Purification and Characterization of Two Potent Heat-Stable Protein Inhibitors of Protein Phosphatase 2A from Bovine Kidney†

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ABSTRACT: Two heat-stable protein inhibitors of protein phosphatase 2A (PP2A), tentatively designated I_1^{PP2A} and I_2^{PP2A}, have been purified to apparent homogeneity from extracts of bovine kidney. The purified preparations of I_1^{PP2A} exhibited an apparent M_r ~30 000 and 250 000 as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and gel permeation chromatography on Sephacryl S-300, respectively. In contrast, the purified preparations of I_2^{PP2A} exhibited an apparent M_r ~20 000 and 80 000 as determined by sodium dodecyl sulfate−polyacrylamide gel electrophoresis and gel permeation chromatography on Sephacryl S-200, respectively. The purified preparations of I_1^{PP2A} and I_2^{PP2A} inhibited PP2A with ^{32}P-labeled myelin basic protein, ^{32}P-labeled histone H1, ^{32}P-labeled pyruvate dehydrogenase complex, ^{32}P-labeled phosphorylase, and protamine kinase as substrates. By contrast, I_1^{PP2A} and I_2^{PP2A} exhibited little effect, if any, on the activity of PP2A with ^{32}P-labeled casein, and did not prevent the autodephosphorylation of PP2A in incubations with the autophosphorylation-activated protein kinase [Guo, H., & Damuni, Z. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90,* 2500−2504]. The purified preparations of I_1^{PP2A} and I_2^{PP2A} had little effect, if any, on the activities of protein phosphatase 1, protein phosphatase 2B, protein phosphatase 2C, and pyruvate dehydrogenase phosphatase. With ^{32}P-labeled MBP as a substrate, kinetic analysis according to Henderson showed that I_1^{PP2A} and I_2^{PP2A} were noncompetitive and displayed a K_i of about 30 and 25 nM, respectively. Following cleavage with *Staphylococcus aureus* V8 protease, I_1^{PP2A} and I_2^{PP2A} displayed distinct peptide patterns, indicating that these inhibitor proteins are the products of distinct genes. The N-terminal amino acid sequences of the purified preparations indicate that I_1^{PP2A} and I_2^{PP2A} are novel proteins.

Protein phosphatase 2A (PP2A)[1] is one of the four major protein serine threonine phosphatases present in the cytoplasm of mammalian cells (Cohen, 1989; Shenolikar & Nairn, 1991), although a form of PP2A has also been purified to apparent homogeneity from extracts of bovine kidney mitochondria (Damuni & Reed, 1987), and nuclear (Jakes et al., 1986) and membrane-associated (Alexander et al., 1989) forms of the enzyme have been detected. Two forms of PP2A, $PP2A_1$ and $PP2A_2$, appear to be ubiquitous (Cohen, 1989; Shenolikar & Nairn, 1991). In addition to a catalytic C subunit of apparent M_r ~36 000, $PP2A_1$ and $PP2A_2$ contain an A (or PR65) subunit of apparent M_r ~ 65 000. $PP2A_1$ also contains a B (or PR52) subunit of apparent M_r ~ 52 000. At least two forms of each of the A, B, and C subunits exhibiting 86% (Mayer et al., 1991; Hemmings et al., 1990), 85% (Hemmings et al., 1990), and 97% (Da Cruz e Silva et al., 1987a,b; Stone et al., 1987; Arino et al., 1988; Snedon et al., 1990) identity in their deduced amino acid sequences, respectively, have been identified by molecular cloning techniques. The different A, B, and C subunit forms may be expressed in a tissue-specific manner (Khew-Goodall & Hemmings, 1988), but their physiological importance has not been established.

Recent studies indicate that the C subunit of PP2A is subject to a variety of posttranslational modifications including phosphorylation on tyrosines (Chen et al., 1992) or threonines (Guo & Damuni, 1993), and methylation on the carboxyl-terminal leucine, Leu^{309} (Lee & Stock, 1993; Xie & Clarke, 1993, 1994; Favre et al., 1994). Phosphorylation of PP2A on tyrosines (Chen et al., 1992) or threonines (Guo & Damuni, 1993; Damuni et al., 1994) inactivated the phosphatase. In contrast, methylation was reported to activate the enzyme (Favre et al., 1994). In this paper, we report on another novel regulation of PP2A. We describe the identification and purification of two unique, potent, and heat-stable protein inhibitors of this enzyme.

EXPERIMENTAL PROCEDURES

Materials. Autophosphorylation-activated protein kinase (Guo et al., 1993; Guo & Damuni, 1993), protamine kinase (Damuni et al., 1989), two forms of a myelin basic protein kinase designated MBPK-1 and MBPK-2 (Reddy et al., 1993), casein kinase II (Damuni, 1990), $PP2A_1$ (Amick et al., 1992a), $PP2A_2$ (Amick et al., 1992a), $PP2A_C$ (Amick et al., 1992a), $PP1_C$ (Amick et al., 1992a), PP2B (Amick et al., 1992a), PP2C (Amick et al., 1992a), pyruvate dehydro-

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[1] Abbreviations: MBP, myelin basic protein; PP1, protein phosphatase 1; $PP1_C$, purified catalytic subunit of protein phosphatase 1; PP2A, protein phosphatase 2A; $PP2A_C$, purified catalytic (C) subunit of protein phosphatase 2A; $PP2A_1$, protein phosphatase $2A_1$; $PP2A_2$, protein phosphatase $2A_2$; I_1^{PP2A}, protein phosphatase 2A inhibitor protein of apparent M_r ~30 000 as estimated by SDS−PAGE; I_2^{PP2A}, protein phosphatase 2A inhibitor protein of apparent M_r ~20 000 as estimated by SDS−PAGE; I_1^{PP1}, protein phosphatase 1 inhibitor-1; I_2^{PP1}, protein phosphatase 1 inhibitor-2; PP2B, protein phosphatase 2B; PP2C, protein phosphatase 2C; PAGE, polyacrylamide gel electrophoresis.

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genase phosphatase (Damuni & Reed, 1988), pyruvate dehydrogenase complex (Damuni & Reed, 1988), inhibitor-2 (Amick et al., 1992a), and MBP (Diebler et al., 1984) were purified as described. A sample of pyruvate dehydrogenase phosphatase was also obtained from Dr. Lester J. Reed, University of Texas at Austin. The catalytic subunit of bovine heart protein kinase A was from Sigma Chemical Co. Protein kinase C was from Calbiochem Corp. The protein tyrosine kinases $p56^{lck}$ and $p60^{c\text{-}src}$ were from Upstate Biotechnology Inc. and Oncogene Science, respectively. The epidermal growth factor receptor kinase domain was from Strategene. Other materials are given elsewhere (Amick et al., 1992b; Reddy et al., 1993).

