# Direct metal analyses of $Mn^{2+}$-dependent and -independent protein phosphatase 2A from human erythrocytes detect zinc and iron only in the $Mn^{2+}$-independent one

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Received 28 January 1999; received in revised form 12 February 1999

**Abstract A $Mn^{2+}$-dependent protein phosphatase 2A which is composed of a 34 kDa catalytic C′ subunit and a 63 kDa regulatory A′ subunit, was purified from human erythrocyte cytosol. C′ and A′ produced V8- and papain-peptide maps identical to those of the 34 kDa catalytic C and the 63 kDa regulatory A subunits of the $Mn^{2+}$-independent conventional protein phosphatase in human erythrocyte cytosol, respectively. Reconstitution of C′A and CA′ revealed that the metal dependency resided in C′ and not in A′. In CA, 0.87 ± 0.12 mol zinc and 0.35 ± 0.18 mol iron per mol enzyme were detected by atomic absorption spectrophotometry, but manganese, magnesium and cobalt were not detected. None of these metals was detected in C′A′. Pre-incubation of C′ with $ZnCl_2$ and $FeCl_2$, but not $FeCl_3$, synergistically stimulated the $Mn^{2+}$-independent protein phosphatase activity. The protein phosphatase activity of C was unaffected by the same zinc and/or iron treatment. These results suggest that C is a $Zn^{2+}$- and $Fe^{2+}$-metalloenzyme and that C′ is the apoenzyme.**



*Key words:* Protein phosphatase 2A; $Mn^{2+}$ dependency; Metal analysis; Zinc; Iron; Human erythrocyte

## 1. Introduction

The reversible phosphorylation of serine/threonine residues in proteins has been known to play a crucial role in cellular signal transduction. Protein-serine/threonine phosphatases (PPs) which catalyze the reverse reaction, have been classified into four groups (1, 2A, 2B, 2C) on the basis of the differences in their biochemical properties [1]. PP1, PP2A and PP2B have highly homologous catalytic domains but differ in their substrate specificities and interactions with regulatory molecules, whereas PP2C shares no sequence homology to the others [2]. The crystal structures of the catalytic subunits of PP1 [3,4] and PP2B [5,6] were recently solved. These structures revealed a common motif of central β-α-β-α-β scaffold containing a dinuclear metal ion center located at the active site. Every active site residue of PP1 and PP2B involved in metal coordination or implicated in catalysis is strictly conserved in PP2A. Although PP2B has been shown to contain one zinc atom and one iron atom per catalytic subunit [7], the metals at the active sites of PP1 and PP2A have not been identified.

Previously, we purified three forms of PP2A from human erythrocyte cytosol, whose subunit structures are CA($\alpha_1\beta_1$), CAB($\alpha_1\beta_1\gamma_1$) and CAB''($\alpha_1\beta_1\delta_1$) where C(α) is a 34 kDa catalytic subunit and A(β), B(γ) and B''(δ) are 63, 53 and 74 kDa regulatory subunits, respectively [8]. Recent molecular cloning of B'' [9] has revealed that the subunit has a strong sequence similarity to the 54 kDa B′ regulatory subunit [10–12] in the central 400 amino acid region and is comprized in the B′ family.

In this paper, in addition to the three $Mn^{2+}$-independent forms of PP2A, a $Mn^{2+}$-dependent form of PP2A was purified from human erythrocyte cytosol. The $Mn^{2+}$-dependent form was composed of a 34 kDa catalytic C′ subunit and a 63 kDa regulatory A′ subunit. V8- and papain-peptide maps of C′ and A′ were indistinguishable from those of C and A, respectively. Direct metal analysis by atomic absorption spectrophotometry detected stoichiometric zinc and substoichiometric iron in CA, but no such metals in C′A′. Although PP2A has been suggested to be a metalloenzyme, this is the first demonstration of zinc and iron in PP2A.

## 2. Materials and methods

### 2.1. Materials

[γ-$^{32}$P]ATP and okadaic acid were obtained from Amersham and Wako, respectively. Other materials were prepared or purchased as described previously [8,13].

