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Activation of Protein Phosphatase 2A by the Fe^{2+}/Ascorbate System[1]

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Freshly isolated protein phosphatase 2A (PP2A) was highly active as to the dephosphorylation of protein substrates, but lost most of its spontaneous activity on prolonged storage, and was converted to a latent form requiring Mn^{2+} or Co^{2+} ions for activity. In this report, we show that the latent form of PP2A can be activated by the Fe^{2+}/ascorbate system. Activation of the phosphatase required both Fe^{2+} ions and ascorbate, and the level of activation was dependent on the concentrations of both Fe^{2+} ions and ascorbate. Both the holoenzyme and catalytic subunit of phosphatase 2A could be activated by the Fe^{2+}/ascorbate system, indicating that direct modulation of the catalytic subunit of the phosphatase by the Fe^{2+}/ascorbate system may cause this activation. Several common divalent metal ions, including Ca^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} ions, cannot cooperate with ascorbate to activate the phosphatase. Dithiothreitol, a SH-containing reducing agent, could replace ascorbate in the Fe^{2+}/ascorbate system to activate the phosphatase, whereas H_2O_2, a strong oxidizer, significantly diminished the phosphatase activation by the Fe^{2+}/ascorbate system. The results indicate that iron ions stabilized in the +2 state by reducing agents can activate the phosphatase. Overall, the present study provides initial biochemical evidence suggesting that Fe^{2+} could be a biologically important metal ion cofactor responsible for PP2A activation.

Key words: ascorbate, Fe^{2+} ions, protein phosphatase.

Reversible protein phosphorylation is now recognized as an important control mechanism for the regulation of many cellular processes. The phosphorylation state of a specific target protein or enzyme is determined by the balance of the activities of protein kinases and phosphatases. The dephosphorylation of protein-bound phosphoserine and phosphothreonine is mediated by a family of serine/threonine-specific protein phosphatases that have been broadly categorized as either type 1 or type 2 phosphatases on the basis of their substrate specificity and sensitivity to inhibitors (*1*). Type-1 phosphatases (PP1) consist of a catalytic C subunit (37 kDa) and one of several regulatory subunits. Various forms of PP1 have been identified, including the inactive Mg•ATP-dependent protein phosphatase (*2*). Type-2 phosphatases (PP2) have been further classified into three groups termed PP2A, PP2B, and PP2C (*3*). The catalytic subunits of PP1, PP2A, and PP2B belong to the same gene family sharing ~40% sequence identity (*4*). PP2A phosphatases are multisubunit enzymes consisting of a catalytic C subunit (37 kDa), an accessory A subunit (60-63 kDa), and one of several regulatory B subunits (54-74 kDa) (*5*). Multiple forms of PP2A resulting from different combinations of the C subunit with the A and/or B subunits from mammalian tissues have been identified and characterized (*5-7*).

PP2A has a broad substrate specificity and has been shown to be capable of dephosphorylating many phosphoproteins involved in the regulation of diverse cell functions (for a review see Ref. *5*). Although the exact physiological functions of PP2A remain to be established, several *in vivo* studies have suggested that it is involved in a variety of signaling pathways controlling cell proliferation and transformation (*8-13*). The activity of PP2A can be modulated by basic proteins and polyamines such as histone H1, protamine, polylysine, and spermine *in vitro* (*6, 14, 15*). Besides these protein factors, divalent metal ions, particularly Mn^{2+} and Co^{2+} ions, have also been shown to activate PP2A at millimolar or submillimolar concentration (*14, 16, 17*), indicating that the phosphatase is a metal-sensitive enzyme. However, since the concentrations of both Mn^{2+} and Co^{2+} ions in tissues are less than 2 μM (*18, 19*), it seems unlikely that PP2A is a Mn^{2+}- or Co^{2+}-bound protein *in vivo*. Previously, we showed that the inactive Mg•ATP-dependent PP1 can be activated by Fe^{2+} ions in the presence of ascorbate (*20*). Because PP1 and PP2A have highly homologous catalytic domains, it is highly possible that the Fe^{2+}/ascorbate system can also have a profound effect on the activity of PP2A. In this report, I show that on prolonged storage PP2A is converted to a latent form, and that this latent form of PP2A can be activated by the Fe^{2+}/

