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^{31}P and ^{17}O NMR of Mn(III)-Containing Acid Phosphatase: Evidence for Direct Metal-Phosphate Interaction and Oxygen Exchange from Water into Inorganic Phosphate

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SUMMARY

The acid phosphatase isolated from sweet potato tubers by us is unique Mn(III)-containing enzyme which hydrolyzes phosphomonoesters and nucleotide phosphates. The present ^{31}P and ^{17}O NMR studies of the Mn(III)-containing acid phosphatase solved two important problems. The broadening of the phosphate ^{31}P resonance signal in the 1:1 enzyme-substrate system shows evidence for direct metal-phosphate interaction in the Mn(III)-containing acid phosphatase. In addition, the ^{17}O NMR evidence for oxygen exchange from water into inorganic phosphate strongly indicates that the Mn(III)-containing acid phosphatase catalyzes an apparent transition state displacement and P-O cleavage as follows: $ROPO_3^{=} + H^{17}OH \longrightarrow ROH + H^{17}OPO_3^{=}$.

INTRODUCTION

To know the active site structure and reaction mechanism of enzyme is one of the major purpose of enzyme chemistry. Recent advance in multi nuclei FT-NMR spectroscopy made it possible to direct observation of individual atom resonances for metalloenzyme. These NMR features are expected to provide information regarding the chemical environment of the active site and the enzymatic reaction mechanism(1,2,3). Acid Phosphatase(EC 3.1.3.2) from sweet potato is a manganese-containing enzyme in which the only Mn(III) ion present at one atom per enzyme molecule(Mw=110000) plays an essential role in the catalytic reaction of hydrolysis of phosphomonoesters and nucleotide phosphate(4). In addition, the resonance Raman and chemical evidences indicated at least tyrosine phenolate and cysteine sulfydryl groups as the Mn(III)-active site ligands(5,6). The present ^{31}P and ^{17}O NMR investigations of the Mn(III)-containing acid phosphatase solved two important problems: The first, direct phosphate substrate-metal interaction in the Mn(III)-active center and the second, oxygen exchange from water into inorganic phosphate during the enzymatic reaction.

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MATERIALS & METHODS

The Mn(III)-containing acid phosphatase was isolated and purified from sweet potato tubers according to our previously reported procedures(4). The enzyme which was dialyzed against 0.1 M Tris-HCl buffer(pH 7.0) over one night, was used for the NMR experiments. Mn(III)-pyrophosphate complex was synthesized according to the published method(7). The ^{31}P and ^{17}O NMR measurements were made on a JEOL FT FX-90 spectrometer operating at 36.2 and 12.1 MHz, respectively. On ^{31}P NMR measurement D_2O was used as a field-frequency lock. Spectra were routinely acquired using the following parameters: spectral width, 20000Hz; acquisition time 0.2 s; and pulse angle, 45°. Although the difficulties in ^{17}O NMR working are due to the ^{17}O quadrupole moment(I=5/2) and low natural isotopic abundance(0.037 %), the sensitivity problem was eliminated by employing ^{17}O-enriched compound, $H_2{}^{17}O$(20 atom %; Prochem. England) in the present study. Spectra were routinely acquired using the following parameters: spectral width, 10000 Hz; acquisition time, 0.42 s; and pulse angle, 12°.

RESULTS & DISCUSSION

Figure 1 shows ^{31}P NMR spectra of the Mn-containing acid phosphatase. The addition of equimolar phosphate per mol of the metalloenzyme clearly resulted in broadening(full width at half-height: 37.8 ppm) of the ^{31}P phosphate resonance line, corresponding well to that(36.2 ppm) for the model pyrophosphate-Mn(III) complex(7). The Mn(III) paramagnetic center of this acid phosphatase dominates the relaxation of nuclei in its vicinity and thus effect of the Mn(III) ion on the ^{31}P nuclear magnetic resonance of phosphate substrate can yield information on the proximity of the phosphate and Mn(III) ion in the phosphate complex of this Mn-containing enzyme(8,9). The Mn(III) sites in the present enzyme and Mn-superoxide dismutase from E.coli are quite characteristic of an S=2 integral spin system(4.10). Indeed, nearly all known Mn(III) complex are high-spin (^5E in Oh), except for low-spin$[Mn(III)\,CN_6]^{3-}$ complex(11,12). Therefore, the presence of paramagnetic broadening of the ^{31}P resonance indicates that the single phosphate bound to the enzyme is clearly very near one of the catalytic Mn(III) site, probably within the first coordination sphere. In the case of Zn(II)-containing alkaline phosphatase, ^{31}P chemical shift data for the enzyme-bound substrate have given direct evidence for diamagnetic Zn(II)-phosphate interaction, and nuclear relaxation study of Na^+- K^+ A T Pase indicated that Mn(II) coordinated to the phosphate in the active site(13,14,15). However, up to the present, no direct Mn(III)-phosphate interaction in active site has been observed because of rarity of Mn(III)-containing enzyme.

