Stimulation of Phosphorylase Phosphatase Activity of Protein Phosphatase $2A_1$ by Protamine Is Ionic Strength Dependent and Involves Interaction of Protamine with Both Substrate and Enzyme†

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ABSTRACT: The effects of protamine on the phosphorylase phosphatase activity of porcine cardiac protein phosphatase $2A_1$ ($PP2A_1$) were complex and ionic strength dependent. Under ionic strength conditions that protamine activation was optimal, activation of $PP2A_1$ by either dilution or heparin was prevented. A time-dependent deactivation of the protamine-stimulated phosphatase activity was observed when $PP2A_1$ was preincubated with protamine. Protamine forms a very tight association with phosphorylase *a*, which is optimal at a 1:1 protamine:phosphorylase *a* monomer molar ratio. Protamine activation of $PP2A_1$ activity, however, is not substrate-directed since the basic polypeptide did not stimulate either the activity of the catalytic subunit or trypsinolysis of [^{32}P]phosphorylase *a*. The interaction of protamine with phosphorylase *a* does not apparently involve the phosphorylation site in the protein substrate (ser 14). The activation of $PP2A_1$ by protamine is proposed to involve part of the basic polypeptide, not associated with phosphorylase *a* monomer, interacting with the regulatory and/or the catalytic subunit(s) of the phosphatase. A minimal model for the activation of $PP2A_1$ by protamine was tested kinetically. In this model, free $PP2A_1$ binds with decreasing affinities to the protamine:phosphorylase *a* complex, free phosphorylase *a*, and free protamine. Protamine decreases the K_m of $PP2A_1$ for the phosphorylase *a* monomer 5-fold and increases the V_{max} 17-fold. Interaction of free protamine with $PP2A_1$ inhibits the phosphatase activity.

Type 2A protein phosphatases are one of two groups of protein phosphatases that dephosphorylate and inactivate phosphorylase *a* (Ballou & Fischer, 1986; Waelkens et al., 1987a; Cohen, 1989; Shenolikar & Nairn, 1991; Bollen & Stalmans, 1992; Walter & Mumby, 1993: Wera & Hemmings, 1995). Multiple forms of PP2A ($PP2A_0$, $PP2A_1$, and $PP2A_2$) that differ in composition by the presence or absence of various subunits have been described (Ballou & Fischer, 1986; Waelkens et al., 1987a; Cohen, 1989; Shenolikar & Nairn, 1991; Bollen & Stalmans, 1992; Walter & Mumby, 1993: Wera & Hemmings, 1995). PP2As are composed of a 37 kDa catalytic C subunit, a 65 kDa accessory A subunit, and one of a heterogeneous class of 54−130 kDa regulatory B subunits (Green et al., 1987; Kamibayashi et al., 1994; Peruski et al., 1993; Ruediger et al., 1992; Stone et al., 1987; Walter & Mumby, 1993). $PP2A_0$ and $PP2A_1$ are each composed of three subunits, termed A, B′, and C ($2A_0$) and A, B, and C ($2A_1$), respectively, while $PP2A_2$ contains only two subunits, A and C. The AC complex has been isolated in association with certain tumor antigens (Ruediger et al., 1992; Walter & Mumby, 1993).

The regulatory properties of PP2A activities are varied and complex. Originally identified as ser/thr protein phosphatases, PP2As display low but detectable protein tyrosine phosphatase (PTPase) activity, which can be stimulated by a variety of procedures (Bollen & Stalmans, 1992; Erickson & Killilea, 1992a; Goris et al., 1988; Usui et al., 1988). A protein that activates the PTPase activity has been isolated and cloned (Calyla et al., 1992, 1994). Phosphorylation of the catalytic subunit ($PP2A_c$) on tyrosine inactivates the ser/thr phosphatase activity (Chen et al., 1992, 1994) while phosphorylation of $PP2A_c$ on threonine has been reported to inactivate both the ser/thr phosphatase and PTPase activities (Damuni et al., 1994). Two heat-stable protein inhibitors of PP2A have been purified from bovine kidney (Li et al., 1995).

Schlender and co-workers were the first to isolate a type 2A ser/thr phosphatase stimulator, later identified as histone H1, from tissue extracts (Schlender et al., 1982, 1985; Schlender & Mellgren, 1984). Other polycationic polypeptides such as protamine and poly-L-lysine in addition to histone H1 were later shown to also stimulate type 2A phosphatases (DiSalvo et el., 1985; Jakes et al., 1986; Khandelwal & Enno, 1985; Pelech & Cohen, 1985; Usui et al., 1988; Wealkens et al., 1988). Although the most effective stimulating polycations (protamine and polylysine) are not present in mammalian cells, and histone H1 is only present in the nucleus, it is hypothesized that these polycations may be mimicking the actions of some as yet undetermined intracellular factor(s) (Schlender et al., 1985; Waelkens et al., 1987a).

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In many studies, it was concluded that activation of type 2A phosphatases was due to interaction of the polycationic polypeptides with the phosphatase and not with the substrate. These conclusions were primarily based on the observations that maximal activation of phosphatase was observed at concentrations of the polypeptides that were well below the concentration of the substrate, and increased concentrations of these polypeptides inhibit the phosphatases (DiSalvo et al., 1985; Schlender et al., 1985). Other studies implicate interaction of the polycationic peptides with protein substrates (Khandelwal & Enno, 1985; King et al., 1994; Waelkens et al., 1987a). Ceramide, a putative second messenger, has also been shown to activate trimeric forms of PP2A (Dobrowsky et al., 1993), $PP2A_2$, and the catalytic subunit (Law & Rossie, 1995).

The results presented here describe studies of the stimulation of the phosphorylase phosphatase activity of porcine cardiac tissue $PP2A_1$ by protamine. Evidence is presented that interactions of protamine with both substrate and phosphatase are involved.

EXPERIMENTAL PROCEDURES

Materials. Protamine chloride (salmon sperm), protamine-agarose, and other biochemicals were purchased from the Sigma Chemical Company, St. Louis, MO. 7-Methyl-6-thioguanosine was synthesized, and stock solutions of the substrate in dimethyl sulfoxide were prepared daily as described previously (Cheng et al., 1995). The chloride contents of the protamine chloride lots were obtained from the vender (Sigma) and used in the calculation of protamine concentrations.