### 2.2. Phosphatase assay and protein determination

The PP activity was measured [8] in a 50 μl mixture containing 50 mM HEPES-NaOH, pH 7.4, 0.5 mM dithiothreitol (DTT), 0.01% (v/v) Triton X-100, 25 mM $MnCl_2$, 250 mM NaCl, 100 μM [$^{32}$P]H2B histone phosphorylated by PKA ($^{32}$P-H2B histone) and an enzyme preparation (standard assay conditions). $Mn^{2+}$-independent PP2A was assayed as described [14]. One unit of enzyme was defined as the amount of enzyme which catalyzed the release of 1 nmol of [$^{32}$P]Pi per minute. Protein was determined by the method of Lowry et al. [15] or Bradford [16].

### 2.3. Buffer solutions

All buffer solutions contained 0.5 mM DTT, 0.005% Triton X-100 and protease inhibitors including 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 0.5 mg/l pepstatin, 0.5 mg/l antipain, 1 mg/l leupeptin and 10 mg/l *N*-tosyl-L-phenylalanyl chloromethyl ketone. In

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*Abbreviations:* PP, protein-serine/threonine phosphatase; PKA, cAMP-dependent protein kinase; DTT, dithiothreitol; BSA, bovine serum albumin; P-H2B histone, H2B histone phosphorylated by PKA


PII: S0014-5793(99)00256-2

addition to the above constituents, buffer A contained 50 mM Tris-HCl, pH 7.4, 10% (v/v) glycerol and 1 mM EDTA and buffer B contained 20 mM HEPES-NaOH, pH 7.0, 10% glycerol and 1 mM EDTA.

*2.4. Purification of $Mn^{2+}$-dependent PP2A (C′A′)*

All procedures were carried out at 0–4°C. The cytosol fraction (36 500 U, 217 g protein, 3 l) was prepared from packed human erythrocytes (700 ml) and was treated with DEAE-Sephadex (110 g wet weight/1 l cytosol) as described previously [8]. The DEAE-Sephadex fraction (13 400 U, 2.83 g protein, 138 ml) was applied to a DEAE-Toyopearl column (30×3.2 cm) equilibrated with 0.03 M NaCl in buffer A. The column was washed with 500 ml of the buffer and the enzyme was eluted with a 3.4 l linear 0.03–0.25 M NaCl gradient in buffer A. The flow rate was 200 ml/h and 18 ml fractions were collected. Four peaks of PP activity appeared at NaCl concentrations of 0.07, 0.11, 0.14 and 0.18 M, as shown in Fig. 1. The fractions in the third unknown peak indicated by a solid bar in Fig. 1 were pooled and stored at −80°C. These initial enzyme preparation steps were repeated three more times. The pooled third unknown peaks were concentrated with the Pellicon Lab Cassette and an Amicon ultrafiltration cell equipped with a YM-10 membrane. The concentrated fraction (8870 U, 228 mg protein, 7.8 ml) was subjected to gel filtration on a Sephadex G-150 column (80×2.5 cm) equilibrated with buffer A. The active fractions (5610 U, 57.2 mg protein) eluted from the column were applied to an AH-Sepharose 4B column (12.5×2.2 cm) equilibrated with 0.1 M NaCl in buffer A. The enzyme was eluted with a 1 l linear 0.1–1.0 M NaCl gradient in buffer A at a flow rate of 60 ml/h. The active fractions (6790 U, 3.80 mg protein) eluting at 0.6 M NaCl were concentrated and re-chromatographed on a Sephadex G-150 column under the same conditions as described above. The active fractions (6540 U, 1.77 mg protein) were loaded onto a H1 histone-Toyopearl column (15.8×2.2 cm) equilibrated with buffer A. The enzyme was eluted at a flow rate of 90 ml/h with a 1.2 l linear 0–0.5 M NaCl gradient in buffer A. The active fractions (4280 U, 1.02 mg protein) eluting at 0.24 M NaCl were concentrated and subjected to gel filtration on a TSK G3000SW column (60×2.15 cm) equilibrated with 0.3 M NaCl in buffer B at a flow rate of 1.0 ml/min, using a Tosoh HPLC system. The pooled active fractions (3630 U, 0.679 mg protein) were concentrated, desalted by ultrafiltration and stored at −80°C.