In order to clarify the reaction mechanism for hydrolysis of phosphomonoesters by the present enzyme, we applied ^{17}O NMR spectroscopy. Figure 2

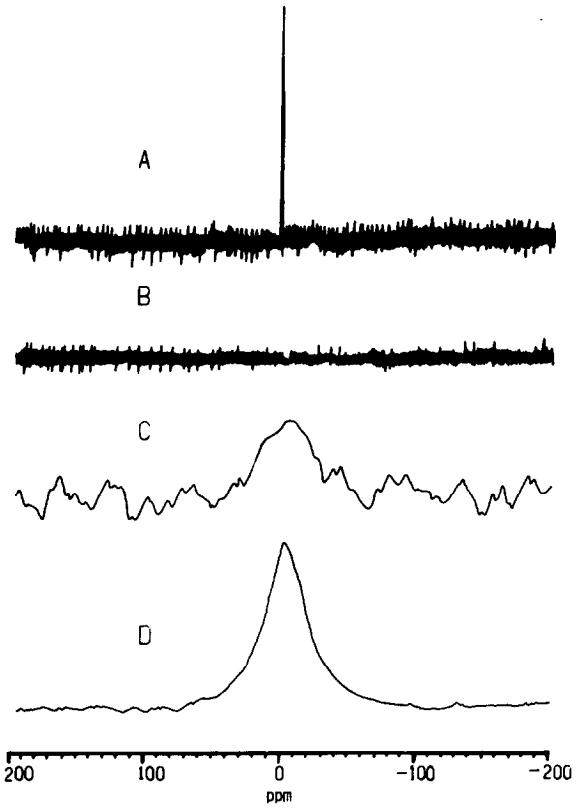

Figure 1 ^{31}P NMR spectra of Mn(III)-containing acid phosphatase in 0.1 M Tris-HCl buffer(pH 7.0) containing 0.1 M NaCl
(A), 1.4 mM phosphoric acid only.
(B), 1.4 mM native enzyme only.
(C), 1.4 mM native enzyme plus 1.4 mM phosphoric acid.
(D), 10 mM Mn(III)-pyrophosphate complex.

shows ^{17}O NMR spectrum of 85 % phosphoric acid(H_3PO_4) which gave the broade signal at 80-90 ppm from standard $H_2{}^{17}O$. When p-nitrophenylphosphate(0.4 M) was hydrolyzed by the acid phosphatase(0.002 mM) in 0.1 M acetate buffer (pH 5.8) containing 20 % enriched $H_2{}^{17}O$ for 60 min, this enzyme reaction sample exhibited a positive ^{17}O signal though broad near 90 ppm, together with the $H_2{}^{17}O$ peak. Taking into account these chemical shifts, the ^{17}O peak near 90 ppm is considered to arise from the ^{17}O-enriched phosphate produced by the enzymatic reaction. There is no possibility that the observed ^{17}O signal is due to natural abundance of ^{17}O phosphate in substrate, under this experimental condition. Therefore, the present ^{17}O NMR evidence for oxygen Mn(III)-containing acid phosphatase catalyzes an apparent transition state displacement and P-O cleavage as follows:

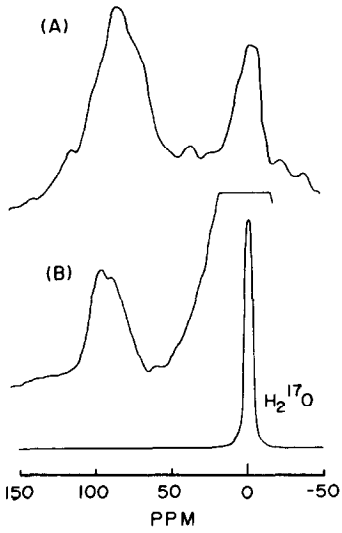

Figure 2 ^{17}O NMR spectra of Mn(III)-containing acid phosphatase at pH 5.8 and 60°C
(A), 85 % phosphoric acid only, 2471 of accumulations.
(B), The reaction mixture of the Mn-enzyme and p-nitrophenylphosphate, 6000 of accumulations.