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Abbreviations: MBP, myelin basic protein; MCO, metal ion-catalyzed oxidation; PP2A, protein phosphatase 2A; PP2Ac, the catalytic subunit of phosphatase 2A.

ascorbate system. The mechanism responsible for this activation was examined.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP was purchased from Amersham. Ferrous sulfate, cobalt(II) chloride, nickel(II) sulfate, copper(II) nitrate, zinc sulfate, calcium chloride, magnesium chloride, manganese(II) chloride, ascorbic acid, ethylenediaminetetraacetic acid (EDTA), dithiothreitol, 2-mercaptoethanol, and hydrogen peroxide were from E. Merck. Other chemicals used were of reagent grade. Distilled water, further purified with a Millipore MilliQ system, was used throughout the present study.

Protein Purification—Phosphorylase *b* (*21*) and phosphorylase *b* kinase (*22*) were purified from rabbit skeletal muscle. The catalytic subunit of cAMP-dependent protein kinase was isolated from pig heart (*23*). Myelin basic protein (MBP) (*24*) was purified from pig brain. Protein phosphatase 2A was purified to homogeneity from rabbit skeletal muscle according to a previous report (*25*), using *p*-nitrophenylphosphate as the substrate, and assayed in the presence of 1 mM Mn^{2+} ions. The catalytic subunit of protein phosphatase 2A (2Ac) was prepared from crude phosphatase 2A preparations treated with 80% ethanol, followed by sequential chromatography on AcA-34 filtration gel and polylysine-Sepharose basically as described by Resink *et al.* (*26*). When analyzed by 10% SDS-PAGE and Coomassie Blue staining, the purified protein phosphatases 2A and 2Ac gave three major protein bands at M_r of 60,000, 54,000, and 38,000, and one major band at M_r of 38,000, respectively (Fig. 1, inset panel). The protein phosphatase 2A preparations used in this study therefore belong to the category of phosphatase $2A_0$ or $2A_1$ according to the definition of Ingebritsen and Cohen (*3*).

Preparation of ^{32}P-Labeled Protein Substrates—^{32}P-Phosphorylase *a* was prepared from phosphorylase *b*, phosphorylase *b* kinase, and [γ-^{32}P]ATP as described in Ref. *27*. ^{32}P-MBP was prepared from purified MBP, the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P]ATP as described in a previous report (*25*).

Enzyme Assays—Protein phosphatase activity was assayed at 30°C by measuring the release of $^{32}P_i$ from the ^{32}P-labeled protein substrate as previously described (*25, 27*). Protein phosphatase 2A or 2Ac was first incubated with divalent metal ions (0-5 mM), ascorbic acid (0-20 mM), and various compounds (0-20 mM) at 30°C for 10 min in buffer A containing 100 mM Tris-HCl, at pH 7.4. ^{32}P-Phosphorylase *a* (final concentration, 1 mg/ml) or ^{32}P-MBP (final concentration, 0.2 mg/ml) was next added to initiate the phosphatase action. The assay time was 10 min. One unit of protein phosphatase activity was taken as amount of enzyme that catalyzes the release of 1 nmol of phosphate/min from ^{32}P-labeled substrates.

Analytic Methods—Protein concentrations were determined by the method of Lowry *et al.* (*28*). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (*29*) using 10% gels. The molecular weight markers used were as follows: myosin (200,000), β-galactosidase (116,300), phosphorylase *b* (97,400), bovine serum albumin (66,300), glutamic dehydrogenase (55,400), lactate dehydrogenase (36,500), and carbonic anhydrase (31,000).

