^{31}P NMR STUDIES ON PHOSPHATE BINDING TO THE Zn^{2+}, Co^{2+} AND Mn^{2+} FORMS OF *ESCHERICHIA COLI* ALKALINE PHOSPHATASE

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Received 30 October 1972
Revised version received 17 January 1973

1. Introduction

E. coli alkaline phosphatase is a zinc metalloenzyme [1, 2]. The requirement of the metal ion for enzymatic reactions catalysed by this enzyme is well established, but the exact role of the metal ion is not clearly understood. Zinc can be replaced by several other metals of which only Co^{2+} produces a fairly active enzyme [2, 4]. It has been shown that the enzyme mechanism involves the formation of a phosphoryl enzyme intermediate and this covalent intermediate has been isolated at low pH and has been shown to be phosphorylated serine residue [5–11]. There exist considerable data suggesting that the metal ion plays a role in the binding of phosphate, as well as in the formation and breakdown of the phosphoryl enzyme [12].

The present study was undertaken to obtain more information concerning phosphate binding to the enzyme and the role of the metal ion in this reaction. The purpose of this communication is to discuss the use of ^{31}P nuclear magnetic resonance (NMR), for an investigation of this problem.

^{31}P NMR signal from orthophosphate was observed with metal-free enzyme, and Zn^{2+}-alkaline phosphatase. Furthermore the measurements were extended to include the effect of paramagnetic ions such as Mn^{2+} and Co^{2+} on the width of the absorption lines. The ^{31}P NMR data give further evidence that metal-free alkaline phosphatase does not bind phosphate tightly, while the addition of metal induces phosphate binding. ^{31}P NMR studies carried out with Zn^{2+}, Co^{2+} and Mn^{2+}-alkaline phosphatases are consistent with the existence of two specific metal binding sites.

2. Material and methods

All reagents were analytical grade. Spectrographically pure metal sulfates were utilized to prepare metal solutions. Chelex 100 (200–400 mesh) from Bio-Rad Laboratories, Richmond, Calif. USA and dithizone from Fischer Laboratories, Springfield, N.J., USA were employed. DEAE A_{23} cellulose was purchased from Whatman, Kent, England and p-nitrophenyl phosphate (Sigma 104) from Sigma Chemical Company, St. Louis, Mo., USA. Metal-free solutions were prepared as described previously [13] and glassware was treated according to Thiers [14].

Alkaline phosphatase was isolated from *E. coli* $K_{12}C_4F_1$ bacterial strain by the methods previously described [15]. The apoenzyme was prepared from Zn^{2+}–enzyme through treatment with Chelex 100 (200–400 mesh) [13]. The apoenzyme was then extensively dialysed against large volumes of metal-free buffer or metal-free deionized water, to ensure removal of any contaminating EDTA [16]. Concentrations of protein were determined by absorbance at 278 nm as described by Malamy and Horecker [17].

Activity was assayed by the methods previously described [16].

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Metal analyses were performed as described earlier [13, 18, 19].

The ^{31}P NMR spectra were recorded with a Varian XL-100 spectrometer operating at a frequency of 40.3 MHz and with deuterium (D_2O) internal lock at 15.4 MHz. For the most dilute solutions, 0.01 M orthophosphate, a Varian T-1024 time average computor was used to accumulate 5 to 20 spectra. 12 mm sample tubes were used.

3. Results

The ^{31}P NMR signal from orthophosphate (0.11 M) at pH 8 consists of a single line, line width $\delta\nu_{1/2}$ = 1.0 Hz. No shift reference was used in these measurements, the variation in the chemical shift can, however, be obtained under the assumption of no change in the lock signal frequency. No shift variations could be detected in this way throughout this work.

On addition of metal-free phosphatase to the orthophosphate solutions, the ^{31}P NMR signal becomes narrower, $\delta\nu_{1/2}$ decreases from 1.0 to 0.45 Hz for a 0.11 M phosphate solution.

