BMB
reports

Effect of metal ions on the secondary structure and activity of calf intestine phosphatase

*Fengjuan Chen, Guoqi Liu, Zhihong Xu & Zhengzhi Zeng**

College of Chemistry and Chemical Engineering and State Key Laboratory of Applied Organic Chemistry, Lanzhou University, Lanzhou 730000, P. R. China.

Cobalt is an essential microelements in many biological processes involving enzymatic activity. We found that Zn^{2+} and Mg^{2+}, which are in the active site of native calf intestine alkaline phosphatase (CIP), can be replaced by Co^{2+} directly in solution. The effect of Co^{2+} concentration on the substitution reaction was examined at ratios of [Co^{2+}]/[CIP] from 0:1 to 8:1. The quantity of Zn^{2+} in CIP decreased progressively as the ratio was increased, but the amount of Mg^{2+} changed in irregular fashion. A series of active site models of the reaction mechanism of CIP are proposed. Low pH was found to promote the replacement of Mg^{2+} by Co^{2+}. To understand how the substitution affects the enzyme, we also solved the secondary structure of CIP after reaction with Co^{2+} in different conditions. [BMB reports 2008; 41(4): 305-309]

INTRODUCTION

Alkaline phosphatase (EC3.1.3.1) is a nonspecific phosphomonoesterase that hydrolyzes phosphomonoesters as well as the phosphate termini of DNA (1). It is characterized by a high pH optimum and broad substrate specificity (2, 3). It exists in organisms of all kinds and plays a vital role in survival under phosphate starvation (4, 5, 6). The X-ray crystal structure of bacterial alkaline phosphatase to 2.0 Å resolution in the presenve of inorganic phosphate has been reported (7). The active site is a tight cluster of two zinc ions (3.9 Å separation) and one magnesium ion (5 Å and 7 Å from the two zinc ions). Recently the x-ray structure of human alkaline phosphatase has also been determined (8, 9).

Calf intestine alkaline phosphatase (CIP) is also a dimeric metalloenzyme containing Zn^{2+} and Mg^{2+}, with a molecular weight of 138000 including 12% carbohydrate (10). Substitution of the metal ions of a metalloenzyme is one of the mildest and most specific procedures presently available to chemically modify an enzyme. Cobalt is an essential component of many biological processes. Moreover, Co^{2+} is the only metal which substitutes for zinc in alkaline phosphatase to yield significant enzymatic activity when measured by steady-state kinetics (11). Some authors have used Co^{2+} to substitute for the native metal ions of alkaline phosphatase (12, 13), starting from metal-free enzyme. Our research focuses on the substitution of cobalt ions for metal ions starting from native CIP. To explore the conformational changes of CIP during the course of substitution, we employed circular dichroism (CD), a well-known spectroscopic technique for obtaining information on the secondary structure of proteins (14,15), together with methods (SELCON3, CONTIN, CDSSTR) for estimating protein secondary structures from CD spectra (16, 17, 18, 19). The assignments obtained from these methods gave six types of secondary structures: regular α-helix, α_R; distorted α-helix, α_D; regular β-strand, β_R; distorted β-strand, β_D; turns, T; and unordered, U. The results suggest that the metal ions in the active site influence the secondary structure and the activity of the enzyme.

*Corresponding author. Tel: 86-931-8912582; Fax: 86-931-8912582; E-mail: zengzhzh@yahoo.com.cn

Received 8 November 2007, Accepted 18 December 2007

Keywords: Activity, Calf intestine alkaline phosphatase, Cobalt ions, Secondary structure

RESULTS AND DISCUSSION

Substitution of the metal ions in CIP

Co^{2+} can replace the metal ions in native CIP directly in solution. Table 1 shows that the native enzyme contains 4.5 g-atoms of zinc and 2.6 g-atoms of magnesium. After reacting with cobalt chloride, the quantity of Zn^{2+} decreased gradually with increasing molar ratio of Co^{2+} to CIP, and Mg^{2+} reached a minimum when the molar ratio was 2:1, then rebound to 1.9g and 1.7 g when the molar ratio increased to 4:1 and 8:1, resepctively. Since the ionic radii of Co^{2+} and Zn^{2+} are similar and these ions share the capacity to accept distorted geometries (20), and the coordinating ability of Co^{2+} is also greater than that of Zn^{2+} ions, Co^{2+} can gradually replace Zn^{2+} with increasing concentrations of Co^{2+}.

