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Purification and Characterization of Protein Phosphatase 2C from Rat Liver

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Abstract: Protein phosphatase 2C (PP2C) is one of the four major serine/threonine phosphatases which is dependent on Mg^{2+} for its activity. PP2C was purified from rat liver cytosol and its characteristics were investigated. The substrate employed for routine assay was [^{32}P]casein phosphorylated by PKA. The purification process involved DEAE chromatography, ammonium sulfate fractionation, phenyl sepharose chromatography, sephacryl S-200 gel filtration, and histone agarose chromatography. The SDS-PAGE of PP2C showed one major single protein band at a position corresponding to a molecular mass of 43 kd and the purification fold was 637. The enzyme showed a pH optimum of 8 and K_M value was 1.9 μM. However, when the substrate was changed to [^{32}P]histone, the pH optimum was shifted to 7 and K_M value was 2.3 μM. Mg^{2+} was essential to the enzyme activity and okadaic acid did not exert any inhibitory effect on the enzyme. To examine residue in the active site of PP2C, effects of some protein-modifying reagents were tested.

Key words: characterization, protein phosphatase 2C, purification

Protein phosphatases, which remove phosphates from proteins, fall into two categories on the basis of the amino acid residues from which the phosphate group is removed (Cohen, 1989). That is, the protein tyrosine phosphatases and protein serine/threonine phosphatases. The dual-specific phosphatases are also identified (Hiraga *et al.*, 1981; Cohen, 1989). The protein serine/threonine phosphatases are subdivided into two groups further (Type 1 and Type 2) depending on whether they dephosphorylate the b subunit of phosphorylase kinase specifically and are inhibited by nanomolar concentrations of two small natural occurring heat and acid stable proteins, termed inhibitor-1 and inhibitor-2 (Type 1, PP1), or they dephosphorylate the a subunit of phosphorylase kinase preferentially and are insensitive to inhibitor protein 1 and 2 (Type 2, PP2). Type 2 phosphatases could, in turn, be subclassified into three distinct enzymes, 2A, 2B, and 2C. PP2B and PP2C have absolute requirements for Ca^{2+} and Mg^{2+}, respectively, while PP2A, like PP1, is active toward most substrates in the absence of divalent cations (Ingebritsen and Cohen, 1983). The type 1 and 2 protein serine/threonine phosphatase family is not structually related to the tyrosine phosphatase family.

A number of studies have indicated a potential role for PP1 and PP2A in the regulation of cell growth. The observation that okadaic acid is a powerful tumor promotor and specific inhibitor of PP1 and PP2A has led to the suggestion that these two enzymes function as tumor suppressors in mammalian cells (Cohen and Cohen, 1989). PP1 and PP2A contain potential phosphorylation sites for cyclin-dependent kinases and PP1, but not PP2A, activity was found to change during the cell cycle of human MG-63 osteosarcoma cells due to phosphorylation of PP1 by cyclin-dependent kinases (Dohadwala *et al.*, 1994). Other studies have shown that PP1 and PP2A play an important role in the cell morphology (Gurland and Gundersen, 1993; Yano *et al.*, 1995). PP2B (calcineurin) is the major Ca^{2+}/calmodulin dependent protein phosphatase and is enriched in neuronal tissue. PP2B was shown to be the target of the immunosuppressive drugs used to prevent host rejection in organ transplantation (Liu *et al.*, 1991). A role for PP2B in the regulation of glutamate release has been presented (Sihra *et al.*, 1995). Another recent study showed that an okadaic acid-insensitive PP2C had an important role in the reversibility of the Ca^{2+}-independent form of CaM kinase II (Fukunaga *et al.*, 1993).

PP2C has been partially purified from canine heart as a casein phosphatase (Binstock and Li, 1979) and purified to apparent homogeneity from rat liver as a glycogen synthase phosphatase (Hiraga *et al.*, 1981), or as a phosphofructokinase phosphatase (Mieskes *et al.*, 1984). Two isoenzymes of PP2C were reported by Cohen

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group from rabbit skeletal muscle and liver (McGowan and Cohen, 1987). Tamura *et al.* have reported that the isolation of a full-length cDNA encoding rat PP2C from a kidney cDNA library detected the mRNA in rat kidney and liver (Tamura *et al.*, 1989). Furthermore, molecular cloning of PP2C isoform has been achieved (Wenk *et al.*, 1992; Kato *et al.*, 1995) and type 2C protein phosphatase of *Leishmania chagasi* has been cloned and expressed (Burns *et al.*, 1993). Recently membrane-bound form of PP2C from *Paramecium tetraurelia* was purified and examined (Klumpp *et al.*, 1994).