RESULTS AND DISCUSSION

Freshly isolated PP2A was highly active as to the dephosphorylation of ^{32}P-MBP (specific activity, 348 U/mg), and this activity was independent of Mn^{2+} ions (*25*). On storage at −30°C for 8 years, the phosphatase lost most of its spontaneous activity (specific activity, 12 U/mg) and was found to be converted into a latent form dependent on Mn^{2+} or Co^{2+} ions for activity. As shown in Fig. 1, after prolonged storage PP2A could be dramatically activated by 1 mM Mn^{2+} or Co^{2+} ions. About 30-35% of the original activity could be restored (specific activity, ~110 U/mg). This is in agreement with a previous report (*30*) that the highly active catalytic subunit of PP2A (PP2Ac) became inactive on prolonged storage but could be reactivated by incubation with Mn^{2+} or Co^{2+} ions. Previously, we found that the inactive Mg·ATP-dependent PP1 can be activated by Fe^{2+} ions in the presence of ascorbate (*20*) and so, it was interesting to examine the effect of the Fe^{2+}/ascorbate system on the activity of the latent form of PP2A. Treatment with 1 mM Fe^{2+} ions alone caused inhibition of the basal phosphatase activity, and treatment with 20 mM ascorbate alone had no effect on the phosphatase activity (Fig. 1). However, when the phosphatase was incubated with both 1 mM Fe^{2+} ions and 20 mM ascorbate for 10 min, the phosphatase activity was dramatically increased to a level comparable to that on activation by Mn^{2+} or Co^{2+} ions under the same assay conditions (Fig. 1). A longer preincubation period (>10 min) did not further increase the activation level caused by Fe^{2+} ions and ascorbate. Furthermore, the addition of the divalent metal ion chelator, EDTA, to the reaction mixture prevented the phosphatase activation by Fe^{2+} ions and ascorbate (Fig. 1). It should be noted that activation of the latent form of PP2A by the Fe^{2+}/ascorbate system could also be observed when

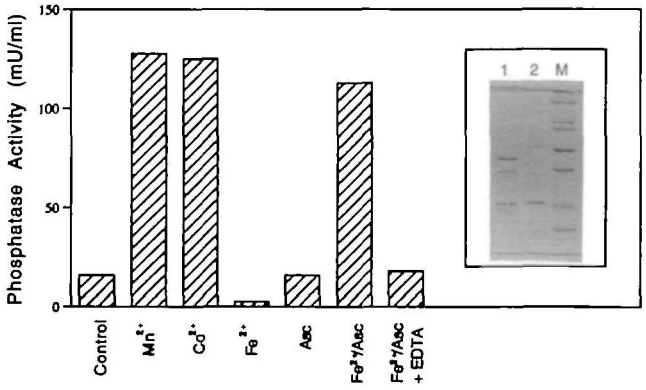

Fig. 1. **Activation of protein phosphatase 2A by the Fe^{2+}/ascorbate system.** Protein phosphatase 2A (1.2 μg/ml) was incubated in buffer A at 30°C for 10 min with the following additions: (1) no addition, (2) 1 mM Mn^{2+} ions, (3) 1 mM Co^{2+} ions, (4) 1 mM Fe^{2+} ions, (5) 20 mM ascorbate, (6) 1 mM Fe^{2+} ions and 20 mM ascorbate, (7) 1 mM Fe^{2+} ions, 20 mM ascorbate, and 10 mM EDTA. The phosphatase activities were then determined using ^{32}P-MBP as the substrate as described under "MATERIALS AND METHODS." Inset, Coomassie Blue staining of highly purified protein phosphatase 2A (lane 1), 2Ac (lane 2), and marker proteins (lane M). Asc, ascorbate. The molecular weights of the marker proteins, from top to bottom, are 200, 116.3, 97.4, 66.3, 55.4, 36.5, and 31 kDa, respectively.

another substrate, ^{32}P-phosphorylase *a*, was used in the assay, and there was no release of $^{32}P_i$ from the ^{32}P-labeled substrates when the phosphatase was omitted from the reaction mixture (data not shown). The results indicate that the activation process is not due to a substrate-directed effect. The results taken together demonstrate that the latent form of PP2A can be potently and specifically activated by the Fe^{2+}/ascorbate system. Further studies revealed that the level of activation of PP2A by the Fe^{2+}/ascorbate system is dependent on the concentrations of both Fe^{2+} ions and ascorbate used in the assay. In the presence of 20 mM ascorbate, the phosphatase activity was activated by Fe^{2+} ions in a dose-dependent manner, whereas in the absence of ascorbate, no activation of phosphatase activity occurred (Fig. 2). The concentrations of Fe^{2+} ions required for half-maximal and maximal activation were about 25 and 600 μM, respectively. The extent of activation decreased significantly when the concentration of Fe^{2+} ions was above 1 mM. Similarly, in the presence of 1 mM Fe^{2+} ions, dose-dependent activation of the phosphatase activity by ascorbate was observed (Fig. 3).