Methods. [^{32}P]Phosphorylase *a* and phosphorylase *a* were prepared and stored as described previously (Cheng et al., 1995). The [^{32}P]-labeled mixture of tryptic penta- and hexapeptides of [^{32}P]phosphorylase *a* was prepared as by Nolan et al. (1964). $PP2A_1$ was purified from porcine heart muscle tissue according to the procedure of Erickson and Killilea (1992b). The specific activity of purified $PP2A_1$, assayed in the presence of 10 μM protamine and 0.2 M NaCl, was 5854 units/mg of protein. The 37 kDa catalytic subunit was prepared from $PP2A_1$ as described (Erickson & Killilea, 1992b). The subunit molecular weight of phosphorylase *a* and the molecular weights of $PP2A_1$ and protamine were taken as 100 000 (Cohen et al., 1971), 160 000 (Erickson & Killilea, 1992b), and 4245 (Ando & Watanabe, 1969), respectively. Each experiment was carried out at least twice with with different preparations of $PP2A_1$ and substrate.

Radiolabled Phosphatase Assays. The phosphorylase phosphatase assay mixture contained 10 μM (1 mg/mL) [^{32}P]-phosphorylase *a* in assay buffer (50 mM Bis-Tris, 5 mM caffeine, 1 mg of bovine serum albumin/mL, 1 mM dithiothreitol, pH 7.0) and phosphatase. Typically, the reaction was initiated by the addition of 0.01 mL of phosphatase to 0.04 mL of [^{32}P]phosphorylase *a* (12.5 μM) in assay buffer containing the indicated concentrations of protamine and NaCl. The polycation solution was freshly prepared as a concentrated stock solution (1 mg/mL in assay buffer) and was added to the [^{32}P]phosphorylase *a* immediately prior to the performance of the assay. To prevent aggregation and precipitation of the substrate by protamine, the [^{32}P]phosphorylase *a* solution contained at least 0.075 M NaCl. Under the conditions of the assay, the phosphatase activity was linear up to the dephosphorylation of 30% of the substrate. All assays were carried out in duplicate. After a 5−10 min incubation at 30 °C (or 25 °C where indicated), reactions were terminated by the addition of 0.05 mL of a 10% trichloroacetic acid solution. One unit of activity was the amount of phosphatase that catalyzed the release of 1 nmol of [^{32}P]phosphate per min. When the [^{32}P]-labeled tryptic peptides of phosphorylase *a* were used as substrate, the procedure described previously (Killilea et al., 1978) was followed with the exception that the [^{32}P]-labeled peptides were added to a concentration equivalent to that originating from 1 mg of intact phosphorylase *a*/mL.

Spectrophotometric Assay for Protein Phosphatase. This assay was used in the kinetic analysis of the protamine activation of the phosphorylase phosphatase activity of $PP2A_1$ (Figures 6 and 7). The assays were carried out at 25 °C in 1.8 mL mixtures containing 50 mM Bis-Tris buffer (pH 7.0), 0.2 M NaCl, 5 mM caffeine, 2 mM dithiothreitol, 2% glycerol, 1% DMSO, 81 μM 7-methyl-6-thioguanosine, 79 μg of purine nucleoside phosphorylase/mL, and the desired amounts of protamine and $PP2A_1$ as described previously (Cheng et al., 1995). After 5 min of incubation at 25 °C, the reactions were initiated by adding the protein phosphatase, and the absorbance at 360 nm was recorded. Initial velocities were determined from the slopes of progress curves acquired using Perkin-Elmer software. Other data were analyzed using a nonlinear regression analysis program. Quantitation of phosphate release was determined using the extinction coefficient of 11 200/M/cm for the phosphate-dependent reaction at 360 nm at pH 7.0 (Cheng et al., 1995).

RESULTS

Preliminary Studies. In studying the effects of the protamine on the phosphorylase phosphatase activity of $PP2A_1$, the order of addition of the components to the assay mixture was found to be important. Preincubation of [^{32}P]-phosphorylase *a* with protamine resulted in a time- and concentration-dependent aggregation and precipitation of the substrate that was visually detectable. The turbid [^{32}P]-phosphorylase *a*-containing complex was easily sedimented by low speed centrifugation. These effects of protamine on phosphorylase *a* have been reported previously (Krebs, 1954; Madsen & Cori, 1954). These processes were eliminated or delayed by including a minimum concentration of 0.06 M NaCl in the assay mixtures. On the other hand, preincubation of $PP2A_1$ with the polycationic polypeptide decreased the protamine stimulated phosphatase activity (see below). For these reasons, the assays were usually carried out by adding protamine to [^{32}P]phosphorylase *a* in the presence of at least 0.075 M NaCl and then rapidly initiating the reactions by the addition of the phosphatase.

Preincubation of $PP2A_1$ with Protamine. Preincubation of $PP2A_1$ with protamine decreased the protamine stimulated phosphatase activity. This phenomenon is shown in Figure 1. Phosphatase was preincubated with the basic polypeptide in the absence of NaCl for the times indicated and the assays were then adjusted to contain 0.06 M NaCl in the phosphatase assays. The residual activity introduced into the assay mixtures was linear with time. The level of protamine used in the preincubation study was chosen so that the level of protamine diluted into the activity assay was 10 μM, which was identical to that of the substrate, [^{32}P]phosphorylase (see