*2.5. Purification of $Mn^{2+}$-independent PP2A (CA)*

Purification of CA was performed using buffer solution without EDTA. The preparation of the cytosol, chromatography on DEAE-Sephadex and DEAE-Toyopearl were performed as described above. The active fractions (11 400 units, 363 mg protein) eluting at 0.18 M NaCl from a DEAE-Toyopearl column were concentrated and applied to an AH-Sepharose 4B column (17×1.5 cm) equilibrated with 0.25 M NaCl in buffer A. The column was washed with 60 ml of the buffer and the enzyme was eluted at a flow rate of 50 ml/h with a 700 ml linear 0.25–1.2 M NaCl gradient in the buffer. The active fractions (4980 U, 46.8 mg protein, 75 ml) eluting at 0.47 M NaCl were dialyzed against 2 l of the same buffer for 6 h and loaded onto an H1 histone-Toyopearl column (4.0×1.5 cm) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 20 ml of the buffer and the enzyme was eluted at a flow rate of 10 ml/h with a 140 ml linear 0.1–0.4 M NaCl gradient in the buffer. The active fractions (3550 U, 9.99 mg protein, 22 ml) eluting at 0.15 M NaCl were mixed with 12 ml of buffer A and re-chromatographed on an H1 histone-Toyopearl column. The active fractions (2910 U, 3.71 mg protein) eluting at 0.19 M NaCl were subjected to a protamine-Toyopearl column (4.0×1.5 cm) equilibrated with 0.1 M NaCl in buffer A. The column was washed with 20 ml of the buffer and the enzyme was eluted at a flow rate of 20 ml/h with a 300 ml linear 0.1–1.5 M NaCl gradient in the buffer. The active fractions eluting at 0.8 M NaCl were concentrated and desalted by ultrafiltration. The purified enzyme had a specific activity of 1530 U/mg protein, nearly equivalent to that of enzyme purified in the presence of 1 mM EDTA as described previously [8], and was stored at −80°C.

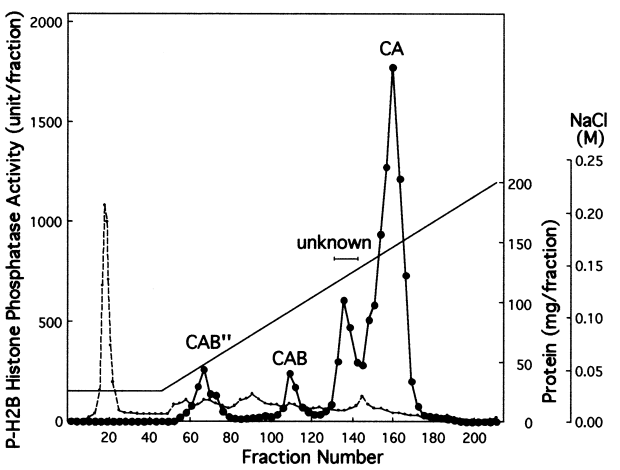


Fig. 1. DEAE-Toyopearl elution profile of P-H2B histone phosphatase activity in human erythrocyte cytosol. The column chromatography, and assays for P-H2B histone phosphatase activity (●) and for protein (·) were performed as described under Section 2.

*2.6. Preparation of C, C′, A and A′*

4.1 Volumes (v/v) of ice-cold 99.5% (v/v) ethanol were added to CA (629 μg, 1.0 ml) or C′A′ (57 μg, 0.2 ml) on ice. After standing for 5 min, the precipitate was collected by centrifugation at 15 000×*g* for 10 min at 4°C. C and C′ were extracted from the precipitate with buffer A without EDTA by homogenization with a Dounce homogenizer followed by centrifugation. The supernatant was dialyzed against the buffer and stored at −80°C. A and A′ were prepared by treatment of CA or C′A′ (60 μg) with 6 M urea in 1 ml of buffer A without EDTA, followed by gel filtration on a Superdex 200 column (118×1.0 cm) equipped with a continuous flow counter current dialyzing apparatus for the removal of urea as described [17]. The purified A and A′ migrated as a single 63 kDa protein band on SDS-PAGE, respectively, and were stored at −80°C.