Assay of Protein Phosphatases. The phosphatase assay mixtures were composed of 50 mM Tris-HCl, pH 7.0, containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin, phosphatase sample, and ^{32}P-labeled substrate as indicated in a final volume of 0.05 mL. Reactions were initiated with the ^{32}P-labeled substrate, and after a 5 min reaction period, 0.1 mL of 10% (w/v) trichloroacetic acid was added. The mixture was centrifuged at 12000*g* for 2 min in a microcentrifuge. A 0.12 mL sample of the supernatant was then added to 1 mL of scintillant, and the radioactivity was determined. Phosphatases were omitted from controls. The activities of $PP1_C$, $PP2A_1$, $PP2A_2$, and $PP2A_C$ were assayed in the presence of 2 mM Mn^{2+}. In some experiments, Mn^{2+} was omitted and 1 mM EDTA was included in the reaction mixtures. The activities of PP2C and pyruvate dehydrogenase phosphatase were determined in the presence of 10 mM Mg^{2+}. Assay of pyruvate dehydrogenase phosphatase activity with pyruvate dehydrogenase complex as substrate also contained 0.1 mM $CaCl_2$. Assays of PP2B activity were carried out in the presence of 0.5 mM Ca^{2+} and 0.5 μM calmodulin. One unit of PP1, PP2A, PP2B, PP2C, and pyruvate dehydrogenase phosphatase activity was defined as the amount of phosphatase that released 1 nmol of phosphoryl groups per minute from ^{32}P-labeled substrate. To ensure linearity, the extent of phosphoryl group release from all the substrates employed was limited to <10%.

Determination of I_1^{PP2A} and I_2^{PP2A} Activity. Assay of I_1^{PP2A} and I_2^{PP2A} activity was based on the measurement of PP2A activity as described above in the absence and presence of the I_1^{PP2A} and I_2^{PP2A} preparations. The reaction mixtures contained 0.002 unit of $PP2A_2$ in 0.04 mL of 50 mM Tris-HCl, pH 7.0, containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 0.1 mg of bovine serum albumin, and a 0.005 mL aliquot of I_1^{PP2A} or I_2^{PP2A} sample. The reaction was initiated with 5 μL of ^{32}P-labeled MBP (0.04 mg/mL), and the assay was continued as described above. One control assay was performed in the absence of I_1^{PP2A} or I_2^{PP2A}, and another in the absence of I_1^{PP2A}, I_2^{PP2A}, and $PP2A_2$. Inhibition of $PP2A_2$ was linear up to 50%. One unit of I_1^{PP2A} and I_2^{PP2A} activity was defined as the amount of the protein that inhibited 1 unit of $PP2A_2$ by 50% in the standard assay.

The activities of autophosphorylation-activated protein kinase (Guo et al., 1993; Guo & Damuni, 1993), protamine kinase (Damuni et al., 1989; Reddy et al., 1990), MBPK-1 (Reddy et al., 1993), MBPK-2 (Reddy et al., 1993), casein kinase II (Damuni, 1990), the catalytic subunit of protein kinase A (Damuni et al., 1989), and protein kinase C (Damuni et al., 1989) were measured as described. One unit of autophosphorylation-activated protein kinase, MBPK-1, and MBPK-2 activity was defined as the amount of enzyme that incorporated 1 nmol of phosphoryl groups into MBP per minute. One unit of protamine kinase, protein kinase A, casein kinase II, and protein kinase C activity was equivalent to the amount of enzyme that incorporated 1 nmol of phosphoryl groups per minute into protamine sulfate, histone H2B, casein, and histone H1, respectively. To ensure linearity, the extent of phosphoryl group incorporation in all the protein kinase reactions was limited to <100 pmol.

Preparation of ^{32}P-Labeled Substrates. ^{32}P-Labeled MBP (Guo & Damuni, 1993), ^{32}P-labeled phosphorylase (Amick et al., 1992a), and ^{32}P-labeled pyruvate dehydrogenase complex (Amick et al., 1992a) were prepared as described earlier except that [γ-^{32}P]ATP had a specific activity of 2000 cpm/pmol. ^{32}P-Labeled histone H1 was prepared by incubation of histone H1 (1 mg) in 50 mM Tris-HCl, pH 7.0, containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 0.5 mM $CaCl_2$, 40 μg/mL phosphatidylserine, 0.2 mM [γ-^{32}P]ATP (2000 cpm/pmol), 10 mM $MgCl_2$, and 50 units/mL protein kinase C in a final volume of 0.25 mL. After 30 min at 30 °C, 1 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged in a microcentrifuge. The supernatant fluid was discarded, and the pellet was washed 10 times with 1-mL portions of trichloroacetic acid followed by 3 times with 1-mL portions of ethanol. The pellet was resuspended in 2 mL of 50 mM Tris-HCl, pH 7.0, containing 10% glycerol and 1 mM benzamidine.

^{32}P-Labeled casein was prepared by incubation of casein (5 mg) in 50 mM Tris-HCl, pH 7.0, containing 10% glycerol, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 14 mM β-mercaptoethanol, 0.2 mM [γ-^{32}P]ATP (2000 cpm/pmol), 10 mM $MgCl_2$, and 50 units/mL of the catalytic subunit of protein kinase A in a final volume of 0.25 mL. After 30 min at 30 °C, the solution was filtered on a column (1.5 × 10 cm) of Sephadex G-50 equilibrated and developed in 50 mM Tris-HCl, pH 7.0, containing 10% glycerol and 1 mM benzamidine.

Incubation of PP2A with the autophosphorylation-activated protein kinase was carried out as described (Guo & Damuni, 1993). Autodephosphorylation of PP2A in these incubations was analyzed by thin-layer chromatography also as described (Guo & Damuni, 1993). Protein was determined as described (Bradford, 1976). Polyacrylamide gel electrophoresis was performed in slab gels (15% acrylamide) with 0.1% sodium dodecyl sulfate and Tris−glycine, pH 8.3 (Laemmli, 1970). Protein bands were detected by staining with Coomassie Blue or silver (Merril et al., 1981). Radioactive bands were identified with Kodak X-Omat AR-5 film. N-Terminal amino acid sequencing was carried out by the Edman method using an Applied Biosystems Model 477A pulsed liquid phase automated protein sequencer fitted with phenylthiohydantoin analyzer Applied Biosystems Model 120A.

