A Mg\(^{2+}\)-dependent, Ca\(^{2+}\)-inhibitable Serine/Threonine Protein Phosphatase from Bovine Brain*

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The Mg\(^{2+}\)-dependent serine/threonine protein phosphatases, also known as type 2C phosphatases (PP2C), belong to a gene family distinct from the other serine/threonine phosphatases and tyrosine phosphatases. Here we report the purification to apparent homogeneity of a novel Mg\(^{2+}\)-dependent, Ca\(^{2+}\)-inhibitable serine/threonine protein phosphatase from bovine brain. It is a type 2C enzyme in view of its Mg\(^{2+}\) requirement, resistance to okadaic acid and calyculin A, inability to use phosphorylase a as substrate, and a segment of amino acid sequence typical of all PP2C type phosphatases known to date. However, it differs from the other PP2C enzymes, particularly the mammalian PP2C\(\alpha\) and -\(\beta\) isoforms, in that its molecular weight, 76,000, is considerably larger and that it is inhibited by Ca\(^{2+}\), NaF, and polyacrylamide gel electrophoresis; MES, 2-(N-morpholino)ethanesulfonic acid.

With the first family and are insensitive to okadaic acid.

The Mg\(^{2+}\)-dependent phosphatases have been identified in animals (9–15), plants (30), and yeast (31), and several have been purified to homogeneity (10–12, 32, 35). cDNA sequences of PP2C\(\alpha\) and -\(\beta\) from mammalian sources showed >90% identity (16–19, 34), whereas those from yeast were 35% identical (13, 20, 33) and showed 21–24% identity with the mammalian ones. The mammalian PP2C\(\alpha\) and -\(\beta\) are monomeric cytosolic proteins with molecular weights in the 42,000–45,000 range, and yeast PP2Cs are 31,500–51,400 monomeric enzymes. PP2Cs of larger sizes, however, have been reported. A rabbit myosin light chain phosphatase has a molecular weight of 70,000 (32), and a bovine pyruvate dehydrogenase phosphatase is a dimer with a 50,000 catalytic subunit complexed to a 97,000 subunit (35). The physiological roles of PP2Cs are unclear (1, 2), although they have been implicated in the regulation of fatty acid and cholesterol biosynthesis (21) and heat shock response (13, 20). Recently, a M\(_v\) = 200,000 PP2C-like phosphatase from HeLa cells has been reported (15) which dephosphorylates the C-terminal region of RNA polymerase II. Conceivably, PP2Cs can exist in different molecular sizes and serve diverse biological functions.

We report here the purification to homogeneity and characterization of a novel Mg\(^{2+}\)-dependent, Ca\(^{2+}\)-inhibitable protein phosphatase (MCP) from bovine brain. Although the Ca\(^{2+}\) inhibition (K\(_i\) = 20–90 \(\mu\)M) may not be related to its cellular control, this unique property permits the differentiation of this PP2C-type phosphatase from other phosphatases during its isolation. While MCP possesses many characteristics common to other PP2Cs, it is a monomer with a larger molecular weight of 76,000 and responds dissimilarly to several substrates and inhibitors.

**EXPERIMENTAL PROCEDURES**

Materials—Chemicals and biological materials were obtained from the following suppliers: DE52 ion exchanger and P81 phosphocellulose filter paper (Whatman); high resolution Sephacryl S-300 and Mono Q (Pharmacia Biotech Inc.); Affi-Gel Blue and Dowex AG 1-X8 (Bio-Rad); [\(~P\)ATP (DuPont NEN), okadaic acid and calyculin A (Biorel Research Laboratory); p-nitrophenyl phosphate, histone H2B, histone H3, casein, MBP, porcine cAMP-dependent protein kinase catalytic subunit, heparin, protamine, polystyrene, sodium orthovanadate, Kemptide (LR-RASLG), and glycogen phosphorylase b (Sigma); syntide-2 (PLARTLS-VAGLPKK) (Bachem); tyrosine phosphatase, pp60\(^{56}\) tyrosine kinase, and Raytidye (Oncogene); gels for PAGE (Novex), PP1 and PP2A (UBI); and bovine brain cDNA library (Clontech). All other chemicals were of reagent grade. The following proteins were kind gifts from the following persons: phosphorylase b kinase, Dr. Gerald Carlson, University of Tennessee, Memphis; protein kinase C, Dr. Kuo-Ping Huang, NICHD, National Institutes of Health; mitogen-activated protein kinase and casein kinase 2, Dr. Lee Graves, University of Washington, Seattle; phosphatase inhibitor 1, Dr. Heng-Chun Li, Mount Sinai Medical School, New York; and recombinant phosphatase inhibitor-2, Dr. Anna de Padil-Roach, Indiana University Medical School, Indianapolis.