Similar P-O cleavage has also occured during the nonenzymatic acid catalyzed hydrolysis of phosphomonoesters(16,17). In enzyme reaction, studies of oxidative phosphorylation were established by Boyer and Bryan(18). For alkaline phosphatase, Na^+- K^+ A T Pase and prosta acid phosphatase, the similar ^{18}O-exchange reaction from water has been demonstrated by mass spectrometry with fine and skillful technique(19,20,21). This simple ^{17}O method will also contribute to the many biochemists for their study of reaction mechanism.

In conclusion, the present ^{31}P and ^{17}O NMR results provide evidences for direct metal-phosphate interaction and for oxygen exchange from water into inorganic phosphate in Mn(III)-containing acid phosphatase.

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REFERENCES

1. Sudmeier,J.L., and Bell,S.J. J.Am.Chem.Soc. 1977, 99, 4499-4500.

2. Bobsein,B.R., and Myers,R.J. J.Am.Chem.Soc. 1980, 102, 2454-2455.
3. Mildvan,A.S., Granot,J., Smith,G.M., and Liebman,M.N. "Methods for determining Metal Ion Environment in Proteins" Elsevier: North-Holland, 1980, pp. 211-236.
4. Sugiura,Y., Kawabe,H., Tanaka,H., Fujimoto,S., and Ohara,A. J.Biol.Chem. 1981, 256, in press.
5. Sugiura,Y., Kawabe,H., and Tanaka,H. J.Am.Chem.Soc. 1980, 102, 6581-6582.
6. Sugiura,Y., Kawabe,H., Tanaka,H., Fujimoto,S., and Ohara,A. J.Am.Chem.Soc. 1981, 103, 963-964.
7. "A Comprehensive Treasite on Inorganic and Theoretical Chemistry" Mellor,J.D., Ed.; Longmans Green & Co: London, 1932, Vol. 13, pp. 447-464.
8. Wien,R.W., Morrisett,J.D., and McCornell,H.M. Biochemistry 1972, 11. 3707-3716.
9. Wertz,J.E., and Bolton,J.R. "Electron Spin Resonance. Elementary Theory and Practical Application" McGraw-Hill: New York, 1972, pp. 38-48.
10. Fee,J.A., Shapiro,E.R., and Moss,T.H. J.Biol.Chem. 1976, 251, 6157-6159.
11. Chawla,I.D., and Frank,M.J. J.Inorg.Nucl.Chem. 1970, 32, 555-563.
12. Sim,P.G., and Sinn,E. J.Am.Chem.Soc. 1981, 103, 241-243.
13. Chiebowski,J.F., Armitage,I.M., Tusa,P.P., and Coleman,J.E. J.Biol.Chem. 1976, 251, 1207-1216.
14. Otvos,J.F., Alger,J.R., Coleman,J.E., and Armitage,I.M., J.Biol.Chem. 1979, 254, 1778-1780.
15. Grisham,C.M., and Mildvan,A.S. J.Biol.Chem. 1974, 249, 3187-3197.
16. Butcher,W.W., and Westheimer,F.H. J.Am.Chem.Soc. 1955, 77, 2420-2424.
17. Eargle,D.H., Licko,V., and Kenyon,G.L. Anal.Biochem. 1977, 81, 186-195.
18. Boyer,P.D., and Bryan,D.M. "Methods of Enzymology" Estabrock,P.W., and Pullman,M.E., Ed.: Academic press: New York, 1967, Vol. 10, pp. 60-71.
19. Dahms,A.S., Kanazawa,T., and Boyer,P.D. J.Biol.Chem. 1973, 248, 6592-6595.
20. Midelfort,G.F., and Rose,I.A. J.Biol.Chem. 1976, 251, 5881-5887.
21. VanEtten,R.L. and Risly,J.M. Proc.Natl.Acad.Sci. 1978, 75, 4784-4787.