_2O and either 10 - 30 μM M^{2+} ion or 2 - 50 μM phosphotriesterase. The samples used to measure the effect of mixed diamagnetic and paramagnetic metal ions on the spin-lattice relaxation rates were prepared by incubating 42 μM phosphotriesterase with 0.67 equivalents of Mn^{2+} and 0 - 1.33 equivalents of the diamagnetic metal ion at 4 °C for 36 hours prior to data acquisition.

Distance Measurements. The distance between the phosphorus center in diethyl *p*-methoxyphenyl phosphate and the phosphotriesterase bound metal ions was estimated by measuring the spin- lattice relaxation rate of the phosphorus atom as a function of phosphotriesterase concentration. A modified form of the Solomon - Bloembergen equation was used to estimate the distance between the ligand nuclei and the metal complex.[15]

$$r = C\,[\,T_{1M}\,f(\tau_c)]^{1/6} \qquad \text{where} \qquad (1)$$

$$C = [(2/15)\,\gamma_I^2\,g^2\,\beta^2\,S(S+1)]^{1/6} \qquad \text{and} \qquad (2)$$

$$f(\tau_c) = 3\tau_c / (\,1 + \omega_I^{\,2}\tau_c^{\,2}\,) \qquad (3)$$

The Solomon - Bloembergen equation (1) relates the distance, in Å, between the two sites to the paramagnetic contribution (T_{1M}) of the M^{2+} - enzyme complex to the spin - lattice relaxation of the ligand nuclei as a function of the correlation time, $f(\tau_c)$. The term C is a collection of constants (equation 2), where γ_I is the gyromagnetic ratio, g is the electronic g - factor, β is the Bohr magneton and S is the electronic spin. The

value of C for the interaction of phosphorus and Mn^{2+}, spin = 5/2 is 601 and spin = 1 is 470.[11] The term $f(\tau_c)$ relates the correlation time for the dipolar interaction, τ_c, to the nuclear Larmor precision frequency, ω_I.

The Luz - Meiboom equation (4) relates the normalized observed relaxation rate to the relaxation times of the ligand nuclei in the metal complex, T_{1M}, and the mean residence time, τ_m, of the ligand in the metal complex.[16]

$$\frac{1}{pT_{1p}} = \frac{1}{T_{1M} + \tau_m} \qquad (4)$$

where

$$\frac{1}{pT_{1p}} = \left[\frac{1}{T_1 \text{(E-para)}} - \frac{1}{T_1 \text{(E-dia)}}\right] \frac{1}{P} \qquad (5)$$

If the relaxation is in fast exchange, then $1/(pT_{1p}) \cong 1/T_{1M}$. The terms $1/T_1$ (E-para) and $1/T_1$ (E-dia) are the observed relaxation rates in the presence of the paramagnetic and diamagnetic M^{2+} - enzyme complexes, respectively, in solution and p is the mole fraction of ligand bound to the M^{2+} - enzyme complex. The Solomon - Bloembergen and the Luz - Meiboom equations were dervied for systems with a single paramagnetic site. Since phosphotriesterase has two spin coupled metals bound together at the active site, only an upper limit for the distance can be determined using these equations.

Results and Discussion

The spin-lattice ($1/T_1$) relaxation rate for the phosphorus center in diethyl *p*-methoxyphenyl phosphate was determined as a function of Mn^{2+}/Mn^{2+}, and Zn^{2+}/Zn^{2+} substituted phosphotriesterase concentrations at two magnetic field strengths. Figure 1 shows a plot of the observed relaxation rate ($1/T_1$) in the presence of varying concentrations of Mn^{2+}/Mn^{2+} phosphotriesterase. The observed spin lattice rate of relaxation was not affected by either Zn^{2+}/Zn^{2+} phosphotriesterase or $MnCl_2$. These data clearly indicate that the phosphorus center in diethyl *p*-methoxyphenyl phosphate is bound at the active site of phosophotriesterase in close approximity of the binuclear metal center.

