The recombinant catalytic subunit of protein phosphatase 1 is produced as an inactive enzyme which can be activated by Mn$^{2+}$ (Zhang, Z., Bai, G., Deans-Zirattu, S., Browner, M. F., and Lee, E. Y. C. (1992) J. Biol. Chem. 267, 1484–1490). In this report, we have investigated the effects of divalent cations on the activity of recombinant catalytic subunit of protein phosphatase 1. Latent phosphatase 1 can be activated by Co$^{2+}$ or Mn$^{2+}$, whereas other metal ions tested including Fe$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, or Ni$^{2+}$ were not effective or were only weakly effective in activating the enzyme. The Mn$^{2+}$-stimulated activity was susceptible to inactivation by EDTA; however, the Co$^{2+}$-activated phosphatase was stable after dilution and chelation of the Co$^{2+}$ with excess EDTA. After stable activation of phosphatase 1 using $^{57}$Co$^{2+}$, a stoichiometric amount of $^{57}$Co$^{2+}$ was shown to be tightly bound to phosphatase 1. These findings demonstrate for the first time the generation of a stable metalloenzyme form of phosphatase 1. Fe$^{2+}$ reversibly deactivated the Co$^{2+}$-stimulated activity, but did not displace the bound Co$^{2+}$. Interestingly, treatment of the enzyme with a combination of Fe$^{2+}$ and Zn$^{2+}$ (but not the individual metal ions) significantly activated phosphatase 1. These results suggest that at least two metal binding sites exist on the enzyme and that protein phosphatase 1 may be an iron/zinc metalloprotein in vivo.

Protein phosphatase 1 (phosphorylase phosphatase), one of the four major Ser/Thr protein phosphatases, has been studied mainly in relation to its central role in the regulation of glyco-
gen metabolism (for reviews, see Bollen and Stalmans (1992), Shenolikar and Nairn (1991), and Shenolikar (1994)). The en-
yzymology of the enzyme is complex and involves multiple forms of the enzyme generated by combinations of a 37-kDa catalytic subunit (PP1) with different regulatory proteins that may also provide for molecular targeting of the enzyme. Several regulatory subunits have been well characterized, including inhibitor 2, the glycogen binding subunit, a nuclear inhibitory subunit, and myosin binding subunits (Bollen and Stalmans, 1992; Shimizu et al., 1994; Chen et al., 1994). Most of the previous studies of the isolated catalytic subunit have been of an active enzyme that is independent of metal ions for its activity. How-
ever, it has been clear for a number of years that there exists a metal ion dependent form or forms of PP1. In the ATP/Mg-de-
pendent enzyme, which is a 1:1 complex of PP1 with inhibitor 2, PP1 is present as an inactive or latent enzyme that is reversibly stimulated by Mn$^{2+}$ (Villa-Moruzzi et al., 1984). All recombinant forms of PP1 expressed in Escherichia coli, including the four known isofoms, are dependent on Mn$^{2+}$ for activity (Zhang et al., 1992, 1993a; Alessi et al., 1993). Zhang et al. (1993b) have suggested that the recombinant enzyme repre-
sents the conformer that is present in the PP1-inhibitor 2 complex. We have recently isolated a form of PP1 catalytic subunit from cardiac muscle which is inactive, but can be converted to a stable active form by exposure to Co$^{2+}$ (Chu et al., 1994).

Thus, there are complex and not completely understood facets of the nature of the differences between these forms of the PP1 catalytic subunit, which are revealed by the effects of divalent cations on its activity. The question of how metals affect phosphorylase phosphatase is an old one. It has long been known that divalent cations, in particular Mn$^{2+}$ and Co$^{2+}$, can activate certain phosphorylase phosphatase prepa-
rations (Merlevede and Riley, 1966; Kato and Bishop, 1972; Kato et al., 1975; Ullman and Perlman, 1975; Khatri and Soderling, 1978; Khandelwal and Kasmani, 1980; Braunig et al., 1980, 1982). It has been suggested that the phosphatase present in these preparations is a metalloenzyme (Burchell and Cohen, 1978; Hsiao et al., 1978; Khatri and Soderling, 1978; Defrey et al., 1979; Mackenzie et al., 1980). However, attempts to demonstrate the presence of bound metal in enzyme preparations have been negative. Metal analysis of a prepara-
tion of liver PP1 by atomic absorption showed only small sub-
stoichiometric amounts of Ca$^{2+}$, Cd$^{2+}$, Co$^{2+}$, Cu$^{2+}$, Fe$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Sn$^{2+}$, or Zn$^{2+}$ (Yan and Graves, 1982). Moreover, activation by $^{54}$Mn$^{2+}$ of the catalytic subunit of ATP/Mg$^{2+}$-de-
pendent protein phosphatase (Villa-Moruzzi et al., 1984) or a preparation containing a high molecular weight form of PP1 (Braunig et al., 1980) did not show significant incorporation of $^{54}$Mn$^{2+}$ into the enzyme.

