Polyamines Alter the Substrate Preference of Nuclear Protein Kinase NII†

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ABSTRACT: When investigating the effects of polyamines on substrate specificity of adenosine cyclic 3′,5′-monophosphate (cAMP) independent nuclear protein kinase NII, we found that the substrate preference of NII was drastically altered in the presence of polyamines. When casein was used as a substrate, spermine stimulated the enzyme activity 7.3-fold at 2 mM, and a 6.5-fold stimulation was observed with 6 mM spermidine. Putrescine was also slightly effective at higher concentrations. With phosvitin as the substrate, however, 2 mM spermine and 4 mM spermidine strongly inhibited the activity by 93% and 80%, respectively, while putrescine showed a weak stimulatory effect. Steady-state kinetics and binding studies suggested that both stimulatory and inhibitory effects of polyamines on NII enzyme activity are probably due to substrate protein–polyamine interactions. The circular dichroism spectrum of phosvitin was apparently altered by spermine, whereas no significant change was observed with casein.

Nuclear protein kinase NII,[1] purified by Thornburg & Lindell (1977), phosphorylates in vitro the high mobility group (Inoue et al., 1980) and other nonhistone chromosomal proteins (Hasuma et al., 1980). As the phosphorylation of nonhistone chromosomal proteins is considered to be intimately involved in gene activation (Stein et al., 1974; Jungmann & Kranias, 1977), the control of this enzyme activity may be an essential factor in regulation of gene expression.

There is a considerable body of evidence that the aliphatic polyamines putrescine, spermidine, and spermine are closely linked to vital cell functions and that a significant amount of spermidine and spermine is present in the nuclei (Cohen, 1971; Dion & Cohen, 1971). In addition, studies have demonstrated that polyamines stimulate the phosphorylation in isolated nuclei (Farron-Furstenthal & Lightholder, 1978) and also have stimulatory effects on nuclear protein kinases from rat liver (Imai et al., 1975; Hara et al., 1981). We now report that spermine and spermidine drastically alter the substrate preference of nuclear protein kinase NII.

Materials and Methods

Materials. Spermidine–Sepharose prepared according to the method of Bartos & Bartos (1979) contained 4.7 μmol of spermidine/mL of swollen gel. [γ-^{32}P]ATP was prepared by the method of Glynn & Chappell (1964). The sources of reagents were the same as in our previous work (Hara et al., 1981).

Isolation of Nuclei. Nuclei were isolated by the method of Chauveau et al. (1956) with slight modification. Male Donryu rats weighing 100–150 g were fasted overnight and then exsanguinated. Livers were perfused with ice-cold buffer A (0.25 M sucrose containing 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 0.5 mM $PhMeSO_2F$) and immediately removed. All subsequent steps were performed at 0–4 °C. Livers were minced and homogenized in 5 volumes of buffer A with five strokes of a motor-driven Teflon–glass homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 1000*g* for 10 min. The pellet was resuspended in buffer A and centrifuged at 1000*g* for 5 min. The crude nuclear pellet was homogenized in 4 volumes of buffer B (2.2 M sucrose containing 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, and 0.5 mM $PhMeSO_2F$) with two strokes and layered over a 5-mL cushion of buffer B in tubes and centrifuged at 20 000 rpm for 45 min in a Beckman SW25-1 rotor. The floating cake was removed with a spatula, and the supernatant was decanted. The final nuclear pellet was washed with buffer A, followed by centrifugation at 1000*g* for 5 min. This washed pellet served as the material for further study.