We propose a series of active site models to account for how Mg^{2+} content changes in the CIP (Fig. 1). When the molar ratio increased from 0:1 to 2:1, Mg^{2+} and Zn^{2+} were displaced by 63.1% and 53.3% respectively (Table 1). The active site of CIP changed from model 1 to models 2 and 3, with model 2 the dominant form. When the molar ratio reached 4:1, Zn^{2+} was displaced by 60%, Mg^{2+} by 26.9%, and models 4 and 5 existed

Table 1. Changes of the metal ions in CIP after reaction with cobalt chloride in different conditions ([Co(II)]/[CIP] varied from 0:1 to 8:1; pH varied from 6.5 to 8.9 at [Co(II)]/[CIP] of 4:1), and enzyme activity of the metal ions were replaced by Co^{2+} . (the samples were dialyzed against 10 mM Tris-HCl (pH 8.9) to eliminate un-bound metal ions)

pH	Co(II) : CIP (molar ratio)	Metal quantity / g·molar^{-1}			$\triangle Zn^{2+}$ / %	$\triangle Mg^{2+}$ / %	Enzyme activity (%native)
		Zn	Mg	Co			
8.9	0 : 1	4.5	2.6	0	0	0	100
8.9	1 : 1	3.6	1.4	1.2	20	46.2	43.4
8.9	2 : 1	2.1	0.96	2.5	53.3	63.1	41.5
8.9	4 : 1	1.8	1.9	2.7	60	26.9	28.1
8.9	8 : 1	0	1.7	4.8	100	34.6	24.1
8.0	4 : 1	2.5	1.7	2.9	44.4	34.6	83.0
6.5	4 : 1	2.8	1.4	2.5	46.2	37.0	42.2
8.0	0 : 1	4.5	2.6	0	0	0	87.5
6.5	0 : 1	4.5	2.6	0	0	0	11.2

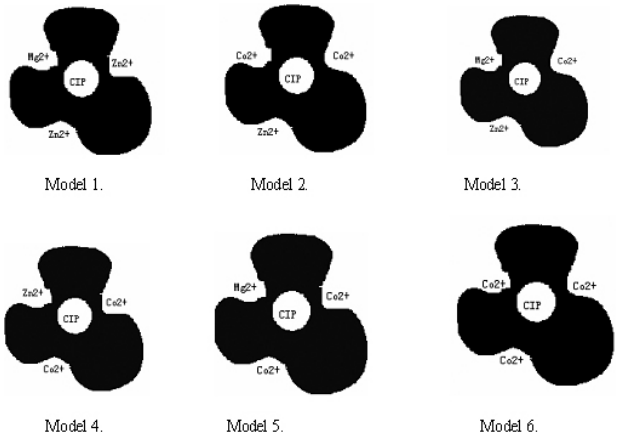

Fig. 1. Active site models of CIP after incubation with different concentrations of Co^{2+} (at a molar ratio of 4:1)