Using recombinant PP1, we have explored the issue of whether it may exist as a metalloprotein. In this study, we demonstrate that the activation of PP1 by $^{57}$Co$^{2+}$ is associated with a stoichiometric incorporation of Co$^{2+}$ into the enzyme. We also report that PP1 is activated by a combination of Fe$^{2+}$/Zn$^{2+}$ and we suggest that PP1 may be an iron/zinc metalloen-
yzme in vivo.

**EXPERIMENTAL PROCEDURES**

Materials—$^{57}$CoCl$_2$ was obtained from ICN. Superase-12 was from Pharmacia Biotech Inc. The recombinant PP1 used in these studies was the PP1a isoform expressed in E. coli as described by Zhang et al. (1992). $^{32}$PPhosphorylation was prepared as described previously (Killilea et al., 1978).

Preparation of Metal-depleted Recombinant PP1—Homogeneous re-
TABLE I
Effects of divalent cations on activation of PP1
The diluted PP1 was incubated with indicated concentrations of divalent cations in the presence of 50 mM MOPS, pH 7.0, 150 mM KCl, 0.5 mg of BSA/ml, 0.5 mM DTT at 30°C for 15 min. Five μl of treated enzyme were added to 45 μl of [32P]phosphorylase a (2.22 μM in 50 mM Mops, pH 7.4, 1.2 mg of theophylline/ml, 0.5 mg of BSA/ml, 1 mM DTT). After 10 min, the reaction was stopped by adding 60 μl of ice-cold 20% trichloroacetic acid, and released 32P was determined as previously described (Chu et al., 1994). The Mn2+-activated phosphatase activity was taken as 100%. Note: Fresh FeCl3 solution was used.

| Additions | Concentrations | Relative activity (%) |
|-----------|----------------|-----------------------|
| None      | —              | 7                     |
| MnCl2     | 1              | 100                   |
| CoCl2     | 1              | 36                    |
| FeCl2     | 1              | 9                     |
| ZnCl2     | 1              | 15                    |
| MgCl2     | 5              | 5                     |
| CaCl2     | 1              | 4                     |
| CuSO4     | 1              | 1                     |
| NiSO4     | 1              | 3                     |
| ZnCl2 + FeCl2 | 1 + 1     | 67                    |
| ZnCl2 + MgCl2 | 1 + 5     | 20                    |
| ZnCl2 + CaCl2 | 1 + 1     | 20                    |
| ZnCl2 + CuSO4 | 1 + 1    | 10                    |
| ZnCl2 + NiSO4 | 1 + 1     | 10                    |
| FeCl2 + MgCl2 | 1 + 5     | 3                     |
| FeCl2 + CaCl2 | 1 + 1     | 3                     |
| FeCl2 + CuSO4 | 1 + 1     | 0                     |
| MgCl2 + CaCl2 | 5 + 1     | 8                     |
| MgCl2 + CuSO4 | 5 + 1     | 0                     |
| CaCl2 + CuSO4 | 1 + 1     | 0                     |

The diluted PP1 stored in the presence of Mn2+ as described by Zhang et al. (1992) was treated with 5 mM EDTA, 100 mM KPO4, 1 mM KCl, 33% glycerol, pH 7.0, for 2 h at 4°C, and extensively dialyzed against 50 mM MOPS, pH 7.0, 500 mM KCl, 0.2 mM EDTA, and 50% glycerol, in order to remove Mn2+ and KPO4.