Purification of Nuclear Protein Kinase NII. Nucleoplasm proteins (700 mg), prepared from the purified nuclei as described previously (Hara et al., 1981), were dialyzed against buffer C (20 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.25 mM $PhMeSO_2F$) containing 0.3 M NaCl and applied to a phosphocellulose column (2.5 × 10 cm) that was equilibrated with buffer C containing 0.3 M NaCl. The column was washed with buffer C containing 0.3 M NaCl and developed with 600 mL of a linear gradient that ranged from 0.3 to 1.0 M NaCl in buffer C. Fifteen-milliliter fractions were collected at a rate of 60 mL/h. Nearly all cAMP-independent protein kinase activities were eluted with a broad peak having several notches, as shown in Figure 1A. Active fractions were directly applied to a hydroxylapatite column (1.6 × 6 cm) equilibrated with buffer C containing 1.0 M NaCl, and the column was washed with 10 mM potassium phosphate buffer, pH 7.4, containing 10% glycerol, 10 mM β-mercaptoethanol, 0.1 mM EDTA, and 0.25 mM $PhMeSO_2F$. Fractions were collected in 15-mL quantities. Subsequently, a 250-mL linear gradient elution was carried out with 10–500 mM potassium phosphate buffer, pH 7.4, containing the same additives as described above. Fractions of 5 mL were collected at a rate of 40 mL/h. This chromatography divided protein kinase activities into two peaks, one in flow through and the other in eluted fractions. Nuclear protein kinase NII was exclusively found in the eluted fractions (Figure 1B). The eluted fractions (75 mL) were precipitated with ammonium sulfate at 80% saturation and dissolved in 3 mL of buffer C containing 1.0 M NaCl. The solution was subjected to a Sephacryl S-300 column (2.5 × 88 cm) equilibrated with buffer C containing 1.0 M NaCl. Fractions of 5 mL were collected at a flow rate of 6 mL/h,

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; ATP, adenosine 5′-triphosphate; cAMP, adenosine cyclic 3′,5′-monophosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; NII, the nomenclature of Desjardins et al. (1972) is employed.

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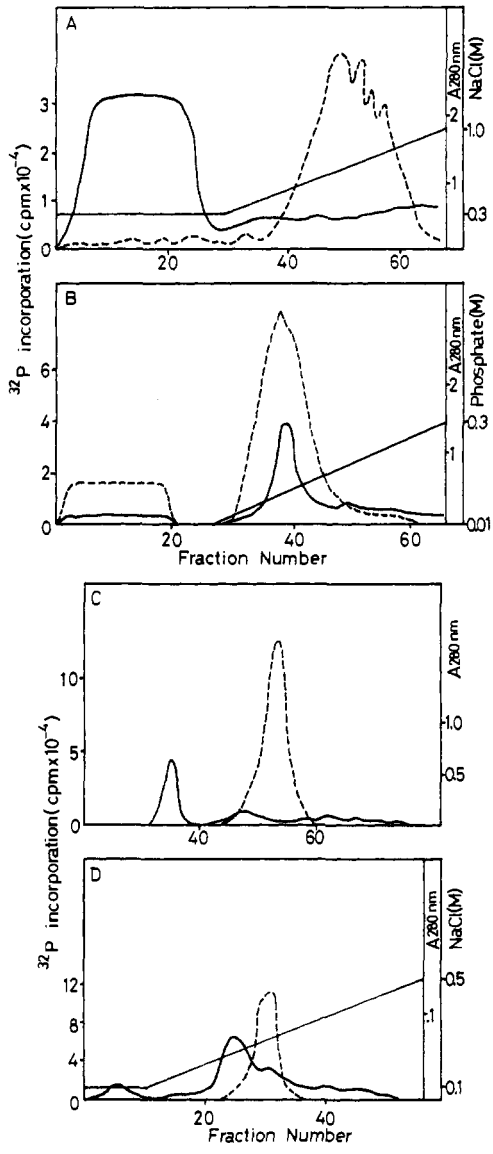

FIGURE 1: (A) Phosphocellulose chromatography. (B) Hydroxylapatite chromatography. (C) Sephacryl S-300 gel filtration. (D) DEAE-Sephadex A-25 chromatography of nuclear protein kinase NII. The fractions were assayed for protein kinase activity (---) and absorbance at 280 nm (—). Assays were carried out in the standard reaction mixture containing 0.15 M NaCl, 2 mg/mL casein, and an aliquot of each fraction at 30 °C for 15 min.

