^{113}Cd Nuclear Magnetic Resonance (NMR) Study of the Inhibitory Effect of Methylvinylether/Maleic Acid (PVM/MA) Copolymer on the Alkaline Phosphatase of *Escherichia coli*

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The inhibitory effect of PVM/MA copolymer on the alkaline phosphatase (AP) of *E. coli* was investigated. Kinetic studies indicated that enzyme inhibition was characterized by a reduction in both the V_{max} and the K_m. Addition of 1 m*M* zinc or magnesium ions to the reaction prevented inhibition of the enzyme by the copolymer. The inhibitory effect of the copolymer on alkaline phosphatase was also investigated using ^{113}Cd NMR after exchange of the active center metal ions with ^{113}Cd. The resulting $Cd(II)_6AP$ exhibited characteristic ^{113}Cd resonances reflecting the environment of the A, B, and C metal binding sites of the enzyme's active center. Addition of copolymer resulted in a ^{113}Cd NMR spectrum which indicated removal of ^{113}Cd from the C site and formation of two distinct forms of the enzyme. Possible explanations for the ^{113}Cd NMR results are discussed.

KEY WORDS: alkaline phosphatase; ^{113}Cd nuclear magnetic resonance (NMR); methylvinylether/maleic acid (PVM/MA) copolymer; enzyme inhibition.

INTRODUCTION

Our interest in the inhibitory effect of methylvinylether/maleic acid (PVM/MA) copolymer (Fig. 1) on alkaline phosphatase activity is based on its application in tartar control dentifrice (1,2). In this product it functions, in combination with pyrophosphate, to inhibit the crystal growth and deposition of insoluble calcium phosphate salts (e.g., hydroxyapatite) on tooth surfaces. Dental calculus (tartar) is composed predominantly of these salts. The PVM/MA copolymer also provides the additional benefit of retarding the hydrolysis of pyrophosphate by phosphatases present in the oral environment.

It was initially observed that PVM/MA copolymer inhibited the pyrophosphatase activity of both the mammalian (1) and bacterial (*E. coli*) forms (3) of alkaline phosphatase. Since PVM/MA is known to bind to divalent cations (4), it was hypothesized that its inhibitory action on alkaline phosphatase may be due to removal of metal ions, particularly zinc, associated with the active sites of this enzyme. The present study investigated the inhibitory effect of PVM/MA copolymer on the alkaline phosphatase (AP) of *E. coli* with a specific emphasis on the mechanism of inhibition.

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Solution-state cadmium-113 NMR is an extremely valuable tool for studying structural changes associated with the metal binding sites of zinc metalloproteins such as alkaline phosphatase, carbonic anyhdrase, and others (5). Cadmium-113 can be substituted for the native metals in these proteins with minimal changes in protein structure. The alkaline phosphatase of *E. coli* is a dimer consisting of two identical monomer subunits, each with a molecular weight of 47,000. The active site in each monomer contains two zinc ions and one magnesium ion located near a serine residue. Substitution of all six metal ions with cadmium-113 [i.e., $^{113}Cd(II)_6AP$] produces a catalytically active enzyme which can be studied by ^{113}Cd NMR to detect changes in protein structure occurring during the process of enzyme phosphorylation (6). ^{113}Cd-NMR of the cadmium-substituted alkaline phosphatase of *E. coli* was employed in the present study to investigate the effect of PVM/MA copolymer on this enzyme.

