^{81}Br AND ^{35}Cl NUCLEAR MAGNETIC RESONANCE STUDIES OF *E. COLI* ALKALINE PHOSPHATASE

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1. Introduction

The halogen ion probe technique introduced by Stengle and Baldeschwieler [1] has been utilized in the study of metal complexes of several proteins [2–8]. Investigations of zinc-containing and zinc-activated enzymes with this method are the subject of two recent publications [9, 10]. The purpose of the present communication is to report on the use of ^{35}Cl and ^{81}Br nuclear magnetic resonance in the study of *E. coli* alkaline phosphatase.

Alkaline phosphatase isolated from *E. coli* cells is a zinc metalloprotein with a molecular weight of about 80,000 [11–13]. The zinc content of the enzyme is in dispute, but two equivalents of zinc are firmly bound to the protein and are necessary for activity [14–19]. Furthermore, the enzyme has been reported to bind specifically other transition metal ions [12]. Zinc binding has been investigated by several authors [20, 21], and on the basis of these studies different conclusions have been reached about the nature of zinc binding to the enzyme. *E. coli* alkaline phosphatase becomes phosphorylated by either substrate or phosphate to form a phosphoryl enzyme intermediate, in which the phosphate is covalently bound to a serine residue [22]. It has been shown that apophosphatase is unable to bind phosphate [23], hence the metal ions of the native enzyme must play a very important role in the phosphate binding and in the formation of the phosphoryl enzyme. To gain more insight into the mode of action of the metal ions in this protein, additional work is required employing various independent methods. Since the halide ion NMR technique has been shown to be valuable for the investigation of zinc - protein interactions [9, 10], this method was applied to *E. coli* alkaline phosphatase for the studies presented here.

The findings of the present investigation are that the ^{35}Cl nuclear magnetic resonance line width in aqueous chloride solutions is slightly broadened upon the addition of apophosphatase, while dramatic increases in line width were observed in the presence of some metallophosphatases. Addition of phosphate reduces the line broadening caused by Zn^{2+}-alkaline phosphatase. The ^{81}Br NMR line width is at pH 7.8 strongly dependent on the zinc content. At higher and lower pH values addition of zinc ions to an aqueous bromide solution containing apophosphatase produces much smaller ^{81}Br linc broadening.

2. Experimental

2.1. *Materials*

Purification, enzyme assay and protein concentration measurements were carried out as previously described [21]. The metal-free protein was prepared

by treating the metalloprotein with Chelex 100 [21]. The preparation of Co^{2+}- and Cu^{2+}-alkaline phosphatases from the apoenzyme was performed by methods previously described [24, 25]. Metal analyses for Zn and Co were performed by atomic absorption spectroscopy; for Cu as described earlier [26]. Metal solutions were prepared from spectrographically pure metal chlorides, and all the other chemicals were of analytical grade. Metal-free solutions were obtained by extraction with dithizone [21], and glassware was treated as described by Thiers [27].

2.2. *NMR measurements*

The ^{35}Cl magnetic resonance signal was recorded with a Varian HA-100D-15 NMR spectrometer at a magnetic field strength of 23.5 kG. The radio-frequency field was obtained from a Varian V-4311 Fixed Frequency Unit. The probe constructed for these measurements was made in essentially the same way as the wide-line probes supplied by Varian. The modulation field was taken from the "manual oscillator" and was amplified in order to obtain a convenient signal-to-noise ratio. The modulation frequency was 1500 Hz. The field was swept by the V-3508 unit (slow sweep), and the spectra were calibrated with the conventional sideband technique. The line widths were taken as the signal breadth at half intensity of the absorption signal and were reproducible within ±10% except for a few very broad signals. The reported line widths usually are the averages of six individual runs. The rate of passage through resonance and the amplitude of the r.f. field were chosen small enough to give no detectable distortion of the signals. It may be seen in fig. 1 that the recorded signals are slightly unsymmetrical in the case of broad curves. This is due to a baseline drift at the highest amplifications caused by thermal fluctuations in the probe which lacked provision for temperature stabilization. Since this drift is very nearly constant it could easily be corrected for. The temperature used was 31 ± 1°. The samples were contained in test tubes an inner diameter of 14 mm.

A Varian V-4200 NMR spectrometer equipped with a 12 inch V-3603 magnet was used for the ^{81}Br measurements at a magnetic field of 13.75 kG and a temp. of 29 ± 2°. The line width was in this case taken as the distance between maximum and minimum slope of the absorption signal. The error in the reported line widths, which are the arithmetic means of 2–3 spectra, is about 10%. Experimental details are given elsewhere [28].

In order to prepare the protein solutions, native alkaline phosphatase and apophosphatase were dialysed against water, then against 5×10^{-3} M tris-HCl buffer, pH 7.5 (metal-free). In the case of the ^{81}Br NMR measurements, the solutions (1 ml) contained about 0.5 M KBr, protein and 0.055 M tris-HCl buffer. Protein and KBr concentrations and pH values are given in table 1. In the case of the ^{35}Cl NMR experiments, samples (2 ml) contained about 0.5 M KCl, protein and 0.055 M tris-HCl buffer.