as depicted in Figure 1C. The active fractions (45 mL) were dialyzed against buffer C containing 0.1 M NaCl and applied to a DEAE-Sephadex A-25 column (1.6 × 3.0 cm) equilibrated with buffer C containing 0.1 M NaCl. After a wash with buffer C containing 0.1 M NaCl, elution was performed with 100 mL of a linear gradient of 0.1–1.0 M NaCl in buffer C, and fractions were collected in 2 mL at a rate of 20 mL/h. Figure 1D shows a typical elution pattern of this column. NII kinase from DEAE-Sephadex chromatography was dialyzed against buffer C containing 0.1 M NaCl and applied to a spermidine column (1.5 × 2.0 cm) equilibrated with buffer C containing 0.1 M NaCl. The column was washed with buffer C containing 0.1 M NaCl and developed with 100 mL of a linear gradient from 0.1 to 1.0 M NaCl in buffer C. Two-milliliter fractions were collected at a rate of 20 mL/h (Figure 2). For the study of casein–polyamine or phosvitin–polyamine interaction, 2 mg of casein or phosvitin was applied to a spermidine column, in the same manner.

Assay of Protein Kinase Activity. The standard reaction mixture (100 μL) contained 50 mM Tris-HCl, pH 7.4, 5 mM

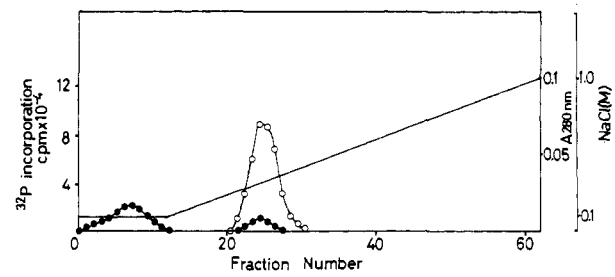

FIGURE 2: Chromatography of nuclear protein kinase NII on spermidine–Sepharose. The fractions were assayed for protein kinase activity (○) and absorbance at 280 nm (●). Assays were performed in the same way as described in Figure 1.

$MgCl_2$, 10 mM β-mercaptoethanol, 2 mg/mL substrate, 0.1 mM [γ-^{32}P]ATP (100 000 cpm/nmol), and enzyme. Casein and other substrates were prepared as described by Thornburg & Lindell (1977). The incubation was carried out at 30 °C for the indicated time. The reaction was terminated by transferring all the mixture onto Whatman 3MM filters (1.5 × 3.0 cm), which were then processed according to the method of Desjardins et al. (1972). The radioactivity was measured in a Beckman liquid scintillation counter.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis. Purified NII kinase was dialyzed against 42 mM acetic acid and lyophilized; it was then dissolved in 40 μL of sample buffer containing 0.125 M Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, and 5% β-mercaptoethanol, heated at 100 °C for 3 min, and applied on a 12% polyacrylamide slab gel in the presence of 0.1% sodium dodecyl sulfate (Laemmli & Favre, 1973). Gels were stained for protein with Coomassie Brilliant Blue R-250.

Circular Dichroism Measurements. Circular dichroism was performed at 20 °C with a Jasco J-20 automatic-recording spectropolarimeter in a 0.5-mm path length cell. Circular dichroism spectra were measured in a solution containing 50 mM Tris-HCl, pH 7.4, 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 0.2 mg/mL phosvitin or casein, and various concentrations of spermine at pH 7.4. The results were expressed as mean residue ellipticity [θ], and the mean residue molecular weights of phosvitin and casein were taken as 161 (Taborsky, 1968) and 119 (Herskovits, 1966), respectively.

Other Procedures. Proteins were determined with Folin's reagent (Lowry et al., 1951) by using bovine serum albumin as a standard.