MATERIALS AND METHODS

Kinetic Studies

E. coli alkaline phophatase (Type III R) was obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals used were reagent grade, obtained from Sigma, J. T. Baker Chemical Co. (Phillipsburg, NJ), or MCB Manufacturing Chemists, Inc. (Darmstadt, Germany). The PVM/MA copolymer (Gantrez) was obtained from GAF Corp. (Wayne, NJ). Reaction mixtures contained 0.1 *M* Tris–HCl, pH 8.0, buffer, 9.5 μg/ml of enzyme, and final concentrations of substrate (*p*-nitrophenyl phosphate) ranging from 2.0×10^{-5} to 2.5×10^{-3} *M*. The reaction mixtures were allowed to equilibrate to 37°C for 15 min. The reactions were initiated by the addition of substrate and allowed to proceed for 5 min. The reaction product (*p*-nitrophenol) was measured spectrophotometrically at 405 nm. In experiments which measured the effect of copolymer and metal ions on the pyrophosphatase activity of alkaline phosphatase, the reaction mixtures contained 0.01 *M* tetrasodium pyrophosphate, 2.9 μg of AP, and 0.1 *M* Tris–HCl in a total volume of 0.5 ml. The copolymer was added at a concentration ranging from 0.1 to 0.5%. The effect of metal ions was studied by adding either $MgCl_2$ or $ZnCl_2$ at concentrations of 0.1 and 0.5 m*M*. Reactions were terminated by the addition of 0.5 ml cold 20% trichloroacetic acid and placed in ice. A 0.5-ml volume of cold 0.034 *M* $CuSO_4$ was then added to stabilize unhydrolyzed pyrophosphate at the resulting low pH. The reaction mixture was centrifuged for 10 min at 2700 rpm and the supernatant was analyzed for content of inorganic phosphate using a modification of the colorimetric method originally described by Fisk and Subbarow (7).

NMR Studies

Alkaline phosphatase was isolated from *E. coli*, strain CW3743, and purified according to the method described by Applebury *et al.* (8). Enzyme concentrations were determined spectrophotometrically at 278 nm using $E^{0.1\%} = 0.72$ (9) and a molecular weight of 94,000 (10). Enzymatic activity

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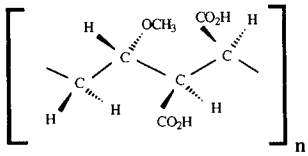

Fig. 1. Regular repeating unit of PVM/MA copolymer.

was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 1.0 *M* Tris–HCl at pH 8.0 and 22°C. The native enzyme had a specific activity of 38 U/mg protein. Apoenzyme was prepared by sequential dialysis of the enzyme solution (10 mg/ml) against 1,10-phenanthroline (10 mg/ml) at pH 5.5; 2 liters of a metal-free solution of 0.01 *M* Tris, 0.01 *M* sodium acetate, 0.1 *M* NaCl, pH 6.5; and finally, 2 liters of 2 *M* ammonium sulfate, pH 9.0, for 24 hr. Ammonium sulfate was removed by dialysis against 2 liters of metal-free 0.01 *M* Tris, 0.01 *M* sodium acetate, 0.1 *M* NaCl solution, pH 6.8. Two changes of the dialysate were made. The enzyme was concentrated in a metal-free Amicon ultrafiltration cell using a PM-30 membrane. The $Cd(II)_6$ form of alkaline phosphatase was prepared by adding a stoichiometric amount of a stock solution of ^{113}Cd-$(CH3COO)_2$ which was prepared from the 96% isotopically enriched cadmium oxide.

Cadmium-113 solution NMR spectra were recorded at 66.56 MHz on a Bruker MSL-300 spectrometer using a 10-mm broadband probe. Spectra were recorded at 297° K, with a 30° pulse width of 2 μsec, a sweep width of 20,000 Hz, and a relaxation delay of 1 sec. Sixteen K points were taken, zero-filled to 32 K before Fourier transformation. A line broadening of 40 Hz was used and 82,000 scans were averaged. Proton decoupling was not employed.

The enzyme sample (2-ml total volume, pH 6.8) contained 0.1 *M* NaCl, 0.01 *M* sodium acetate, 0.01 *M* Tris–HCl, 1.5 m*M* enzyme, 2 equivalents of PO_4 per dimer, and 2 drops of D_2O as a field lock. The spectra were recorded 72 hr after the addition of phosphate to the $^{113}Cd(II)_6$ enzyme.

After recording the initial data, the PVM/MA copolymer (pH adjusted to 6.8) was added to the enzyme sample at a final concentration of 1% by weight, and the ^{113}Cd NMR spectrum was recorded.

RESULTS

PVM-MA Copolymer Effects on Enzymatic Activity

The effect of 0.5% PVM/MA copolymer on the rate of *p*-nitrophenylphosphate hydrolysis induced by purified *E. coli* alkaline phosphatase is shown in Fig. 2. Double-reciprocal plots of initial reaction velocity (V_o) vs substrate concentration showed that reaction rates at alkaline pH exhibited classical Michaelis–Menton kinetics. Addition of the copolymer resulted in a uncompetitive type of inhibition with a large reduction in the V_{max} and decrease in the K_m.