*2.7. Metal analysis*

The enzymes (C′A′ or CA, 0.10 ml, 0.10–0.38 mg/ml) in Teflon beakers were wet ashed with sulfuric acid, nitric acid and hydrogen peroxide [18]. Metal contents were determined by atomic absorption spectrophotometry using a Hitachi model Z8000 spectrophotometer.

## 3. Results and discussion

When human erythrocyte cytosol was applied to column chromatography on DEAE-Toyopearl, the PP activity toward P-H2B histone was eluted into four peaks as shown in Fig. 1. Three different forms of PP2A in the first, second and fourth peak were separately purified and their subunit structures were determined to be CAB'', CAB and CA, respectively [8]. The protein phosphatase in the third peak designated unknown in Fig. 1 was purified by successive column chromatographies on Sephadex G-150, AH-Sepharose, H1 histone-Toyopearl and TSK G3000SW. The purified unknown phosphatase had a specific activity of 5340 U/mg protein and was purified 17 000-fold from the cytosol fraction with an overall yield of 1.8%. On non-denaturing PAGE, the purified enzyme migrated as one main protein band, which corresponded to PP activity (data not shown).

The purified unknown phosphatase preferentially dephosphorylated the α subunit of phosphorylase kinase, which was phosphorylated at both the α and β subunits by cAMP-dependent protein kinase (PKA). In addition, phosphorylase phosphatase activity of the unknown phosphatase was unaffected by inhibitor 2 under the conditions in which the activity of rabbit skeletal muscle PP1 was strongly inhibited (data not shown). Furthermore, the enzyme was very sensitive to okadaic acid. Using phosphorylase *a* as substrate, the $IC_{50}$ value of okadaic acid for the unknown phosphatase

was 8.2 nM. These results classify the purified phosphatase into PP2A.

The Stokes radius of the purified PP2A was determined by gel filtration on Sephadex G-150 to be 4.2 ± 0.1 (mean ± S.E.M., $n = 3$) nm. The $s_{20,w}$ value of the enzyme was measured by sucrose density gradient centrifugation analysis to be 5.7 ± 0.1 (mean ± S.E.M., $n = 3$) S. From these parameters, the apparent molecular weight of the purified PP2A was calculated to be 103 000 as described [19].

The SDS-PAGE of the purified enzyme yielded two Coomassie blue stained bands of 63 and 34 kDa and were tentatively referred to as A′ and C′, respectively (Fig. 2A). The 34 kDa C′ subunit had the same mobility on SDS-PAGE and displayed the same V8- and papain-peptide maps as those of the 34 kDa catalytic C subunit of conventional $Mn^{2+}$-independent PP2A (Fig. 2A and B). Likewise, the 63 kDa A′ subunit showed the same mobility on SDS-PAGE and the same peptide maps as those of the 63 kDa regulatory A subunit of conventional PP2A (Fig. 2A and B). Assuming that Coomassie blue staining of each subunit was equal, the molar ratio of C′ and A′ estimated from the densitometric scan of the stained gel was 1.00: 0.97. The sum of the molecular masses of C′ and A′ was in good agreement with the molecular weight of 103 000 for the purified enzyme, indicating that the subunit structure of the purified PP2A was C′A′.