Results

Purification of Nuclear Protein Kinase NII. A typical purification of the protein kinase is summarized in Table I. A 1850-fold purification from nucleoplasm proteins was achieved for protein kinase activity. The recovery of nuclear protein kinase NII was 6.6%, but this figure was no doubt an underestimation as more than half of the total activity in the starting material was ascribed to other protein kinases. The purity and homogeneity were determined by polyacrylamide gel electrophoresis, in the presence of sodium dodecyl sulfate. The three major bands had molecular weights of 42 000, 38 000, and 23 000, as determined by the mobility relative to standards of known molecular weights (Figure 3). The molar ratio of subunits was 1:1:2 for the 42 000, 38 000, and 23 000 molecular weight subunits, respectively. A molecular weight of 120 000 determined from gel filtration data was consistent with that calculated from the subunit molecular weights and their molar ratio. The purified kinase preferred phosvitin and casein to histone and was not stimulated by either cAMP or cGMP. These data are in agreement with findings of

Table I: Purification of Nuclear Protein Kinase NII

	total proteins (mg)	sp act.[a] (nmol mg^{-1} min^{-1})	purifcn (x-fold)	total act. (nmol min^{-1})	recovery (%)
nucleoplasm proteins	700	1.2	1	840 (121)[b]	100
phosphocellulose	95	8.8	7	836 (0)	99.5
hydroxylapatite	14.9	16.4	14	244 (0)	29.0
Sephacryl S-300	0.68	133.1	111	90.5 (0)	10.8
DEAE-Sephadex	0.09	689.8	575	62.1 (0)	7.4
spermidine–Sepharose	0.025	2220.0	1850	55.5 (0)	6.6

[a] Protein kinase activity was measured in the standard reaction mixture containing 10 μL of each sample and 0.15 M NaCl at 30 °C for 15 min with casein as a substrate. [b] Values in parentheses indicate endogenous phosphorylation assayed in the absence of casein.

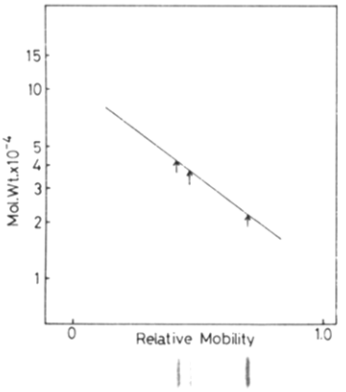

FIGURE 3: Sodium dodecyl sulfate gel electrophoresis of the purified protein kinase. Approximately 20 μg of the protein kinase from the spermidine column was analyzed with a 12% polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate as described under Materials and Methods. The calibration curve was established with the following standards: heavy chain (55 000) and light chain (23 000) of immunoglobulin G and actin from rabbit muscle (42 000).

Thornburg & Lindell (1977).

Interaction of Polyamines with Nuclear Protein Kinase NII. We have reported that polyamines markedly stimulate the purified NII from rat liver with casein as a substrate (Hara et al., 1981). However, Thornburg et al. (1979) briefly mentioned that NII activity was strongly inhibited by polyamines with phosvitin. Therefore, we examined the effect of 2 mM spermine on the purified enzyme toward various substrates. In the absence of spermine, ^{32}P incorporations into albumin, protamine, histone, α-casein, casein, and phosvitin were 0, 0.28, 0.64, 3.13, 3.92, and 8.17 pmol/min, respectively, when assayed in the standard reaction mixture containing 10 ng of enzyme and 2 mg/mL of each substrate. Addition of 2 mM spermine stimulated the phosphorylation of histone, α-casein, and casein 2.5-, 4.4-, and 7.3-fold, respectively, while the phosvitin phosphorylation was inhibited by 93%. There was no alteration in the phosphorylation of albumin and protamine with 2 mM spermine. Since, among these substrates tested, polyamine showed contrasting effects on the phosphorylation of casein and phosvitin, the effects of various polyamines on the protein kinase activity were studied in greater detail with casein and phosvitin (Figure 4). When casein was used as the substrate, a stimulation of 6.5- and 7.3-fold was observed with 6 mM spermidine and 2 mM spermine, respectively. Putrescine was to some extent effective at higher concentrations. On the contrary, when phosvitin was used as a substrate, 2 mM spermine and 4 mM spermidine inhibited the activity by 93% and 80%, respectively, whereas putrescine had a weak stimulatory effect and a 1.4-fold stimulation occurred at 10 mM (Figure 4B). Casein as well as phosvitin solution becomes turbid when polyamine is added. However, the turbidity of the phosvitin solution disappeared