Preparation of \(^{32}\)P-Labeled Protein Substrates—\(^{32}\)P-Ser-Histone H2B was prepared by incubating 10 mg of the protein with 500 micromolars of cAMP-dependent protein kinase catalytic subunit in 1 ml of
solution containing 1.5 mM [γ-32P]ATP (300 μCi), 5 mM MgCl₂, 50 mM Hepes, pH 7.0, at 30 °C for 2 h. Incorporation, usually 0.8–1 mol of 32P per mol of histone H2B, was measured by a thin layer chromatography method (23). The reaction mixture was then passed through a Dowex AG 1-X8 column to remove free ATP. MBP, histone H1, and α-casein were phosphorylated similarly by cAMP-dependent protein kinase.

32P-Histone phosphatase kinase b kinase was prepared under conditions minimizing autoposphorylation by incubating 6 μM phosphorylase b kinase with 500 micromolar of cAMP-dependent protein kinase catalytic subunit for 4 min at 30 °C in a 1-ml solution containing 1 mM EGTA, 0.4 mM EDTA, 5 mM MgCl₂, 0.2 mM [γ-32P]ATP in 50 mM Hepes, pH 7.0. The phosphate incorporation (ω and β) was 2.0 mol/mol of protein (ωβγ). [32P]Phosphorylase a was prepared according to the method described by King et al. (25).

32P-Labeled histone H3 and -MBP were phosphorylated by protein kinase C according to the method of Masmoudi et al. (26).

32P-Labeled MBP was phosphorylated by mitogen-activated protein kinase by incubating 2 mg of MBP with 5 mM MgCl₂, 0.2 mM ATP (100 μCi), 30 μg of mitogen-activated protein kinase in 50 mM Tris buffer, pH 7.2, at 30 °C for 2 h. 32P-labeling of MBP was determined by the standard assay used to identify MCPP turned out to be quite reliable. After the MCPP was purified and its properties were studied, we were able to compare the 32P-γ assay with other assays using specific phosphatase inhibitors (cf. Table IV). As can be seen from Fig. 1A, at the DEAE-cellulose chromatography step, MCPP detected by the standard assay eluted with the main PP2C activity peak that was measured by chromatography step, MCPP activity profile obtained by this method agreed well with that using 1 μM okadaic acid and 1 mM orthovanadate (see Table III) to inhibit both PP1 and PP2A. Calcineurin (PP2B) did not contribute much in this assay when Ca²⁺ was present because exogenous calmodulin was not added and histone H2B is a poor substrate for this enzyme. After the Sepharyl S-300 step, MCPP activity can be measured readily by the 32Ca²⁺ method. MCPP activity profile obtained by this method agreed well with that using 1 μM okadaic acid and 1 mM orthovanadate (see Table III) to inhibit both PP1 and PP2A and PP2Ca and -β. The peak fraction from the Mono Q step yielded the pure form of MCPP.

**RESULTS**

Purification of MCPP—Table I summarizes the results of a typical purification described under “Experimental Procedures.” The ±Ca²⁺ assay used to identify MCPP turned out to be quite reliable. After the MCPP was purified and its properties were studied, we were able to compare the ±Ca²⁺ assay with other assays using specific phosphatase inhibitors (cf. Table IV). As can be seen from Fig. 1A, at the DEAE-cellulose chromatography step, MCPP activity profile obtained by this method agreed well with that using 1 μM okadaic acid and 1 mM orthovanadate (see Table III) to inhibit both PP1 and PP2A. Calcineurin (PP2B) did not contribute much in this assay when Ca²⁺ was present because exogenous calmodulin was not added and histone H2B is a poor substrate for this enzyme. After the Sepharcl S-300 step, MCPP activity can be measured readily by the ±Ca²⁺ method. MCPP activity profile obtained by this method agreed well with that using 1 μM okadaic acid and 1 mM orthovanadate (see Table III) to inhibit both PP1 and PP2A and PP2Ca and -β. The peak fraction from the Mono Q step yielded the pure form of MCPP.

| Step | Total volume | Total protein | Specific activity | Yield |
|------|--------------|---------------|------------------|-------|
| 1. Extract | 2500 | 27,900 | | |
| 2. DEAE | 180 | 990 | 0.82 | 100.0 |
| 3. Affi-Gel blue | 14 | 154 | 2.3 | 43.6 |
| 4. Sephacryl S-300 | 15 | 7.5 | 18.6 | 17.2 |
| 5. Mono Q | 3 | 0.15 | 161 | 3.0 |

