Biochimica et Biophysica Acta, 384 (1975) 277—282

BBA Report

BBA 61301

^{19}F NMR STUDIES OF THE BINDING OF A FLUORINE-LABELED PHOSPHONATE ION TO *E. COLI* ALKALINE PHOSPHATASE

HANS LILJA [a], HEDVIG CSOPAK [b], BJÖRN LINDMAN [a] and GEORG FÖLSCH [c]

[a] *Division of Physical Chemistry 2, The Lund Institute of Technology, P.O. Box 740, S-220 07 Lund 7,* [b] *Department of Biochemistry, Chalmers Institute of Technology, Fack, S-402 20 Göteborg 5 and* [c] *Department of Medical Chemistry, University of Göteborg, Fack, S-400 33 Göteborg 33 (Sweden)*

(Received December 16th, 1974)

Summary

The interaction of a fluorinated phosphonate with Zn^{2+}- and Mn^{2+}-alkaline phosphatase as studied by ^{19}F NMR revealed a stoichiometry of 1 : 1 for the binding of the phosphonate anion to the enzyme.

In the presence of two metal ions, one fluorinated phosphonate ion was found to interact strongly with the enzyme, while a different interaction was observed when the number of metal ions per enzyme exceeded two. Phosphate replaced enzyme bound phosphonate, as is shown by the ^{19}F NMR spectra.

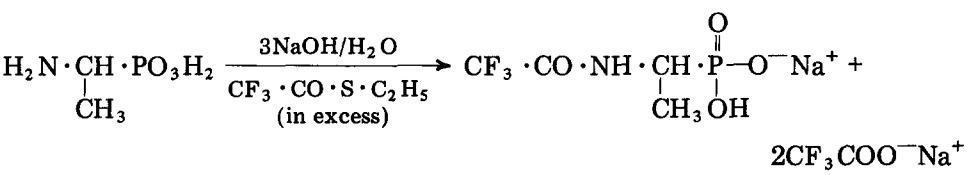
No direct interaction between the fluorinated phosphonate and the metal ion responsible for enzyme activity was indicated by the ^{19}F NMR data. This observation supports the idea of a considerable distance between metal ion and substrate binding site in *Escherichia coli* alkaline phosphatase.

The interaction of phosphonates with *E. coli* alkaline phosphatase has been studied by several authors [1—6]. It has been found that phosphonates are effective competitive inhibitors of the substrates of alkaline phosphatase from *E. coli* [1—6]. Therefore, it is of interest to investigate the possibility of using phosphonates for studying the active site of the enzyme.

The high sensitivity of ^{19}F NMR to changes in the chemical environment makes it an attractive tool for studying conformational changes in proteins and ligand binding of proteins [7—9]. Using the Fourier transform method ligand concentrations comparable to the protein concentration may easily be explored making it possible to use ^{19}F NMR to investigate conveniently the stoichiometry of ligand binding to proteins.

Abbreviation: TFP, *N*-trifluoroacetyl-1-aminoethylphosphonic acid.

This report describes an attempt to use the ^{19}F NMR spectrum of a fluorine-labeled phosphonate to obtain information on the stoichiometry of substrate binding to *E. coli* phosphatase. The fluorinated phosphonate inhibitor abbreviated here as TFP was prepared from 1-aminoethyl phosphonic acid as shown below:



All chemicals were of analytical grade. Metal solutions were prepared from spectrographically pure metal chlorides. Chelex 100 (200-400 mesh) was obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; diphenylthiocarbazone (dithizone) from Fisher Laboratories, Springfield, N.J., U.S.A.; 1,10-phenanthroline from Merck, Darmstadt, W. Germany; Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), *p*-nitrophenyl phosphate (Sigma 104) and 2-aminoethylphosphonic acid from Sigma Chemical Co., St. Louis, Mo., U.S.A. All operations were performed in metal-free water obtained by slow passage through a mixed-bed ion exchange resin. Metal-free solutions were prepared by extraction with dithizone in CCl_4 [10]. Glassware was soaked in 2 M nitric acid for weeks and rinsed with de-ionized water.