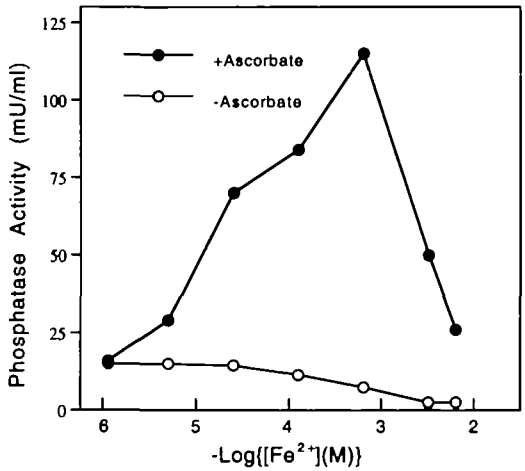

Fig. 2. **Dose-dependent activation of protein phosphatase 2A by Fe^{2+} ions in the presence of ascorbate.** Protein phosphatase 2A (1.2 μg/ml) was incubated with various concentrations of Fe^{2+} ions in the presence and absence of 20 mM ascorbate in buffer A at 30°C for 10 min. The phosphatase activities were then determined using ^{32}P-MBP as the substrate as described under "MATERIALS AND METHODS."

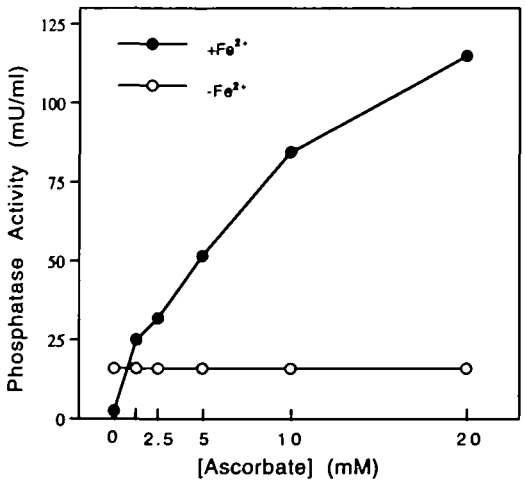

Fig. 3. **Dose-dependent activation of protein phosphatase 2A by ascorbate in the presence of Fe^{2+} ions.** Protein phosphatase 2A (1.2 μg/ml) was incubated with various concentrations of ascorbate in the presence and absence of 1 mM Fe^{2+} ions in buffer A at 30°C for 10 min. The phosphatase activities were then determined using ^{32}P-MBP as the substrate as described in "MATERIALS AND METHODS."

Since the A (60 kDa) and B (54 kDa) subunits of PP2A have been shown to play important regulatory roles in modulating the activity of the catalytic subunit of PP2A (PP2Ac) (*7, 31*), it was interesting to determine which subunit(s) is (are) affected by the Fe^{2+}/ascorbate system during the activation process. To address this issue, we examined whether or not PP2Ac can be activated by the Fe^{2+}/ascorbate system. The PP2Ac used in this experiment was highly active as isolated and had been stored at −30°C for 2 years. As shown in Table I, similar to its holoenzyme form, PP2Ac could be dramatically activated by the Fe^{2+}/ascorbate system. This result strongly suggests that direct modulation and concurrent activation of the catalytic subunit of PP2A by the Fe^{2+}/ascorbate system occur during this activation process. It was noticed that treatment with 20 mM ascorbate alone, which had no effect on the activity of PP2A (Fig. 1), partially activated PP2Ac (Table I).

To determine whether or not divalent metal ions besides Fe^{2+} can cooperate with ascorbate to activate PP2A, several common divalent metal ions were included in the reaction mixture in the presence and absence of ascorbate. As shown in Table II, although the Ni^{2+} ion alone could partially

TABLE I. **Activation of protein phosphatase 2Ac by the Fe^{2+}/ascorbate system.**

Addition	Phosphatase activity (mU/ml)
None	12.0
Fe^{2+}	1.4
Ascorbate	49.2
Fe^{2+} + ascorbate	105.7
Fe^{2+} + ascorbate + EDTA	49.8

Protein phosphatase 2Ac (0.66 μg/ml) was incubated in buffer A at 30°C for 10 min with the following additions: (1) no addition, (2) 1 mM Fe^{2+} ions, (3) 20 mM ascorbate, (4) 1 mM Fe^{2+} ions and 20 mM ascorbate, (5) 1 mM Fe^{2+} ions, 20 mM ascorbate, and 10 mM EDTA. The phosphatase activities were then determined using ^{32}P-MBP as the substrate as described under "MATERIALS AND METHODS."

