

Kinetic Study on Dephosphorylation of Myelin Basic Protein by Some Protein Phosphatases

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The dephosphorylation specificity of protein phosphatase 2A (PP2A), calcineurin (PP2B) and protein phosphatase 2C (PP2C) were studied *in vitro* using myelin basic protein (MBP) as a model substrate which was fully phosphorylated at multiple sites by protein kinase C (PKC) or cyclic AMP-dependent protein kinase (PKA). In order to determine the site specificity of phosphatases in myelin basic protein, the protein was digested with trypsin and the radioactive phosphopeptide fragments were isolated by high performance liquid chromatography (HPLC) on reversed-phase column. Subsequent analysis and/or sequential manual Edman degradation of the purified phosphopeptides revealed that Thr-65 and Ser-115 were most extensively phosphorylated by PKA and Ser-55 by PKC. For the dephosphorylation kinetics, the phosphorylated MBP was treated with calcineurin or PP2C with various time intervals and the reaction was terminated by direct tryptic digest. Both Thr-65 and Ser-115 residues were dephosphorylated more rapidly than any other ones by phosphatases. However it can be differentiated further by first-order kinetics that the PP2B dephosphorylated both Thr-65 and Ser-115 with almost same manner, whereas PP2C dephosphorylated somewhat preferentially the Ser-115.

Introduction

The myelin basic protein (MBP) is an excellent substrate for phosphorylation by several protein serine/threonine kinases,¹ such as cyclic AMP-dependent protein kinase (PKA),² a calcium-calmodulin dependent protein kinase,³ and a calcium-phospholipid dependent protein kinase (PKC).⁴ Although several favored phosphorylation sites have been identified, they are quite controversial.⁵⁻⁹ Recently, immunological method, which employed monoclonal antibody with specificity for a known phosphorylation site in MBP, was used to localize MBP phosphorylation.¹⁰

Where there is a protein kinase, there is a protein phosphatase. Protein phosphatases, in combination with protein kinases, are presumed to play crucial roles in regulating the level of phosphorylation of the major proteins involved in the control of general metabolism. They fall into approximately three classes according to the amino acid residues where the dephosphorylation occurs: protein serine/threonine phosphatase removes phosphates from both serine and threonine residues.^{11,12} Protein tyrosine phosphatase dephosphorylates phosphotyrosine residues. Dual-specific phosphatase can do both tyrosine and serine/threonine residues. A newly characterized protein tyrosine phosphatase, also known as mitogen-activated protein kinase (MAP kinase) phosphatase, turned out to be a dual-specific phosphatase by dephosphorylating critical tyrosine and threonine residues.¹³

The majority of all serine/threonine-specific protein phosphatases have been divided into four major groups, PP1, PP2A, PP2B, and PP2C.¹⁴ PP1 and PP2A are known to dephosphorylate many protein *in vitro* and are likely to have pleiotropic actions *in vivo*. PP1 is involved in the control of glycogen metabolism and muscle contractility,¹⁵ whereas PP2A regulates enzymes involved in glycolysis, lipid metabolism and catecholamine synthesis.¹⁵ It had been shown

that PP1 is a metalloprotein¹⁶ and crystal structure of PP1 was recently described.^{17,18} Calcineurin (PP2B) is the major calcium/calmodulin dependent protein phosphatase which largely resides in neuronal tissue. The specificity of PP2B is much more restricted than PP1, PP2A, and PP2C and the most effective substrates so far identified are proteins that regulate other protein kinases and phosphatases.¹⁹ A role for PP2B in the regulation of glutamate release had been presented.²⁰ PP2C also dephosphorylates many proteins *in vitro*.¹² It had been implicated that the phosphorylation of microtubule-associated protein tau by PP1 and PP2C is related with Alzheimer disease.²¹

The kinetic properties of those protein phosphatases have been studied: dephosphorylation of PKA regulatory subunit by PP2B;²² rat brain Na⁺ channel α subunit by PP1, PP2A and PP2B;²³ synthetic phosphopeptides by PP2A,^{24,25} PP2B²⁶ and PP2C.²⁷ For all of those experiments, however, just whole substrates were dephosphorylated in kinetic study without investigating the site specificity or synthetic/purified peptide fragments were used for the site-specific dephosphorylation kinetic study, which implies that there was no way to see what was really happened to intact substrates.