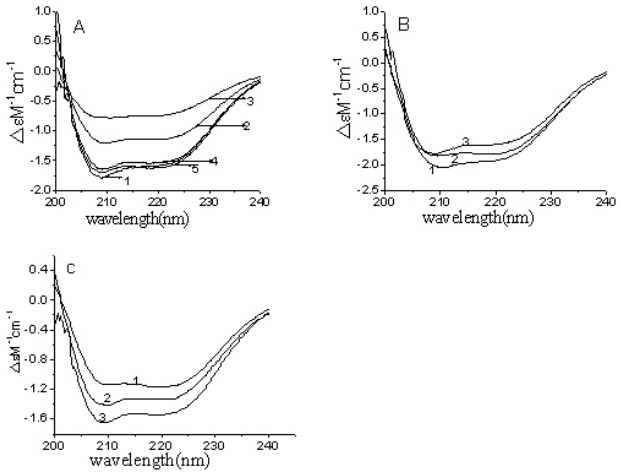

Fig. 2. The CD spectra were taken at 25°C. The final concentration of enzyme was 0.95 mg/ml ($\triangle\varepsilon M^{-1}$ cm^{-1} is given per residue molar absorbance unit), after the active site ions were replaced at different concentrations of Co^{2+}. (A) The molar ratios of [Co^{2+}]/[CIP] were 0:1, 1:1, 2:1, 4:1 and 8:1 (curves 1 to 5). (B) pH effect on CD spectra of native CIP solution at pH 6.5 to 8.9 (curves 1 to 3). (C) in a solution of constant Co^{2+} concentration (4:1) at pHs from 6.5 to 8.9 (curves 1 to 3)

in equilibriom with the former dominant so that the content of Mg^{2+} in the CIP increased. At 8:1, models 5 and 6 appeared together; at this point Co^{2+} was in great excess and could replace the Mg^{2+}, so that model 6 was the dominant form, and the Mg^{2+} content of the enzyme was lower than at 4:1.

The effects of pH on the substitution reaction are shown in Table 1. It can be seen that low pH favors the replaceent of Mg^{2+} by Co^{2+}, but that the Zn^{2+} content increases as the pH decreases. This difference may result from conformational changes of CIP in solution at different pHs.

Structure studies

Fig. 2A presents the changes of CD spectra. The native CIP solution gives double negative peaks, and the peak at 208 nm is higher than the peak at 222nm. The intensity and shape of the CD spectra changed when the Zn^{2+} and Mg^{2+} in CIP were replaced by Co^{2+}. The average fractions of secondary structure estimated from the CD data are given in Table 2. The regular α-helix content was 7.2% and the distorted α-helix content was 10.3% in the native enzyme, while the two helical structures were reduced to 0.5% and 4.9%, respectively, when the molar ratio reached 2:1. There was also a substantial increase in the two kinds of β-strand structures as the molar ratio increased from 0:1 to 2:1, which indicates that the β-strand conformation may be more stable than the α-helix when the Mg^{2+} in CIP decreases. At molar ratio 4:1, the Mg^{2+} in CIP increased compared with 2:1 and the content of regular α-helix was similar to that of native CIP. When the molar ratio increased to 8:1, the metal ions in CIP consisted almost entirely of Co^{2+} and Mg^{2+}, and the two helical structures and regular β-strand

Table 2. Average estimates of the Secondary Structure Fractions of CIP Solutions obtained with the CDPro Software Package after reaction with cobalt chloride in different conditions

pH	Co^{2+} : CIP Molar ratio	Fraction of secondary structure, %					
		α_R	α_D	β_R	β_D	T	U
8.9	0:1	7.2	10.3	19.3	10.5	20.5	32.2
8.9	1:1	4.2	9.7	30.8	14.0	18.1	23.2
8.9	2:1	0.5	4.9	27.3	12.8	21.9	32.6
8.9	4:1	7.1	8.8	15.9	9.3	22.6	36.3
8.9	8:1	8.6	10.4	23.3	10.2	20.4	27.0
8.0	4:1	5.8	8.2	21.8	11.6	19.7	32.9
6.5	4:1	2.8	3.9	22.4	12.9	23.1	35.0
8.0	0:1	7.7	10.9	18.7	10.4	22.5	29.8
6.5	0:1	11.0	11.8	17.7	10.0	22.9	27.4

increased to 8.6%, 10.4% and 23.3%, respectively, because Co-CIP and Zn-CIP have different conformations.