3. Results and discussion

Fig. 1 depicts the ^{35}Cl NMR spectra of aqueous KCl solutions under various conditions. The ^{35}Cl NMR line width in a 0.5 M KCl solution is very narrow, about 13 Hz, due to the symmetrical solvation and the rapid tumbling of the chloride ion. From the spectrum shown in fig. 1b it can be estimated that the ^{35}Cl line width in the presence of apophosphatase is 23 Hz. This 10 Hz broadening caused by apophosphatase can arise from the presence of 3% residual zinc, and from the weak binding of Cl^- to a variety of side chains of the protein molecule. During the NMR measurements no significant change in the stability of the apoenzyme was observed, since Zn^{2+} addition restored enzymatic activity completely.

The ^{35}Cl NMR spectrum of a 0.5 M aqueous KCl solution exhibits a dramatic broadening when Zn^{2+}-alkaline phosphatase is present in the solution. A representative spectrum is shown in fig. 1c. This increase in the chlorine resonance width, for comparable protein concentrations, is 72 Hz for Zn^{2+}-alkaline phosphatase, while only 10 Hz for the apoenzyme. Experiments showed that the ^{35}Cl line broadening produced by Zn^{2+}-alkaline phosphatase is orders of magnitude larger than that produced by Zn^{2+} alone (cf. [9]). This enhanced broadening might be the result of either Cl^- occupying a coordination site of the zinc ion bound to the enzyme or of a metal-induced conformational change.

Titration of an apoalkaline phosphatase solution containing 0.5 M KCl with Zn^{2+}, using the ^{35}Cl NMR

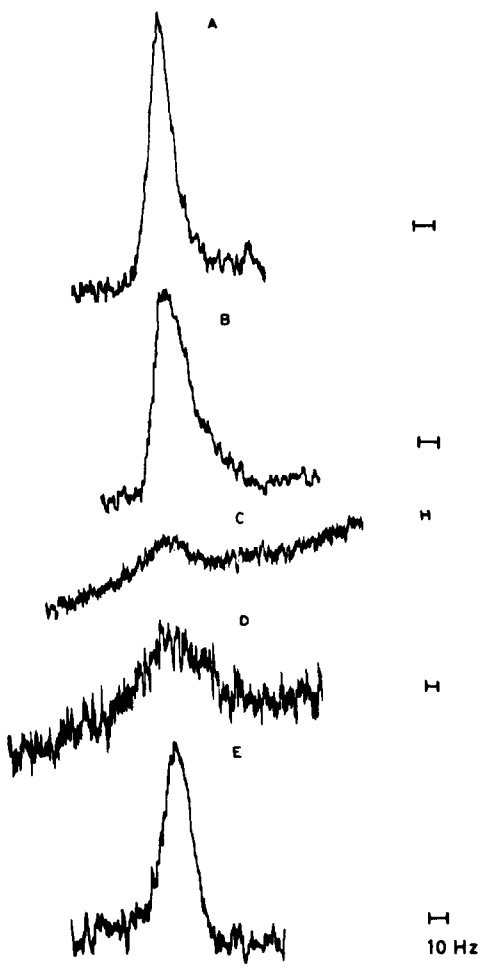

Fig. 1. ^{35}Cl nuclear magnetic resonance absorption of 0.5 M KCl solution.
a) no protein, line width = 13 Hz;
b) 1.1×10^{-4} M apoalkaline phosphatase in 0.055 M tris buffer (pH 7.6), line width = 23 Hz. This experiment was performed in the presence of 0.4 M KCl;
c) 1.1×10^{-4} M Zn^{2+}-alkaline phosphatase (zinc/enzyme = 4.8) in 0.055 M tris buffer (pH 7.6), line width = 85 Hz. This experiment was performed in the presence of 0.4 M KCl;
d) 8.75×10^{-5} M Cu^{2+}-alkaline phosphatase (copper/enzyme = 4.2) in 0.055 M tris buffer (pH 7.0), line width = 60 Hz;
e) 8.75×10^{-5} M Co^{2+}-alkaline phosphatase (cobalt/ enzyme = 4.5) in 0.055 M tris buffer (pH 7.2), line width = 21 Hz.

line width as the measured parameter, yields a titration curve differing markedly from pervious experiments of this type [1–3, 7, 10, 29]. Thus the first additions of Zn^{2+} give no increase in line width compared to apophosphatase. Above a molar ratio Zn^{2+} to enzyme about one the line width starts increasing. (The point where the resonance signal begins to broaden seems to depend on the pH used.) This may indicate that the first zinc equivalent is not accessible for chloride binding. On the other hand, if the Cl^- binding is a result of metal-induced conformational changes, then the first zinc atom does not cause such alterations. A further possibility is that cooperative interactions occur.