Purification of I_1^{PP2A} and I_2^{PP2A}. Unless indicated otherwise, all operations were performed at 2−5 °C. Bovine kidney was obtained from a local slaughterhouse and transported to the laboratory on ice. The kidney cortex was removed and homogenized in a Waring blender at high setting for 1 min with 2 volumes of buffer A (25 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose, 1 mM EDTA, 1

Table 1: Purification of I_1^{PP2A} and I_2^{PP2A}[d]

step	vol (mL)	protein (mg)	sp act.[a]	recovery (%)
(1) extract	4200	130400	0.08[b]	100
(2) poly(ethylene) glycol supernatant	4350	97000	0.10	93
(3) heat-treatment	2900	4560	2.0	87
(4) trichloroacetic acid precipitation	310	820	6.3	50
(5) DEAE-cellulose	700	135	12	16
(6) Q-Sepharose	350	40	24	9
(7) poly(L-lysine)−agarose	75	6	125	7
(8) ethanol precipitation	2	6	120	6.9
(9) Sephacryl S-200				
I_1^{PP2A}	18	0.4[c]	385	1.5
I_2^{PP2A}	18.2	0.2[c]	1200	2.3

[a] Units per milligram of protein. Inhibitor activity was determined with $PP2A_2$ and ^{32}P-labeled MBP as described under Experimental Procedures. [b] Activity was determined after chromatography of a 1 mL aliquot on a column (1.5 × 3 cm) of poly(L-lysine)−agarose equilibrated with buffer B. The column was washed with buffer B containing 0.35 M NaCl, and inhibitor activity was then eluted with buffer B containing 0.8 M NaCl. [c] Protein was determined after concentration of each of the solutions on a small column (1.5 × 1.5 cm) of Q-Sepharose as described in the text. [d] Two kilograms of bovine kidney was used.

mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, and 14 mM β-mercaptoethanol). The homogenate was centrifuged for 30 min at 14000*g* in a Beckman JA-10 rotor and the pellets were discarded. To the extract (step 1) was then added, with stirring, 0.08 volume of 50% w/v poly(ethylene) glycol followed by NaCl to a final concentration of 0.5 M. After 30 min, the mixture was centrifuged, and the pellets were discarded. The supernatant (step 2) was heated with constant stirring at 100 °C for 10 min. The solution was then cooled to 4 °C on ice and centrifuged, and the pellets were discarded. To the supernatant (step 3) was added, with stirring, 20 g/L trichloroacetic acid. After 10 min, the solution was centrifuged, and the supernatant was discarded. The pellets were resuspended in 500 mL of 70% ethanol, and the mixture was centrifuged and the supernatant was discarded. This procedure was repeated 4 times, and the final pellets were resuspended in 300 mL of buffer B (buffer A containing 10% glycerol instead of 0.25 M sucrose). The pH of the solution was then adjusted to 7.4 by dropwise addition, with stirring, of 1 M Tris, and the mixture was centrifuged for 30 min at 30000*g* in a Beckman JA-14 rotor. The supernatant (step 4) was applied onto a sintered glass funnel containing DEAE-cellulose (10 × 5 cm) equilibrated with buffer B. The column was washed under mild suction (4 L/h) with 8 L of buffer B containing 0.2 M NaCl. About 30% of inhibitor activity was detected in this wash (not shown). The remaining activity was eluted with buffer B containing 0.5 M NaCl. The active fractions from this eluate were pooled (step 5), diluted with 5 volumes of solution A (1 mM EDTA, 1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, and 14 mM β-mercaptoethanol), and applied onto a sintered glass funnel containing Q-Sepharose (5 × 4 cm) equilibrated with buffer B. The column was washed under mild suction with 8 L of buffer B containing 0.2 M NaCl, and inhibitor activity was eluted with buffer B containing 0.5 M NaCl. The active fractions were pooled (step 6), diluted with 4 volumes of solution A, and then applied onto a column (2.5 × 5 cm) of poly(L-lysine)−agarose equilibrated with buffer B. The column was washed with 1 L of buffer B followed by 1 L of buffer B containing 0.2 M NaCl. Inhibitor activity was eluted with buffer B containing 0.8 M NaCl. The active fractions were pooled (step 7), mixed with 2 volumes of 99% ethanol, stirred for 5 min at room temperature, and then centrifuged for 15 min at 39000*g* in a Beckman JA-20 rotor. The supernatant was discarded, and the pellets were resuspended in buffer B (step 8). The solution was then applied onto a calibrated column (2.5 × 90 cm) of Sephacryl S-200 equilibrated and developed with buffer B containing 0.1 M NaCl and 0.01% Brij 35. The active fractions containing I_1^{PP2A} and I_2^{PP2A} were pooled separately (step 9) and diluted with 4 volumes of buffer B, and then each diluted pool was applied onto a column (1.5 × 1.5 cm) of Q-Sepharose equilibrated with buffer B. The columns were washed with 50 mL of buffer B, and I_1^{PP2A} and I_2^{PP2A} were eluted with buffer B containing 1 M NaCl. The active fractions from each column were pooled separately and then dialyzed against 20 volumes of buffer B with three changes in 16 h. The solutions were then aliquoted and stored at −20 °C. A summary of the purification is provided in Table 1. The purified preparations of I_1^{PP2A} and I_2^{PP2A} were stable at −20 °C for at least 6 months.

RESULTS

Identification of Heat-Stable Protein Inhibitors. During the course of studies on the regulation of PP2A, we detected fractions that inhibited PP2A activity following chromatography of bovine kidney extracts on poly(L-lysine)−agarose. By contrast to PP2A which was eluted from poly(L-lysine)−agarose with buffer containing 0.35 M NaCl, inhibitor activity was recovered with buffer containing 0.8 M NaCl. Inhibitor activity was destroyed following incubation with trypsin, indicating that the inhibitor(s) was(were) proteinaceous in nature. By contrast to the inactivation of PP2A by protein kinases (Chen et al., 1992; Guo & Damuni, 1993), inhibition was spontaneous and was independent of ATP and Mg^{2+}. Subsequent analysis revealed that the PP2A inhibitor protein(s) was(were) heat-, acid-, and ethanol-stable. In addition, following gel permeation chromatography on Sephacryl S-200, two peaks of inhibitor activity, tentatively designated I_1^{PP2A} and I_2^{PP2A}, were separated. To characterize the properties of these PP2A inhibitor proteins on a firm molecular basis, a procedure was developed to purify them to apparent homogeneity as described under Experimental Procedures (Table 1).