When a concentrated apoenzyme + orthophosphate solution was titrated with Zn^{2+} the ^{31}P line width increased significantly and a reasonably sharp titration point at ca. 2 Zn^{2+} per enzyme molecule was found (fig. 1). Since a large amount of apoenzyme was needed in this experiment the sample volume was kept as low as possible, about 1 ml, which prohibited the spinning of the sample due to the vortex formation. The resolution was therefore not very good in this experiment ($\delta\nu_{1/2}$ = 3.7 Hz with no Zn^{2+} added).

Diluted metal-free alkaline phosphatase solutions (ca. 10^{-5} M) in the presence of orthophosphate (0.11 M) were titrated with Co^{2+} and Mn^{2+} solutions, recording the ^{31}P NMR signal (figs. 2 and 3). Both titrations gave fairly sharp titration points at ca. 2 metal ions per enzyme molecule. The line width of the ^{31}P NMR signal is small and almost constant upon addition of metal ions up to the titration point and then the line width increases rapidly but not as much as on addition of these metal ions to an enzyme-free solution. The ^{31}P orthophosphate signal from a 0.11 M solution is broadened 0.45 Hz by 10^{-6} M Co^{2+} and 7 Hz by 10^{-6} M Mn^{2+}, at pH 8.0 with no enzyme present.

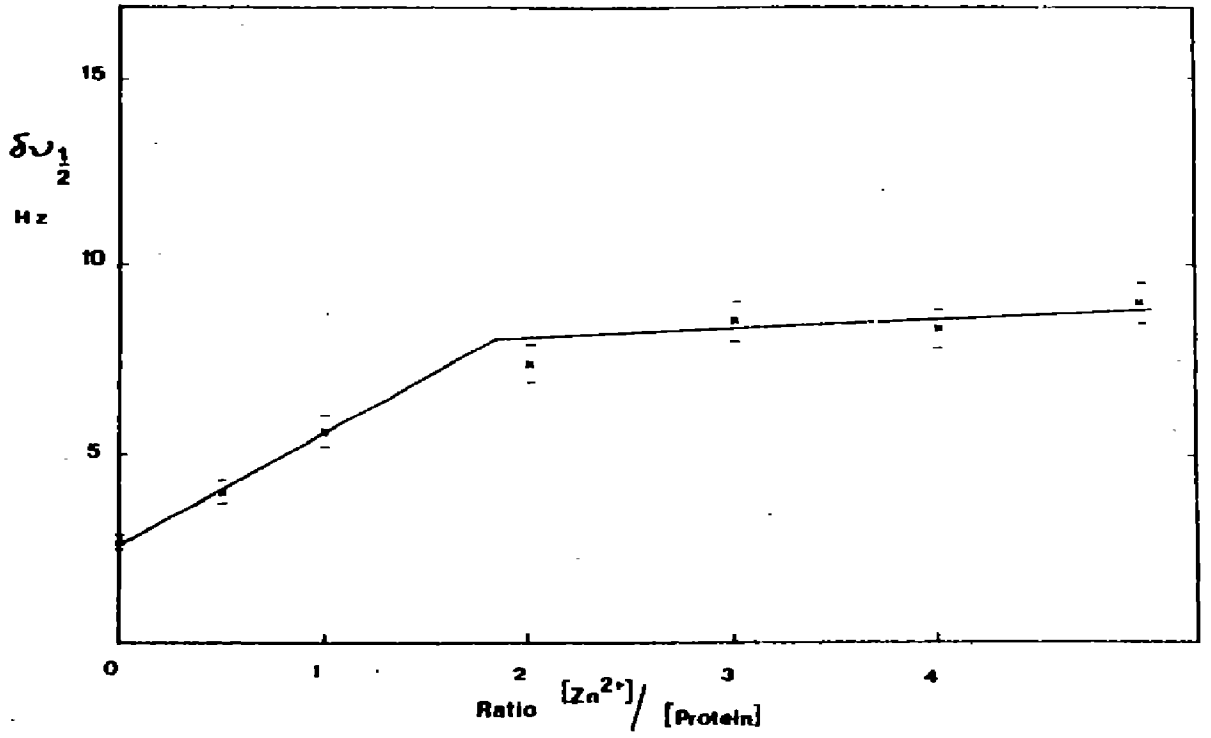

Fig. 1. Plot of the phosphate ^{31}P NMR signal half width, $\delta\nu_{1/2}$, versus zinc ion concentration for solutions containing 0.01 M orthophosphate and 1.0×10^{-3} M apoalkaline phosphatase, at pH 8.0 in 0.4 M Tris buffer. ^{31}P NMR spectra were obtained at 20°.