2 K. F. Qin and C. Y. Huang, unpublished data.
is considerably smaller than the \(-5 \text{ mM}\) reported for rabbit PP2Cs (12). The enzyme was totally inactive when Mg\(^{2+}\) was removed by a chelator like EDTA. Mn\(^{2+}\) could support MCPP activity as effectively as Mg\(^{2+}\), but the other cations tested, Ca\(^{2+}\), Co\(^{2+}\), Zn\(^{2+}\), Ni\(^{2+}\), Fe\(^{3+}\), and Al\(^{3+}\) could not. All the cations tested were chloride salts, and all reagents had been passed through Chelex 100 columns. In the presence of 1 mM Mg\(^{2+}\), the percent inhibition observed with 1 mM cation in descending order was Zn\(^{2+}\), 98%; Fe\(^{3+}\) and Al\(^{3+}\), 89%; Co\(^{2+}\), 86%; Ni\(^{2+}\) and Ca\(^{2+}\), 85%. The inhibitory effect of these cations increased with decreasing Mg\(^{2+}\) concentration. Although these cations all have similar inhibitory effects on MCPP, the inhibition by Ca\(^{2+}\) distinguishes MCPP from most phosphatases. \(K_i\) for Ca\(^{2+}\) is on the order of 90 \(\mu\text{M}\) when histone H2B is the substrate. With MBP as substrate, however, \(K_i\) for Ca\(^{2+}\) is \(-20 \mu\text{M}\). Calmodulin has no effect on this phosphatase.

pH Optimum of MCPP—The pH optimum of MCPP was determined by using histone H2B as substrate in the standard assay. MES buffer was used for pH values from 5.0–7.0 and Hepes buffer from pH 6.6–8.5. MCPP activity reached optimal values at pH values greater than 7.5, indicating that it is an alkaline protein phosphatase.

Substrate Specificity—The rates of MCPP-catalyzed dephosphorylation using various substrates and, whenever possible, at comparable concentrations (in terms of phosphoryl groups)
and -

| Substrate                      | Concentration of 32P-labeled substrate | Specific activity -Ca²⁺ | +1 mM Ca²⁺ |
|-------------------------------|----------------------------------------|--------------------------|------------|
| Histone H2B                   | 20 μM                                  | 189                      | 0          |
| Histone H3                    | 20 μM                                  | 165                      | 0          |
| MBP                           | 30 μM                                  | 1978                     | 0          |
| Casein                        | 20 μM                                  | 80                       | 0          |
| Kemptide                      | 10 μM                                  | 88                       | 0          |
| Syntide 2                     | 35 μM                                  | 150                      | 0          |
| Phosphorylase kinase          | 2 μM                                   | 0.8                      | 0          |
| Phosphorylated by protein kinase C |                                        |                          |            |
| MBP                           | 40 μM                                  | 302                      | 0          |
| Histone H3                    | 20 μM                                  | 478                      | 0          |
| Phosphorylated by mitogen-activated protein kinase |          |                          |            |
| MBP                           | 10 μM                                  | 0                        | 0          |
| Phosphorylated by casein kinase 2 |                                        |                          |            |
| Casein                        | 10 μM                                  | 1                        | 0          |
| Phosphorylated by phosphorylase b kinase |          |                          |            |
| Phosphorylase a               | 10 μM                                  | 0                        | 0          |
| Phosphorylated by tyrosine kinase |                                        |                          |            |
| Raytide                       | 5 μM                                   | 0                        | 0          |
| MBP                           | 10 μM                                  | 0                        | 0          |
| Casein                        | 5 μM                                   | 0                        | 0          |
| p-Nitrophenyl phosphate       | 10 nM                                  | 0                        | 0          |

### Table III

Effect of different inhibitors on MCPP

| Inhibitor    | Inhibition | Concentration* | IC₅₀ b |
|--------------|------------|----------------|-------|
| NaF          | Yes        | 10 mM          |       |
| Polysine     | Yes        | 4 μg/ml        |       |
| Protamine    | Yes        | 35 μg/ml       |       |
| Okadaic acid | No         | 10 μM          |       |
| Calycin A    | No         | 100 nM         |       |
| Orthovanadate| No         | 1 μM           |       |
| Heparin      | No         | 100 μg/ml      |       |
| Inhibitor 1  | No         | 20 μM          |       |
| Inhibitor 2  | No         | 15 μM          |       |

* Concentration of inhibitor causing 50% inhibition under standard assay conditions as described under "Experimental Procedures."

### Amino Acid Sequence Homology with Known PP2C-type Phosphatases—Partial cDNA sequence of MCPP obtained by us revealed a segment that is homologous to the same region found in every PP2C reported so far. Fig. 4 compares a 24-residue segment of MCPP with segments from 12 other PP2Cs. Residues identical with those of MCPP are underlined. Conservative substitutions (relative to MCPP) are in capital letters. The number given at the end of each sequence marks the position of the last residue. All alignments are obtained from the BLAST Server.

