Vol. 208, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 8, 1995 Pages 274-279

A Metal-Dependent Form of Protein Phosphatase 2A

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Received January 18, 1995

SUMMARY: Highly purified bovine heart protein phosphatase 2A catalytic subunit lost virtually all of its activity during storage at -70°. When the enzyme was preincubated with Co^{2+}, over 35% of the original activity was restored. Freshly prepared protein phosphatase 2A purified from bovine heart was stimulated at least 3 to 4-fold by pretreatment with Co^{2+} or Mn^{2+}. Activation by Co^{2+} appeared to be irreversible whereas activation by Mn^{2+} was partially reversed after the cation was chelated with excess EDTA/EGTA. The sensitivity of Co^{2+}-stimulated protein phosphatase 2A to okadaic acid or inhibitor-2 was similar to that of spontaneously active protein phosphatase 2A. The enzyme was converted to a latent form by treatment with phosphate or pyrophosphate. The latent form was completely reactivated by preincubation with Co^{2+}. These results demonstrate that protein phosphatase 2A, like phosphatase 1, can exist in a metal ion-dependent form and may represent a new mechanism for the regulation of protein phosphatase 2A activity.

Serine/threonine-specific protein phosphatases are critical enzymes involved in the regulation of many cellular processes. Two major types of serine/threonine protein phosphatases, type 1 (PP1) and type 2 (PP2) were classified on the basis of their substrate specificity and sensitivities to heat-stable protein inhibitors (1, for recent reviews see refs. 2,3). PP1 preferentially dephosphorylates the β-subunit of phosphorylase kinase, and is also characterized by its unique sensitivity to heat-stable protein inhibitor 1 and inhibitor 2. The PP2 types preferentially dephosphorylate the α-subunit of phosphorylase kinase, and are not sensitive to inhibitor 1 or inhibitor 2. PP2 is further classified into three distinct enzymes PP2A, PP2B and PP2C, based on their metal ion requirements. PP2B and PP2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively, while PP2A is active in the absence of divalent cations.

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Abbreviations: PP1, protein phosphatase 1; PP1c, catalytic subunit of protein phosphatase 1; PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of protein phosphatase 2A; DTT, dithiothreitol; MOPS, 3-[N-morpholino] propanesulfonic acid; Pi, inorganic phosphate; PPi, inorganic pyrophosphate.

0006-291X/95 $5.00

Another very useful criterion for distinguishing among the serine/threonine phosphatases is based on sensitivity to okadaic acid (4). PP2A is almost completely inhibited by 3 nM okadaic acid whereas PP1 is unaffected at this concentration and μM concentrations are required for complete inhibition. PP2B is inhibited by okadaic acid only at much higher concentrations and PP2C is not inhibited (5).

The catalytic subunits of phosphatase 1 (PP1c) and phosphatase 2A (PP2Ac) are quite similar proteins. The overall sequence identity between PP1c and PP2Ac is 43% (6,7). It is well established that PP1c exists in two forms: an active form and an inactive or latent form (2,3). The inactive holoenzyme complex of PP1c and inhibitor 2 can be activated by phosphorylation of the inhibitor-2 subunit by glycogen synthase kinase 3 (8). Free PP1c can be activated by Co^{2+} or Mn^{2+} (9,10). Although PP2A is usually isolated in a metal-independent state (3), Ingebritsen et al. (11) reported that PP2A in fresh skeletal muscle or liver extracts was variably stimulated by Mn^{2+}. They further noted that during purification, PP2A became more dependent upon Mn^{2+} for activity. More recently it has been reported that PP2A purified from bovine heart (12,13) or from yeast (14) could be stimulated several fold by Mn^{2+}. In the present study, we present evidence that PP2A, like PP1, can exist in a latent form which requires Co^{2+} or Mn^{2+} for activity.

MATERIALS AND METHODS

Materials: Antibodies against peptides C-TPPRNSAKAKK and C-VTRRTPDYFL corresponding to the carboxyl terminus of PP1 and PP2A respectively were produced as described (15). Inhibitor 2 was a generous gift from Dr. Ernest Y.C. Lee. Okadaic acid was obtained from Moana BioProducts (Honolulu, Hawaii). PP1c was purified from rabbit skeletal muscle (16) and PP2Ac was purified from bovine heart through the heparin-Sepharose step (17).