Homogeneity and Composition. The procedure for the purification of I_1^{PP2A} and I_2^{PP2A} has been employed successfully 10 times. This procedure can be completed within 3 days and should facilitate future studies on I_1^{PP2A} and I_2^{PP2A}. The purified preparations of I_1^{PP2A} and I_2^{PP2A} exhibited an apparent M_r ~250 000 and ~80 000 as estimated by gel

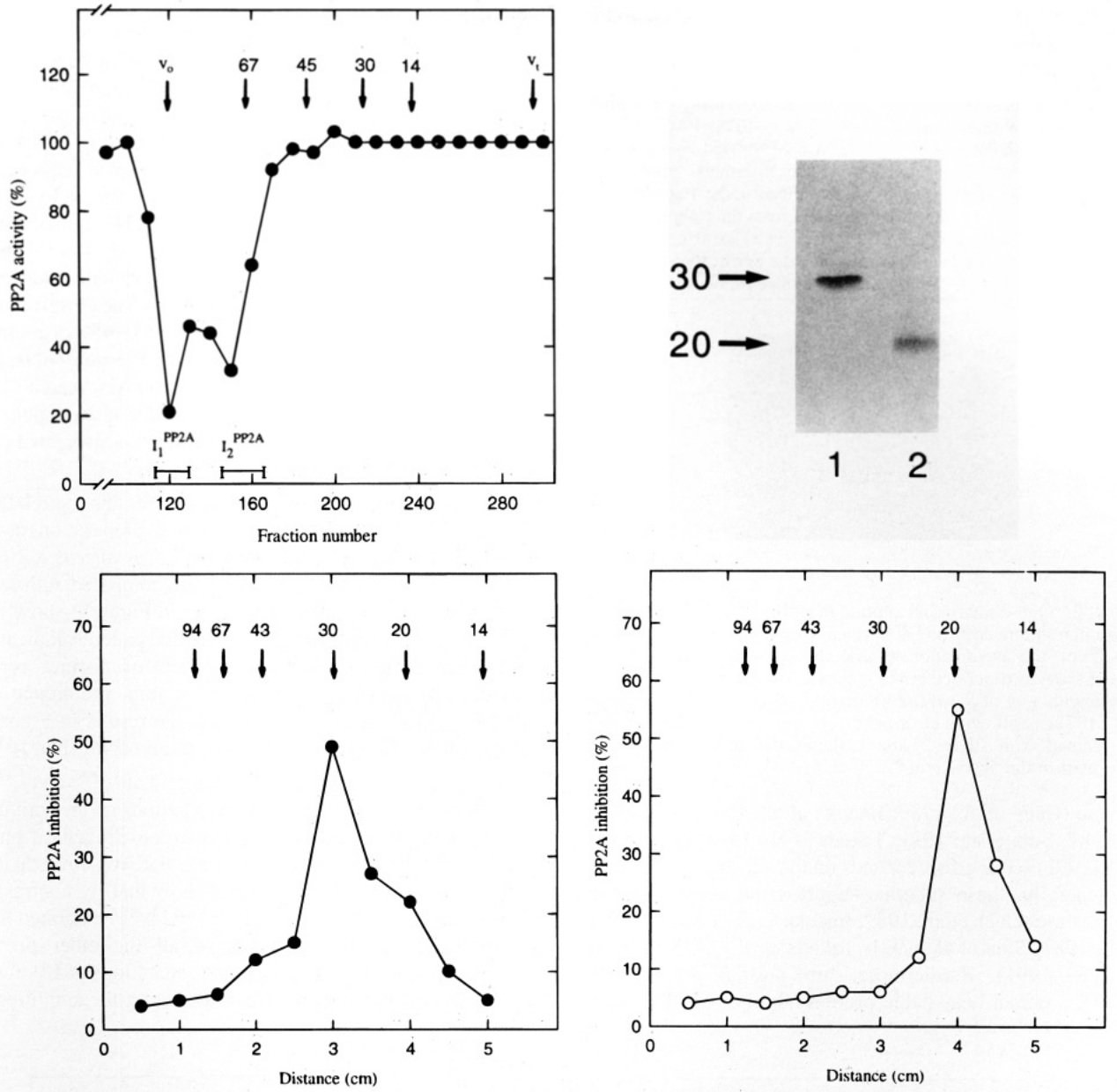

FIGURE 1: Gel permeation chromatography of I_1^{PP2A} and I_2^{PP2A} on Sephacryl S-200. In the top left panel, the solution from step 8 (Table 1) was applied onto a calibrated column (2.5 × 90 cm) of Sephacryl S-200 equilibrated and developed in buffer B containing 0.1 M NaCl and 0.01% Brij 35. The flow rate was 20 mL/h, and 2 mL fractions were collected. The activity of $PP2A_2$ was determined as described under Experimental Procedures in the presence of 0.005 mL of the indicated fractions. The percent activity of PP2A (●) was determined relative to control incubations in which 0.005 mL of the gel filtration buffer was used instead of the fraction aliquots. The protein standards were bovine serum albumin, ovalbumin, carbonic anhydrase, and ribonuclease. Blue dextran and flavin mononucleotide were used to determine v_o and v_t, respectively. Active fractions containing I_1^{PP2A} and I_2^{PP2A} were pooled as indicated by the bars, and each pool was concentrated on Q-Sepharose as described in the text. The top right panel shows the SDS−PAGE pattern of 2 μg of I_1^{PP2A} (lane 1) and 2 μg of I_2^{PP2A} (lane 2) of the concentrated pools. The gel was stained with Coomassie Blue. The arrows denote the positions corresponding to the apparent M_r's ~30 000 and 20 000. In the botton panel, each lane was cut into 5 mm pieces and soaked overnight in 1 mL of buffer B, and $PP2A_2$ activity was determined as described under Experimental Procedures in the presence of a 0.005 mL aliquot of the gel slice solutions. The percent inhibition of PP2A [I_1^{PP2A} (●) and I_2^{PP2A} (○)] was determined relative to control incubations in which 0.005 mL of buffer B instead of the gel slice solutions was employed. The protein standards were phosphorylase, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and ribonuclease.

permeation chromatography on Sephacryl S-300 (not shown) and Sephacryl S-200 (Figure 1), respectively. In contrast, the purified preparations of I_1^{PP2A} and I_2^{PP2A} exhibited an apparent M_r ~30 000 and ~20 000 as estimated by SDS−PAGE (Figure 1), respectively. The apparent discrepancies in the apparent M_r's as determined by gel permeation chromatography and SDS−PAGE suggest that I_1^{PP2A} and I_2^{PP2A} may be asymmetric and/or aggregate in solution. The apparent M_r's of these proteins do not appear to have been altered during the purification procedure because inhibitor activity corresponding to the apparent M_r's of I_1^{PP2A} and I_2^{PP2A} was detected following gel permeation chromatography of extracts on Sephacryl S-200 and Sephacryl S-300 (not shown). Incubation with trypsin (1:10 w/w) for 30 min at 30 °C completely inactivated the purified preparations of I_1^{PP2A} and I_2^{PP2A}.

N-Terminal Amino Acid Sequence. The A and B subunits of PP2A have a marked influence on the activity of this

Table 2: N-Terminal Amino Acid Sequences[a]

I_1^{PP2A}	GTDEDVLELV
I_2^{PP2A}	SDGADATSTK

[a] About 5 μg of I_1^{PP2A} and I_2^{PP2A} was electrophoretically transferred onto Immobilon-P transfer membranes following SDS−PAGE. After being stained with Ponseau S, the I_1^{PP2A} and I_2^{PP2A} bands were cut out from the membrane strips and subjected to N-terminal amino acid sequencing by the Edman method as described under Experimental Procedures. Identical results were obtained from three separate runs of three different preparations of I_1^{PP2A} and I_2^{PP2A}. The inhibitor protein preparations employed were from step 9 of the purification (Table 1) after concentration on Q-Sepharose as described in the text.