### Effect of Known Phosphatase Inhibitors—The effects of different known inhibitors of protein phosphatases have been examined, and the results are summarized in Table III. NaF, a common inhibitor of PP1, PP2A, and PP2B, but not of PP2Ca and -β, inhibits MCPP. Polysine and protamine, which activate PP2A, are potent inhibitors of MCPP, although they do not inhibit PP1, PP2B, and PP2Ca and -β. Orthovanadate, a potent inhibitor of tyrosine phosphatase that inhibits PP1, PP2A, PP2C (2) at near millimolar levels, has no effect on MCPP at concentrations up to 1 mM. The phosphatase types 1 and 2A inhibitors okadaic acid and calyculin A (27) do not inhibit. Phosphatase (PP1) inhibitors 1 and 2 and heparin also have no effect.

### Distribution of MCPP in Different Tissues—Since MCPP was isolated from bovine brain, it is of interest to see whether this Ca²⁺-inhibitable enzyme or its isofoms may exist in other tissues and other animals. Therefore, we examined the distribution of MCPP activity in various mouse organs by using calycin A to inhibit PP1 and PP2A and employing the standard ±Ca²⁺ assay with histone H2B as substrate to detect the presence of this type of phosphatase. As can be seen from Table IV, the Ca²⁺-inhibitable phosphatase activity can be demonstrated in the four selected mouse organs, brain, kidney, lung, and liver, and the results agree very well with those obtained from using polysine to inhibit MCPP.
We have purified to homogeneity a new Mg$^{2+}$-dependent protein phosphatase from bovine brain. It is named Ca$^{2+}$-inhibitable protein phosphatase to differentiate it from other mammalian Mg$^{2+}$-dependent phosphatases which have been called PP2Ca, PP2C$\beta$, or named after their substrates such as phosphofructokinase phosphatase (14), myosin light chain phosphatase (32), and pyruvate dehydrogenase phosphatase (35). Although PP2C-type phosphatases have been identified in various sources, only a handful have been purified to homogeneity, and MCPP is the first 76-kDa enzyme obtained in pure form.

The catalytic and structural characteristics of MCPP indicate that it can be classified as a type 2C phosphatase. 1) It requires Mg$^{2+}$ (or Mn$^{2+}$) for activity. 2) It catalyzes the dephosphorylation of phosphoseryl/threonyl residues of proteins and peptides phosphorylated by cAMP-dependent protein kinase. 3) Its catalytic activity is highest with MBP (10 times better than any other substrate tested so far) may also imply a special function for this activity. 4) MCPP is 54.58% identical with and 96% similar to 6 mammalian PP2Cs in (or MHC-I) for activity. 2) It catalyzes the dephosphorylation of phosphoseryl/threonyl residues of proteins and peptides phosphorylated by cAMP-dependent protein kinase. 3) Its catalytic activity is highest with MBP (10 times better than any other substrate tested so far) may also imply a special function for this activity. 4) MCPP is 54.58% identical with and 96% similar to 6 mammalian PP2Cs (Fig. 4). In this segment, MCPP is found in other PP2C-type enzymes (32). 2) It is inhibited by polycations like okadaic acid and calyculin A, heparin, and PP1 inhibitors (Table II). 3) It is sensitive to inhibitors of SET protein by protein kinase C (Table II). 4) It does not attack phosphorylase a or b, although a SET protein was found in 12 other PP2C-type enzymes (32). 5) A segment of the intracellular Ca$^{2+}$-dependent phosphatase from turkey gizzard smooth muscle which do not inhibit PP2C may also be involved in amplifying the Ca$^{2+}$ signal in vivo. In this regard, it is interesting to note that bovine pyruvate dehydrogenase phosphatase is activated by Ca$^{2+}$ with $K_{d}$ of 24–62 µM range in the absence of EGTA buffer (36).

The role of MCPP in the cell, like the other PP2Cs, is not clear at this time. The rather wide distribution of MCPP, as shown in the survey presented in Table IV, suggests that MCPP or its isoforms constitute a new subclass of PP2C and likely serve important cellular functions. It should be of interest to note that a SET protein encoded by a set gene (38) copurified with MCPP until the Mono Q chromatography step. In a case of acute undifferentiated leukemia, the set gene was fused to a can gene as a result of chromosomal translocation (38). Phosphorylation of the SET protein by protein kinase C was blocked by the presence of trace amounts of MCPP, indicating that SET is an excellent substrate for the phosphatase. Since the set gene is expressed in all tissues of the mouse, particularly during embryogenesis, SET may play a key role in the cell and MCPP may regulate the function of SET. The fact that MCPP activity is highest with MBP (10 times better than any other substrates tested so far) may also imply a special function for this phosphatase in the brain.

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