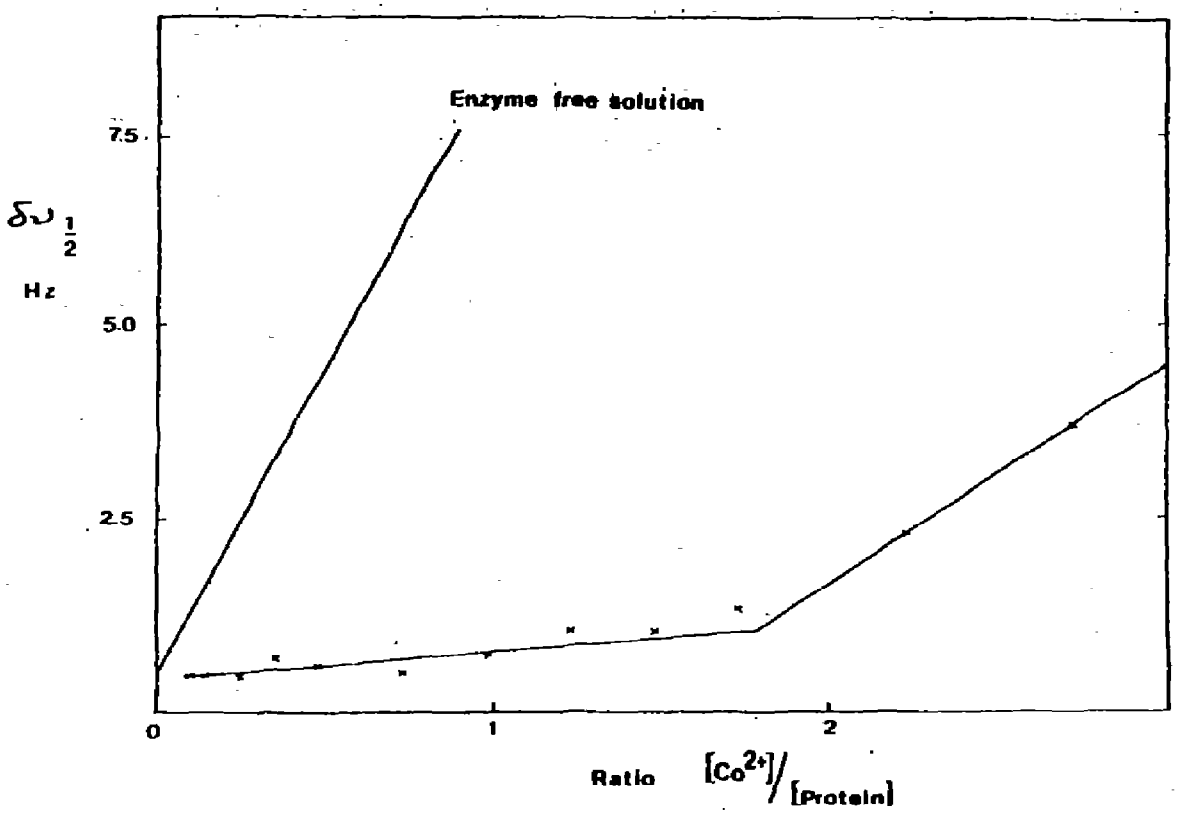

Fig. 2. Plot of the phosphate ^{31}P NMR signal half width, $\delta\nu_{1/2}$, versus Co^{2+} concentrations for solutions containing 0.11 M orthophosphate and ca. 10^{-5} M apoalkaline phosphatase, at pH 8.0 in 0.4 M Tris buffer. ^{31}P NMR spectra were obtained at 20°.