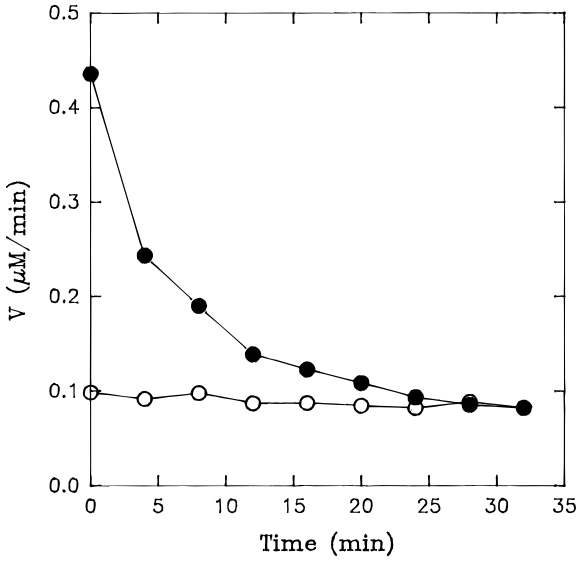


FIGURE 1: Effect of preincubation of $PP2A_1$ with protamine. $PP2A_1$ (0.7 μg/mL) was preincubated at 30 °C in the absence (open circles) and in the presence of 50 μM protamine (closed circles) in assay buffer. At the times indicated 0.01 mL samples were removed and immediately added to 0.04 mL of phosphatase assay mixtures containing [^{32}P]phosphorylase *a* (12.5 μM) in assay buffer containing 0.075 M NaCl. Reactions were carried out for 6 min at 30 °C.

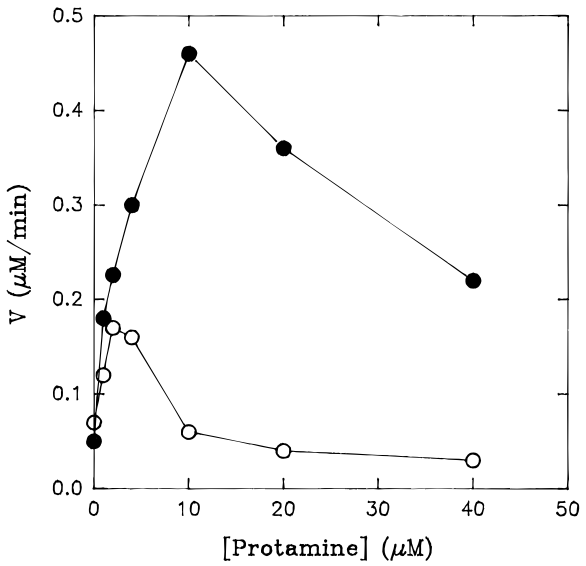


FIGURE 2: Effect of protamine levels on the phosphorylase phosphatase activity of $PP2A_1$ in the absence (open circles) and in the presence (closed circles) of 0.06 M NaCl. Assays were carried out at 30 °C for 6 min in 0.05 mL reaction mixtures containing [^{32}P]phosphorylase *a* (10 μM), protamine (0, 1, 2, 4, 10, 20, or 40 μM) and $PP2A_1$ (0.16 μg/mL) in assay buffer. Reactions were initiated by the addition of $PP2A_1$ in 0.01 mL of assay buffer.

below). Protamine decreased the polycation-stimulated enzyme activity in a time-dependent manner to the level of the basal phosphatase activity. The presence of salt in the preincubation mixtures did not prevent this effect. Since the deactivation by protamine was dependent on the time that the polycation was preincubated with the enzyme, this effect was obviously enzyme directed. Decreases in polycationic polypeptide-stimulated type 2A phosphatase activities has also been reported for phosphatases isolated from rabbit skeletal muscle (Waelkens et al., 1987a,b). In the latter study it was reported also that the basal activity was not affected and the rate of decrease in polycation stimulated activity was dependent on the concentration of polycations in the preincubation mixtures.

Stimulation of $PP2A_1$ by Protamine. The effects of increasing concentrations of protamine on the phosphorylase phosphatase activity of $PP2A_1$, in the absence and in the presence of 0.06 M NaCl, are shown in Figure 2. In the absence of salt, the phosphorylase phosphatase activity was stimulated 2-fold by 2 μM protamine. As the levels of the basic polypeptide were further increased in the assay mixtures, decreased stimulation of phosphatase activity was observed. In the presence of 0.06 M NaCl, maximal stimulation by protamine was observed at 10 μM. Thus, in the presence of NaCl, the stimulation of phosphorylase phosphatase activity of $PP2A_1$ was increased over that observed in the absence of NaCl by protamine. Also, the concentration of protamine that gave maximum stimulation in the presence of salt was also elevated (to 10 μM). The interpretation of these results is as follows. In the absence of salt, low concentrations of the basic polypeptide were stimulatory, but as the concentrations were increased, aggregation and precipitation of the substrate took place with concomitant unavailability of the substrate for dephosphorylation. In the presence of NaCl, the phosphatase reactions were stimulated since aggregation and precipitation of the substrate was prevented.

The concentration of protamine (10 μM), that caused maximal stimulation in the presence of 0.06 M NaCl (Figure 2) was similar to the substrate concentration (10 μM phosphorylase *a* monomer) and significantly higher than the $PP2A_1$ concentration (0.001 μM) used in the assays. This indicated that the stimulation of the phosphatase activity might involve interaction of the basic polypeptide with the substrate, phosphorylase *a*. The decrease in activity observed at higher levels of protamine (Figure 2) could indicate that free excess protamine was interacting with and causing inhibition of $PP2A_1$ activity.

In contrast to the dephosphorylation of phosphorylase *a*, the dephosphorylation of trypsin-derived phosphorylated penta- and hexapeptide mixture from [^{32}P]phosphorylase *a* (Nolan et al., 1964) by $PP2A_1$ was not stimulated by protamine (not shown). Under the conditions used, the basal activity of $PP2A_1$ dephosphorylated the [^{32}P]tryptic-derived peptide mixture at 60% the rate of dephosphorylation of the intact protein substrate. The presence of protamine decreased the dephosphorylation of the tryptic phosphopeptides by 25%. This decreased activity may be the result of free protamine inhibiting the basal activity of $PP2A_1$.