The molar concentration of PP2C *in vivo* is similar to that of PP1 and PP2A (McGowan and Cohen, 1987), and like other phosphatases it is capable of dephosphorylating a number of proteins *in vitro*. However, the biochemical study of PP2C has yet to be carried out. To examine the substrate specificity of PP2C, we purified PP2C and investigated its general properties using some model proteins phosphorylated by PKA as substrates. In addition, effects of modification of a cysteine residue in PP2C with 5,5'-dithiobis-(2-nitrobenzoic acid) and modification of a histidine residue with diethylpyrocarbonate were examined.

Materials and Methods

Materials

DEAE-cellulose, phenyl sepharose CL-4B, sephacryl S-200, sephadex G-25, histone agarose, and cAMP-dependent protein kinase catalytic subunit (PKA), hydrolysed and partially dephosphorylated casein (product #4765), histone (Type II-AS), and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma (St. Louis, USA). Diethyl pyrocarbonate (DEPC) was from Fluka (Buchs, Switzerland). [γ-^{32}P]ATP was purchased from DuPont NEN (Boston, USA). All other chemicals were of reagent grade commercially available.

Substrates preparation

[^{32}P]*Casein Preparation*: [^{32}P]Casein was prepared by incubating 16 mg/ml casein with 60 U PKA for 8 h at 30°C in 0.1 mL of reaction mixture containing 50 mM Tris-HCl (pH 6.9), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (McGowan and Cohen, 1987). The reaction was stopped by cooling the reaction mixture to 0°C and subjected to gel filtration on Sephadex G-25 (1×7 cm). Incorporation of [^{32}P] was about 0.3 mol/mol of protein. The [^{32}P]casein (4 μM) is stored at 0-4°C and is used as substrate stock.

[^{32}P]*Histone Preparation*: [^{32}P]Histone was prepared by incubating 15 mg/ml histone with 60 U PKA for 4 h at 30°C in 0.1 ml of reaction mixture containing 50 mM Tris-HCl (pH 6.9), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 10 mM $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP (Hiraga *et al*, 1981). The reaction was stopped by adding 0.3 ml of 30% trichloroacetic acid. After microcentrifugation, the precipitated phosphorylated histone was washed using water and ethanol : ether (1 : 4) solution two times repeatedly. After the protein was dried, it was dissolved (20 μM) and stored at 0-4°C.

[^{32}P]*Myelin Basic Protein Preparation*: Four mg MBP was phosphorylated by 80 U PKA for 6 h at 30°C in 0.15 ml solution containing 0.1 mM [γ-^{32}P]ATP (10 μCi), 50 mM Tris-HCl (pH 6.9), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, and 10 mM $MgCl_2$ (Yang, 1986). The reaction was stopped and simultaneously the protein was recovered by directly pouring onto a Sephadex G-25 column with a flow rate of 50 μl/min. [^{32}P]MBP was aliquoted and stored in a freezer for later use.

Assay of protein phosphatase 2C

The PP2C activity was assayed in 0.1 ml of 50 mM Tris-HCl buffer pH 7.9 for [^{32}P]casein (pH 7 for [^{32}P]histone) containing 0.1 mM EGTA, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 250 nM okadaic acid (McKintosh, 1993). Enzyme source and substrate were added to start the phosphatase reaction. The final concentration of [^{32}P]casein was 0.2 μM and that of [^{32}P]histone was 1 μM. The total reaction volume was 0.1 ml. This mixture was incubated at 30°C for 5 min for [^{32}P]casein or 20 min for [^{32}P]histone substrate. The reaction was quenched by addition of 30 μL of 15 mg/ml BSA solution and 70 μl of 30% trichloroacetic acid solution. After microcentrifugation the supernatant was taken and the released inoganic phosphate was determined by the molybdate assay (Foulkes *et al.*, 1981). The supernatant was mixed with 200 μl of 1.25 mM KH_2PO^4 in 0.5 M H_2SO_4, 500 μl of isobutanol : toluene (1 : 1) and 100 μl of 5% ammonium molybdate. After vigorous shaking the upper organic phase containing the inorganic ^{32}P was removed and counted in a scintillation counter. The assay were only linear up to 10-20% conversion to the dephosphorylated form. Therefore extents of dephosphorylation were kept within this limit. One unit of activity was defined as the release of 1.0 pmol [^{32}P]phosphate/min at 30°C.