In this paper, the site specificity of dephosphorylation of bovine MBP phosphorylated exhaustively by PKA was scrutinized by comparing two types of protein phosphatases, PP2B and PP2C. Both site specificity and natural property of intact substrate were preserved throughout the experiment by direct digesting method. Covering defects of synthetic/resolved peptides, this approach showed a reasonable methodology in site-specific protein phosphorylation/dephosphorylation kinetics.

Experimental

Materials. [γ -³²P]ATP was obtained from DuPont-New England Nuclear. CM-cellulose, DEAE-cellulose, Sephacryl

S-200, Sephadex G-25, histone agarose, sequencing grade porcine trypsin, calcineurin, calmodulin, and catalytic subunit of protein kinase (PKA) were purchased from Sigma. Edman degradation reaction membrane (GEN 920033) and coupling buffer (GEN 920020) were from Millipore. Phenylisothiocyanate (PITC) was from Janssen Chimica. Trifluoroacetic acid (TFA) was from Merck. All other chemicals used were of analytical or HPLC grade.

Preparation of Bovine MBP. MBP was isolated from bovine brain essentially according to the methods of Oshiro and Eylar with slight modifications.²⁸ In brief, fresh 500 g bovine brain was homogenized and delipidated in cold Folch solution (chloroform/methanol 2 : 1). About three hours later this solution was filtered through Whatman No. 41 paper followed by acid extraction of residual protein by adding HCl solution down to pH 2. After centrifugation at 10,000 x g for about 20 minutes, the supernatant of this solution was neutralized with 15 N NH₄OH up to pH 5.5. The neutral solution was then sedimented as 50% ammonium sulfate saturation was made. This precipitate was collected by centrifugation and treated with acidified acetone, which produced flocculent precipitate. After gathered by centrifugation, this product was lyophilized and loaded to CM-cellulose column with gradient of 50 mM-1 M NaCl. About 1 g of MBP was isolated from 500 g of bovine brain. Purified MBP showed a single band on SDS-PAGE with 18.5 kd in size.

Assay of PP2C. Radioactive phospho-labeled [³²P]MBP by PKA, instead of [³²P] casein, a typical non-specific substrate for PP2C,²⁹ was engaged in PP2C assay with the same condition as for [³²P] casein in 0.1 mL containing 50 mM Tris-HCl (pH 7.9), 0.1 mM EGTA, 10 mM MgCl₂, 5 mM 2-mercaptoethanol and 250 nM okadaic acid. Enzyme source and substrate were incubated together for 10 min and the reaction was stopped by addition of 70 μL of 30% trichloroacetic acid and 30 μL of bovine serum albumin (4 mg/mL). After centrifugation the upper soluble part was decanted in order to assay the radioactive phosphate released from MBP by organic extraction method.³⁰

Purification of PP2C. All procedures were based on the previously described method except a few steps.²⁹ In essence, four rat livers were homogenized in 40 mL buffer containing 60 mM Tris-HCl (pH 7.4), 6 mM EDTA, and 0.25 M sucrose. The homogenate was centrifuged and the supernatant was subjected to DEAE-cellulose equilibrated with buffer A containing 20 mM Tris-HCl (pH 7.4), 0.1 mM EGTA, 5 mM 2-mercaptoethanol, 50 mM NaCl, and 2% glycerol. Gradient profile, from 50 mM to 850 mM NaCl, was maintained throughout the column work. Active fractions were pooled together and ammonium sulfate was added until the solution reached to 33% saturation. After centrifugation the supernatant was fractionated again by adjusting the saturation to 60% ammonium sulfate. Precipitated protein was dissolved in buffer A, then filtered and loaded onto Sephacryl S-200. The active part was subjected to second DEAE-cellulose column equilibrated with buffer A containing extra 10 mM MgCl₂, which was kept within every buffer used afterwards. The active fractions, after dialysis against water to eliminate salts, applied to histone agarose for the final purification step. About 0.2 mg of PP2C was purified from four rat livers. The PP2C appeared as

a single band in 43 kd region on SDS-PAGE.