Effects of Protamine on the Activity of the Catalytic Subunit of $PP2A_1$. The effects of increased concentrations of protamine on the phosphorylase phosphatase activity of the isolated 37 kDa catalytic subunit of $PP2A_1$ are shown in Figure 3. In the absence of 0.06 M NaCl, up to 10 μM protamine did not stimulate activity, and decreased activity was observed at higher concentrations of the basic polypeptide. In the presence of 0.06 M NaCl, the activity of the catalytic subunit was inhibited 55% and protamine concentrations of 10−20 μM stimulated the activity, but only to about the level observed in the absence of the salt and protamine. These results were in contrast to the observations observed for intact native $PP2A_1$ and are discussed below.

Effects of NaCl Levels on $PP2A_1$ Activity. The effects of increased NaCl concentrations on the protamine stimulation

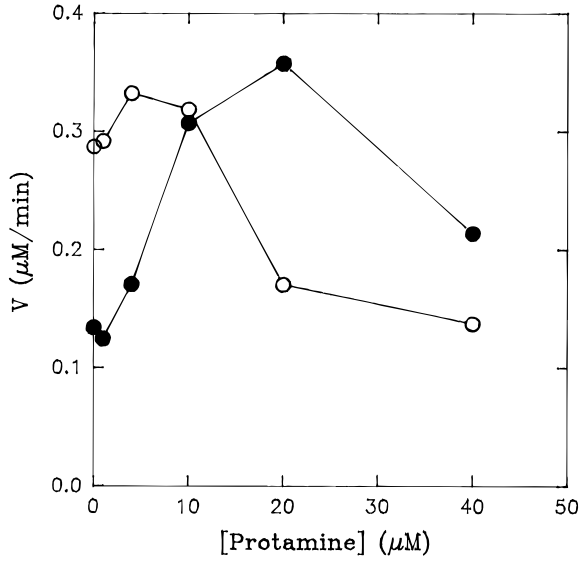


FIGURE 3: Effect of protamine levels on the phosphorylase phosphatase activity of the catalytic subunit of $PP2A_1$ in the absence (open circles) and in the presence (closed circles) of 0.06 M NaCl. Conditions were as described in Figure 2 with the exception the catalytic subunit of $PP2A_1$ (0.3 units/mL) replaced $PP2A_1$ in the reaction mixtures.

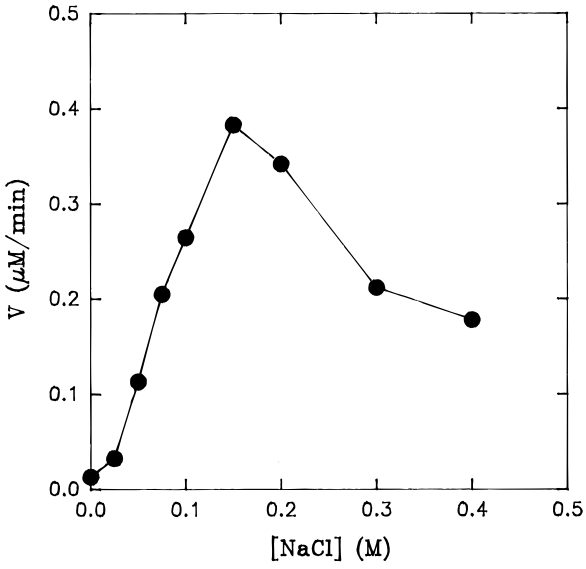


FIGURE 4: Effect of NaCl on the stimulation of $PP2A_1$ activity by protamine. Assays were carried out at 30 °C for 5 min in 0.05 mL reaction mixtures containing [^{32}P]phosphorylase *a* (10 μM), protamine (10 μM) and $PP2A_1$ (0.4 units/mL) in assay buffer containing 0, 0.025, 0.05, 0.075, 0.10, 0.15, 0.20, 0.30, and 0.40 M NaCl.

of the dephosphorylation of [^{32}P]phosphorylase *a* by $PP2A_1$ are shown in Figure 4. The extent of protamine stimulation of $PP2A_1$ activity increased to a maximal level (56−85-fold depending on the $PP2A_1$ preparation used) at 0.15−0.2 M NaCl. At higher concentrations of NaCl, progressive inhibition or less activation was observed. Preincubation of [^{32}P]-phosphorylase *a* with protamine in the presence of 0.15 M NaCl for up to 30 min did not alter the stimulation of $PP2A_1$ activity by the basic polypeptide. This indicated that when the optimal conditions of ionic strength were used, the basic polypeptide did not aggregate and precipitate the [^{32}P]-phosphorylase *a* and thus did not make the substrate unavailable for dephosphorylation.

The increased salt levels decreased the basal phosphorylase phosphatase activity of $PP2A_1$ and in the presence of 0.15 M NaCl this activity was less than 2% of the activity expressed in the presence of protamine. Since the level of the basal activity of $PP2A_1$ increases on dilution (Erickson & Killilea, 1992b; Ingebritsen et al., 1983; Lee et al., 1977), the effect of 0.15 M NaCl on this process was examined. In the absence of the NaCl, the basal activity of the $PP2A_1$ preparation used increased on dilution to a maximal value about 10% of that determined for the protamine-stimulated activity assayed in the presence of 0.15 M NaCl. This increase was suppressed by 0.15 M NaCl in the assays. These observations are further discussed below.

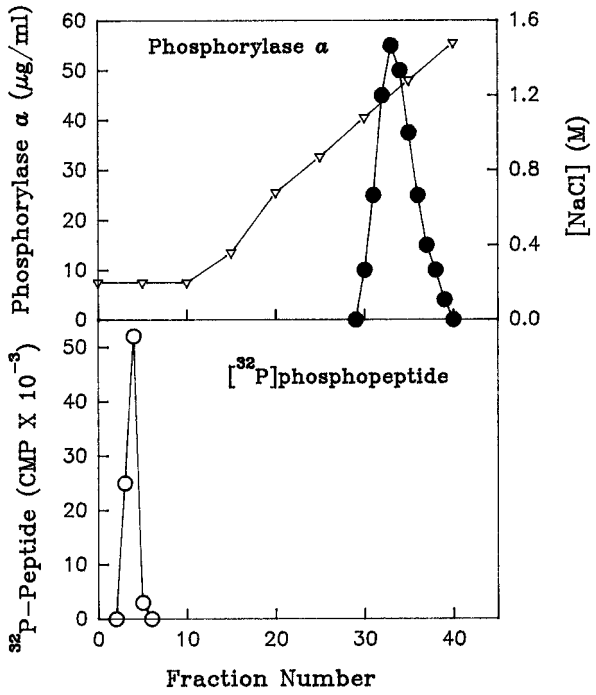
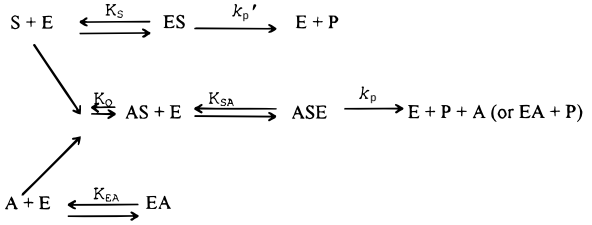