TABLE II. **Effects of various divalent metal ions on the activity of protein phosphatase 2A in the presence and absence of ascorbate.**

Addition	Phosphatase activity (mU/ml)	
	−Ascorbate	+Ascorbate
None	16.0	16.0
Fe^{2+}	2.6	112.9
Mg^{2+}	16.0	16.0
Ca^{2+}	16.0	16.0
Zn^{2+}	3.6	3.6
Cu^{2+}	3.0	3.0
Ni^{2+}	41.6	30.0

Protein phosphatase 2A (1.2 μg/ml) was incubated with various divalent metal ions (1 mM each) in the presence and absence of 20 mM ascorbate at 30°C for 10 min. The phosphatase activity was then determined using ^{32}P-MBP as the substrate as described under "MATERIALS AND METHODS."

activate the phosphatase, the Fe^{2+} ion appeared to be the only one that could cooperate with ascorbate to activate the phosphatase among the metal ions tested. This result indicates that it is the unique chemical nature of Fe^{2+} ions that renders the Fe^{2+}/ascorbate system capable of activating PP2A. The effect of the combined addition of ascorbate with Mn^{2+} or Co^{2+} ions on the phosphatase was also examined, and it was found that the addition of ascorbate did not significantly alter the Mn^{2+}- or Co^{2+}-stimulated phosphatase activity, although a small increase in the phosphatase activity (~10-20%) could always be observed in the presence of ascorbate (data not shown).

One possible mechanism responsible for the observed phosphatase activation may be protein oxidation, since it is well-established that the Fe^{2+}/ascorbate system, originally developed by Udenfriend *et al.* (*32*), is one of the nonenzymatic metal-catalyzed oxidation (MCO) systems that mediate protein oxidation. To examine this possibility, another MCO system, Fe^{2+}/H_2O_2 (*33*), was used to treat PP2A. As shown in Table III, neither H_2O_2 alone nor H_2O_2 plus Fe^{2+} ions could activate the phosphatase. These results taken together with the fact that most enzymes that are sensitive to the Fe^{2+}/ascorbate system can be converted to catalytically inactive or less active forms after modification (*33-35*) suggest that the protein oxidation mechanism is not responsible for the observed activation of the phosphatase by the Fe^{2+}/ascorbate system. Meanwhile, it was also noticed that the level of phosphatase activity by the Fe^{2+}/ascorbate system decreased significantly when H_2O_2 was included in the reaction mixture (Table III). Since H_2O_2 is a strong oxidizer, which will antagonize the reducing capability of ascorbate, it seems likely that a reducing environment is required for activation of PP2A by the Fe^{2+}/ascorbate system. To further address this issue, other reducing agents, such as SH compounds, were therefore used to examine whether or not they can replace ascorbate in the Fe^{2+}/ascorbate system to activate PP2A. It was found that although dithiothreitol alone had no effect on the phosphatase activity, this thiol agent at concentrations of >10 mM could indeed mimic ascorbate in cooperating with Fe^{2+} ions to activate the phosphatase in a dose-dependent manner (Table III). On the other hand, another thiol agent, 2-mercaptoethanol, at concentrations of up to 20 mM was ineffective under identical assay conditions (Table III). The reason why the phosphatase responds differently to the two thiols is not clear; nevertheless, the result supports the notion that a reducing environment is required for activation of PP2A by Fe^{2+} ions.

TABLE III. **Effects of various compounds on the Fe^{2+}/ascorbate-catalyzed activation of protein phosphatase 2A.**

Addition	Phosphatase activity (mU/ml)
None	16.0
Fe^{2+}	2.6
Fe^{2+} + ascorbate	112.9
H_2O_2 (20 mM)	16.0
Fe^{2+} + H_2O_2 (20 mM)	2.6
Fe^{2+} + ascorbate + H_2O_2 (20 mM)	46.8
Dithiothreitol (20 mM)	16.0
Fe^{2+} + dithiothreitol (5 mM)	16.0
Fe^{2+} + dithiothreitol (10 mM)	26.3
Fe^{2+} + dithiothreitol (20 mM)	93.5
2-Mercaptoethanol (20 mM)	16.0
Fe^{2+} + 2-mercaptoethanol (5-20 mM)	2.6

Protein phosphatase 2A (1.2 μg/ml) was incubated with various compounds in different combinations, as indicated, at 30°C for 10 min, and then the phosphatase activity was determined using ^{32}P-MBP as the substrate as described under "MATERIALS AND METHODS." The concentrations of Fe^{2+} ions and ascorbate used in the assays were 1 and 20 mM, respectively.