Assay for Phosphatase Activity—PP1 was preincubated with divalent cations as indicated. The preincubated enzyme was then assayed directly (without chelation of the cation) for phosphatase activity by measuring the release of [32P]Pi from [32P]phosphorylase a (Chu et al., 1994). One unit of phosphatase activity was defined as 1 μmol of [32P]Pi, released/min.

Determination of Co2+ Binding to PP1—Latent PP1 (10 μg) was incubated in 50 μl of 50 mM MOPS, pH 7.0, 500 mM KCl, 0.2 mM EDTA, 50% glycerol, and 1 mM 106CoCl2 (specific radioactivity, 600 dpm/μmol) for 15 min at 30°C. Then the sample was diluted 5-fold with 50 mM MOPS, pH 7.0, 2 mM EDTA, 0.5 mg of BSA/ml. The bound Co2+ was separated from unbound Co2+ on a Superose-12 gel filtration column equilibrated with 50 mM MOPS, pH 7.0, 150 mM KCl, 0.2 mM EDTA, 1 mM DTT, 10% glycerol at 4°C.

Protein Determinations—Protein concentration was determined by the method of Bradford (1976) using BSA as a standard.

RESULTS
Effects of Divalent Cations on Activation of PP1—After metal depletion with EDTA/P, (see “Experimental Procedures”) recombinant PP1 required divalent metal cations for activity (Table I). Of the cations examined individually, only Co2+ and Mn2+ were efficient in activating the enzyme. Treatment of PP1 with 1 mM Ca2+, Cu2+, Fe2+, Ni2+, or 5 mM Mg2+ did not activate the enzyme, while 1 mM Zn2+ had only a slightly stimulating effect (Table I). Similar results were obtained when metal-dependent PP1 purified from bovine heart myofibrils (Chu et al., 1994) was tested under the same conditions (data not shown). As shown in Table I, PP1 was activated to a greater extent by 1 mM Mn2+ than 1 mM Co2+ when the preincubation was carried out in the presence of 150 mM KCl. It is worth noting that Co2+ and Mn2+ were equally effective in the activation of PP1 when the preincubation was carried out in the presence of 500 mM KCl, indicating that Co2+-activation was more dependent on ionic strength than Mn2+ (data not shown). Very surprisingly, combined treatment of PP1 with Zn2+ and Fe2+ significantly activated the enzyme (Table I). Preincubation with other metal ion combinations had little or no effect on phosphatase activity. As shown in Fig. 1, maximal activation of the enzyme by Zn2+ in the presence of 0.1 or 1 mM Fe2+, or by Fe2+ in the presence of 0.1 or 1 mM Zn2+, was achieved at a metal concentration of 1 mM. The A528 was approximately 0.1 mM for either cation. Activation by the combination of Zn2+/Fe2+ did not appear to be sensitive to ionic strength (data not shown).

Effect of EDTA and P, on Co2+- or Mn2+-activated PP1—The reversibility of the activation of recombinant PP1 was investigated by studying the effects of EDTA and P, on Co2+- or Mn2+-activated PP1. More than 80% of the Mn2+-stimulated activity was reversed by chelation of the Mn2+ with 5 mM EDTA (Fig. 2A). It was noted that much of the Mn2+-stimulated activity was lost simply by dilution of the Mn2+ (data not shown). Mn2+-activated PP1 was totally inactivated within 5 min by 100 mM P,. Thus, the effect of Mn2+ on PP1 was properly characterized as a stimulation of the enzyme. Activation by Fe2+/Zn2+ was also reversed by chelation of the metal ions (data not shown). Co2+-activated PP1 was resistant to EDTA or P, treatment (Fig. 2B). Incubation at 30°C for up to 2 h with 5 mM EDTA did not reverse Co2+ activation. Inorganic phosphate (100 mM) or a combination of 100 mM P, and 5 mM EDTA only partially reversed the Co2+-activated PP1 even after 2 h incubation. These results indicate the effect of Co2+ on PP1 is an activation, as we previously observed with the latent cardiac PP1 (Chu et al., 1994).