FIGURE 5: Chromatography of phosphorylase *a* (top panel) and [^{32}P]-labeled phosphopeptides (bottom panel) on protamine-agarose. The phosphorylase *a* (0.3 mg/mL) and [^{32}P]tryptic peptides derived from [^{32}P]phosphorylase *a* (0.3 mg/mL) were applied in separate runs at ambient temperature to a protamine-agarose column (0.7 and 2.3 cm) equlibrated with assay buffer containing 0.2 M NaCl. The flow rate was 14 mL/h and 1 mL fractions were collected. The column was eluted with a 32 mL salt gradient (0.2−2.0 M NaCl in buffer). The recoveries of phosphorylase *a* and the [^{32}P]-tryptic peptide material were 87% and 100%, respectively.

Protamine−Phosphorylase a Interactions. Physical evidence for a strong interaction between phosphorylase *a* and the basic polypeptide was provided by subjecting the substrate to chromatography on protamine-agarose. Phosphorylase *a* readily bound to the column and was only eluted by 1.25 M NaCl (Figure 5). On the other hand, the immobilized protamine did not retain the phosphorylated penta- and hexapeptide mixture derived from [^{32}P]phosphorylase *a* by trypsin (Figure 5). These results complement the observations above that while this [^{32}P]peptide material is a substrate for the basal activity of $PP2A_1$, the action of $PP2A_1$ on this substrate was not stimulated by protamine.

As mentioned above, protamine, in assay buffer containing 0.15 M NaCl, did not aggregate and precipitate the protein substrate. This assay mixture also contains caffeine. If the methylxanthine was omitted, however, equimolar phosphorylase *a* monomer:protamine mixtures immediately appear turbid, and on standing in the cold, crystallization occured. The resulting crystals are rod shaped. This result would indicate that under the proper conditions of ionic strength,

Scheme 1



protamine not only interacts with but also must form very specific interactions with phosphorylase *a*. The presence or absence of caffeine did not alter the chromatographic properties of phosphorylase *a* on protamine-agarose (not shown). The crystallization of phosphorylase *b*−protamine complexes has been previously described (Madsen & Cori, 1954).

Kinetics of the Activation of the Phosphorylase Phosphatase Activity of PP2A₁ by Protamine. Having determined the levels of NaCl that resulted in the optimal stimulation of $PP2A_1$ activity by protamine, the effects of increased levels of protamine on the $PP2A_1$ activity were examined. For these studies, except where indicated, the continuous spectrophotometric assay was used. These effects were initially examined using three different levels of the substrate (2, 4, and 6 μM phosphorylase *a* monomer) and varying levels of protamine (0.25, 0.5, 1, 2, 4, 6, and 10 μM). In each case increasing levels of protamine resulted in increased $PP2A_1$ activity that was maximal at a 1:1 molar ratio of protamine to phosphorylase *a* monomer (not shown). At higher levels of protamine decreased activity was observed indicating that the excess protamine was binding to $PP2A_1$ and inhibiting its activity.

These results and the previous observations suggest that (1) protamine and phosphorylase *a* form a very tight complex; (2) $PP2A_1$ displays basal phosphorylase phosphatase activity that can be stimulated by protamine; and (3) free $PP2A_1$ combines with the phosphorylase *a*-protamine complex, free phosphorylase *a*, and free protamine with varying affinities. The free protamine and free phosphorylase *a* compete with the protamine−phosphorylase *a* complex for free $PP2A_1$. Since both the phosphorylase−$PP2A_1$ and protamine−phosphorylase−$PP2A_1$ complexes are catalytically active and free protamine inhibits $PP2A_1$, the minimal model (Scheme 1) for the effects of protamine on the activity of $PP2A_1$ is proposed, where phosphorylase *a* = substrate (S), phosphorylase *b* = product (P), protamine = activator (A), K_s is dissociation constant for ES, K_0 is dissociation constant for AS, K_{SA} is dissociation constant for ASE, K_{EA} is dissociation constant for EA, k_p is the rate constant for ASE, and k_p' is the rate constant for ES.

In this model where $V_{max} = k_p[E_0]$ and $V_{max}' = k_p'[E_0]$, the velocity equation can be written as follows:

$$V = \frac{V_{max}[S][A]/K_0K_{SA} + V_{max}'[S]/K_S}{1 + [S]/K_S + [A]/K_{EA} + [S][A]/K_0K_{SA}} \quad (1)$$

The relationships among S, A, and SA are the following:

$$[S] = [S]_t - [SA] \quad (2)$$

$$[A] = [A]_t - [SA] \quad (3)$$

$$[S][A] = K_0[SA] \quad (4)$$

The relationships shown in eqs 2−4 are substituted into eq 1.

$$V = \frac{V_{max}[SA]/K_{SA} + V_{max}'([S]_t - [SA])/K_S}{1 + ([S]_t - [SA])/K_S + ([A]_t - [SA])/K_{EA} + [SA]/K_{SA}} = \frac{V_{max}'[S]_t/K_S + (V_{max}/K_{SA} - V_{max}'/K_S)[SA]}{1 + [S]_t/K_S + [A]_t/K_{EA} + (1/K_{SA} - 1/K_S - 1/K_{EA})[SA]} \quad (5)$$