In order to estimate the upper limit for the distance from the ligand nuclei to the metal center, the correlation time for dipolar interaction, τ_c, must be determined. With the assumption there is no magnetic field dependence on the correlation time, τ_c can be determined from the frequency dependence on the normalized relaxation rate, $1/(pT_{1p})$ (equation 3). Table I shows the normalized relaxation rate, $1/(pT_{1p})$, of diethyl *p*-methoxyphenyl phosphate determined in the presence of Mn^{2+} / Mn^{2+} phosphotriesterase. The mole fraction of bound ligand was determined using the dissociation constant of 1.57 mM for Mn^{2+}/Mn^{2+} phosphotriesterase.[12] The ratio of pT_{1p} for the diethyl *p*-methoxyphenyl phosphate - Mn^{2+}/Mn^{2+} phosphotriesterase complex at 162

MHz and 81 MHz is 3.39. Since the ratio is > 1, it is valid to assume the ligand is in fast exchange and $1/(pT_{1p}) \cong 1/T_{1M}$. [17] A value of 3.9×10^{-9} sec for τ_c can be calculated using the data in Table I.

The distance determined from the Solomon - Bloembergen equation is dependent on the electronic spin of the metal complex. If the metals in the binuclear metal center of Mn^{2+}/Mn^{2+} phosphotriesterase are independent, then the normal Mn^{2+} spin of 5/2 can be used to calculate the metal ligand distance. Using a value of C of 601 (S = 5/2), a root mean sixth average distance of 5.1 Å for the phosphorus - metal distance is calculated. However, EPR experiments on Mn^{2+}/Mn^{2+} phosphotriesterase indicate the two metals are antiferromagnetically coupled with the spin best modeled as S = 1.[5] Using a spin of 1, the value of C in the Solomon - Bloembergen equation becomes 470 for the interaction of phosphorus and Mn^{2+} yielding a distance of 4.0 Å for the phosphorus - metal distance. The distance thus calculated is the average distance of the phosphorus atom in diethyl *p*-methoxyphenyl phosphate

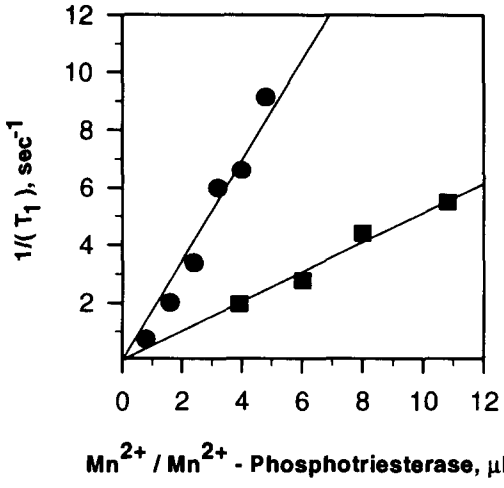

Figure 1. ^{31}P $1/T_1$ of diethyl *p*-methoxyphenyl phosphate as a function of Mn^{2+} / Mn^{2+} phosphotriesterase concentration: measurements made at 81 MHz (●) and at 162 MHz (■).

Table 1. ^{31}P Relaxation rates of diethyl *p*-methoxyphenyl phosphate in the presence of Mn^{2+}/ Mn^{2+} phosphotriesterase.

Nucleus	$1/(pT_{1p})$ (s^{-1})		τ_c (s)	r (Å)	
	81 MHz	162 MHz		S = 5/2	S = 1
^{31}P	6200	1830	3.9×10^{-9}	5.1	4.0

to the binuclear metal center in phosphotriesterase. If the value for τ_c is incorrect, then the upper limit for the distance increases to only 4.2 Å. Distances of 2.9 - 3.0 Å have been measured for the Mn^{2+} - ^{31}P ligand separation in protein kinase.[18] These distances have been attributed to inner sphere coordination of the ligand to the protein bound metal. Distances of 5 - 6.5 Å would indicate outer sphere coordination.[19] Based on the calculated distance of 4 - 5 Å for the phosphorus - Mn^{2+} distance in the phosphotriesterase - diethyl *p*-

methoxyphenyl phosphate complex, it is likely that the phosphoryl oxygen is directly coordinating with the binuclear metal center of phosphotriesterase.