Evidence for a Stable Co2+-PP1 Complex—Although Co2+-activated PP1 is stable, it is not clear whether the Co2+ activation involved formation of a stable Co2+-PP1 complex or a transient binding of Co2+ which results in the induction of a stable active conformation. Therefore, we examined the Co2+-binding properties of PP1 by direct binding studies using 57Co2+. For analysis of Co2+ binding, the sample of recombinant PP1 was depleted of endogenous Mn2+ by EDTA/P, treatment (see “Experimental Procedures”). The enzyme was incubated with 57CoCl2 and passed through a Superose-12 gel filtration column to separate free and bound Co2+. After chromatography, PP1 was fully activated, and 57Co2+ was associated with the enzyme (Fig. 3). The fact that the bound 57Co2+ was not removed by chromatography in the presence of EDTA indicates the formation of a stable metalloenzyme complex. This is supported by a calculated stoichiometry of 0.93 mol of 57Co2+ bound/mol of PP1 for the fractions in which the enzyme activity and radioactivity co-eluted. These results imply that Co2+ is incorporated into PP1 during the enzyme activation
and PP1 contains one stable Co²⁺-binding site. Effect of Fe²⁺ on Co²⁺-activated PP1—Fe²⁺ itself had very little effect on activation of PP1. However, as shown in the insert of Fig. 4, Fe²⁺ could inactivate the Co²⁺-activated PP1. The possibility that displacement of ⁵⁷Co²⁺ by Fe²⁺ is responsible for the enzyme inactivation was examined by determining the ⁵⁷Co²⁺ content of the enzyme before and after treatment with Fe²⁺. From Fig. 4, it can be seen that ⁵⁷Co²⁺ remained bound, even though phosphatase activity was almost completely lost. The enzyme activity was fully recovered when the Fe²⁺ was chelated by EDTA indicating that the Fe²⁺ effect was reversible (data not shown). The fact that ⁵⁷Co²⁺ was not displaced by Fe²⁺ after the enzyme inactivation suggested that Co²⁺ and Fe²⁺ do not share a common binding site.

DISCUSSION
As noted in the Introduction, the issue of whether the catalytic subunit of protein phosphatase 1 is a metalloenzyme is an old issue that has not been satisfactorily resolved to date, although the effects of Mn²⁺ on the enzyme activity have been well documented (see Bollen and Stalmans, 1992 for review). Attempts to show the binding of Mn²⁺ to PP1 have been negative (Brautigan et al., 1980; Villa-Moruzzi et al., 1984). Our data provide an explanation for this in that Mn²⁺ binding is reversible, whereas Co²⁺ binding results in the formation of a stable metalloprotein complex. Thus, the use of Co²⁺ rather than Mn²⁺ has proven in this study to be more revealing. The data presented here provides the first direct evidence that PP1 is a metalloenzyme by demonstration of a stable 1:1 complex of enzyme and Co²⁺. Previously, direct metal analysis of liver PP1 preparations showed the presence of only substoichiometric amounts of metal (Yan and Graves, 1982). While the formation of a cobalt metalloenzyme form is probably not physiological (see below), the results establish that PP1 has the ability to bind a metal ion in a stable and stoichiometric manner. Our studies show that cobalt ion will be a useful tool for the study of the role of metal ions on PP1 activity.

Although Co²⁺ can bind to and activate PP1 in vitro, it seems unlikely that PP1 is a Co²⁺-bound protein in vivo, because the concentration of Co²⁺ in tissues is less than 1 μM (Iyengar and Woitzez, 1988). The observations that PP1 purified from rabbit skeletal muscle is susceptible to P inhibition² and that Co²⁺-activated PP1 is relatively resistant to P inhibition (see “Re-