Multiplying eq 2 by eq 3,

$$[S][A] = ([S]_t - [SA])([A]_t - [SA]) \quad (6)$$

The relationship in eq 4 is substituted into eq 6.

$$K_0[SA] = [S]_t[A]_t - ([S]_t + [A]_t)[SA] + [SA]^2$$

$$[SA]^2 - ([S]_t + [A]_t + K_0)[SA] + [S]_t[A]_t = 0$$

$$[SA] = (^1/_2)\{[S]_t + [A]_t + K_0 - \{([S]_t + [A]_t + K_0)^2 - 4[S]_t[A]_t\}^{1/2}\} \quad (7)$$

The relationship shown in eq 7 is substituted in eq 5 to give eq 8:

$$V = \{2V_{max}'[S]_t K_{SA}/K_S\{[S]_t + [A]_t + K_0 - \{([S]_t + [A]_t + K_0)^2 - 4[S]_t[A]_t\}^{1/2}\} + V_{max} - V_{max}'K_{SA}/K_S\}/\{2K_{SA}(1 + [S]_t/K_S + [A]_t/K_{EA})/\{[S]_t + [A]_t + K_0 - \{([S]_t + [A]_t + K_0)^2 - 4[S]_t[A]_t\}^{1/2}\} + (1 - K_{SA}/K_S - K_{SA}/K_{EA})\}$$

To test kinetically this minimal model for the activation of $PP2A_1$ by protamine, two studies were undertaken in which initial velocity determinations were made under conditions that the concentrations of protamine and phosphorylase *a* were varied and eq 8 was fitted to the data. In the first study (Figure 6, Panel A), four fixed levels of protamine were used, and the levels of phosphorylase *a* were varied. In the second study (Figure 6, panel B), four fixed levels of phosphorylase *a* were used, and the levels of protamine were varied. In each case, maximal activity of $PP2A_1$ was observed at a phosphorylase *a* monomer:protamine molar ratio of 1:1. At concentrations of phosphorylase *a* (Figure 6, panel A) and protamine (Figure 6, panel B) that exceeded this ratio progressive decreases in activity were observed. The data determined from the two studies are summarized in Table 1. The results obtained were comparable.

The results show that protamine and phosphorylase *a* bind very tightly (K_0 = 0.69 nM). Free $PP2A_1$ binds tighter to the protamine-phosphorylase *a* complex than to free phosphorylase *a* or free protamine as shown by the average values (Table 1) for K_{SA} (2.26 μM), K_S (11.29 μM), and K_{EA} (18.1 μM), respectively.

Without protamine, phosphorylase *a* can be converted to phosphorylase *b* by $PP2A_1$, but this conversion is less efficient, having a 5-fold higher K_m value and a 17-fold lower V_{max} than that catalyzed by $PP2A_1$ in the presence of protamine (Table 1). This fact would explain the decreased $PP2A_1$ activity observed at high substrate concentration

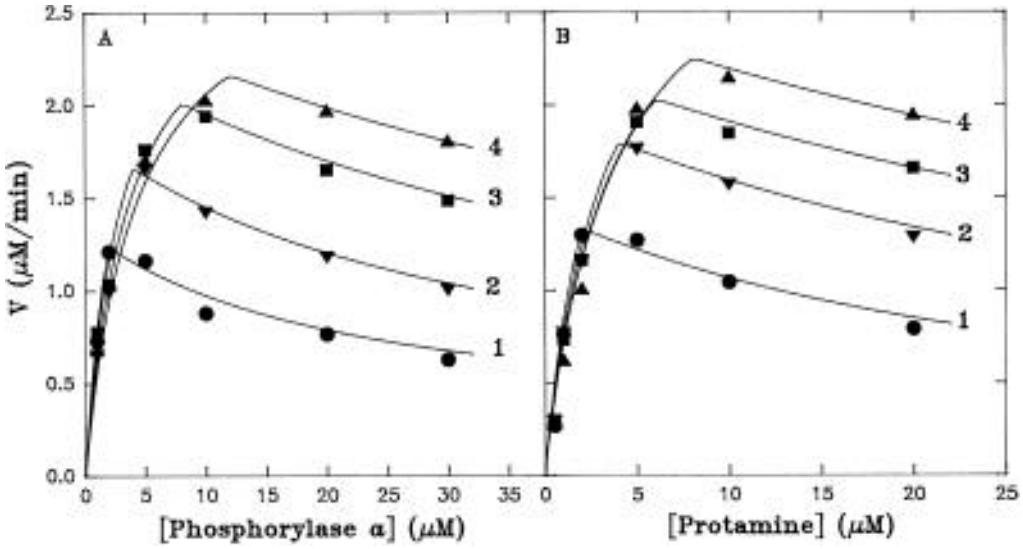


FIGURE 6: (A) Plots of initial velocities of $PP2A_1$ versus different concentrations of protamine at 1, 2, 5, 10, 20, and 30 μM phosphorylase *a*. The concentrations of protamine were 2, 4, 8, and 12 μM for curves 1−4, respectively. (B) Plots of initial velocities of $PP2A_1$ versus different concentrations of phosphorylase *a* at 0.5, 1, 2, 5, 10, and 20 μM protamine. The concentrations of phosphorylase *a* were 2, 4, 6, and 8 μM for curves 1−4, respectively. The solid lines were calculated from experimental data using eq 8 (see text). The continuous spectrophotometric assays were carried out at 25 °C in 1.8 mL mixtures containing 50 mM Bis-Tris (pH 7.0), 0.2 M NaCl, 5 mM caffeine, 2 mM dithiothreitol, 2% glycerol, 1% DSMO, 81 mM 7-methyl-6-thioguanosine, and 79 mg of purine nucleoside phosphorylase/mL. Reactions were initiated, after 5 min preincubation of reaction components at 25 °C, by the addition of 0.01 mL $PP2A_1$ (0.076 mg/mL) in assay buffer.