Assay of protein phosphatase: Protein phosphatase activity was determined by measuring the release of [^{32}P]Pi from [^{32}P] phosphorylase *a* (17). One unit of protein phosphatase activity was defined as the amount of enzyme which released 1 μmol [^{32}P]Pi/min. To determine the effects of divalent cations, the enzyme was diluted with 50 mM MOPS buffer, pH 7.0, containing 0.5 M KCl, 0.2 mM EDTA, 0.5 mg bovine serum albumin/ml, and 1 mM DTT, then incubated with 0.4 mM Me^{2+} at 30°C for 15 min. The sample was diluted with buffer containing 50 mM imidazole-HCl, pH, 7.0, 0.12 mg theophylline/ml, 1 mM DTT. The enzyme activity was measured as described above. PP2Ac was inactivated by preincubating the enzyme with different concentrations of Pi or PPi at 30°C for 15 min. The mixture was set on ice for 1 hr and then dialyzed against 50 mM MOPS buffer containing 0.5 M KCl, 0.2 mM EDTA, 1 mM DTT overnight to remove Pi or PPi.

RESULTS

PP2Ac was purified from bovine heart as previously described to near homogeneity (17). On SDS gel electrophoresis, the preparation had a major protein band at 36 kDa which reacted

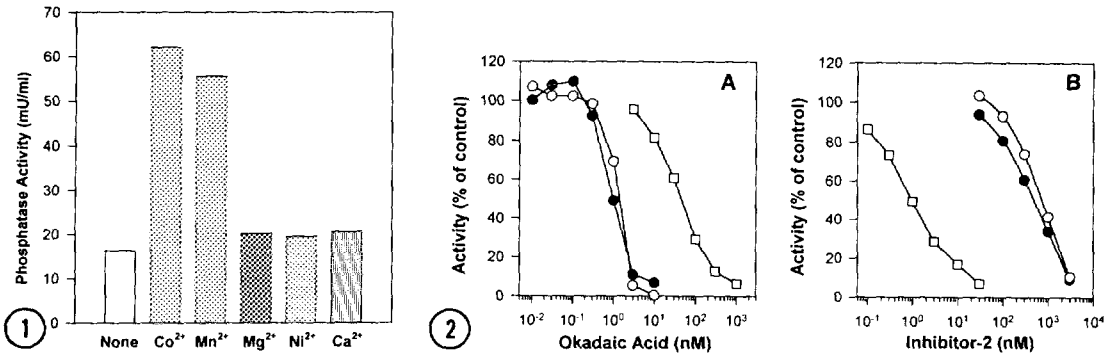

Figure 1. The activation of PP2Ac by divalent cations. The catalytic subunit of PP2A was pre-treated with 0.2 mM Co^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, or Ca^{2+} for 15 min at 30°C. The spontaneous and cation stimulated activity was measured as described under "Materials and Methods".

Figure 2. Effect of okadaic acid (A) or inhibitor 2 (B) on Co^{2+}-stimulated PP2Ac. Phosphatase activity of PP2Ac (○), Co^{2+}-treated PP2Ac (●) or PP1c (□) was measured in the presence of varying concentrations of okadaic acid or inhibitor-2.

on a Western blot with an antibody specific for the carboxyl terminus of PP2Ac (15). The preparation did not react with an antibody which reacts with the four known isoforms of PP1 catalytic subunit (10). When PP2Ac was freshly prepared the specific activity was approx. 815 munits/mg protein. After storage at -70°C for 6 years the enzyme was virtually inactive under standard phosphatase reaction conditions. Since PP1c can be activated by preincubation with Co^{2+} or Mn^{2+}, we preincubated the inactive PP2Ac with Co^{2+} as described in "Materials and Methods". After preincubation, much of the original activity was restored (295 munits/mg protein).