Purification of PP2C

Purification steps were based on the previous description by McGowan and Cohen with some modifications (1987). All operations were carried out at 4°C.

Step 1. Preparation of Enzyme Source: Four rat livers (12 g) were chopped with scissors and homogenized in 3 vol. of 60 mM Tris-HCl (pH 7.4), 6 mM EDTA, and 0.25 M sucrose with glass-Teflon homo-

genizer. The crude extract was then subjected to centrifugation at 100,000×g for 60 min and the supernatant was retained.

Step 2. DEAE-Cellulose Ion Exchange Chromatography: The supernatant fluid (50 mL) was loaded on a 2.8×10 cm column of DEAE-cellulose equilibrated with buffer A (20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 5 mM 2-mercaptoethanol, 50 mM NaCl, and 2% (v/v) glycerol). The column was washed with 600 ml of buffer A and the enzyme was eluted with 160 ml linear NaCl gradient from 50 mM to 700 mM. The volume of each fraction was 6 ml.

Step 3. Ammonium Sulfate Fractionation: Active fractions were pooled and solid ammonium sulfate (192 g/L) was added to bring the degree of saturation to 33%, and after standing for 30 min, the suspension was centrifuged for 20 min at 10,000×g. The supernatant was decanted and diluted to 0.5 M ammonium sulfate.

Step 4. Phenyl Sepharose Chromatography: The resuspended enzyme solution was loaded on a 0.7×8 cm column of phenyl sepharose equilibrated with buffer A containing 0.5 M ammonium sulfate. The column was washed with 60 ml buffer A containing 0.5 M ammonium sulfate, developed with 40 ml linear ammonium sulfate gradient from 0.5 M to 0 M, and then washed with 30 ml of buffer A. The volume of each fraction was 2 ml.

Step 5. 60% Ammonium Sulfate Fractionation: Active fractions were pooled and solid ammonium sulfate was added to bring to 60% saturation. After standing for 30 min, the suspension was centrifuged for 20 min at 10,000 ×g. The supernatant was discarded and the precipitate was resuspended in 500 μl buffer A.

Step 6. Sephacryl S-200 Gel Filtration: The suspended solution was loaded on a 1×50 cm sephacryl S-200 column equilibrated with buffer A containing 0.1 M NaCl. The flow rate was 2.4 ml/h and fractions of 0.8 ml were collected. The PP2C activity was eluted as a single peak.

Step 7. Histone Agarose Column: Active fractions were pooled, diluted to lower the salt concentration and magnesium chloride was added to make final concentration 10 mM. The sample was applied to a 0.7×5 cm histone agarose column equilibrated with buffer A containing 10 mM $MgCl_2$. The column was washed with 40 mL equilibrating buffer and the enzyme was eluted with 20 ml linear NaCl gradient from 50 mM to 800 mM. The volume of each fraction was 1.5 ml.

Other methods

Protein Determination: The protein concentration was determined according to Bradford (1976). The standard protein used here was bovine serum albumin.

Polyacrylamide Gel Electrophoresis: PAGE was conducted using 10% polyacrylamide gel and 0.1% sodium dodesyl sulfate. The proteins were stained with silver nitrate (Hames, 1990).

Modification of PP2C: DTNB or DEPC was added to reaction mixture excluding substrate, and the mixture was preincubated for 5 min at 30℃. The substrate was then added and the reaction mixture was incubated for another 5 min for appropriate time for each substrate.