Table 1: Kinetic Parameters Determined for Protamine-Stimulated $PP2A_1$

kinetic parameter	data set I[a]	data set II[b]
V_{max}	2.55 ± 0.21 μM/min	2.76 ± 0.15 μM/min
V_{max}'	0.16 ± 0.04 μM/min	0.15 ± 0.05 μM/min
K_0	0.67 ± 0.62 nM	0.69 ± 0.62 nM
K_{SA}	2.12 ± 0.29 μM	2.40 ± 0.37 μM
K_S	11.88 ± 1.21 μM	10.71 ± 3.99 μM
K_{FA}	20.09 ± 3.81 μM	16.11 ± 3.72 μM

[a] Figure 6A. [b] Figure 6B.

(Figure 6, panel A). In a separate study, the kinetic properties of the basal activity of $PP2A_1$ were determined using the more sensitive radiolabeled assay at 25 °C. Assays were carried out using 0, 5, 10, 15, and 20 μM [^{32}P]-phosphorylase *a* and K_m and V_{max} values of 16.18 ± 2.91 μM and 0.21 ± 0.02 μM/min were determined, respectively. These values are comparable to those determined for K_s and V_{max}' (Table 1). The small differences in the kinetic values were probably due to the use of different phosphorylase *a* preparations in the radiolabeled and spectrophotometric assays.

The kinetics of the activation by protamine were further examined under conditions that the levels of protamine and phosphorylase *a* were at or less than their respective K_{EA} and K_S values, respectively. In the two cases studied (1, 2, 5, and 10 μM phosphorylase *a* monomer and 8 μM protamine, and 1, 2, 5, 10, and 20 μM phosphorylase *a* monomer and 12 μM protamine) K_m values of 2.28 and 2.27 μM and V_{max} values of 2.41 and 2.33 μM/min were determined, respectively. These values are consistent with the values of K_{SA} and V_{max} determined for the model (Table 1).

The inhibition by excess protamine (Figures 2 and 6, panel B) could be explained by the formation of a dead-end $PP2A_1$−protamine complex. The latter would also be consistent with the observation that protamine inhibited dephosphorylation of the phosphorylated tryptic peptides by the basal activity of $PP2A_1$. In all these initial rate studies the inhibition of the phosphatase activity by protamine was immediate. This is in contrast to the effect observed when $PP2A_1$ was preincubated with protamine (Figure 1). A possible explanation for this result is that protamine can induce a time-dependent conformational change in $PP2A_1$ that results in a progressive loss in the protamine-stimulated activity but not the basal activity of the phosphatase. The latter was still detected after the 30 min preincubation period since when phosphorylase *a* was added to initiate the reaction all the protamine and substrate combined to form the phosphorylase *a*−protamine complex due to the strong interaction between the two components (K_0 = 0.67 nM) and so excess protamine was not present to inhibit the basal activity.

When the kinetic studies were repeated using different $PP2A_1$ and phosphorylase *a* preparations similar results were obtained. The values determined from one such study, for example, were V_{max} = 2.77 μM/min, $V_{max'}$ = 0.25 μM/min, K_0 = 0.15 nM, K_{SA} = 2.38 μM, K_S = 13.58 μM, K_{EA} = 26.8 μM. These values are consistent with those summarized in Table 1.

The dissociation constant determined for the protamine−phosphorylase *a* complex indicates a very tight interaction between these two polypeptides. Despite this tight interaction, the $PP2A_1$ reaction goes to completion at both optimal and suboptimal levels of protamine. An example of this is shown in Figure 7. This would indicate that free phosphorylase *a* can compete successfully with phosphorylase *b* for the protamine after phosphorylase *a*−protamine is converted to phosphorylase *b*−protamine by $PP2A_1$. This could be the result of a lesser affinity of phosphorylase *b* than phosphorylase *a* for protamine. Evidence that this was obtained from chromatography studies. Phosphorylase *b* eluted from immobilized protamine (not shown) at a lower NaCl concentration (0.8 M) than phosphorylase *a* (1.25 M). These

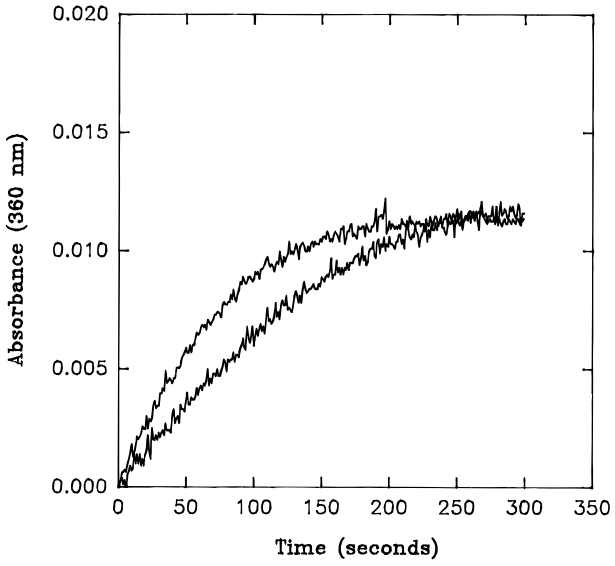


FIGURE 7: Time course of the release of inorganic phosphate by $PP2A_1$ in the presence of different concentrations of protamine in the phosphatase-coupled enzyme system. The continuous spectrophotometric assays were carried out as described in Figure 6 with the exception that the reaction mixture contained 1 μM phosphorylase *a*, and 1 μM (upper curve) and 0.4 μM (bottom curve) protamine, respectively.

observations could suggest that the driving force for completing the reaction may be due to phosphorylase *a* competing with phosphorylase *b* for protamine after phosphorylase *a* is converted to phosphorylase *b*.