Phosphorylation of MBP As Substrate. Four mg MBP was phosphorylated by PKA (80U) for 6 hr at 30 °C in 0.15 mL solution containing 0.1 mM [γ -³²P]ATP (10 μCi), 50 mM Tris-HCl (pH 6.9), 0.1 mM EGTA, 0.1% 2-mercaptoethanol, and 10 mM MgCl₂. The protein was recovered and simultaneously the reaction was ended by directly pouring into Sephadex G-25 column with flow rate of 50 μL/min. [³²P]MBP was aliquoted and stored in freezer for the later use.

Time Course of Dephosphorylation of MBP. The 250 μg [³²P]MBP was dephosphorylated by 10 units of PP2B (about 4.5 μg) in 0.16 mL reaction buffer (50 mM Tris-HCl of pH 7.4, 0.5 mM dithiothreitol, 10 mM MgCl₂, 50 units of calmodulin, and 1 mM CaCl₂) or by 3.5 μg of PP2C at 30 °C as in assay condition for various time periods. Enzymes were activated by incubation at 30 °C for 10 min in reaction buffer and then added to the substrate incubated at same temperature. Dephosphorylation was stopped by simply initiating the tryptic digestion.

Peptide Maps. The phosphorylated/dephosphorylated MBP as described above was subjected to the trypsin digestion: For each 250 μg MBP aliquot, 10 μg of sequencing grade trypsin was employed in ammonium bicarbonate buffer (pH 8.0) containing 5 mM CaCl₂ for 18 hr at 37 °C. The digest was lyophilized, taken with a small volume of 0.1% TFA, applied to a reversed-phase C18 Vydac column in 0.1% TFA in Milli-QTM water, and eluted with a gradient of 0-25% acetonitrile containing 0.1% TFA at a flow rate of 0.75 mL/min for 60 min. The radioactive peptides were collected and subjected to automated amino acid sequence analyzer or manual Edman degradation.

Amino Acid Sequence Analysis And Determination of Phosphorylation Site. The amino acid sequence analysis of the peptides isolated from tryptic digests of [³²P]MBP was performed on an Applied Biosystems model 473A Sequencer. Radiosequencing of the multiple phosphorylation sites of the phosphopeptide isolated from tryptic digest was performed by sequential manual Edman degradation fundamentally based on the methods of Laursen and Machleidt.³¹ In brief, 60 μL aliquots of phosphopeptide peak fractions were incubated with Sequelon-AATM reaction membrane at 56 °C for 20 min and then freshly prepared 5 μL of carbodiimide solution in Attachment Buffer (10 mg/mL) was applied to the dry disk in ambient temperature followed by washing with methanol and deionized water. The membrane was next incubated with 100 μL of PITC at 56 °C for 5 min, followed by incubation with 100 μL of coupling buffer at 56 °C for 15 min. The protected N-terminal amino acid was then cleaved from the membrane-linked peptides by 100 μL of TFA at 56 °C for 10 min. At each reaction cycle, the TFA extracts were subjected to ³²P counting in liquid scintillation counter for determination of the phosphorylation site. The original numbering system for the amino acid sequence of bovine MBP³² was used throughout the present studies although this sequence has been later modified.³²

Results and Discussion

Phosphorylation Site Identification. The incorpo-

ration rate of phosphate in [32 P]MBP phosphorylated by PKA as described previously in Experimental was usually 70%. Tryptic digests of [32 P]MBP were resolved into about 25 peptide peaks (Figure 1) and two major phosphopeptide peaks (Figure 2), designated as P4 and P9 on C18 reversed-phase column chromatography using a LKB Bromma HPLC system. Other peaks had a tendency to increase in area only when the trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK) was used. Previous report showed that [32 P]MBP exhaustively phosphorylated by PKA was resolved into at least 15 major phosphopeptide peaks including products of incomplete digestion when the TPCK-treated trypsin was used.⁶ This result points out that both quality and capacity of trypsin is of importance for precise peptide mapping.