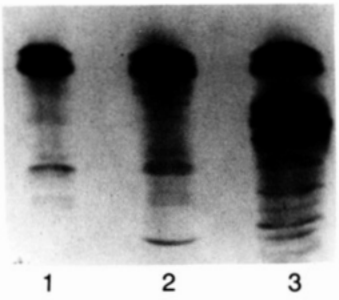

FIGURE 2: One-dimensional peptide mapping of I_1^{PP2A} and I_2^{PP2A}. Nine micrograms each of I_1^{PP2A} (lane 2) and I_2^{PP2A} (lane 3) from step 9 of the purification (Table 1), after concentration on Q-Sepharose as described in the text, was incubated separately for 60 min with 4 μg of *S. aureus* V8 protease as described (Cleveland et al., 1977). Following electrophoresis, the gel (20% acrylamide) was stained with silver. Lane 1 shows the control incubation performed in the absence of I_1^{PP2A} and I_2^{PP2A}.

enzyme (Chen et al., 1989; Imaoka et al., 1983; Mumby et al., 1987; Sola et al., 1991; Takeda et al., 1985; Usui et al., 1988, 1991). The effect depends on the substrate employed, but generally, these subunits suppress the activity of the phosphatase (Chen et al., 1989; Imaoka et al., 1983; Mumby et al., 1987; Sola et al., 1991; Takeda et al., 1985; Usui et al., 1988, 1991). Furthermore, some purified preparations of I_2^{PP1}, a potent heat-stable protein inhibitor of PP1, were reported to inhibit PP2A (Brautigan et al., 1986; Serra et al., 1990) although other preparations were without effect (e.g., Bollen et al., 1988). Therefore, as a first step to determine the relationship, if any, of I_1^{PP2A} and I_2^{PP2A} to the A and B subunits of PP2A, I_2^{PP1}, and other previously described proteins, the purified preparations of I_1^{PP2A} and I_2^{PP2A} were subjected to N-terminal amino acid sequencing, and the derived sequence (Table 2) was compared to known sequences. These analyses showed that the N-terminal amino acid sequences of I_1^{PP2A} and I_2^{PP2A} (Table 2) were distinct from that of any protein in the GenBank data base, indicating that I_1^{PP2A} and I_2^{PP2A} were novel proteins. The closest match (70% identity) to I_1^{PP2A} were residues 451−457 of methylmalonyl-CoA carboxyltransferase from *Propionibacterium shermanii* (Thornton et al., 1993). The closest match (70% identity) to I_2^{PP2A} was between residues 18 and 26 of putative class II human histocompatibility leukocyte-associated protein II (Vaesen et al., 1994).

Peptide Mapping. As a first step to examine the relationship of I_1^{PP2A} and I_2^{PP2A}, the purified preparations were subjected to cleavage with *Staphylococcus aureus* V8 protease, and the resulting peptides were compared following SDS−PAGE. The results presented in Figure 2 show that I_1^{PP2A} and I_2^{PP2A} displayed distinct peptide patterns, indicating that these proteins were the products of distinct genes. Because *S. aureus* V8 protease exhibits preference for aspartic and glutamic acid residues, the results in Figure 2 suggest that I_1^{PP2A} is richer in these amino acids than I_2^{PP2A}.

Specificity. To examine the specificity of I_1^{PP2A} and I_2^{PP2A}, the effect of the highly purified preparations on the activities of $PP1_C$, PP2B, PP2C, and pyruvate dehydrogenase phosphatase with ^{32}P-labeled MBP as a substrate was investigated. The results presented in Figure 3 show that by contrast to their effects on PP2A activity, I_1^{PP2A} and I_2^{PP2A} exhibited little or no effect on the activities of all the other protein phosphatases tested. The purified preparations of I_1^{PP2A} and I_2^{PP2A} also exhibited little effect, if any, on the activities of

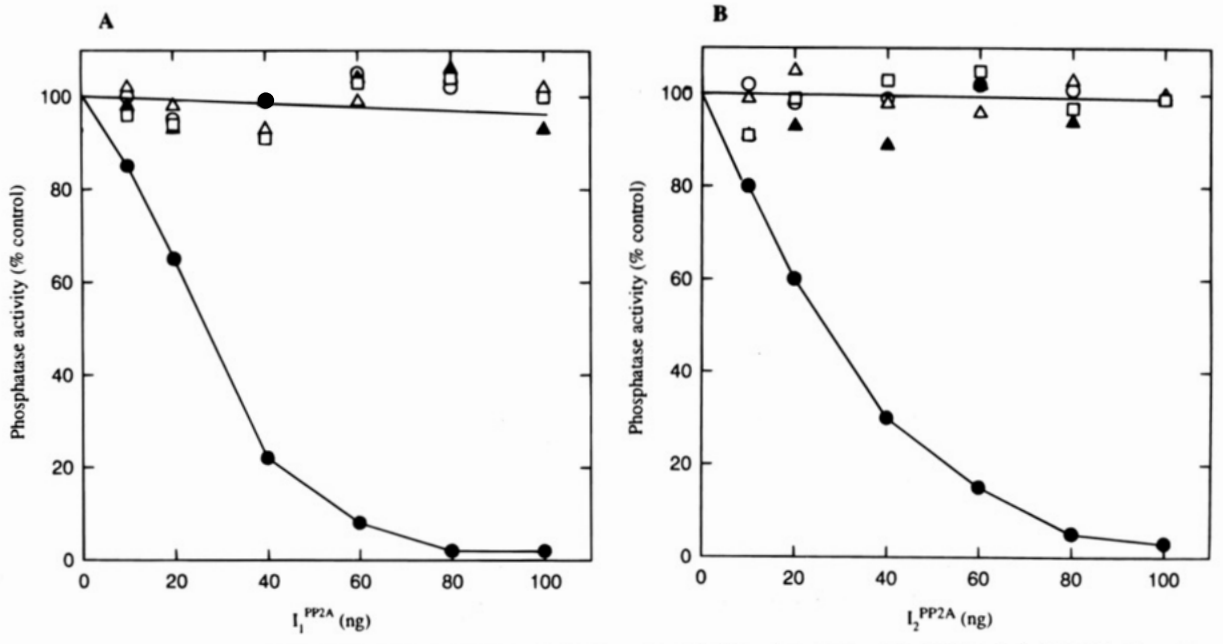

FIGURE 3: Specificity of I_1^{PP2A} and I_2^{PP2A}. The activities of 0.005 unit of $PP2A_2$ (●), $PP1_C$ (○), PP2B (▲), PP2C (△), and pyruvate dehydrogenase phosphatase (□) were determined with ^{32}P-labeled MBP as substrate as described under Experimental Procedures in the presence of the indicated concentrations of I_1^{PP2A} (A) and I_2^{PP2A} (B). The I_1^{PP2A} and I_2^{PP2A} employed were from step 9 of the purification (Table 1) after concentration on a small column (1.5 × 1.5 cm) of Q-Sepharose as described in the text.