Fig. 2B shows the pH-dependent changes of the secondary structure of native CIP in solution, and Table 2 shows that the content of regular α and distorted α-helix declined when pH increased from 6.5 to 8.9, while the two kinds of β-strand increased. Fig. 2C illustrates the significant change in the CD spectra of CIP when incubated with Co^{2+} at different pHs. Table 2 also reveals that the percentage of regular and distorted α-helical structure decreased from 7.1% and 8.8% to 2.8% and 3.9%, resepctively, while the two kinds of β-strand increased. This is because the Mg^{2+} in the active site of CIP plays a significant role in maintaining the structure of the enzyme. A decrease of Mg^{2+} causes changes in the micro-environment that disrupt the conformation of CIP.

Studies on *Escherichia coli* alkaline phosphatase (ECAP) have shown the importance of the divalent metal ions: Mg^{2+} does not directly participate in the mechanism but is important for stabilization of the enzyme, whereas the two Zn^{2+} ions are directly involved in catalysis (21, 22). The Mg^{2+} can affect the structure and function of molecular chromophores (23) as well as of many enzymes (24, 25). Mg^{2+} plays an important role as a site-specific effector since it binds to the non-native structure, probably to the active site region, stabilizing transient conformations and guiding conformational reconstitution into productive pathways (26).

Changes of the activity of CIP

The changes of the activity of CIP are shown in Table 1. Activity gradually decreased due to the changes of Zn^{2+}. The alkaline phosphatase reaction involves an attack of a serine alkoxide on the phosphorus of the substrate to form a transient covalent enzyme-phosphate complex followed by hydrolysis of the serine phosphate (27). When Co^{2+} replaces Zn^{2+} and Mg^{2+}, it forms a very strong coordinate bond with Ser-102, so the activity of the enzyme decreases (28). When the pH was decreased from 8.0 and 6.5, and the molar ratio maintained at 4:1, activity declined to minimum of 8.3%. This suggests that CIP was denatured at low pH and could not be renatured completely at high pH (29). Activity at pH 8.0 was higher than at pH 8.9. The change of the enzyme activity was related to the content of Zn^{2+}, which implies that Zn^{2+} plays an important role in CIP activity.

MATERIALS AND METHODS

Materials

CIP was obtained from TCI (Tokyo, Japan). The specific activity of the purified enzyme was 3.0 u/mg. *p*-Nitrophenyl phosphate (*p*NPP) was from Promega. All other chemicals were local products of analytical grade.

Protein concentration

Enzyme concentration was determined using an absorption coefficient $A^{1\%}_{278}$ of 7.60 (10).

CIP activity assay

Enzyme activity was determined at 25°C by following the increase of absorbence at 402 nm per minute accompanying the hydrolysis of substrate (*p*NPP) with a molar absorption coefficient of 1.73×10^4 $M^{-1}cm^{-1}$. The reaction was initiated by addition of the enzyme and stopped by addition of 0.5 ml of 0.5M NaOH.

Reaction of CIP with cobalt chloride

We used doubly distilled water in all the experiments, and the enzyme concentration was 5.7 mg/ml. The molar ratios of Co^{2+} to CIP were 0:1, 1:1, 2:1, 4:1, 8:1. Measurements of the effect of pH on the substituent reaction were done in 50 mM Tris-HCl buffer, at a molar ratio of Co^{2+} to CIP of 4:1. The pH titration was started at pH 8.9, following which the solution was titrated to pH 8.0 and pH 6.5 by the addition of 0.1 M HCl. During the titration, pH was monitored with a Pinnacle 545 pH meter.

All samples were incubated at 4°C for a week, then dialyzed against 10 mM Tris-HCl (pH 8.9) to eliminate un-bound metal ions. The final enzyme concentration was 0.95 mg/ml; this was

used to determine the metal ion content of CIP, circular dichroism (CD) spectra and enzyme activity.