Amino acid sequence analysis of two major phosphopeptide peak fractions of P4 and P9 revealed that their amino acid sequences as 65-TTHYGSLPQK and 114-FSWGAEQK respectively. The Ser-115 site agreed exactly

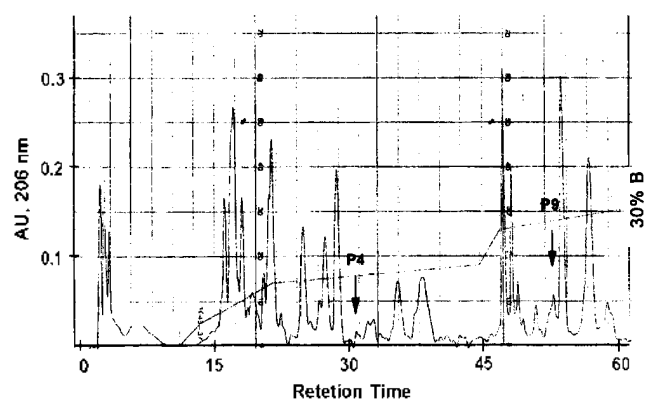


Figure 1. Tryptic digest pattern of MBP on reversed-phase C18 column chromatography. Gradient was modified for faster elution and better resolution of phosphopeptides with flow rate of 0.75 ml/min for 60 min. P4 and P9 represent two major phosphopeptides.

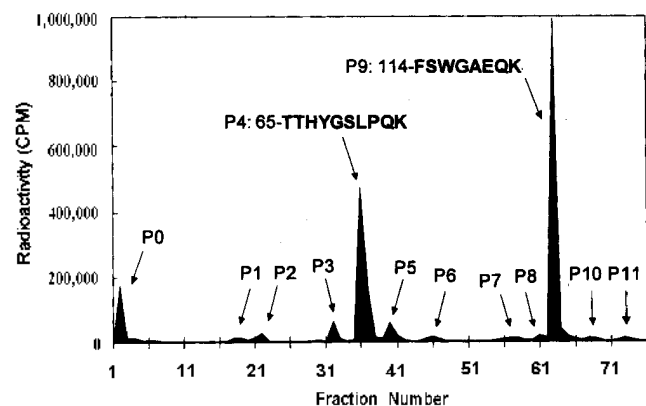


Figure 2. Separation of tryptic digests of [32 P]MBP phosphorylated by PKA on C18 reversed-phase chromatography. MBP was exhaustively 32 P-phosphorylated by 80 units of the kinase and then subjected to complete trypsin digestion. P0 represents inorganic phosphates which tailed after in recovery step using Sephadex G-25. Amino acid sequence of P4 and P9, the two major phosphopeptides, were analyzed.

to the previous report of the preferable site for PKA⁶ or PKC.⁷ However P4 contained three phosphorylation sites and did not report any phosphorylating site so far. Thus further identification was performed for P4 fraction by using sequential manual Edman degradation (Figure 3). Phosphopeptide P4 gave forth radioactivity in first cycle which indicated that Thr-65 was the site of phosphorylation. In order to substantiate the Ser-115 phosphorylation site, manual Edman degradation reaction was also carried out for P9 phosphopeptide. In similar approach the Ser-55 was the most favorable phosphorylating site for PKC.²⁴

Time Course of Dephosphorylation of MBP. Previous studies on the time course of dephosphorylation/phosphorylation of phosphatases/kinases have been performed without preservation of the original configuration of a substrate by using synthetic or resolved phosphopeptides before treating with kinases or phosphatases when examining the site specificities.^{6,23-26} Since the protein folding itself, apart from the folding due to disulfide bonds, can surely affect the nature and the susceptibility of protein toward phosphorylation and dephosphorylation, it is a crucial point to maintain the original configuration of intact protein when site-specific kinetics is to be investigated. Furthermore, frequently the peptides that have shown promise as phosphatase substrates were much larger than those typically recognized by protein kinases. This implies that either the primary sequence scanned by phosphatases is larger than that typically scanned by kinases, or that greater length is required to support the formation of higher order structures required for recognition. Thus site-specific dephosphorylation kinetic study, compared to phosphorylation kinetics, is likely more sensitive to tertiary structure of substrates.