After being developed, the Fe^{2+}/ascorbate system has been commonly used as a nonenzymatic MCO tool for studying the mechanisms of oxidative modification of lipids, nucleic acids, and proteins *in vitro* (*33-35*). Enzymes such as glutamine synthetase (*34*), malic enzyme (*35*), and glucose-6-phosphate dehydrogenase (*36*) have been shown to be selectively modified through Fe^{2+}-catalyzed oxidation at their respective metal binding site(s), and this modification concomitantly causes a rapid decrease in the enzyme activity. As compared to these typical metal-sensitive enzymes, PP2A, like the ATP·Mg-dependent PP1 (*20*), appears to respond to the Fe^{2+}/ascorbate system in a distinct way. First, the Fe^{2+}/ascorbate system does not inactivate, but activates PP2A (Figs. 1-3). Second, it appears that the phosphatase activation does not result from the typical protein oxidation catalyzed by the Fe^{2+}/ascorbate system (Table III). On the other hand, our results indicate that a reducing environment is required for the activation of PP2A by Fe^{2+} ions. Since an aqueous Fe^{2+} solution undergoes spontaneous oxidation to Fe^{3+} in air, the addition of reducing agents such as ascorbate and dithiothreitol would be expected to protect Fe^{2+} from spontaneous oxidation or to reverse its oxidation. In this context, it is tempting to believe that the role of ascorbate or dithiothreitol here is to stabilize the +2 state of iron, and that iron in its divalent state can activate PP2A. This notion is supported by the observation that better protection of Fe^{2+} from oxidation by increasing the ascorbate or dithiothreitol concentration leads to a greater increase in PP2A activity (Fig. 3 and Table III). The failure of detection of PP2A activation by Fe^{2+} in previous studies (*37, 38*) may have been simply due to the spontaneous oxidation of Fe^{2+} to Fe^{3+} under the assay conditions used. It seems likely that Fe^{2+} activates PP2A in the same way as the other two transition metal ions (Mn^{2+} and Co^{2+}) do. The only difference here is that Mn^{2+} and Co^{2+} are more resistant to air oxidation than Fe^{2+}, and they do not need reducing environment for their stabilization. However, a reducing atmosphere is needed to keep iron in its divalent state.

Although PP2A has long been recognized as a metal-sensitive enzyme, and the effects of Mn^{2+} and Co^{2+} on its enzyme activity have been well documented (*14, 16, 17*), whether PP2A is a metalloenzyme and what are the physiologically important metal ions responsible for PP2A activation remain questions that need to be answered. Recently, the crystal structures of PP2B (also known as calcineurin) and the catalytic subunit of PP1 (PP1c) were determined, respectively (*39-41*). Structural analysis of the two phosphatases revealed the existence of two metal ion binding sites in the active center of both enzymes. For PP2B, structural analysis data together with the results of previous atomic absorption experiments of on the purified enzyme (*42*) have confirmed that it is a metalloenzyme containing stoichiometric amounts of iron and zinc. For PP1c, in addition to Mn^{2+}, which was included during enzyme preparation, a stoichiometric amount of iron ions was also detected in PP1c crystals (*40*). In another experi-

ment, Chu *et al.* (*43*) found that recombinant PP1c can be activated by the combination of Fe^{2+} and Zn^{2+}. These observations together with our previous finding that the inactive ATP·Mg-dependent PP1 can be activated by Fe^{2+} in the presence of ascorbate (*20*) suggest that the iron ion is an important physiological "cofactor" for both PP1 and PP2B. Since PP1, PP2A, and PP2B have highly homologous catalytic domains, and the predicted catalytic site residues that interact with metal ions are conserved in these three enzymes (*39, 40*), it is highly possible that PP2A is also a metalloenzyme containing an iron ion at its active site. Further experiments, such as crystal structural analysis of PP2A, are needed to clarify this point. Nevertheless, the present finding that Fe^{2+} in a reducing environment can activate PP2A strengthens this hypothesis and provides initial biochemical evidence suggesting that Fe^{2+} could be a biologically important metal ion cofactor responsible for PP2A activation.

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