In separate experiments, using pyrophosphate as the substrate, it was also noted that the inhibitory effect of copolymer on alkaline phosphatase is dependent upon the concentration of copolymer used (Fig. 3). Copolymer induced reductions in enzymatic activity were 40, 51, and 65% at

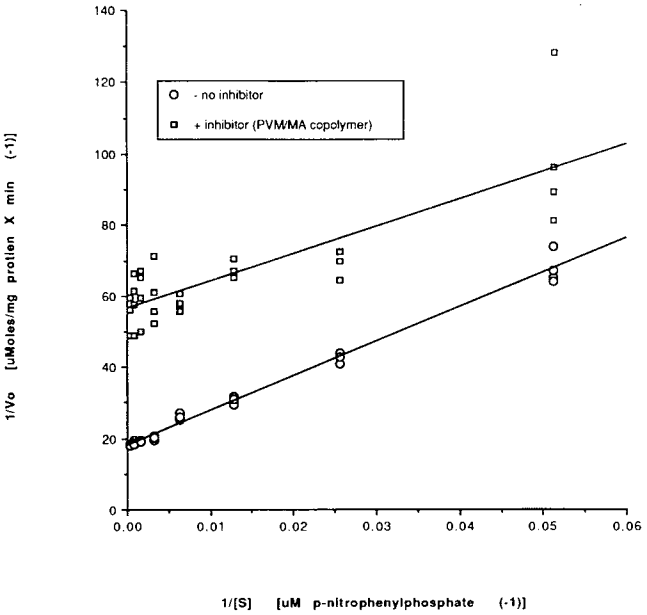

Fig. 2. Double-reciprocal plot of initial reaction velocity, V_o, vs substrate concentration, {*S*}, showing the effect of PVM/MA copolymer on the hydrolysis of *p*-nitrophenylphosphate by the alkaline phosphatase of *E. coli*. Reaction mixtures contained 0.1 *M* Tris–HCl, pH 8.0, 9.5 ug of enzyme/ml of reaction, and substrate as indicated.

copolymer concentrations of 0.1, 0.25, and 0.5%, respectively. For purposes of comparison the effect on alkaline phosphatase of a second polymer, polyacrylic acid (PAC), which has been shown to be a weaker chelator of magnesium ion than PVM/MA copolymer (4), was also determined. The results indicated a much weaker inhibition of the enzyme by PAC than that seen with the PVM/MA copolymer.

The possibility of metal complexation as a mode of polymer-induced inhibition of the enzyme was further investigated by adding magnesium and zinc to reactions to determine if the inhibition of alkaline phosphatase would be affected (Table I). The results demonstrated that PVM/MA copolymer-induced inhibition of the pyrophosphatase activity of the enzyme could be prevented by addition of either 0.1 m*M* $ZnCl_2$ or $MgCl_2$. It was also noted, however, that

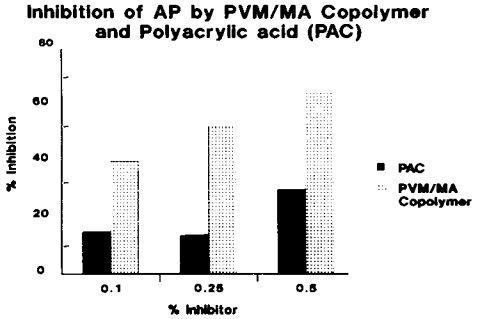

Fig. 3. Effect of PVM/MA copolymer and polyacrylic acid (PAC) on the pyrophosphatase activity of alkaline phosphatase. Reaction mixtures contained 0.1 *M* Tris–HCl, pH 8.0, 0.01 *M* tetrasodium pyrophosphate; 1.75 μg alkaline phosphatase/0.5 ml of reaction; and copolymer as indicated.