To further characterize the effect of divalent cations on PP2Ac, a fresh preparation was purified from bovine heart (17). The PP2Ac was preincubated with 0.2 mM free Co^{2+}, Mn^{2+}, Mg^{2+}, Ca^{2+}, or Ni^{2+} at 30°C for 15 min, and the phosphatase activity was assayed in the presence of 0.2 mM free cation (Fig. 1). The activity of the Co^{2+} pre-treated PP2Ac was about 4 times greater than that of untreated enzyme. Mn^{2+} also stimulated the PP2Ac activity greater than 3 fold. The other metals tested, Mg^{2+}, Ca^{2+} or Ni^{2+}, did not stimulate the PP2Ac. If Mn^{2+} was chelated after pretreatment and the phosphatase was assayed in the absence of Mn^{2+}, more than 60% of the stimulated activity was lost, while removal of Co^{2+} did not result in loss of the stimulated activity (data not shown).

Although the PP2Ac appeared to be free of PP1c, we considered the possibility that it might contain some latent PP1 that was activated by preincubation with Co^{2+}. Since metal-independent PP2A is much more sensitive to okadaic acid, and much less sensitive to inhibitor 2 than PP1, the effects of these inhibitors were tested on spontaneously active and Co^{2+}-activated

PP2Ac. Both the spontaneously active and the Co^{2+}-activated PP2Ac were very sensitive to okadaic acid, with IC_{50}s of approx 1 nM (Fig. 2A). Spontaneous activity and metal-dependent activity were almost completely inhibited by 3 nM okadaic acid, a concentration at which PP1 is not affected. The concentration of inhibitor 2 required to inhibit either metal-independent or metal-dependent activity of the PP2Ac preparation was comparable to that previously reported for PP2A (16). The IC_{50} was >400 nM and the enzyme was not inhibited by 30 nM inhibitor 2, a concentration which almost fully inhibits PP1c (Fig. 2B). These results indicated that the Co^{2+}-activated phosphatase activity was due to PP2A.

PP1 can be inactivated by incubation with inorganic phosphate (Pi) and pyrophosphate (PPi) and the activity can be restored by treatment with Co^{2+} or Mn^{2+} (reviewed in ref 7). Some of the earlier studies cited were done on enzyme preparations which presumably contained a mixture of PP1 and PP2A; therefore we tested the effect of Pi and PPi on the activity of highly purified PP2Ac. The activity of PP2Ac decreased as Pi or PPi concentration was increased. More than 80% of activity was inhibited after incubation with 100 mM Pi and the IC_{50} was 4.5 mM (Fig. 3A). The enzyme was completely inactivated by 1 mM PPi and the IC_{50} was 17 μM (Fig. 3B). The activity of Pi or PPi-inactivated enzyme was fully restored by treatment with 0.2 mM Co^{2+} (Fig. 4). These results indicate that active PP2A can be converted into a latent form and this process could be reversed by treatment with Co^{2+}.

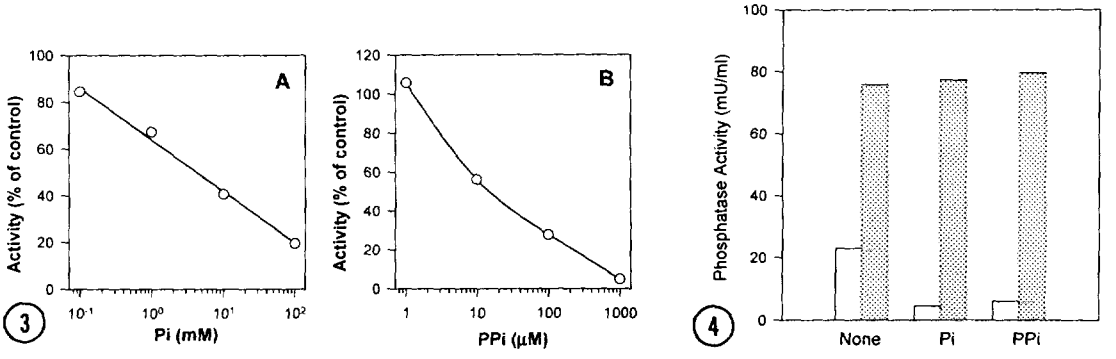

Figure 3. Inactivation of PP2Ac by Pi or PPi. PP2Ac was incubated with varying concentrations of Pi (A) or PPi (B), dialyzed and then assayed as described in **Materials and Methods**.

Figure 4. Reactivation of inactivated PP2Ac by Co^{2+}. PP2Ac was treated with 100 mM Pi or 0.15 mM PPi for 15 min at 30°C and 1 h at 0°C, then diluted 50-fold. The treated PP2A was incubated with 0.2 mM Co^{2+} for 15 min at 30°C and assayed for Co^{2+}-treated activity as described under "Materials and Methods". Open and filled bars represent without and with Co^{2+}-treatment, respectively.