Results and Discussion

Purification of PP2C

After the homogenate was centrifuged, the supernatant was applied to DEAE-cellulose column. When the enzyme was eluted with linear NaCl gradient from 50 mM to 700 mM, the PP2C activity was eluted around 300 mM salt concentration (Fig. 1A). During the DEAE-cellulose column work, recovery was 114% and the purification factor was 4. The pooled fractions from the previous step were fractionated with ammonium sulfate. The recovery of PP2C in 33% saturated supernatant was 45% and the purification fold was 1.4. The supernatant obtained was diluted to lower the concentration of ammonium sulfate to 0.5 M. This diluted enzyme was then applied to phenyl sepharose column. Proteins began to be eluted when the ammonium sulfate concentration of eluting buffer was lower than 0.2 M, and the PP2C activity was shown at latter portion of the main peak of protein (Fig. 1B). Since the enzyme activity peak did not overlap with the main protein peak, high purification factor was expected. However, the apparent purification factor did not exceed 4 fold.

The pooled fractions from previous step were fractionated again with ammonium sulfate. This time the precipitate formed from 60% saturation was collected by centrifugation. The recovery was 41% and the purification factor was 0.7. For enhanced recovery and stability of the enzyme, concentrating sample using membrane would be another choice. The precipitated PP2C was dissolved in 500 μL of buffer A and applied to sephacryl S-200 gel filtration chromatography. The PP2C activity was eluted as a single peak (Fig. 1C). During the gel filtration step, the recovery was 116% and the purification factor was 4. The pooled fractions of Sephacryl S-200 were diluted to have the same salt composition with buffer A containing 10 mM magnesium chloride, applied to histone agarose column equilibrated with the same buffer. A linear NaCl gradient from 50 mM to 800 mM was developed and the PP2C was eluted around 350 mM sodium chloride concentration (Fig. 1D). During the histone agarose column work the

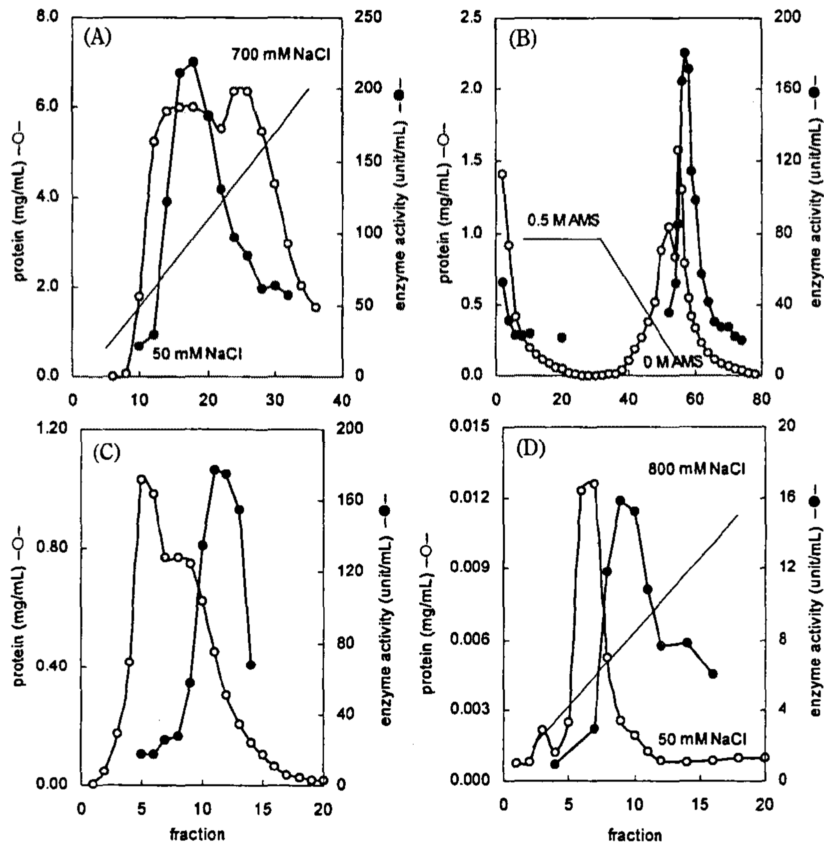

Fig. 1. Chromatographic purification of PP2C from rat liver. A, The elution profile of PP2C from DEAE cellulose column; B, The elution profile of PP2C from phenyl sepharose column; C, The elution profile of PP2C from sephacryl S-200 gel filtration column; D, The elution profile of PP2C from histone agarose column.

recovery was 60% and purification factor was 12. In this step, Mg^{2+} was required for PP2C to bind on beads. In preliminary experiments, it was observed that the enzyme did not bind significantly to the column when Mg^{2+} was not present in the equilibrating buffer. This indicates that PP2C requires Mg^{2+} in order to bind effectively to the potential substrates. [^{32}P]Histone was used as a substrate for PP2C assays (Mieskes *et al.*, 1984; Donella-Deana *et al.*, 1990) and histone agarose was chosen earlier as an affinity column for substrate analogue (Hiraga *et al.*, 1981). A more powerful affinity column like a thiophosphorylated myosin-P-light-chain sepharose was usefully applied for the purification of PP2C (McGowan and Cohen, 1987).