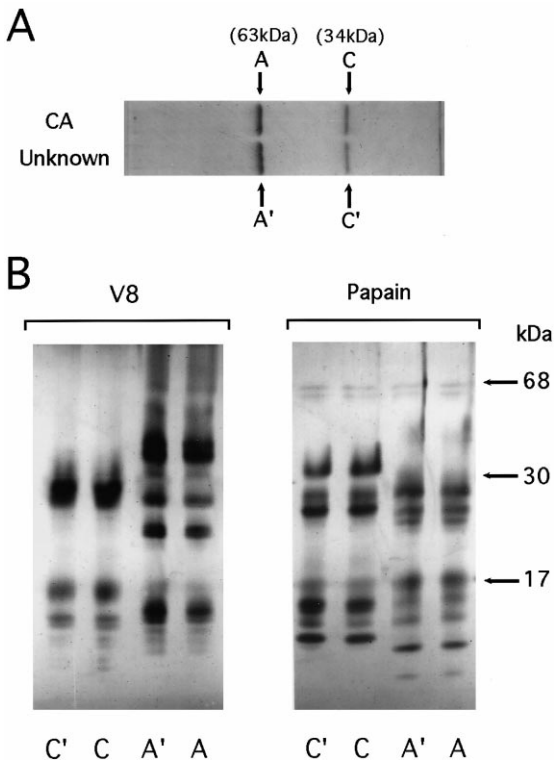


Fig. 2. SDS-PAGE of the unknown phosphatase purified from human erythrocytes and peptide maps of the subunits. A: The purified unknown phosphatase and CA (1.1 μg protein each) were applied to 10% SDS-PAGE and detected by Coomassie blue staining as described [8]. B: The stained bands of C′, C, A′ and A were cut out and applied to 15% SDS-PAGE with 0.20 μg/lane V8 protease or 0.025 μg/lane papain. Proteolytic digestion was then allowed to take place for 30 min in the stacking gel as described [20]. After electrophoresis, peptide bands were visualized by silver staining [21]. Bovine serum albumin (BSA) (68 kDa), carbonic anhydrase (30 kDa) and myoglobin (17 kDa) were used as standards.

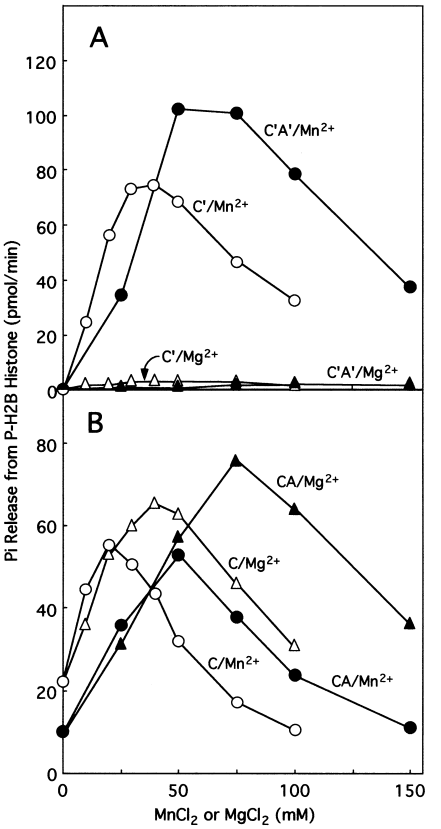


Fig. 3. $Mn^{2+}$ and $Mg^{2+}$ requirement for PP activity of C′A′, C′, CA and C. The rate of Pi release from P-H2B histone was measured as described under Section 2 except that 250 mM NaCl was excluded and 25 mM $MnCl_2$ was replaced by the indicated concentration of $MnCl_2$ (●, ○) or $MgCl_2$ (▲, △). A: C′A′ (1.7 U/ml) (●, ▲) and C′ (1.3 units/ml) (○, △) were used. B: CA (1.3 U/ml) (●, ▲) and C (0.58 U/ml) (○, △) were used.

Although the physicochemical properties of C′A′ so far tested were indistinguishable from those of CA, the metal requirement of C′A′ was clearly distinct from that of CA. $Mn^{2+}$ was essential for the P-H2B histone phosphatase activity of C′A′ (Fig. 3A). $Mn^{2+}$ could not be replaced by $Mg^{2+}$ (Fig. 3A). By contrast, CA, the conventional PP2A showed the intrinsic PP activity without $Mn^{2+}$ (Fig. 3B). The intrinsic PP activity was stimulated by $Mn^{2+}$ or $Mg^{2+}$ (Fig. 3B). The $Mn^{2+}$ requirement for C′A′ activity was also observed when phosphorylase *a*, phosphorylase kinase *a* and P-H1 histone were used as substrates (data not shown).