DISCUSSION

It is well established that PP1 exists in two interconvertible forms, an active and a latent form (2,3). The latent form of PP1c can be activated by Co^{2+} or Mn^{2+} (9,10). Although the structure of the catalytic subunit of PP2A is more than 40% identical with the catalytic subunit of PP1, it has generally been accepted that PP2A is not dependent on Mn^{2+} or other metal ions for activity (3). Early studies noted that PP2A from fresh skeletal muscle or liver extracts was variably stimulated by Mn^{2+} (11). Recently, Yang et al. (12) and Scheidtmann et al. (13) found that the activity of the three forms of PP2A purified from bovine heart was stimulated by Mn^{2+} and to a lesser extent by Mg^{2+}, and Peng et al. (14) reported that yeast PP2A was stimulated by Mn^{2+}.

In the present study we found that PP2Ac purified from bovine heart totally lost its spontaneous activity when stored at -70°C for several years. Much of the activity was restored by treatment with Co^{2+}. Using freshly prepared PP2Ac, we found that the catalytic subunit of PP2A, like the catalytic subunit of PP1, exists in reversible active and latent forms. Like PP1c, the latent form of PP2Ac could be activated by treatment with Co^{2+} or Mn^{2+}. The active form could be converted into a latent form by treatment with Pi or PPi. Thus, a possible new mechanism for the regulation of PP2A activity is through the controlled interconversion of active and inactive conformations *in vivo*.

ACKNOWLEDGMENT

This work was supported by NIH grant HL 36573.

REFERENCES

1. Ingebritsen, T.S., and Cohen, P. (1983) Science 221, 331-338.
2. Bollen, M., and Stalmans, W. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 227-281.
3. Mumby, M.C., and Walter, G. (1993) Physiol. Rev. 74, 673-699.
4. Cohen, P., Klumpp, S., and Schelling, D.L. (1989) FEBS Lett. 250, 596-600.
5. Bialojan, C., and Takai, A. (1988) Biochem. J. 256, 283-290.
6. Berndt, N., Campbell, D.G., Caudwell, F.B., Cohen, P., da Cruz e Silva, E.F., da Cruz e Silva, O.B., and Cohen, P.T.W. (1987) FEBS Lett. 223, 340-346.
7. Cohen, P., and Cohen, P.T.W. (1989) J. Biol. Chem. 264, 21435-21438.
8. Ballou, L.M., Brautigan, D.L., and Fischer, E.H. (1983) Biochemistry 22, 3393-3399.
9. Villa-Moruzzi, E., Ballou, L.M., and Fischer, E.H. (1984) J. Biol. Chem. 259, 5857-5863.

10. Chu, Y., Wilson, S.E., and Schlender, K.K. (1994) Biochim. Biophys. Acta 1208, 45-54.

11. Ingebritsen, T.S., Stewart, A.A., and Cohen, P. (1983) Eur. J. Biochem. 132, 297-307.

12. Yang, S.-I., Lickteig, R.L., Estes, R.C., Rundell, K., Walter, G. and Mumby, M.C. 1991 Mol. Cell. Biol. 11, 1988-1995.

13. Scheidtmann, K.H., Mumby, M.C., Rundell, K., and Walter, G. (1991) Mol. and Cell. Biol. 11, 1996-2003.

14. Peng, Z.-Y., Wang, W., Wilson, S.E., Schlender, K.K., Trumbly, R.J., and Reimann, E.M. (1991) J. Biol. Chem. 266, 10925-10932.

15. Wang, W., Lane, R.D., and Schlender, K.K. (1992) Biochem. Biophys. Res. Commun. 185, 657-662.

16. Brautigan, D.L., Shriner, C.L., and Gruppuso, P.A. (1985) J. Biol. Chem. 260, 4295-4302.

17. Schlender, K.K., Wilson, S.E., and Mellgren, R.L. (1986) Biochim. Biophys. Acta 872, 1-10.

18. Brautigan, D.L., Gruppuso, P.A., and Mumby, M. (1986) J. Biol. Chem. 261, 14924-14928.