² L. Cai and K. K. Schlender, unpublished results.
While this manuscript was under review, the crystal results also do not support a role for Co$^{2+}$. The fact that the amount of Mn$^{2+}$ in skeletal muscle ranges from 1 to 2 $\mu$M (Versieck, 1985) and that $^{54}$Mn$^{2+}$ did not show significant binding to PP1 catalytic subunit (Brautigan et al., 1980) do not favor the idea that PP1 is activated by Mn$^{2+}$ in vivo. Our findings that a combination of Fe$^{2+}$/Zn$^{2+}$, but not the individual metals, can activate PP1 raises the possibility that PP1 is an iron/zinc metalloenzyme. The activation by Fe$^{2+}$/Zn$^{2+}$ was found to have an $A_2$ of approximately 0.1 m of both cations. Fe$^{2+}$ and Zn$^{2+}$ are present in skeletal muscle in millimolar and near millimolar concentrations respectively (Versieck, 1985; Iyengar and Woittiez, 1988). Interestingly, metal analysis of a purified preparation of the rabbit liver PP1 revealed that although Zn$^{2+}$ and Fe$^{2+}$ were present in substoichiometric amounts, there was considerably more Zn$^{2+}$ and Fe$^{2+}$ detected than some other metal cations tested (Yan and Graves, 1982).

It is likely that the substoichiometric levels of Zn$^{2+}$ and Fe$^{2+}$ in the purified enzyme are due to the loss of metal cation during enzyme purification. This speculation is consistent with the fact that the Fe$^{2+}$/Zn$^{2+}$ co-activated PP1 loses phosphatase activity when the cations are removed by chelation.

Fe$^{2+}$ itself cannot effectively activate PP1 but it can reversibly inactivate the Co$^{2+}$-activated enzyme. Even though the Co$^{2+}$-activated enzyme was inactivated in the presence of Fe$^{2+}$, the bond Co$^{2+}$ was not removed. These results indicate that the deactivation does not result from the displacement of Co$^{2+}$. It may result from Fe$^{2+}$ binding at another site and/or an Fe$^{2+}$-induced conformational change in the enzyme. These results are consistent with two metal binding sites on PP1. Another family of phosphatases, the mammalian purple acid phosphatases, are metalloproteins (Vincent and Averill, 1990a, 1990b). Comparison of the primary structures of purple acid phosphatases and Ser/Thr protein phosphatases have lead Vincent and Averill to speculate that PP1 and phosphatase 2A are iron/zinc metalloenzymes with active sites isostructural with those of the purple acid phosphatase. Our data provide the first experimental evidence to support the postulate that PP1 is an iron/zinc metalloenzyme. It is interesting to note that we recently established that the catalytic subunit of phosphatase 2A can also exist in a divalent cation-dependent form (Cai et al., 1995).

While the suggestion of Vincent and Averill (1990b) that the Ser/Thr protein phosphatase may contain two metal sites was based on weak sequence homologies of the purple acid protein phosphatase with the Ser/Thr protein phosphatases represented by PP1, protein phosphatase 2A, and protein phosphatase 2B (calcineurin), the recent elucidation of the crystal structure of protein phosphatase 2B has now confirmed the existence of iron and zinc in the active site (Griffith et al., 1995). While this manuscript was under review, the crystal structure of recombinant PP1 (a isoform) was reported (Goldberg et al., 1995). This structure shows the presence of two metal ions in the catalytic site. Since the enzyme was prepared in the presence of Mn$^{2+}$, the ions were presumed to be Mn$^{2+}$. These studies confirm our findings that PP1 is a metalloenzyme and strengthen the view that PP1 may have bound zinc and iron ions at the active site. Given the structural similarities of protein phosphatase 2B with PP1 and protein phosphatase 2A, it seems likely that the latter will possess similar metal ion sites. On the other hand, Zhuo et al. (1993, 1994) reported that Mn$^{2+}$ or Ni$^{2+}$ activation of a bacteriophage $\gamma$Ser/Thr protein phosphatase ($\gamma$ PPase) had an apparent single $K_m$ for each of the divalent metals. The latter results are consistent with one metal ion binding site involved in the activation of $\gamma$ PPase. Further studies will be necessary to identify the physiologically important metal ions responsible for PP1 activation.