Preparation of *N*-trifluoroacetyl-1-aminoethylphosphonic acid sodium salt (TFP) was performed as follows. An excess of ethylthiol trifluoroacetate (1 ml, prepared [11] from ethyl mercaptan and trifluoroacetic anhydride, b.p. 90—91°C) was added to a solution of 1-aminoethylphosphonic acid (250 mg, i.e. 2 mmol; obtained from Calbiochem AG, Switzerland) in 1 M NaOH (4 ml). The mixture was shaken vigorously for 5 min at room temperature, and then 1 ml 1 M NaOH was added. The addition of 1 ml 1 M NaOH was repeated after another 5 min of shaking followed by shaking overnight. The solvent was removed in vacuo (water bath, 30°C), and traces of water distilled off in vacuo together with the added dioxane (10 ml). The white solid residue was then heated with an additional 10 ml anhydrous dioxane (10 ml). The insoluble sodium salt of trifluoroacetic acid was removed by filtration (0.68 g, m.p. 208°C; formed from the excess of ethylthiol trifluoroacetate and sodium hydroxide), and the solution of the *N*-trifluoroacetyl derivative was concentrated to a small volume. Addition of diethyl ether gave a white precipitate of the derivative, 0.50 g. Recrystallization from tetrahydrofuran and diethyl ether and drying in vacuo over KOH gave the monosodium salt of the compound (0.31 g, 64% yield), melting at 128°C followed by solidification and a new melting with decomposition at around 225°C. The nitrogen content was found to be 5.78%. For $C_4H_6F_3NNaO_4P$ (M_r = 248.1) the calculated amount is 5.76%. Thin-layer chromatography of the compound on Kieselgel G (Merck) plates showed complete N-acylation of the ninhydrin-positive starting material, i.e. 1-aminoethylphosphonate. This material has R_F 0.41 in 1-butanol/acetic acid/water (4:1:1) and R_F 0.50 in methanol/acetic acid/water (4:1:1), and was absent in the reaction product. The latter gave no colour with ninhydrin spray reagent but was detected by chlorin/iodide/starch

reagent [12] and had R_F 0.55 and R_F 0.83 in the above two solvent systems. The presence of a *N*-trifluoroacetyl group was further confirmed by the infrared spectrum of the compound (KBr pellet), showing strong amide absorbance bands at 5.9 and 6.4 μm. Alkaline treatment of the compound (0.2 M NaOH, 10 min at room temperature) removed the *N*-trifluoroacetyl group, and 1-aminoethylphosphonic acid was formed (identified by thin-layer chromatography).

It should be noted, that the use of a greater excess of ethylthiol trifluoroacetate in the preparation gives the compound as the free acid (found: N = 6.32%; calcd.: N = 6.34%), which does not crystallize well. If a small amount of ethylthiol trifluoroacetate is used, the dioxane-insoluble disodium salt (found: N = 5.22%; calcd.: N = 5.29%) is formed. This salt can be solubilized in dioxane by the addition of trifluoroacetic acid.

Enzyme preparation, preparation of apoenzyme, concentration of protein and activity measurements, metal analyses and phosphate analyses were done as previously described [10, 13–15].

^{19}F NMR spectra were recorded at 94 MHz using a Varian XL-100-15 spectrometer for heavy water solutions containing TFP, enzyme and buffer. All the spectra were obtained using the Fourier transform method. The number of transients was 10 000—60 000 with an acquisition time of 0.3 s. Deuteron lock was employed. Measurements were made at 4°C on 2 ml solutions which at the start of the titration normally contained 1 mM apoalkaline phosphatase, 1 mM TFP and 0.7 M buffer. Since the experiments showed incorporation of metal ions in the enzyme to be slow at 4°C, samples were kept for a short time at room temperature after each addition of metal ion solution.

Solutions containing equimolar quantities of apoalkaline phosphatase and *N*-trifluoroacetyl-1-aminoethylphosphonate (TFP) were titrated with Mn^{2+} and with Zn^{2+} at various pH values while following the ^{19}F NMR spectrum. Furthermore, we investigated the effect on the NMR spectrum of addition of excess TFP or of addition of orthophosphate at different metal contents.