The SDS-PAGE of PP2C showed a nearly single protein band at a position corresponding to a molecular mass of 43 kDa (Fig. 2). Molecular weight of PP2C from rabbit skeletal muscle and liver was 43 kDa (McGowan and Cohen, 1987). For the PP2C from rat liver, the mass was estimated 40 kDa by gel filtration and 48 kDa by SDS-PAGE (Hiraga *et al.*, 1981). A summary of the purification steps is shown in Table 1. A 637 fold-purification was achieved from the high speed supernatant and the yield of overall procedure was about 1.2%.

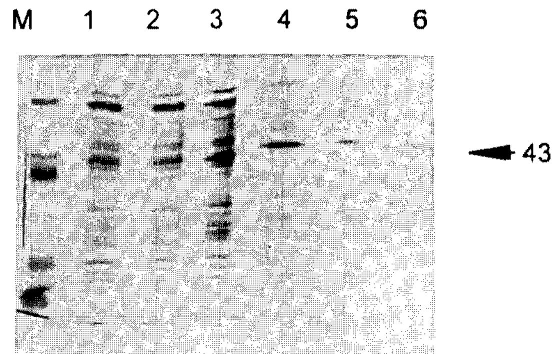

Fig. 2. SDS-PAGE of purified PP2C. M, molecular weight marker, 66, 45, and 24 kDa; Lane 1, 2, and 3, crude PP2C with activity right before histone agarose column work; Lane 4, 5, and 6, purified PP2C, which were used for further characterization.

General properties of purified PP2C

General properties of PP2C, such as time dependence, enzyme amount dependence, pH dependence, and substrate dependence were examined. Furthermore the effects of Mg^{2+} and okadaic acid were also investigated. In

Table 1. Summary of the purification procedure of PP2C from rat liver

	Protein (mg)	Activity (U)	Yield (%)	Sp. activity (U/mg)	Purification Factor
Homogenate	1824	13944	100	8	1
DEAE cellulose	257	8359	60	33	4
Ammonium Sulfate (33%)	86	3776	27	44	6
Phenyl Sepharose	9.3	1611	12	174	23
Ammonium Sulfate (60%)	6.8	656	4.7	97	13
Sephacryl S-200	1.4	568	4.1	409	54
Histone Agarose	0.04	171	1.2	4844	637

U definition: One unit of activity was difined as the amount of enzyme catalyzing the release of 1.0 pmol [^{32}P]phosphate / min at 30°C.

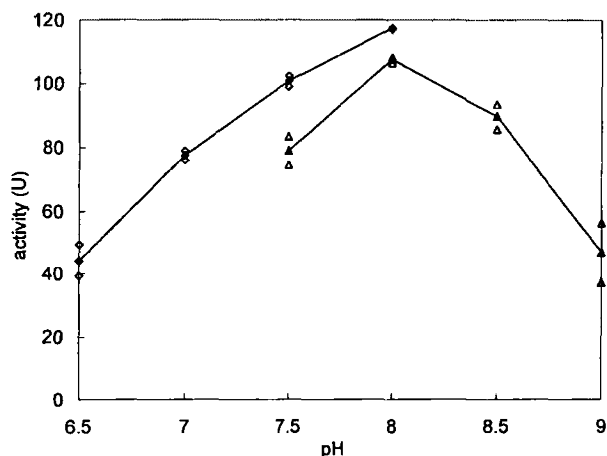

Fig. 3. pH dependence of PP2C activity for [^{32}P]casein. HEPES buffer (pH 6.5-8.0) and Tris buffer (pH 7.5-9.0) systems are used in this assay.