^{113}Cd NMR experiments with mixed metal complexes of phosphotriesterase have shown that Zn^{2+} ions have an affinity for the M_α site, whereas, Cd^{2+} ions have a greater affinity for the M_β site of the binuclear metal center. [7] It has also been observed in EPR experiments, that the spin quantitation increases in mixed metal diamagnetic - paramagnetic phosphotriesterase complexes. [5,6] To ascertain if distinctions could be made between the metal sites, the ^{31}P spin lattice relaxation of diethyl *p*-methoxyphenyl phosphate was determined with Mn^{2+}/Zn^{2+} and Mn^{2+}/Cd^{2+} mixed metal complexes of phosphotriesterase. Figure 2 shows the effect of increasing the diamagnetic ion content on the observed relaxation rate ($1/T_1$). In the Mn^{2+}/Zn^{2+} complex, $1/T_1$ increases 2 fold in the presence of 0.33 equivalents of Zn^{2+} ion. This can occur by an increase in either the electron spin (S) and/or the electron spin relaxation time (τ_e) of the metal center. These results indicate that in the Mn^{2+}/Zn^{2+} mixed metal complexes a greater paramagnetic effect is observed on the relaxation of bound ligand. The decrease in $1/T_{1p}$ at high concentrations of diamagnetic ions probably indicates a displacement of the paramagnetic ions from the protein. These results are consistent with the paramagnetic relaxation data indicating the Mn^{2+}/Mn^{2+} binuclear metal center is spin coupled which results in an overall decrease in the paragmagnetic effect observed on the spin lattice relaxation of the diethyl *p*-methoxyphenyl phosphate. No effect on $1/T_{1p}$ is observed in the presence of 0 - 1 equivalents of Cd^{2+}. This could be due to more efficient relaxation in the Mn^{2+}/Zn^{2+} complex or that the phosphorus center binds closer to the M_β site (assuming the Zn^{2+} ions bind preferentially at the M_α site) in the diethyl *p*-methoxyphenyl phosphate - phosphotriesterase complex.

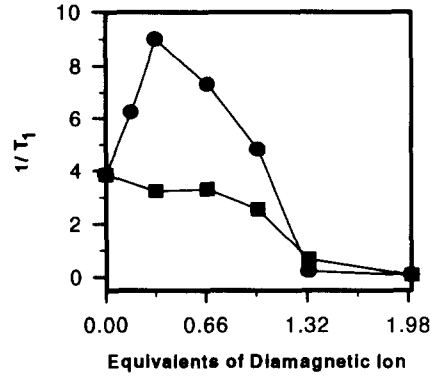

Figure 2. Effect on the observed ^{31}P relaxation rate of diethyl *p*-methoxyphenyl phosphate as a function of increasing diamagnetic ion concentration: Zn^{2+} (●) and Cd^{2+} (■).

In summary, we have shown the substrate, diethyl *p*-methoxyphenyl phosphate, is bound at the active site of phosphotriesterase in close proximity to the binuclear metal center. An exact distance from the ligand to the protein-metal complex is difficult to determine due to the complexities associated with a coupled binuclear metal center. However, if it is assumed that the metal center appears mononuclear to the bound ligand since the metals are spin coupled, then a distance of <4.2 Å can be estimated. It is likely that the phosphoryl oxygen

is directly coordinating with the binuclear metal center of phosphotriesterase in an inner sphere complex with no intervening ligand.

Acknowledgment. This work was supported in part by NIH (GM33894). We thank Dr. George Omburo for preliminary experiments.

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(*Received in USA* 3 *July* 1995; *accepted* 14 *November* 1995)