Nuclear magnetic relaxation is the result of a time-dependent interaction where the spins are involved and the rate of relaxation is given by the strength of interaction and by the correlation time giving the time-scale of the motion causing relaxation. When a ligand is attached to a macromolecule more efficient relaxation is expected since the correlation time is influenced by the slow tumbling of the macromolecule. If the ligand is in the neighbourhood of a paramagnetic center in the macromolecule, the interaction strength will increase for a nucleus like ^{19}F for which dipole-dipole interactions constitute an important relaxation mechanism.

On the basis of these considerations and by noting that the NMR line width is proportional to the transverse nuclear magnetic relaxation rate our experimental line widths and their interpretations will be described.

Binding studies by several methods show that Mn^{2+} competes with Zn^{2+} for binding sites on alkaline phosphatase [16, 17]. Mn^{2+}-alkaline phosphatase lacks significant catalytic activity but induces phosphate binding and forms the phosphoryl enzyme which has been implicated as a major step in the catalytic reaction pathway [18—25]. These facts and the advantageous effect of a paramagnetic center as mentioned above prompted the investigation of

the binding of phosphonate to the Mn^{2+}-enzyme.

In all cases with Mn^{2+}-alkaline phosphatase a single ^{19}F NMR signal of TFP was obtained showing that exchange of TFP between sites on the protein and the bulk solution is rapid compared to differences in relaxation rate and chemical shifts. Therefore, line widths ($\Delta\nu$) and chemical shifts (δ) constitute weighted averages over the different sites, i.e. $\Delta\nu = \Sigma P_i \Delta\nu_i$ and $\delta = \Sigma P_i \delta_i$. ($P_i$ is the fraction of TFP ions at sites of type i.)

The line width data obtained with the Mn^{2+}-enzyme are exemplified in Table I. The observations can be summarized as follows (spectra displaying some of these points are given in Fig. 1): (a) Addition of an equimolar amount of apoenzyme to a buffer solution containing 1 mM TFP has no detectable effect on the line width. Thus, TFP does not appear to bind to the metal-free enzyme. (b) On addition of Mn^{2+} there is a slow increase in the line width up to 2 Mn^{2+} per enzyme molecule followed by a much more rapid increase with higher metal content. This is interpreted as binding of TFP to the enzyme as Mn^{2+} is added with the first two metal ions binding differently to the enzyme. The slow increase in line width at low metal contents indicates a large distance between TFP and the first two metal ions. This is supported by the observation that in the absence of enzyme the effect of Mn^{2+} addition on the line width is an order of magnitude greater than when enzyme is present. (c) Addition of TFP in excess of one molecule per enzyme to a solution containing the enzyme with two Mn^{2+} leads to a marked decrease in line width. Thus, it appears that only one TFP is bound per enzyme molecule in this case. The rate of line width decrease with increasing TFP concentration was also consistent with the binding of one TFP per enzyme molecule. (d) Addition of orthophosphate at different metal ion-to-enzyme ratios gives a decrease in line width. Therefore, it appears that orthophosphate binds to the same site as TFP but with a greater affinity.

The experiments performed with the Zn^{2+}-enzyme are all consistent with the picture obtained with the Mn^{2+}-enzyme. However, in the case of the Zn^{2+}-enzyme, exchange of TFP between the free state and the enzyme-bound state

TABLE I

^{19}F NMR HALF HEIGHT LINE WIDTHS AT 4°C FOR SOLUTIONS CONTAINING Mn^{2+}-ALKALINE PHOSPHATASE, Hepes BUFFER AND FLUORINE-LABELED PHOSPHONATE ION (TFP).