DISCUSSION

The activation of the phosphorylase phosphatase activity of $PP2A_1$ by protamine was determined to be complex. Both the basal and protamine-stimulated activities of the enzyme were sensitive to ionic strength. In the presence of 0.15 M NaCl the basal activity of $PP2A_1$ was less than 2% that expressed in the presence of 10 μM protamine. This could indicate that the basal activity of the phosphatase could be very low in the cell under physiological ionic strength conditions. It was also observed (not shown) that the activation of $PP2A_1$ by preincubation with heparin, which was shown to be the result of removal of the B subunit from the ABC heterotrimer complex of $PP2A_1$ (Kamibayshi et al., 1991) was also prevented by 0.15 M NaCl. The maximal values of activity determined, in the absence of the NaCl, for $PP2A_1$ on dilution and by preincubation with heparin (15−30 μg/mL) were essentially the same. These observation could support the possibility that the increase on dilution of the basal activity of the phosphatase may be due to the dissociation of the B subunit from the ABC complex as has been previously suggested (Erickson & Killilea, 1992b; Ingebritsen et al., 1983; Lee et al., 1977).

One of the effects of ionic strength on the protamine stimulated activity of $PP2A_1$ is to prevent aggregation and precipitation of the substrate, phosphorylase *a*, by protamine. However, with the increased ionic strength, the protamine still interacted with phosphorylase *a* as evidenced by the tight binding of the latter protein to immobilized protamine. This interaction, at optimal ionic strength, also appears to be very specific since (a) maximal activation of phosphorylase phosphatase activity of $PP2A_1$ is expressed at a protamine to phosphorylase *a* monomer ratio of 1:1 and (b) crystallization of the complex takes place in the absence of caffeine.

The specific interaction(s) between the polycationic polypeptide and phosphorylase *a* does not apparently involve ser 14, the phosphorylation site in the substrate, since (a) protamine does not stimulate the dephosphorylation of this phosphorylated serine residue in [^{32}P]-labeled tryptic penta- and hexapeptides derived from [^{32}P]phosphorylase *a* and (b) these [^{32}P]-labeled tryptic peptides were not retained by immobilized protamine. These observations would indicate that secondary/tertiary protein structure(s) in intact phosphorylase *a*, other than the immediate vicinity of the phosphorylation site, are involved in the binding of protamine to the substrate. Possible regions could include residues 109−128 and 118−128, which contain 11 out of 20 and 8 out of 11 acidic amino acid side chains, respectively (Titani et al., 1977). These observations complement the results of other studies (Agostinis et al., 1987; King et al, 1994) that have shown that the dephosphorylation of phosphopeptides, corresponding to phosphorylation sites in pyruvate kinase, inhibitor-1, and opsin by type 2A phosphatases, is not stimulated by basic polypeptides. Like phosphorylase *a*, the dephosphorylation of the phosphorylated forms of these native proteins is stimulated by basic polypeptides (Agostinis et al., 1987; King et al., 1994).

While the physical and kinetic modeling studies offer compelling evidence for specific and very tight binding of protamine to phosphorylase *a*, the stimulation of $PP2A_1$ activity cannot be explained as being similar to substrate-directed phosphatase stimulators such as glucose, glucose 6-phosphate, and caffeine. If this were the case, protamine would have been expected to stimulate not only the phosphorylase phosphatase activity of the intact catalytic subunit but also the tryptic release of phosphorylated penta- and hexapeptides corresponding to the phosphorylation site of phosphorylase *a*. Phosphatase and trypsin action on phosphorylase *a* has been demonstrated to be very sensitive to inhibition and stimulation by modulators, such as AMP and glucose, which stabilize the R- and T-state of the substrate in which the phosphorylation site is buried or exposed, respectively, to the action of phosphatase or trypsin (Mananu & Madsen, 1981; Madsen, 1986). Protamine did not stimulate either the activity of the catalytic subunit or the release of [^{32}P]peptide material from [^{32}P]phosphorylase by trypsin (not shown). These results would thus indicate that protamine stimulation of $PP2A_1$ activity is not a substrate-directed effect and must be due to additional interaction(s) of protamine with $PP2A_1$.

Since protamine could stimulate $PP2A_1$ activity, but not that of its isolated catalytic subunit, the polycationic polypeptide must be also directly interacting with $PP2A_1$. This could involve part of the protamine, not bound to phosphorylase *a*, interacting with the catalytic subunit and/or with one or both of the two regulatory subunits in the intact enzyme. The subunit(s) involved will need to be identified. However, activation of type 2A protein phosphatases by basic polypeptides does not apparently involve subunit dissociation since it has been reported that in gel filtration studies release of the catalytic subunit was not observed (Pelech & Cohen, 1985), and immobilized preparations of protamine, polylysine, and histone H1 have been used in the purification of the intact trimeric and dimeric forms of PP2A from a variety

of tissues [e.g., see Gergely et al. (1986), Imaoka et al. (1983), Tung et al. (1985), Waelkens et al. (1987b)].

The inability of protamine to activate the catalytic subunit could be due to the fact that the catalytic subunit had already been previously fully activated by the ethanol treatment used to activate and release the subunit from the parent enzyme, $PP2A_1$. The inability of the protamine to significantly activate the catalytic subunit is also consistent with studies (Depoali-Roach & Klingberg, 1985; Schlender et al., 1086; Waelkens et al., 1987a) that have reported that the native catalytic subunit must be subjected to mild trypsinolysis before protamine stimulation can be observed. Trypsinolysis reduces the activity of the catalytic subunit, and this lost activity can then be restored by polycationic polypeptides (Depoali-Roach & Klingberg, 1985; Schlender et al., 1986; Waelkens et al., 1987a). A similar phenomenon was apparently observed here for the protamine activation of the salt-inhibited intact catalytic subunit.

On the basis of these observations, the following model for the activation of the phosphorylase phosphatase activity of $PP2A_1$ by protamine is proposed. Under the appropriate conditions of ionic strength, protamine associates in a 1:1 complex with each subunit of the substrate, phosphorylase *a*. This complex must have a specific orientation to allow for the interaction and concomitant activation of the phosphorylase phosphatase activity of $PP2A_1$ by the substrate-bound protamine. Such specific interactions among the substrate, polycationic polypeptide, and phosphatase would explain the activation observed when intact phosphorylase *a*, but not when its tryptic derived penta- and hexaphosphopeptides, was the substrate, and, when the parent $PP2A_1$, but not the catalytic subunit (or trypsin), were used. Based on this model, the interactions between protamine and the substrate would also be essential to prevent inhibition of $PP2A_1$ by free protamine.

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