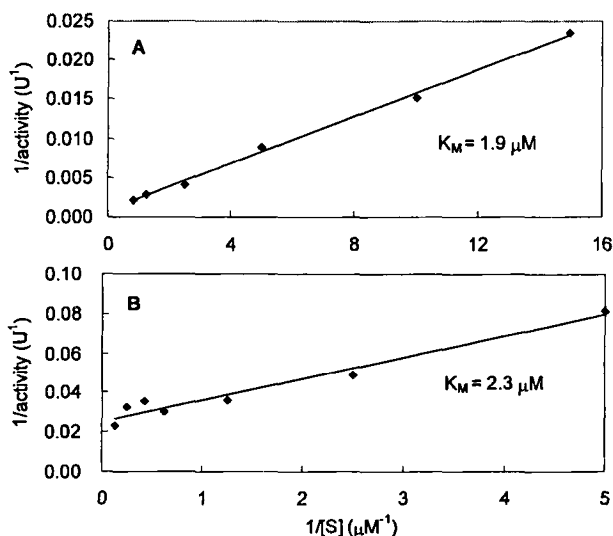

Fig. 4. Lineweaver-Burk plots of substrate dependence of PP2C. A, [^{32}P]Casein; B, [^{32}P]Histone.

time dependence assay, PP2C activity was linearly proportional to the incubation time up to 20 minutes when using [^{32}P]casein as a substrate. However when using [^{32}P]histone, the linearity improved up to about 1 hour (data not shown). PP2C was assayed at several different pH values using two substrate systems. As shown in Fig. 3, the enzyme showed a relatively broad pH optimum of 8 μm this assay condition using [^{32}P] casein as substrate. When [^{32}P]histone was used as substrate the pH optimum was shifted to 7 (data not shown). Another pH optimum of 7.5 was observed when the PP2C from canine heart used [^{32}P]synthase as substrate (Binstock and Li, 1979). In Fig. 4, Lineweaver-Burk plots of substrate dependence are shown. Result presented in Fig. 4A shows that the K_M value of PP2C for [^{32}P]casein was determined to be 1.9 μM. When [^{32}P]histone was used as substrate, the K_M value of PP2C was estimated to be 2.3 μM (Fig. 4B). The activity of PP2C toward [^{32}P]casein was about 2 times larger than that of PP2C toward [^{32}P]histone when assayed simultaneously. As to the PP2C from canine heart, the K_M value for [^{32}P]synthase was about 0.6 μM (Binstock and Li, 1979). Comparison of kinetic

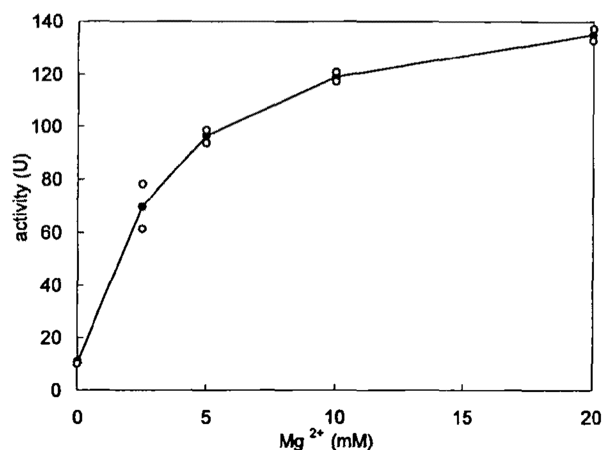

Fig. 5. Effect of Mg^{2+} on PP2C activity. In the standard assay condition, 10 mM Mg^{2+} was maintained. The substrate used here was [^{32}P]casein.

data of various substrates would be helpful to understand the biochemical interaction of substrate and PP 2C *in vivo*. Fig. 5 shows that Mg^{2+} is essential for its activity. As referred in purification section, Mg^{2+} is crucial for PP2C to bind to the substrates. Since Mg^{2+} effect was