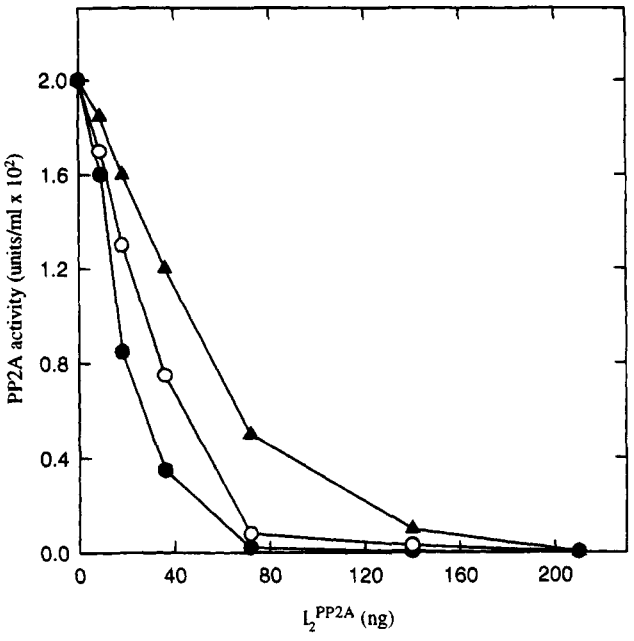

FIGURE 4: Inhibition of PP2A by I_2^{PP2A}. The activities of $PP2A_1$ (●), $PP2A_2$ (○), and $PP2A_C$ (▲) were determined with ^{32}P-labeled MBP as described under Experimental Procedures in the presence of the indicated concentrations of I_2^{PP2A} from step 9 of the purification (Table 1) after concentration of the solution on Q-Sepharose as described in the text.

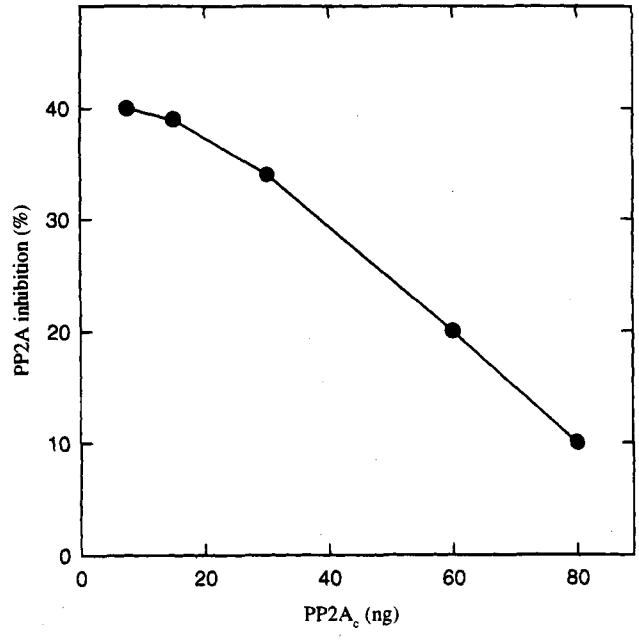

FIGURE 5: Effect of PP2A on I_2^{PP2A}. $PP2A_C$ activity was determined at the indicated concentrations in the presence of 38 ng of I_2^{PP2A} as described under Experimental Procedures. For each $PP2A_C$ concentration, the percent inhibition was determined relative to the incubation performed in the absence of I_2^{PP2A}. The I_2^{PP2A} employed was from step 9 of the purification (Table 1) after concentration on Q-Sepharose as described in the text.

11 different protein kinase preparations including the autophosphorylation-activated protein kinase, protamine kinase, MBPK-1, MBPK-2, casein kinase II, protein kinase A, protein kinase C, $p60^{c\text{-}src}$, and $p56^{lck}$ with MBP, protamine sulfate, protamine, histone H2B, and histone H2A as substrates. Together, these results indicate that I_1^{PP2A} and I_2^{PP2A} are specific for PP2A and indicate that I_1^{PP2A} and I_2^{PP2A} act, at least in part, by binding to the phosphatase.

Inhibition of $PP2A_1$, $PP2A_2$, and $PP2A_C$. The effects of I_1^{PP2A} and I_2^{PP2A} on the activities of purified preparations of the native forms of PP2A, $PP2A_1$, and $PP2A_2$, as well as purified preparations of the dissociated C subunit of the enzyme, $PP2A_C$, were evaluated. The results presented in Figure 4 show that all the forms of PP2A tested were inhibited by I_2^{PP2A}. Similar results were obtained when I_1^{PP2A} was employed (not shown). Previous studies showed that with MBP as substrate, the activity of PP2A is stimulated by the A and B subunits (Sola et al., 1991). Therefore, because the activities of $PP2A_1$, $PP2A_2$, and $PP2A_C$ were normalized with MBP as substrate, the apparent differences in the sensitivities of the PP2A preparations to I_1^{PP2A} (not shown) and I_2^{PP2A} (Figure 4) are likely due to the different concentrations of the C subunit in the assays, and that I_1^{PP2A} and I_2^{PP2A} bind, at least in part, to the C subunit of the phosphatase. The results presented in Figure 5 support this idea and show that at a fixed concentration of I_2^{PP2A}, the extent of inhibition was inversely dependent on the concentration of $PP2A_C$ in the incubations. Similar results were obtained with I_1^{PP2A} (not shown).

Kinetic Analysis. Preliminary experiments indicated that I_1^{PP2A} and I_2^{PP2A} behaved as noncompetitive inhibitors of PP2A with respect to ^{32}P-labeled MBP as the substrate (not shown). In addition, the results indicated that I_1^{PP2A} and I_2^{PP2A} bind tightly to PP2A. Therefore, the data were plotted according to Henderson (1972). The results show that the apparent K_i (given by the slope) for I_1^{PP2A} (Figure 6A) and I_2^{PP2A} (Figure 6B) is independent of substrate concentration. These results demonstrate that I_1^{PP2A} and I_2^{PP2A} are noncompetitive inhibitors. The apparent K_i for I_1^{PP2A} (Figure 6A) and I_2^{PP2A} (Figure 6B), calculated from the slope of each line, was 30 and 25 nM, respectively (Figure 6). Whether I_1^{PP2A} and I_2^{PP2A} display similar kinetics of inhibition with substrates other than MBP remains to be determined. Because heated samples (100 °C for 5 min) of $PP2A_1$, $PP2A_2$, and $PP2A_C$ were without effect on $PP2A_2$ activity (not shown), these phosphatase preparations did not contain I_1^{PP2A} or I_2^{PP2A}.