4. Discussion

The use of ^{31}P NMR to investigate the chemical nature of phosphorylated molecules is generally based on the interpretation of three spectral features: i) the chemical shift; ii) the spin–spin coupling pattern and iii) the relaxation times, T_1 and T_2. In general chemical shifts give information about the local environments of the phosphorous nuclei. Spin–spin coupling patterns give information about the identity of neighboring groups. Relaxation times reveal the effect of dynamic processes, e.g. chemical exchange or molecular tumbling in solution. For a comprehensive discussion of the relaxation phenomena, see [20 and 21].

In order to study the phosphate binding to *E. coli* alkaline phosphatase, one may utilize the line width of ^{31}P NMR spectra, to obtain information about the spin–spin relaxation time, T_2. When there are two sites for the nuclei under observation and the population of one at these sites is much less than the other the observable relaxation rate is given by eq. 1:

$$\frac{1}{T_2} = k + \frac{P_m \cdot q}{\tau_M}\left[\frac{\frac{1}{T_{2M}}\left(\frac{1}{\tau_M}+\frac{1}{T_{2M}}\right)+\Delta\omega_M^2}{\left(\frac{1}{\tau_M}+\frac{1}{T_{2M}}\right)^2+\Delta\omega_M^2}\right] \quad (1)$$

where $\frac{1}{T_{2M}}$ is the relaxation rate of nucleus in the less populated site, and τ_M is the residense time in this site For a recent discussion of application of this equation in biochemical studies c.f. [21].

For nuclei with $I = \frac{1}{2}$ the relaxation time T_{2M} is usually assumed to be dominated by dipole–dipole type interactions which is the situation when the nuclei in the less populated site is close to a paramagnetic ion, but eq. 1 is equally valid when other types of relaxation mechanisms dominates.

The phosphate binding to the metal-free alkaline phosphatase has been investigated by equilibrium dialysis experiments, and Applebury et al. [12] have found that the apoenzyme does not bind phosphate