Solution composition	Line width (Hz)
1 mM TFP	3
1 mM TFP, 1 mM apoenzyme, pH 8.2	4.5
1 mM TFP, 1 mM apoenzyme, 1 mM Mn^{2+}, pH 8.2	10
1 mM TFP, 1 mM apoenzyme, 2 mM Mn^{2+}, pH 8.2	14
1 mM TFP, 1 mM apoenzyme, 3 mM Mn^{2+}, pH 8.2	30
1 mM TFP, 1 mM apoenzyme, 4 mM Mn^{2+}, pH 8.2	62
1 mM TFP, 1 mM apoenzyme, 4 mM Mn^{2+}, 1 mM PO_4^{3-}, pH 8.2	30
1 mM TFP, 1 mM apoenzyme, 4 mM Mn^{2+}, 2 mM PO_4^{3-}, pH 8.2	17
1 mM TFP, 1 mM apoenzyme, 2 mM Mn^{2+}, pH 7.8	20
2 mM TFP, 1 mM apoenzyme, 2 mM Mn^{2+}, pH 7.8	12 (13)*
4 mM TFP, 1 mM apoenzyme, 2 mM Mn^{2+}, pH 7.8	10 (9.5)*
1 mM TFP + 1 mM Mn^{2+}	114

*Figures in parentheses are calculated line widths assuming the strong binding of one TFP per enzyme molecule and rapid exchange between free and bound TFP.

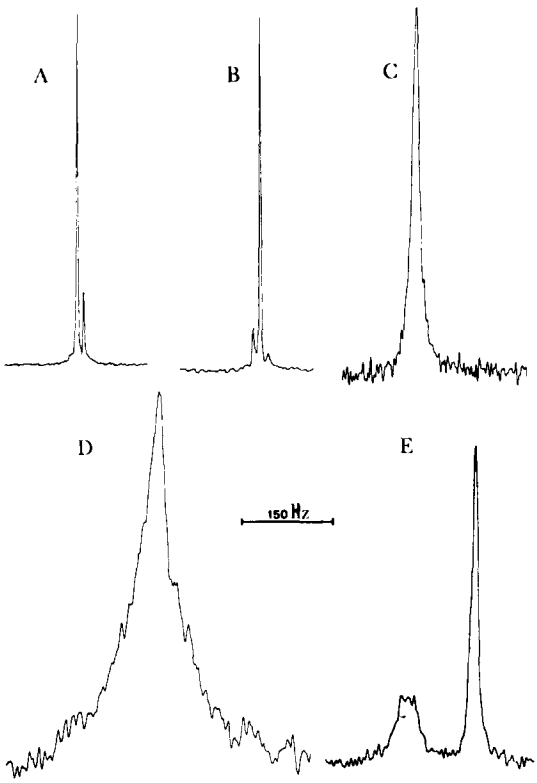


Fig. 1. ^{19}F NMR spectra at 4° of the fluorine-labeled phosphonate ion (TFP) illustrating line broadening effects of alkaline phosphatase under various conditions. In addition to 0.7 M Hepes buffer the sample compositions were: (1) 1.5 mM TFP, (b) 1 mM TFP and 1 mM apoenzyme, (c) 1 mM TFP, 1 mM apoenzyme enzyme and 2 mM Mn^{2+}, (d) 1 mM TFP, 1 mM apoenzyme and 4 mM Mn^{2+}, (e) 2 mM TFP, 1 mM apoenzyme and 2 mM Zn^{2+}.

is sufficiently slow to produce two separate TFP signals in the ^{19}F NMR spectrum. Since the exchange rate depends on both pH and metal content interpretation becomes complicated and further studies are needed before the TFP binding to the zinc enzyme can be fully characterized. Among our observations with the Zn^{2+}-enzyme the following can be mentioned: (a) Zn^{2+} addition to the apoenzyme TFP solution causes binding of TFP to the enzyme. (b) TFP binding affinity is markedly reduced above pH 8 and orthophosphate replaces TFP at the enzyme. (c) The slow TFP exchange facilitates deductions on the stoichiometry of TFP binding. Thus, the TFP spectrum in the presence of excess TFP may consist of two signals, one broad corresponding to bound TFP and one narrow corresponding to free TFP (Fig. 1e). Addition of TFP in excess of one per enzyme molecule to the enzyme with two Zn^{2+} only affects the intensity of the narrow signal. Also the integrated intensity of the broad signal is consistent with a binding of only one TFP to the enzyme with 2 Zn^{2+}. (d) With two metal ions per enzyme molecule the line width is nearly the same for the two metalloenzymes investigated. Since Zn^{2+} is diamagnetic and Mn^{2+} paramagnetic our conclusion is that there is a considerable distance between the TFP binding site (and thus the substrate binding site) and the metal ions.