Physical measurements

The Inductively Coupled Plasma (ICP) was recorded with a Thermo Jarrell ASH Corporation IRIS Advantage, and the content of metal ions per mole of CIP was calculated from a molecular weight of CIP of 138000. The secondary structure of CIP was determined with a Jasco J-810 spectropolarimeter fitted with a 150-W xenon lamp. Quartz cells of 0.2-mm path length were used for all CD measurements, and the spectra were recorded in the far-UV region (200-240 nm) with a response time of 8 s and scan speed of 50 nm/min. Three scans were accumulated and averaged for each spectrum after the background of the buffer solution or blank quartz slide was subtracted. In order to obtain the secondary structure fractions of CIP, the CDPro. software package was used; this consists of the three programs, SELCON3, CONTIN, and CDSSTR, and a program for determining tertiary structure class (CLUSTER). One of the major advantages of the CDPro software package is that the programs have been modified to accept any given set of reference proteins (CD spectra and secondary structure fractions), and seven such reference sets are provided. Moreover, input data files for these three programs are identical. More information about CDPro is available at the following website: http://lamar.colostate.edu/sreeram/CDPro. The activity analysis was performed with a Varian Cary 100 UV-Vis spectrophotometer. All of these spectral analyses were completed at a room temperature of 25^{o}C.

Acknowledgements

The present investigation was supported by a grant from the National Natural Science Foundation of China (NO. 20171019).

REFERENCES

1. Coleman, J. E. and Gettins, P. (1983a) Molercular properties and mechanism of alkaline phosphatase: In Metal Ions in Biology. Spiro TG (ed) John Wiley and sons, New York, pp 153-217
2. Coleman, J. E., Nakamura, I..and Chlebowski, J. F. (1983) 65ZnII, 115mCdII, 60CoII, and MgII binding to alkaline phosphatase of Escherichia coli. Structural and functional effects. *J. Biol. Chem.* **258**, 386-395
3. Fernely, H. N (1971) Mammalian alkaline phosphatase in enzymes. Boyer PD (ed) Academic Press, New York, pp 417-447
4. Prada, P. D., Loveland-Curtze, J. and Brenchley, J. E. (1996) Production of two extracellular alkaline phosphatases by a psychrophilic arthrobacter strain. *Appl. Envir. Microbiol.* **62**, 3732-3738
5. Zappa, S., Rolland, J., Flament, L. D., Gueguen, Y., Boudrant, J. and Dietrich, J. (2001) Characterization of a highly thermostable alkaline phosphatase from the euryarchaeon pyrococcus abyssi. *Appl. Envir. Microbiol.* **67**, 4504-4511
6. Ayantika, G.,and Parames, C. S. (2007) Anti-oxidative effect of a protein from cajanus indicus L against acetaminophen-induced hepato-nephro toxicity. *J. Biochem. Mol. Biol.* **40**, 1039-1049
7. Kim, E. E., and Wycko, H. W. (1991) Reaction mechanism of alkaline phosphatase based on crystal structures: two-metal ion catalysis. *J. Mol. Biol.* **218**, 449-464
8. Llinas, P., Masella, M., Stigbrand, T., Ménez, A., Stura, E. A. and Du, M.H.L. (2006) Structural studies of human alkaline phosphatase in complex with strontium: implication for its secondary effect in bones. *Protein Sci.* **15**, 1691-1700
9. Llinas, P., Stura, E. A., Ménez, A., Kiss, Z., Stigbrand, T., Millan, J. L. and Du, M.H.L. (2005) Structural studies of human placental alkaline phosphatase in complex with functional ligands. *J. Mol. Biol.* **350**, 441-451
10. Fosset, M., Chappelet-Tordo, D. and Lazdunski, M. (1974) Intestinal alkaline phosphatase physical properties and quaternary structure. *Biochemistry* **13**, 1783-1787
11. Plock, D. J. and Vallee, B. L. (1962) Interaction of alkaline phosphatase of E. coli with metal ions and chelating agents. *Biochemistry* **1**, 1039-1043
12. Ciancaglini, P., Pizauro, J. M., Leone, F. A. (1997) Dependence of divalent metal ions on phosphotransferase activity of osseous plate alkaline phosphatase. *J. Inorg. Biochem.* **66**, 1-55
13. Richard, A., Anderson, R. A. and Vallee B. L. (1977) Selective cobalt oxidation as a means to differentiate metal-binding sites of cobalt alkaline phosphatase. *Biochemistry* **16**, 4388-4393
14. Mostafa, R. T., Seyed, H. M. and Bijan, R.(2006) Conformational study of human serum albumin in pre-denaturation temperatures by differential scanning calorimetry, circular dichroism and UV spectroscopy. *J. Biochem. Mol. Biol.* **39**, 530-536
15. Zahra, S., Saman, H., Bijan, R., and Mohsen, N. G. (2006) Interaction of native and apo-carbonic nnhydrase with hydrophobic adsorbents: a comparative structure- function study. *J. Biochem. Mol. Biol.* **39**, 636-641
16. Sreerama, N. and Woody, R. W. (2000) Estimation of protein secondary structure from circular dichroism spectra: comparison of CONTIN, SELCON, and CDSSTR methods with an expanded reference set. *Anal. Biochem.* **287**, 252-260
17. Greenfield, N. J. (1995) Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. *Anal. Biochem.* **235**, 1-10
18. Sreerama, N. and Woody, R. W. (1993) A Self-Consistent method for the analysis of protein secondary structure from circular dichroism. *Anal. Biochem.* **209**, 32-44
19. Zheng, J. Y. Celeste, A. C., Vipin, K. R., Cheng, T. C., Joseph, J. D., and Roger, M. L.(2004) Secondary structure of organophosphorus hydrolase in solution and in Langmuir-Blodgett film studied by circular dichroism spectroscopy. *J. Phys. Chem. B.* **108**, 17238-17242
20. Valle, B. L. and Williams, R. J. P. (1968) Metalloenzymes: the entatic nature of their active sites. *Pro. Natl. Acad. Sci. U.S.A.* **59**, 498-505
21. Simpson, R. T., Vallee, B. L. and Tait, G. H. (1968) Alkaline phosphatase of escherichia coli: composition. *Biochemistry* **7**, 4336-4342
22. Anderson, R., Bosron, W., Kennedy, F. and Vallee ,B.