Inhibition of PP2A with Various Substrates. The effects of I_1^{PP2A} and I_2^{PP2A} on PP2A activity with a variety of substrates were examined next. The results presented in Table 3 show that the purified preparations of I_1^{PP2A} and I_2^{PP2A} inhibited PP2A with ^{32}P-labeled MBP, ^{32}P-labeled histone H1, ^{32}P-labeled pyruvate dehydrogenase complex, and ^{32}P-labeled phosphorylase as substrates (Table 3). In addition, the purified preparations of I_1^{PP2A} and I_2^{PP2A} inhibited PP2A activity with purified preparations of the protamine kinase (Amick et al., 1992a) and autophosphorylation-activated protein kinase (Guo et al., 1993) as substrates (not shown). By contrast, I_1^{PP2A} and I_2^{PP2A} exhibited little effect, if any, on PP2A activity when ^{32}P-labeled casein was employed as a substrate (Table 3). In addition, I_1^{PP2A} and I_2^{PP2A} (up to 1 μg) exhibited little effect, if any, on the autodephosphorylation of $PP2A_1$ and $PP2A_2$ in incubations with the autophosphorylation-activated protein kinase (Guo & Damuni, 1993) (not shown). Inhibition of PP2A by I_1^{PP2A} and I_2^{PP2A} with ^{32}P-labeled MBP and ^{32}P-labeled pyruvate dehydrogenase complex as substrates was prevented when casein was included in the incubations (not shown). The molecular basis of this effect of casein remains to be determined.

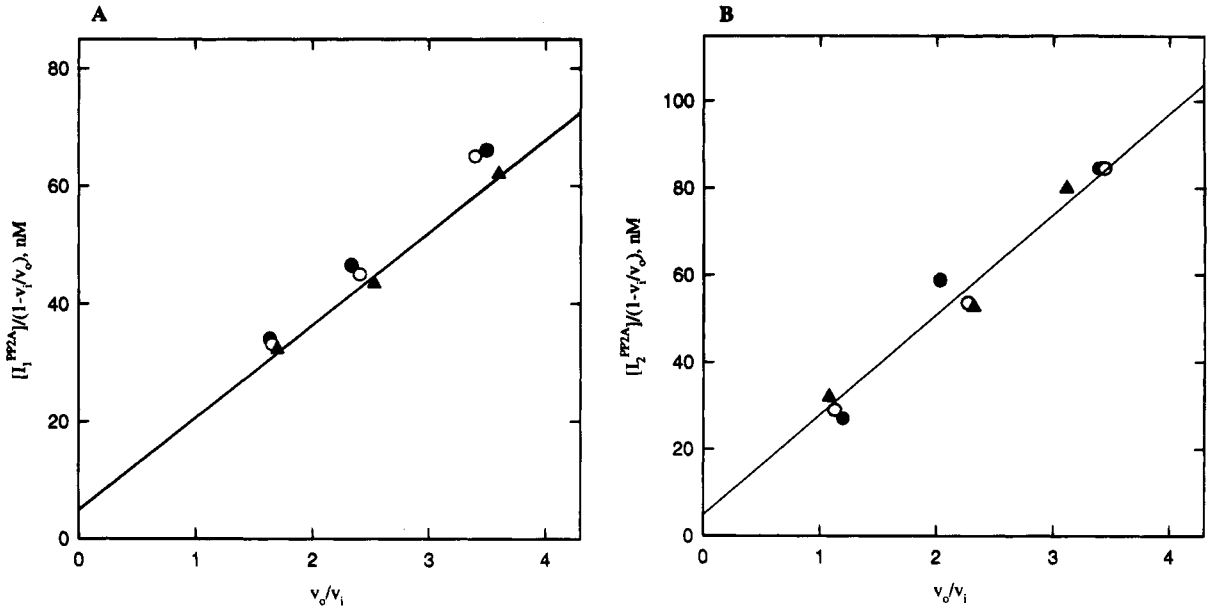

FIGURE 6: Henderson plot showing noncompetitive inhibition of PP2A activity by I_1^{PP2A} (A) and I_2^{PP2A} (B). The ^{32}P-labeled MBP concentrations employed were 0.035 mg/mL (●), 0.014 mg/mL (○), and 0.002 mg/mL (▲). The activities of PP2A in the absence and presence of I_1^{PP2A} and I_2^{PP2A} are denoted by v_o and v_i, respectively.

Table 3: Inhibition of PP2A by I_1^{PP2A} and I_2^{PP2A}

^{32}P-labeled substrate	$PP2A_2$ act. (% control)[a]	
	I_1^{PP2A}	I_2^{PP2A}
MBP	3	0
histone H1	1	3
phosphorylase	2	1
PDC	3	1
casein	95	100

[a] The activity of $PP2A_2$ was measured in the absence (control) and presence of I_1^{PP2A} and I_2^{PP2A} each at 600 ng with the indicated substrates as described under Experimental Procedures. About 0.002 unit of $PP2A_2$ was employed with each of the indicated substrates. This corresponded to 6.6, 6.6, 132, 66, and 100 ng of $PP2A_2$ with ^{32}P-labeled MBP, histone H1, pyruvate dehydrogenase complex (PDC), phosphorylase, and casein, respectively. The concentrations of the ^{32}P-labeled substrates in the assays were 0.04 mg/mL MBP, 0.04 mg/mL histone H1, 1 mg/mL phosphorylase, 1 mg/mL pyruvate dehydrogenase complex, and 1 mg/mL casein. Similar results were obtained when $PP2A_1$ or $PP2A_C$ and 0.1 mg/mL casein or phosphorylase were employed. The I_1^{PP2A} and I_2^{PP2A} were from step 9 of the purification (Table 1) after concentration on Q-Sepharose as described in the text.

DISCUSSION

In this paper, we have described the purification to apparent homogeneity of two novel thermostable protein inhibitors of PP2A, tentatively designated I_1^{PP2A} and I_2^{PP2A} (Table 1, Figure 1). These protein inhibitors exhibited unique N-terminal amino acid sequences (Table 2) which distinguished them from previously identified proteins. On the basis of the different peptide patterns obtained following cleavage with *S. aureus* V8 protease (Figure 2), I_1^{PP2A} and I_2^{PP2A} are considered the products of distinct genes. The results presented indicate that I_1^{PP2A} and I_2^{PP2A} are specific (Figure 3), noncompetitive (Figure 6), and potent inhibitors of PP2A with K_i values of about 30 and 25 nM, respectively (Figure 6). However, further studies are needed to define the molecular mechanism of action of I_1^{PP2A} and I_2^{PP2A}. In this connection, evidence was presented recently indicating that residues 267−270 (YRCG) of the C subunit of PP2A play an important role in the sensitivity of this phosphatase to okadaic acid. Thus, Lee and co-workers (Zhang et al., 1994) demonstrated that replacement of PP1 residues 274−277 (GEFD) with YRCG resulted in a PP1 that was inhibited by okadaic acid with a potency similar to PP2A. Whether this PP1 mutant is sensitive to I_1^{PP2A} and/or I_2^{PP2A} remains to be determined.