The stoichiometric features of the alkaline phosphatase substantiated with fluorinated phosphonate are in good agreement with our previous findings with phenylphosphonate and Cu^{2+}-alkaline phosphatase. The changes in the electron paramagnetic resonance spectra of Cu^{2+}-alkaline phosphatase induced by phenylphosphonate demonstrate that the combination of one phosphonate with the Cu^{2+}-enzyme is sufficient to produce the change completely [26]. These data and the present ^{19}F NMR studies with fluorinated phosphonate are in accordance with the model of a single strong phosphonate binding site on the alkaline phosphatase.

In conclusion since ^{19}F NMR studies on TFP provide information on the TFP binding site in alkaline phosphatase, further understanding of the function of the enzyme is possible. A more complete study of the zinc enzyme by this method is in progress and will be published later with the chemical shift data.

The authors wish to thank Professors Bo G. Malmström and Sture Forsén for support and helpful discussions. We are most grateful to Mr Jan-Erik Norne for valuable advice and assistance. The skillful technical assistance of Mrs Annette Arvidsson is also acknowledged. This study was supported by research grants 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.

References

1 Lazdunski, C. and Lazdunski, M. (1966) Biochim. Biophys. Acta 113, 551—572
2 Lazdunski, C. and Lazdunski, M. (1969) Eur. J. Biochem. 7, 294—300
3 Halford, S.E., Bennet, N.G., Trentham, D.R. and Gutfreund, H. (1969) Biochem. J. 114, 243—251
4 Gottesman, M., Simpson, R. and Vallee, B. (1969) Biochemistry 8, 3776—3783
5 Reid, T.W. and Wilson, I.B. (1971) Biochemistry 10, 380—387
6 Szajn, H., Csopak, H. and Fölsch, G., manuscript in preparation
7 Raftery, M.A., Huestis, W.H. and Millett, F. (1971) Cold Spring Harbor Symp. Quant. Biol. Vol. Vol. XXXVI, pp. 541—550
8 Muller, N. and Mead, Jr., R.J. (1973) Biochemistry 12, 3831—3835
9 Dwek, R.A. (1973) Nuclear Magnetic Resonance in Biochemistry: Applications to Enzyme Systems, Clarendon Press, Oxford
10 Csopak, H. (1969) Eur. J. Biochem. 7, 185—192
11 Hauptschein, M., Stokes, C.S. and Nodiff, E.A. (1952) J. Am. Chem. Soc. 74, 4005—4010
12 Rydon, H.N. and Smith, P.W.G. (1952) Nature 169, 922—923
13 Csopak, H. and Szajn, H. (1973) Arch. Biochem. Biophys. 157, 374—379
14 Csopak, H., Falk, K.E. and Szajn, H. (1972) Biochim. Biophys. Acta 258, 466—472
15 Norne, J.E., Csopak, H. and Lindman, B. (1974) Arch. Biochem. Biophys. 162, 552—559
16 Harris, M.I. and Coleman, J.E. (1968) J. Biol. Chem. 243, 5063—5073
17 Lazdunski, C., Petitclerc, C. and Lazdunski, M. (1969) Eur. J. Biochem. 8, 510—517
18 Schwartz, J.H. (1963) Proc. Natl. Acad. Sci. U.S. 49, 871—876
19 Engström, L. (1962) Biochim. Biophys. Acta 56, 606—609
20 Engström, L. (1962) Ark. Kem. 19, 129—235
21 Pigretti, M.M. and Milstein, C. (1965) Biochem. J. 94, 106—117
22 Barrett, H.H., Butler, R. and Wilson, I.B. (1969) Biochemistry 8, 1042—1047
23 Levine, D., Reid, J.W. and Wilson, I.B. (1969) Biochemistry 8, 2374—2380
24 Reid, J.W., Pavlic, M., Sullivan, D.J. and Wilson, I.B. (1969) Biochemistry 9, 3184—3188
25 Neumann, H. (1969) Eur. J. Biochem. 8, 164—173
26 Csopak, H. and Falk, K.E. (1974) Biochim. Biophys. Acta 359, 22—32