In order to verify that there were no further phosphatase activities right after trypsin treatment in the incubation periods, MBP was tryptic-digested with and without phos-

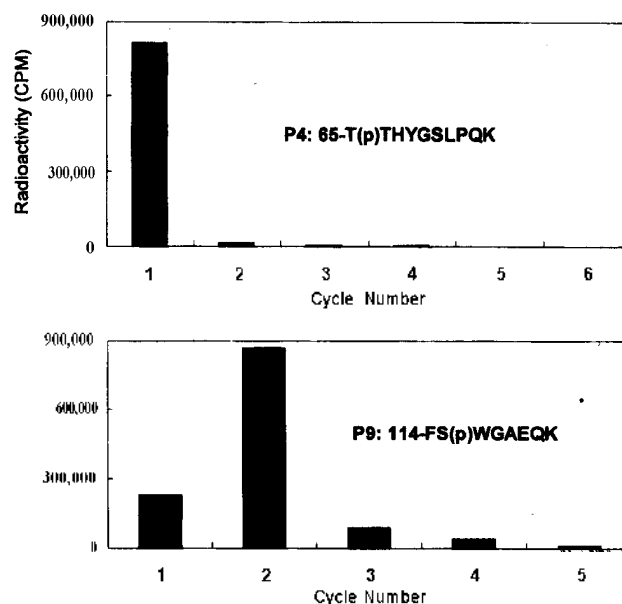


Figure 3. Phosphorylation site determination by sequential manual Edman degradation. In P4, Thr-65 was exclusively phosphorylated. P9 was also analyzed to corroborate phosphorylation site as Ser-115.

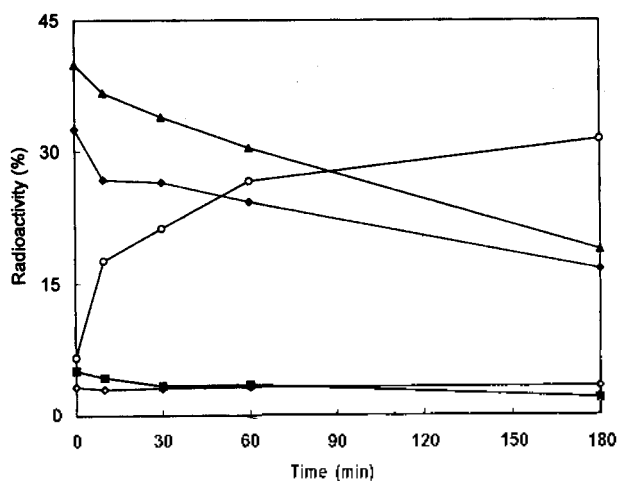


Figure 4. Time course of dephosphorylation of major peaks. [32 P]MBP was dephosphorylated by PP2B for various time periods. P0 (O), P3 (◇), P4 (●), P5 (■), P9 (▲). P0, unfettered inorganic phosphates, increased as time went by whereas others fell down.

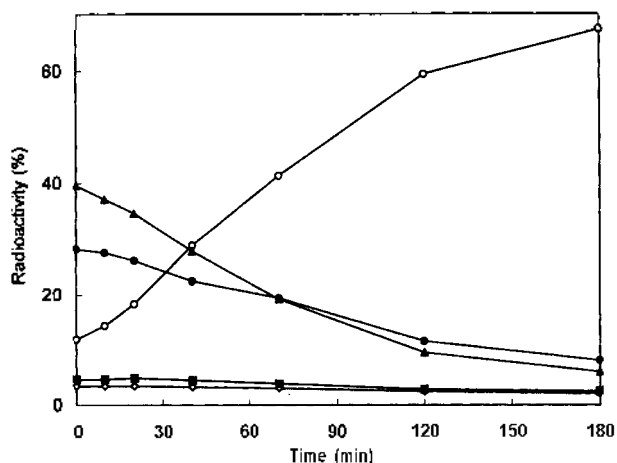


Figure 5. Time course of dephosphorylation of major peaks. [32 P]MBP was dephosphorylated by PP2C for various time periods. P0 (O), P3 (◇), P4 (●), P5 (■), P9 (▲).