As is shown by the spectrum in fig. 1d, Cu^{2+}-alkaline phosphatase also has a strong line broadening effect on the chlorine resonance. The increase of the ^{35}Cl line width at 8.75×10^{-5} M protein concentration is 47 Hz. The shape of the apophosphatase titration curve with Cu^{2+} is similar to the one described above for Zn^{2+}. In contrast to the Zn^{2+}- and Cu^{2+}-enzymes and to Co^{2+}-carbonic anhydrases [29], no broadening due to addition of Co^{2+} to an apoalkaline phosphatase solution was detected at the experimental conditions employed (fig. 1e). There are several possible interpretations of this fact: 1) the association of cobalt with the protein is not sufficiently strong under the experimental conditions. However, this possibility can be ruled out since activity measurements gave the value corresponding to Co^{2+}-enzyme. Furthermore, the optical spectrum was identical with that of Co^{2+}-alkaline phosphatase; 2) the most important aspect is that the metal must be accessible for chloride binding in order to affect the chlorine resonance. Thus, it is plausible that Co^{2+} does not have available a coordination site for chloride. This is in accordance with the fact that addition of a Co^{2+} salt to an aqueous solution of an alkali chloride produces only small changes in the ^{35}Cl magnetic relaxation rate. On the other hand, the addition of Cu^{2+} and Zn^{2+} salts substantially broadens the ^{35}Cl magnetic resonance signal (unpublished measurements; cf. above).

Table 1 summarizes the ^{81}Br line width measurements made on 0.5 M KBr solutions containing apophosphatase and different metallophosphatases. As may be seen, the effect of apophosphatase is relatively smaller in this case. The Cu^{2+}- and Co^{2+}-alkaline phosphatases produce, at the pH used, the same line

Table 1
^{18}Br NMR line widths for KBr solutions in the presence of apophosphatase and different metallophosphatases.

Enzyme		Protein concn. (M)	KBr concn. (M)	pH	Line width (gauss)
		–	0.5	6.0	0.25
Apo-		1.05×10^{-4}	0.5	6.0	0.34
Zn^{2+}-	1 Zn^{2+}/E	1.05×10^{-4}	0.5	6.0	0.34
	3 Zn^{2+}/E	1.05×10^{-4}	0.5	6.0	0.31
Apo-		1.27×10^{-4}	0.5	7.8	0.33
Zn^{2+}-	0.8 Zn^{2+}/E	1.27×10^{-4}	0.5	7.8	0.32
	1.5 Zn^{2+}/E	1.27×10^{-4}	0.5	7.8	0.36
	3 Zn^{2+}/E	1.27×10^{-4}	0.5	7.8	0.41
Apo-		7.05×10^{-5}	0.4	7.5	0.28
Cu^{2+}-	1 Cu^{2+}/E	7.05×10^{-5}	0.4	7.5	0.28
	2 Cu^{2+}/E	7.05×10^{-5}	0.4	7.5	0.31
	5 Cu^{2+}/E	7.05×10^{-5}	0.4	7.5	0.29
Co^{2+}-	2 Co^{2+}/E	7.05×10^{-5}	0.4	7.8	0.28
	5 Co^{2+}/E	7.05×10^{-5}	0.4	7.8	0.28

pH was adjusted by 0.05 M tris-HCl buffer. Temperature 29 ± 2°. E stands for enzyme.

broadenings as the apophosphatase. Addition of Zn^{2+} ions to a 0.5 M KBr solution containing apophosphatase gives a strong line broadening at pH 7.8. At other pH values the effect is smaller or absent. The small effect of apophosphatase is probably the result of bromide binding to the protein unrelated to the metallic sites (cf. [30]).

Orthophosphate is a competitive inhibitor of *E. coli* alkaline phosphatase. However, it also undergoes a catalytic exchange reaction with $H_2{}^{18}O$ [22, 31]. Thus, it may also be regarded as a simple phosphatase substrate. Titration of the Zn^{2+}-alkaline phosphatase solution with K_2HPO_4 in an 0.5 M NaCl solution was performed, using the ^{35}Cl NMR line width as the measured parameter. Addition of phosphate reduces the line width markedly, which implies that phosphate either coordinates to zinc or binds to the enzyme in some other way, preventing access of chloride ions.

From the experiments reported here, we conclude that the halide ion probe technique offers a valuable method for studying several important aspects of the alkaline phosphatase molecule. The variation of relaxation rate with the metal content of the enzyme gives information of the interaction between alkaline phosphatase and metal ions. Furthermore, competition between phosphate and chloride may be indicated by the influence of phosphate on the broadening of ^{35}Cl NMR line width produced by Zn^{2+}-alkaline phosphatase. Our present data do not reveal the exact affinity or stoichiometry of phosphate binding to the enzyme. Nevertheless, this study indicates that the halide probe technique can be used to obtain information concerning enzyme-substrate interactions.

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