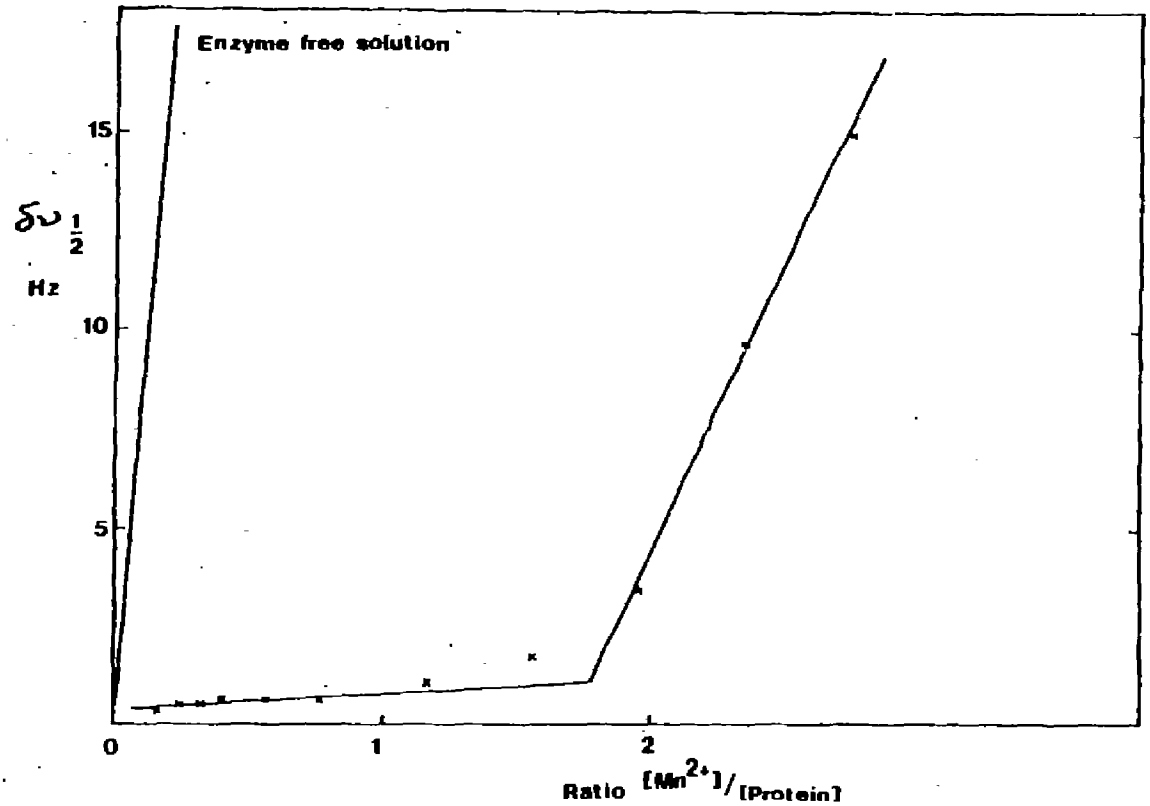

Fig. 3. Plot of the phosphate ^{31}P NMR signal half width, $\delta\nu_{1/2}$, versus Mn^{2+} concentration for solutions containing 0.11 M orthophosphate and ca. 10^{-5} M apoalkaline phosphatase, at pH 8.0 in 0.4 M Tris buffer. ^{31}P NMR spectra were obtained at 20°.

specifically. Our observation that the addition of metal-free alkaline phosphatase to the phosphate sample does not change the ^{31}P NMR spectrum significantly is in accordance with the dialysis data. The possibility that τ_M in eq. 1 is very large (i.e. that slow exchange conditions exist) is rendered unlikely by the fact that no detectable line broadening was observed of the phosphate signal in the presence of the metal-free enzyme at elevated temperatures.

Our data from the Zn^{2+} titration shows that addition of stoichiometric amounts of zinc to the metal-free alkaline phosphatase creates a phosphate binding site(s), since a line broadening due to the enhanced diamagnetic relaxation [22] was observed. The formation of a weak complex between the phosphate and the "free" zinc ions is likely to occur. A diamagnetic complex of this kind is not expected to markedly influence the relaxation rate of the phosphorous nucleus, in contrast to the situation in a paramagnetic complex. This latter situation is evident in the experiments summarised in figs. 2 and 3.

The titrations of metal-free alkaline phosphatase with Co^{2+} and Mn^{2+} show that two metal ions are bound to the apoenzyme (figs. 2 and 3). For these titrations no significant line broadening of the ^{31}P phosphate signal was observed before more than two metal ions per apoenzyme was added. Preliminary variable temperature experiments indicates that this is due to slow exchange between free and enzyme bound phosphate ions, since the line width increases with increasing temperature. This will be further investigated using different magnetic fields strengths. That there is slow phosphate ion exchange for Mn^{2+} and Co^{2+}-enzymes, is not in contradiction with the observed broadening in the zinc titration experiment. The fact that large broadening is observed in the zinc titration experiment does not necessarily imply that fast exchange conditions exist since the mole fraction bound phosphate ions, Pm, (eq. 1) is 10^3 times higher in the zinc experiment than in the manganese and cobalt experiments.

From figs. 2 and 3 it can be seen that the ^{31}P NMR signal broadening is not, even after the titration point, as large for the solution with apoenzyme as for the

enzyme-free solution. This indicates that a second type of binding site(s) may exist in which the paramagnetic ions can, to some extent, interact with the phosphate ions.

It is at this stage not possible to evaluate the number of phosphates, which bind tightly to the metalloalkaline phosphatase. Nevertheless, the results obtained with ^{31}P NMR measurements are potentially of great value because they give direct evidence of two specific metal binding sites in *E. coli* alkaline phosphatase.

Acknowledgements

The authors wish to thank Professors Bo G. Malmström and Sture Forsén and Drs. Tore Vänngård and K.E. Falk for untiring support and helpful discussions. This study was supported in part by a grant from the Swedish Natural Science Research Council. The Knut and Alice Wallenberg Foundation is gratefully acknowledged for financial support to the XL-100 NMR spectrometer.

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