Table I. Effect of Metal Ions and PVM/MA Copolymer on the Pyrophosphatase Activity of Alkaline Phosphatase

Conditions	Percentage enzymatic activity[a]
AP	100.0
AP + copolymer (0.5%)	54.2
AP + 0.1 m*M* $MgCl_2$	109.7
AP + copolymer + 0.1 m*M* $MgCl_2$	106.9
AP + 0.5 m*M* $MgCl_2$	34.7
AP + copolymer + 0.5 m*M* $MgCl_2$	102.8
AP + 0.1 m*M* $ZnCl_2$	80.2
AP + copolymer + 0.1 m*M* $ZnCl_2$	134.0
AP + 0.5 m*M* $ZnCl_2$	9.3
AP + copolymer + 0.5 m*M* $ZnCl_2$	74.1

[a] Reaction mixtures contained 0.1 *M* Tris–HCl, pH 8.0; 0.01 *M* tetrasodium pyrophosphate; 2.9 μg AP/0.5 ml reaction; and copolymer, $MgCl_2$ or $ZnCl_2$ as indicated.

addition of higher concentrations (0.5 m*M*) of these metal ions inhibited the pyrophosphatase activity of AP.

NMR Studies

Prior to initiating an experiment with the cadmium-substituted alkaline phosphatase preliminary experiments were conducted to characterize the extent of a cadmium–copolymer interaction. The known metal binding ability of PVM/MA copolymer suggested that such an interaction is likely. Therefore, the solution NMR spectra of free ^{113}Cd and ^{113}Cd combined with copolymer were recorded for use in interpreting subsequent NMR experiments with the cadmium-substituted AP. The results indicated the formation of a ^{113}Cd–copolymer complex which exhibited a chemical shift at −37 ppm relative to $Cd(ClO_4)_2$.

The NMR results with $^{113}Cd(II)_6$ alkaline phosphatase before and after addition of the PVM/MA copolymer are shown in Fig. 4. At pH values below 8, the $^{113}Cd(II)_6$ alkaline phosphatase exists as the phosphorylated enzyme intermediate (E-P) with phosphate covalently bound to serine 102 at the active site of each monomer (11). The E-P form of the $^{113}Cd(II)_6$ alkaline phosphatase (in the presence of 0.1 *M* Cl and PO_4 in solution) has a ^{113}Cd NMR spectrum with resonances at 153, 70, and 2 ppm, corresponding to the A, B, and C metal binding sites, respectively (11). The cadmium-113 NMR spectrum of the enzyme sample prepared in the present study exhibited resonances at 157, 70, and 2.8 ppm; typical of the $^{113}Cd(II)_6$ alkaline phosphatase at pH 6.8. No peak at 20 ppm corresponding to the free ^{113}Cd was present. This was not surprising since a stoichiometric amount of Cd was added to the apoenzyme to form the $Cd(II)_6$ alkaline phosphatase.

The addition of PVM/MA copolymer to the prepared $^{113}Cd(II)_6$ enzyme resulted in the appearance of three new peaks (at 140, 56, and −37.4 ppm), and the disappearance of the peak at 2.8 ppm. The resonance peak at −37.4 ppm indicates the formation of a copolymer–^{113}Cd complex. Since no free cadmium was present in the solution, the copolymer–cadmium complex was formed by removal of the metal from the protein. This was evident by the disappearance of the C-site ^{113}Cd resonance at 2.8 ppm. The formation