To know whether the $Mn^{2+}$ requirement for C′A′ activity is attributable to the catalytic subunit C′ or the regulatory subunit A′ or both, C′ was isolated by treatment of C′A′ with 80% ethanol [22]. C′ thus obtained showed a $Mn^{2+}$ requirement for activity as observed with C′A′ (Fig. 3A). On the other hand, the catalytic subunit C prepared by the 80% ethanol treatment of CA, showed $Mn^{2+}$ and $Mg^{2+}$ curves similar to those of CA (Fig. 3B). However, the optimal concentrations of $Mn^{2+}$ and $Mg^{2+}$ for C′ and C were lower than those for C′A′ and CA, respectively (Fig. 3). To test the effect of the regulatory subunits A′ and A on the divalent cation requirement of C′ and C, A′ and A were isolated separately by gel filtration of C′A′ and CA in the presence of 6 M urea [17]. Reconstruction of heterodimeric forms of PP2A clearly showed that neither A′ nor A had any effect on the $Mg^{2+}$

and $Mn^{2+}$ requirement of C′ and C (Table 1). Reconstruction of heterodimeric forms of PP2A was confirmed by the shift of the activity peak on Sephadex G-150 gel filtration (data not shown) and the inhibitory effect of A′ and A on the PP activity of C′ and C (Table 1). These results indicate that the difference in the divalent cation requirement of the heterodimeric forms of PP2A is attributable to the catalytic subunits, C′ and C.

It has been suggested from the predicted primary structure that the catalytic subunit of PP2A may contain two metal ions at the active site, possibly zinc and/or iron [23]. To test this possibility, zinc, iron, manganese, magnesium and cobalt in CA and C′A′ were analyzed by atomic absorption spectrophotometry. The metal analysis of CA showed that 1 mol CA contains $0.87 \pm 0.12$ (mean ± S.E.M., $n = 3$) mol zinc and $0.35 \pm 0.18$ (mean ± S.E.M., $n = 3$) mol iron but no manganese, cobalt or magnesium. On the other hand, such metals were not detected in C′A′. These results support the notion that the catalytic subunit C in the conventional form of PP2A is a zinc- and iron-metalloenzyme and is transformed into C′ by removal of the metals. PP2B has been shown to contain one zinc atom and one iron atom per catalytic subunit [7]. The two-metal active site in the crystal structures of the catalytic subunits of PP1 [3,4] and PP2B [5,6] explains the function of a set of highly conserved residues and should lead to the elucidation of the reaction mechanism shared by the structural family of metallo-phospho-esterases including PP2A and purple acid phosphatase. The crystal structure of a PP1 catalytic subunit also showed the surface location of the metal binding sites suggesting that the metals could be labile, which might explain why they are difficult to identify biophysically [24]. These results may explain why substoichiometric iron was detected in CA.

To know whether or not C′ is an apoenzyme of C, the effect of pre-incubation of C′ and C with $Zn^{2+}$, $Fe^{2+}$ and $Fe^{3+}$ in the presence of 1 mM ascorbate on their $Mn^{2+}$-independent phosphorylase phosphatase activity was investigated (Table 2). The $Mn^{2+}$-independent PP activity of C′ was stimulated 2–3-fold by pre-incubation with 5 μM $ZnCl_2$ or 15 μM $FeCl_2$, but only slightly stimulated by pre-incubation with 15 μM $FeCl_3$. A synergistic stimulatory effect on the $Mn^{2+}$-independent PP activity was observed when C′ was pre-incubated with $ZnCl_2$ and $FeCl_2$, but not with $ZnCl_2$ and $FeCl_3$ (Table 2). The PP activity of $Zn^{2+}/Fe^{2+}$-activated C′ was 16% of that of $Mn^{2+}$-activated C′. On the other hand, the same pre-incubation of C with these metal ions showed no effect on the $Mn^{2+}$-independent PP activity (Table 2). These results suggest that C is a $Zn^{2+}$- and $Fe^{2+}$-metalloenzyme and that C′ is the apoenzyme.