phatases for 18 hr. which did not show any differences in the amount of liberated inorganic phosphates between them (data not shown). Based on this result, [32 P]MBP was treated with PP2B and PP2C for various time periods as described in Experimental Procedures. Synchronized profiles of time course of dephosphorylation are represented in Figure 4 and Figure 5 for PP2B and PP2C, respectively. In every chromatographic run, P0-P9 were eluted almost at the same individual retention times on reversed-phase column. For the peak area calculation, 3-5 points corresponding to each peak were added up and then divided by the total radioactivity to give percent radioactivity. Usually the four major phosphopeptides (P3, P4, P5, and P9) and emancipated phosphates (P0) occupied majority of the total phosphorylation rate of MBP up to ~90%. In Figure 4, PP2B appeared to dephosphorylate both P4 and P9 with almost same velocity, whereas, in Figure 5, P9 was more favored than P4 by PP2C.

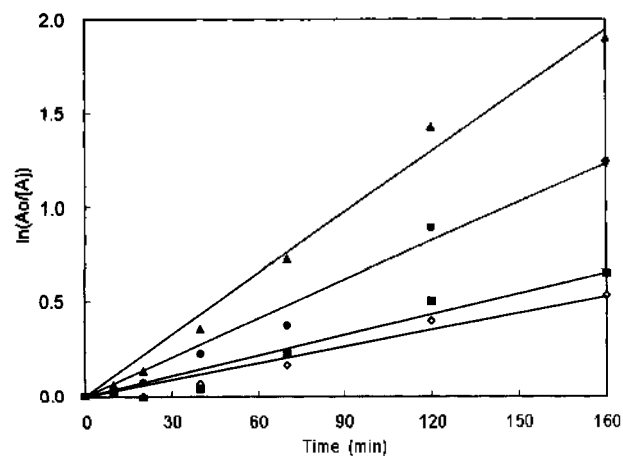


Figure 6. Logarithmic calculation of time course of dephosphorylation of [32 P]MBP by purified PP2C. [A] stands for the concentration of Ser Thr-P; A0 is the concentration of A at zero time. P0 (O), P3 (◇), P4 (●), P5 (■), P9 (▲).

To elicit kinetic terms, those data were manipulated for first-order kinetics. In order to examine whether the dephosphorylation reaction really was first-order reaction, linear regression values (R^2) were calculated for PP2C case (Figure 6). The R^2 values of P4 and P9 were regularly around 0.9 for first-order calculation whereas the values were far below than 0.9 for the second-order calculation (data not shown). The ratio of first-order rate constants of dephosphorylation for P4 and P9 in case of PP2B was 0.93 ($k_{p4}=0.0039$, $R^2=0.853$; $k_{p9}=0.0042$, $R^2=0.989$) while in the case of PP2C the ratio was 0.39 ($k_{p4}=0.0042$, $R^2=0.907$; $k_{p9}=0.0108$, $R^2=0.989$).

Donella-Deana *et al.*,²⁶ reported that an extended N-terminal stretch appeared to be necessary, albeit not sufficient, condition for an optimal dephosphorylation of both phosphoserine and phosphotyrosine peptides by PP2B. This finding corroborates the view that higher-order structure is an important determinant for the substrate specificity of PP2B. The specificity of PP2C towards phosphopeptides appeared to be narrower than that of PP2B and PP2A with a striking preference for phosphothreonyl peptides over their phosphoserine homologues.²⁷ The results obtained however were not so clear-cut and did not disclose any obvious correlation between structural features responsible for the dephosphorylation of physiological protein substrates.

In this study, inconsonant with the previous results obtained using synthetic peptides, phosphoserine group in P9 phosphopeptide was more favored by PP2C than by PP2B. Because this work, concerning with both site-specificity and preservation of intact substrate, exhibited a reasonable and distinct approach, it is now possible to investigate the site-specific dephosphorylation/phosphorylation kinetics more precisely.

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