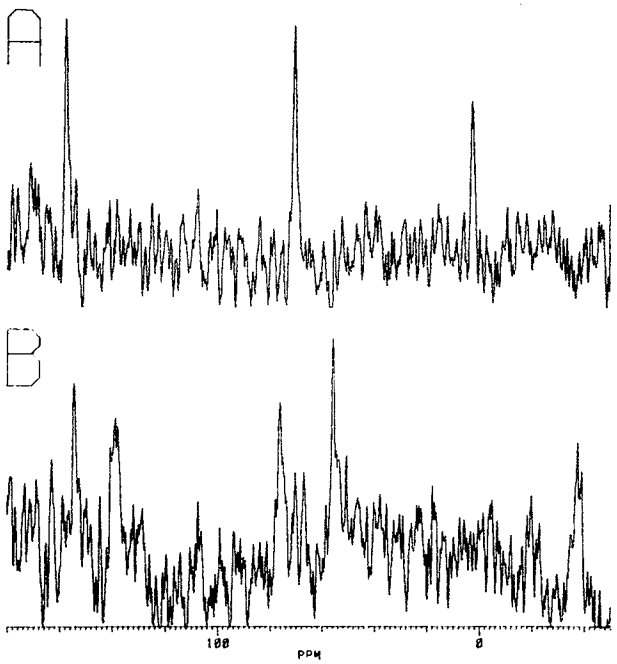

Fig. 4. ^{113}Cd NMR spectrum of $113Cd(II)_6$ alkaline phosphatase: (A) at pH 6.8 in the E-P form; (B) after addition of PVM/MA copolymer. The sample (1.5 ml) contained 0.01 *M* sodium acetate, 0.01 *M* Tris–HCl, 1.5 m*M* enzyme, 0.1 *M* NaCl, and 2 equivalents of phosphate per dimer. Spectra were recorded at 297 K and 82,000 scans were collected using a pulse angle of 30° and relaxation delay of 1 sec.

of two additional resonances (140 and 56 ppm) upfield from the original A and B site peaks indicates that there are two distinct forms of the enzyme present, in approximately equal proportions, with different environments for the A and B sites.

DISCUSSION

Alkaline phosphatase catalyzes the nonspecific hydrolysis and transphosphorylation of various phosphate esters. A reaction mechanism has been proposed by Coleman *et al.* (6,12). Briefly, it involves initial coordination of the substrate with the A-site zinc metal to form the noncovalent enzyme–substrate complex. The substrate is then hydrolyzed and the phosphate covalently binds to serine 102, lying in close proximity to the A and B sites, thus forming the phosphorylated enzyme intermediate, E-P. Phosphoserine is then hydrolyzed allowing the free phosphate to form a coordination complex (E · P) with the A site zinc ion. The E · P and E-P intermediates exist in equilibrium, the E-P being the predominant species at acid pH and the E · P form most prevalent at alkaline pH, where the enzyme is maximally active. Finally, in the rate-limiting step (at alkaline pH), the phosphate (product) is dissociated from the enzyme. An apparent feature of the alkaline phosphatase mechanism, at alkaline pH, is that it displays the phenomenon of negative cooperativity. During phosphorylation of one enzyme monomer a conformational interaction occurs which prevents phosphorylation of the active site in the second monomer.

The inhibitory effect of PVM/MA copolymer on alkaline

phosphatase observed in the present study occurred at copolymer concentrations greater than 0.1% and in a concentration-dependent manner. This effect either may be a direct effect on the enzyme or possibly is related to the metal chelating properties of the polymer. The inhibitory effect of metal complexing agents such as EDTA on alkaline phophatase is well known (13,14).

It was observed in these experiments that PVM/MA-mediated inhibition of the pyrophosphatase activity of alkaline phosphatase could be prevented by the addition of either 0.1 m*M* $MgCl_2$ or $ZnCl_2$. The effect of magnesium, in this case, is not due to direct stimulation of the enzyme by magnesium. Although magnesium is known to be a stimulator of alkaline phosphatase, particularly of the mammalian enzyme, its effect on the *E. coli* enzyme is very modest, as can be seen in Table I. Moreover, Mg, at sufficiently high concentrations (e.g., 0.5 m*M*) has been shown to inhibit the pyrophosphatase activity of alkaline phosphatase (15), an effect also apparent in this study (Table I) with the *E. coli* enzyme. This is thought to be due to formation of a magnesium–pyrophosphate complex and, therefore, loss of free pyrophosphate as a substrate. Zinc has also been reported to have an inhibitory effect on alkaline phosphatase (14,16) and the present results were in agreement with this. The ability of 0.1 m*M* $MgCl_2$ and, to a lesser extent, 0.1 m*M* $ZnCl_2$ to prevent PVM/MA copolymer-induced inhibition of alkaline phosphatase is probably not due to replacement of metal ions removed from the enzyme by the copolymer. Mg would not completely restore activity if it were added to a zinc-deficient enzyme (14). Rather, the addition of metals and copolymer simultaneously probably prevented the inhibition by forming a metal–copolymer complex and, thereby, reducing the available anionic sites on the copolymer for interaction with the enzyme.