Ingebritsen et al. [25] reported that PP2A in fresh skeletal muscle or liver extracts was variably stimulated by $Mn^{2+}$. They further noted that during purifications, PP2A became more dependent upon $Mn^{2+}$ for its activity. Highly purified bovine heart PP2A catalytic subunit lost virtually all of its activity and became $Mn^{2+}$- or $Co^{2+}$-dependent during storage at −70°C for 6 years [26]. Yu [27] also obtained $Mn^{2+}$- and $Co^{2+}$-dependent PP2A after prolonged storage of PP2A and observed the activation of the latent form of PP2A by the addition of $Fe^{2+}$ and reducing agents such as ascorbate or DTT to the reaction mixture, suggesting that $Fe^{2+}$ could be a biologically important cofactor responsible for PP2A activation. These phenomena may also be explained by the notion that the $Mn^{2+}$-independent catalytic subunit C of PP2A is a metalloenzyme and converts to $Mn^{2+}$-dependent catalytic subunit C′ by removal of the bound metals. Since freshly prepared human erythrocyte cytosol showed the C′A′/CA ratio in the DEAE-Toyopearl elution profile (Fig. 1) to be $8.1 \pm 0.4$ (mean ± S.E.M., $n = 3$)/100 even in the absence of EDTA, the interconversion of C′A′ and CA in the cell may have the physioligical significance which remains to be explored.

Table 1
$Mg^{2+}$ and $Mn^{2+}$ requirement for PP activity of catalytic subunits and reconstituted holoenzymes of PP2A

| Subunit | PP activity (pmol Pi released/min) in the presence of | | |
|---|---|---|---|
| | None | $Mg^{2+}$ | $Mn^{2+}$ |
| C′ | 0 | 0 | 22 |
| C′+A′ | 0 | 0 | 11 |
| C′+A | 0 | 0 | 10 |
| C | 38 | 84 | 40 |
| C+A′ | 24 | 60 | 31 |
| C+A | 26 | 58 | 31 |

C′ (0.13 μg protein, 0.31 U) and C (0.13 μg protein, 0.78 U) were pre-incubated at 0°C for 10 min with or without A′ and A (0.24 μg protein each) in 80 μl of 50 mM Tris-HCl, pH 7.4, 0.5 mM DTT, 0.005% Triton X-100, 10% glycerol and protease inhibitors. The rate of Pi release from P-H2B histone was measured with 5 μl of the pre-incubation mixture as described under Section 2 except that 250 mM NaCl and 25 mM $MnCl_2$ were excluded or replaced with 50 mM $MgCl_2$ or $MnCl_2$.

Table 2
Effect of zinc and iron pre-incubation of C′ and C on their phosphorylase phosphatase activity

| Preincubated with | Phosphorylase phosphatase activity (pmol Pi released/min) of | |
|---|---|---|
| | C′ | C |
| None | 0.066 | 5.74 |
| $ZnCl_2$ | 0.117 | 5.82 |
| $FeCl_2$ | 0.183 | 5.81 |
| $FeCl_3$ | 0.094 | 5.77 |
| $ZnCl_2$+$FeCl_2$ | 0.508 | 5.97 |
| $ZnCl_2$+$FeCl_3$ | 0.168 | 5.90 |

C′ and C (0.049 U each) were pre-incubated at 30°C for 15 min with 5 μM zinc and/or 15 μM iron chlorides in 12 μl of 50 mM MOPS-NaOH, pH 7.0, 0.5 mM DTT, 1 mM ascorbate, 150 mM KCl and 0.5 mg/ml BSA and their phosphorylase phosphatase activity was measured as described under Section 2 in a 50 μl mixture containing 45 mM imidazole-HCl, pH 7.4, 1 mM DTT, 6 mM theophylline, 0.5 mg/ml BSA, 1 mM ascorbate, 2 μM $^{32}$P-phosphorylase *a* and 5 μl of the pre-incubation mixture containing C′ which released 3.17 pmol Pi per minute in the presence of 5 mM $MnCl_2$.

*Acknowledgements:* We are grateful to Dr Jean Lucas-Lenard for her critical reading of the manuscript and to Dr Shigeki Takemori for his discussion. We also thank Mieko Kawamura and Ryoko Takemoto for their secretarial and technical assistance. This work was supported in part by grants-in-aid for Cancer Research and Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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