(1975) Role of magnesium in escherichia coli alkaline phosphatase. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2989-2993
23. Azem, A., Diamant, S. and Goloubinoff, P. (1994) Effect of divalent cations on the molecular structure of the GroEL oligome. *Biochemistry* **33**, 6671-6675
24. Jakob, U., Meyer, I., Bugl, H., Andre, S., Bardwel, J. C. A. and Buchner, J. (1995) Structural organization of procaryotic and eucaryotic Hsp90. *J. Biol. Chem.* **270**, 14412-14419
25. Sun, G. and Budde, R. J. A. (1997) Requirement for an additional divalent metal cation to activate protein tyrosine kinases. *Biochemistry* **36**, 2139-2146
26. Subramaniam, V., Bergenhem, N.C., Gafni, A. and Steel, D. G. (1995) Phosphorescence reveals a continued slow annealing of the protein core following reactivation of escherichia coli alkaline phosphatase. *Biochemistry* **34**, 1133-1136
27. Trentham, D. R. and Gutfreund, H. (1968) The kinetics of the reaction of nitrophenyl phosphates with alkaline phosphatase from Escherichia coli. *Biochem. J.* **106**, 455-460
28. Wang, J., Stieglitz, K. A. and Kantrowitz, E. R. (2005) Metal specificity is correlated with two crucial active site residues in escherichia coli alkaline phosphatase. *Biochemistry* **44**, 8378-8386
29. Sailendra, N. S. and Nandini, G. (1996) Reversible unfolding of eschericha coli alkaline phosphatase: activity site can be reconsitituted by a number of pathways. *Arch. Biochem. Biophys.* **330**, 174-180