The relationship of I_1^{PP2A} and I_2^{PP2A} to a previously identified rat liver protein inhibitor of PP2A (Serra et al., 1989) is uncertain. Nonetheless, three observations indicate that I_1^{PP2A} and I_2^{PP2A} are different from the rat liver protein. First, in contrast to I_1^{PP2A} and I_2^{PP2A} which were stable in ethanol (Table 1), the inhibitor protein from rat liver was denatured in ethanol (Serra et al., 1989). Second, although I_1^{PP2A} and I_2^{PP2A} were stable in 2% trichloroacetic acid (Table 1), they were denatured in 15% trichloroacetic acid (not shown). In contrast, the rat liver inhibitor protein was stable in 15% trichloroacetic acid (Serra et al., 1989). Third, I_1^{PP2A} and I_2^{PP2A} inhibited PP2A activity with phosphorylase as a substrate (Table 3). By contrast, the rat liver inhibitor protein exhibited no effect on PP2A activity with this substrate (Serra et al., 1989). Earlier studies showed that I_1^{PP1} (Ingebritsen & Cohen, 1983) and the protein inhibitor of the mitochondrial branched-chain α-keto acid dehydrogenase phosphatase (Damuni et al., 1985, 1986) were without effect on PP2A activity. In addition, partially purified preparations of two heat-stable protein inhibitors of the protein tyrosine phosphatases PTP-4 and PTP-5 exhibited little effect, if any, on PP2A activity (Ingebritsen, 1987). However, some apparently homogeneous preparations of I_2^{PP1} inhibited PP2A (Brautigan et al., 1986; Serra et al., 1990) whereas other preparations did not (e.g., Bollen et al., 1988). The reason for these discrepancies is unclear. One possibility raised by the results presented herein is that some of the I_2^{PP1} preparations (Brautigan et al., 1986; Serra et al., 1990) may have been contaminated with I_1^{PP2A} and/or I_2^{PP2A} whereas other preparations (e.g., Bollen et al., 1988) were not.

The effects of the PP2A A and B subunits on the activity of the phosphatase are different from those of I_1^{PP2A} and I_2^{PP2A}. For example, the A and B subunits suppress the activity of PP2A with casein in a substrate (Chen et al., 1989;

Imaoka et al., 1983; Takeda et al., 1985; Usui et al., 1988, 1991), and markedly reduce the autodephosphorylation of PP2A in incubations with the autophosphorylation-activated protein kinase (Damuni, unpublished observations). By contrast, I_1^{PP2A} and I_2^{PP2A} exhibited little effect, if any, on the activities of $PP2A_1$, $PP2A_2$, and $PP2A_C$ with casein as a substrate (Table 3), and had little effect on the autodephosphorylation of PP2A in the incubations with autophosphorylation-activated protein kinase. Similarly, I_1^{PP2A} and I_2^{PP2A} inhibited PP2A activity with MBP and histone H1 as substrates. In contrast, with these substrates, the A and B subunits enhance the activity of the phosphatase (Sola et al., 1991). The reason I_1^{PP2A} and I_2^{PP2A} did not inhibit PP2A activity with casein as substrate is uncertain. It is possible that when casein binds to PP2A it prevents the inhibitor proteins from binding to the phosphatase. Alternatively, casein may bind I_1^{PP2A} and I_2^{PP2A}.

The results presented in this paper provide the basis for examining further the structure, function, and regulation of I_1^{PP2A} and I_2^{PP2A}. On the basis of the specific activities of the purified preparations, and assuming that extracts contain equivalent quantities of I_1^{PP2A} and I_2^{PP2A}, we estimate the concentrations of these inhibitor proteins in the kidney extracts to be about 0.1 and 0.05 μM, respectively. These values are similar to the estimated concentration of about 0.1 μM for PP2A in these extracts. Considered also with the potency of I_1^{PP2A} and I_2^{PP2A} (Figure 6), these values suggest that I_1^{PP2A} and I_2^{PP2A} could operate under conditions *in vivo*. In this connection, a recent report indicated that, in response to insulin, PP2A was inactivated in rat skeletal muscle cells in culture during myogenesis (Srinivasan & Begum, 1994). The molecular basis of this insulin effect was not determined, although, consistent with earlier proposals (Chen et al., 1992; Guo & Damuni, 1993), it was suggested that phosphorylation of PP2A may be involved (Srinivasan & Begum, 1994). The results presented in this paper raise the alternative possibility that insulin-mediated inactivation of PP2A may result from the activation of I_1^{PP2A} and/or I_2^{PP2A}. Such an activation would be analogous to the catecholamine-stimulated inhibition of PP1 which results from the activation of I_1^{PP1} by phosphorylation catalyzed by protein kinase A (Cohen, 1989). However, whether I_1^{PP2A} and/or I_2^{PP2A} are themselves present in skeletal muscle and whether they are regulated by phosphorylation are unknown.

The recent discoveries on the phosphorylation (Chen et al., 1992; Guo & Damuni, 1993) and methylation (Lee & Stock, 1993; Xie & Clarke, 1993, 1994; Favre et al., 1994) of PP2A, together with the observations reported herein, indicate the presence in cells of a network of enzymes and regulatory proteins for controlling the activity of PP2A. However, whether the sensitivity of PP2A to I_1^{PP2A} and I_2^{PP2A} is regulated by the tyrosine (Chen et al., 1992) and/or threonine (Guo & Damuni, 1993) phosphorylation, and/or by the methylation (Lee & Stock, 1993; Xie & Clarke, 1993, 1994; Favre et al., 1994) of the C subunit of the phosphatase, remains to be determined. In this connection, we have shown recently that okadaic acid and microcystin-LR not only inhibit PP2A activity but also inhibit directly the methylation of the C subunit of PP2A at Leu^{309} by its specific methyltransferase (Li & Damuni, 1994). Whether I_1^{PP2A} and/or I_2^{PP2A} also inhibit this methylation of the C subunit of PP2A remains to be determined. The specificity and potency of I_1^{PP2A} and I_2^{PP2A} indicate that these novel protein inhibitors may serve as new powerful tools not only for distinguishing protein phosphatases but perhaps also for more rigorously evaluating the physiological function of PP2A.

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