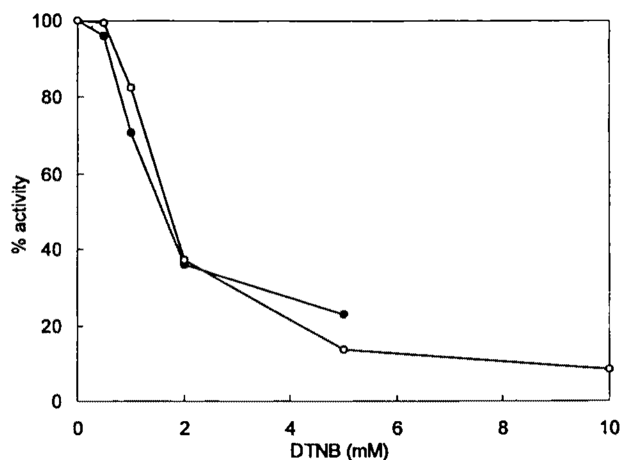

Fig. 6. Effect of DTNB on PP2C activity. [^{32}P]casein (●) and [^{32}P]histone (○).

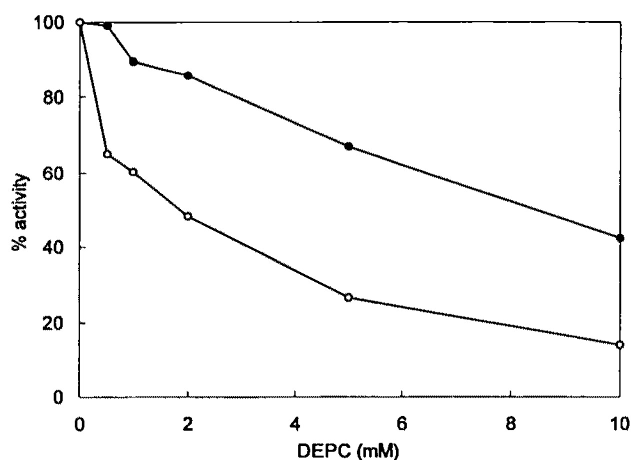

Fig. 7. Effect of DEPC on PP2C activity. [^{32}P]casein (●) and [^{32}P]histone (○).

known to be partially substituted by Mn^{2+} (Hiraga *et al.*, 1981), effect of Mn^{2+} was examined. The maximum activity was attained at 1 mM Mn^{2+} and this activity was about 30% of the activity obtained at 10 mM Mg^{2+} (data not shown). The activity shows no dependency on okadaic acid up to 250 nM (data not shown), indicating that the purified PP2C was almost independent of the toxin and the enzyme source was not contaminated with PP2A or PP1.

Substrate Specification of PP2C

For the study of substrate specificity, MBP was phosphorylated by PKA and this phospho-MBP was dephosphorylated by PP2C. In order to specify the phosphorylation site, the radioactive phospho-labeled MBP was digested with trypsin and the phosphopeptides were resolved by C18 reversed-phase column HPLC (Hwang *et al.* 1997). Amino acid sequence analysis of two major phosphopeptides revealed the sequences as 65-TTHYGSLPQK and 114-FSWGAEQK. By sequential manual Edman degradation, Thr-65 and Ser-115 were the preferable site for PKA (Hwang *et al.*, 1997; Kishimoto *et al.*, 1985). When phospho-MBP was dephosphorylated by PP2C, the Ser-115 was more favorable site than the Thr-65. Whereas Ser-115 and Thr-65 were dephosphorylated by almost same velocity by PP2B in parallel experiment (Hwang *et al.* 1997). This observation indicates the substrate specificity of PP2C is different from that of PP2B.

Modification of PP2C

Modification of cysteine residues of PP2C protein was performed with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), and that of histidine residues was made with diethylpyrocarbonate (DEPC). The inhibitory effect (IC_{50}=1~2 mM) of DTNB on PP2C was similar for [^{32}P]casein and [^{32}P]histone as shown in Fig. 6. This result indicates that PP2C might have some cysteine residues involved in the catalytic activity. On the other hand, the inhibitory effect of DEPC on PP2C revealed subtle substrate difference between [^{32}P]casein and [^{32}P]histone as shown in Fig. 7. The inhibitory effect of DEPC to [^{32}P]casein substrate was trivial (IC_{50}>10 mM), whereas DEPC effect to [^{32}P]histone was relatively strong (IC_{50}=2 mM). This indicates that the extent of interaction of DEPC to PP2C seems to be dependent on the substrate. The result therefore implies that histidine residue of PP2C may be related with substrate binding site. Although the chemical modification study obtained so far was limited and indirect, it offers an interesting clue for the crucial residues in PP2C.

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