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REFERENCES

Alessi, D. R., Street, A. J., Cohen, P., and Cohen, P. T. W. (1993) Eur. J. Biochem. 213, 1035–1066
Bülling, M., and Stalmans, W. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 227–281
Brautigan, D. M. (1976) Annu. Rev. Biochem. 45, 54–75
Brautigan, D. L., Picton, C., and Fischer, E. H. (1980) Biochemistry 19, 5787–5794
Brautigan, D. L., Ballou, L. M., and Fischer, E. H. (1982) Biochemistry 21, 1977–1982
Burchell, A., and Cohen, P. (1978) Biochem. Soc. Trans. 6, 220–222
Cai, L., Chu, Y., Wilson, S. E., and Schlender, K. K. (1995) Biochem. Biophys. Res. Commun. 208, 274–279
Chen, Y. H., Chen, M. X., Campbell, D. G., Shanahan, C., Cohen, P., and Cohen, P. T. W. (1994) FEBS Lett. 351, 51–55
Chu, Y., Wilson, S. E., and Schlender, K. K. (1994) Biochim. Biophys. Acta 1208, 45–54
Defreyne, G., Verplaetse, J., Goris, J., Lontie, R., and Merlievede, W. (1979) Biochem. Soc. Trans. 7, 1050–1051
Goldberg, J., Huang, H.-b., Kwon, Y.-g., Greengard, P., Nairn, A. C., and Kuriyan, J. (1995) Nature 376, 745–753
Griffith, J., P., Kim, J. L., Kim, E. E., Sintchak, M. D., Thomson, J. A., Fitzgibbon, M. J., Fleming, M. A., Caron, P. R., Hsiao, K., and Navia, M. (1995) Cell 82, 507–522
Hsiao, K.-J., Sandberg, A. R., and Li, H.-C. (1978) J. Biol. Chem. 253, 6901–6907
Iyengar, V., and Woittiez, J. (1988) Clin. Chem. 34, 474–481
Kato, K., and Bishop, J. S. (1972) J. Biol. Chem. 247, 7420–7429
Kato, K., Kubayashi, M., and Sato, S. (1975) J. Biochem. (Tokyo) 77, 811–815
Khandelwal, R. L., and Kasmani, S. A. S. (1980) Biochim. Biophys. Acta 613, 95–105
Khatra, B. S., and Soderling, T. R. (1978) Biochim. Biophys. Acta. Commun. 85, 647–654
Klein, D. S., Aylward, J. H., Melgren, R. L., and Lee, E. Y. C. (1978) Arch. Biochem. Biophys. 191, 638–646
Mackenzie, C. W., Bulbulian, G. J., and Bishop, J. S. (1980) Biochim. Biophys. Acta 614, 413–424
Merlievede, W., and Riley, G. A. (1966) J. Biol. Chem. 241, 3517–3524
Shenolikar, S., and Nairn, A. C. (1994) Adv. Second Messenger Phosphoprotein Res. 31, 121
Shimizu, H., Ito, M., Miyahara, M., Ichikawa, K., Okubo, S., Konishi, T., Naka, M., Tanaka, T., Hirano, K., Hartshorne, D. J., and Nakano, T. (1994) J. Biol. Chem. 269, 30407–30412
Ullman, B., and Perfman, R. L. (1975) Biochim. Biophys. Acta 403, 393–411
Versieck, J. (1985) Crit. Rev. Clin. Lab. Sci. 22, 97–184
Villa-Maruvzi, E., Bälou, L. M., and Fischer, E. H. (1984) J. Biol. Chem. 259, 5857–5963
Vincent, J. B., and Averill, B. A. (1990a) FASEB J. 4, 3009–3014
Vincent, J. B., and Averill, B. A. (1990b) FEBS Lett. 263, 265–268
Yan, S. C. B., and Graves, D. J. (1982) Mol. Cell. Biochem. 42, 21–29
Zhang, Z., Bai, G., Deans-Zirrato, S., Browner, M. F., and Lee, E. Y. C. (1992) J. Biol. Chem. 267, 1848–1849
Zhang, Z., Bai, G., Shima, S., Zhao, S., Nagao, M., and Lee, E. Y. C. (1993a) Arch. Biochem. Biophys. 303, 402–406
Zhang, Z., Bai, G., Zhao, S., and Lee, E. Y. C. (1993b) Adv. Protein Phosphatases 7, 183–197
Zhuo, S., Clemens, J. C., Hakes, D. J., Barford, D., and Dixon, J. E. (1993) J. Biol. Chem. 268, 17754–17761
Zhuo, S., Clemens, J. C., Stone, R. L., and Dixon, J. E. (1994) J. Biol. Chem. 269, 26234–26238