The effect of the copolymer on the ^{113}Cd NMR spectrum of the $Cd(II)_6$ alkaline phosphatase indicated two apparent changes. The first was that cadmium was removed from the C site of the enzyme resulting in loss of the Cd resonance at 2 ppm. The second effect was that a second set of upfield cadmium resonances were apparent for the A and B metal binding sites. This suggests that two forms of the enzyme are present with different environments for the A- and B-site cadmium ions; two ^{113}Cd chemical shifts for the A site (154 and 140 ppm) and two set for the B site (75 and 56 ppm).

The splitting of the A and B cadmium resonances resembles the splitting of the A and B peaks seen when both the covalent E-P and the noncovalent E · P forms of the Cd enzyme are present (11) except that in this case the upfield shift of the B-site resonance is greater. At pH 6.8, however, the Cd enzyme exists mainly as the phosphoserine derivative, and there appears to be no reason why addition of PVM/MA would affect this equilibrium. One possible explanation of the results is that the PVM/MA binds to the A-site Cd, on an approximately half-molar basis, thereby yielding a second form of the enzyme, with a resultant splitting of the A and B peaks. Binding of PVM/MA to the A-site metal is possible since the A-site metal ion is thought to be in a five-coordinate state, with two coordination positions open to the solvent in the unliganded state (12). One of these positions normally becomes occupied by phosphate in the E · P enzyme. It is difficult to reconcile the idea of a common binding site for both PVM/MA and phosphate with the kinetic data, since it was clearly apparent that PVM/MA inhibition of the enzyme was not of the competitive type. It is possible, however, that since PVM/MA copolymer is a large polyanionic molecule, it binds to the enzyme at multiple sites, resulting in a binding that is not easily reversible. This could block access of the substrate to the A metal binding site, resulting in inhibition of catalytic activity.

An alternative possibility is that the reduced enzymatic activity resulting from addition of PVM/MA is due to removal of metal from either the A or the B site of the enzyme. Removal of metal from the C site alone may not appreciably affect activity (6). However, the A-site Zn is an absolute requirement for catalytic activity and occupancy of the B site by metal has been shown to enhance activity greatly (11, 17). An initial thought concerning the Cd NMR data was that PVM/MA removed cadmium from the C site and also partially from the B site. This would result in the formation of $Cd(II)_2$ AP, a species in which only one monomer is phosphorylated and which exhibits both A- and B-site cadmium resonances (140 and 55 ppm). Both A- and B-site resonances occur (when only an A-site resonance would be expected) due to migration of some of the A-site Cd of the unphosphorylated monomer to the B site of the phosphorylated monomer (11). This could account for the second set of A- and B-site cadmium resonances seen. One problem with this interpretation is that this migration phenomenon occurs when phosphate is added to the $Cd(II)_2$ AP and it is questionable whether this form could be formed by removal of Cd from the phosphorylated $Cd(II)_6$ AP. It is apparently difficult to remove metal from the B site of the $Cd(II)_6$ AP, a form in which both monomers are phosphorylated, with a resultant stabilization in the Cd binding at both the A and the B sites. ^{113}Cd and ^{31}P NMR data have been reported which suggested that once the enzyme is phosphorylated, the A site becomes most labile (11,12).

Another factor to consider regarding metal ion removal from the Cd enzyme is that it may not be possible to extrapolate these results to the native enzyme since bound zinc at the A and B sites and Mg at the C site are smaller than cadmium and generally bind with a higher affinity (17,5). Removal of the native metals, therefore, would probably be more difficult. It has, however, been noted that cadmium at the B site is very resistant to exchange with Zn when the A site is also occupied by Cd (17). If, indeed, the copolymer removed zinc from the native enzyme, this would certainly account for the reduced enzymatic activity observed. Removal of Mg from the C site alone would probably not appreciably affect enzymatic activity (6,12), at least not to the degree observed.

It was clear from the kinetic data that the copolymer-induced inhibition of AP could not be classified as competitive since the V_{max} was definitely reduced. The NMR data suggested that this may have resulted from interaction of the copolymer with metals at the active site of the enzyme, as was evident by the existence of apparently two forms of the enzyme. This is a likely possibility considering the affinity of the copolymer for complexing with divalent metal ions and the removal of cadmium from the C site of $Cd(II)_6$ AP. While the precise mechanism of enzyme inhibition is not entirely

clear, the Cd NMR data define molecular changes occurring as a result of enzyme–copolymer interaction. The results also raise some interesting possibilities about the exact nature of the interaction.

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