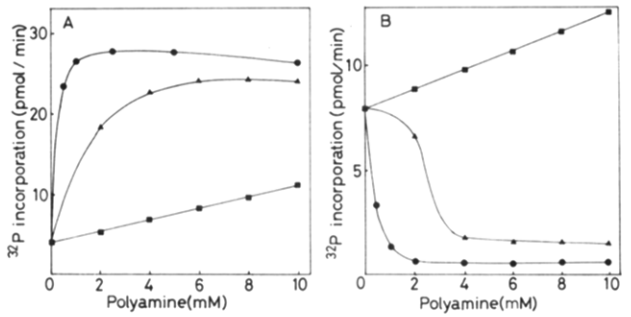

FIGURE 4: Effect of polyamines on the protein kinase activity with casein or phosvitin as a substrate. Protein kinase activity was assayed for 10 min in the standard reaction mixture containing 10 ng of enzyme, 2 mg/mL casein (A) or phosvitin (B), and the indicated concentrations of polyamine: putrescine (■), spermidine (▲), spermine (●).

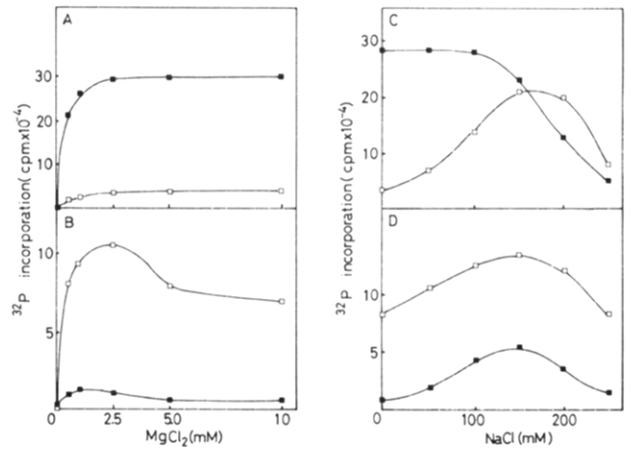

FIGURE 5: Effect of various concentrations of $MgCl_2$ and NaCl on the stimulation and inhibition of the protein kinase activity by spermine. Protein kinase activity was measured for 10 min in the standard reaction mixture containing 10 ng of enzyme and 2 mg/mL casein (A and C) or phosvitin (B and D) in the presence (■) and absence (□) of 2 mM spermine. $MgCl_2$ (A and B) and NaCl (C and D) concentrations were varied as indicated. Phosvitin was dialyzed against 10 mM EDTA and 1 M NaCl and then water, to study the effect of Mg^{2+} on spermine inhibition.

when the solution was incubated at 30 °C or dialyzed overnight, as described in Table II. Since a similar inhibition was observed even in the absence of turbidity, the inhibitory effect of polyamine is not due to the precipitation of phosvitin.

To analyze the mechanism by which polyamines exert contrasting effects on protein kinase NII, we first examined the effect of Mg^{2+} on spermine activation and inhibition. When casein was used as a substrate, the enzyme showed an absolute requirement for Mg^{2+}, and the maximal activity was exhibited at concentrations over 2.5 mM Mg^{2+}, in the presence or absence of 2 mM spermine, as shown in Figure 5A. Spermine was unable to completely substitute for Mg^{2+}, as was also found with cAMP-independent protein kinases from

Table II: Effects of the Preincubation of NII Enzyme, α-Casein, and Phosvitin with Spermine on the Phosphorylation

	NII enzyme preincubated with 5 mM spermine	substrate	preincubated with 5 mM spermine	^{32}P incorpn[c] (pmol/min)	A/B (%)
expt 1[a]	+	α-casein		2.82 (A)	92
	−			3.07 (B)	
	+	phosvitin		7.97 (A)	96
	−			8.30 (B)	
expt 2[b]		α-casein	+	5.64 (A)	188
			−	3.00 (B)	
		phosvitin	+	1.90 (A)	23
			−	8.26 (B)	

[a] NII enzyme was preincubated with (+) or without (−) 5 mM spermine in the buffer containing 20 mM Tris-HCl, pH 7.4, and 10 mM β-mercaptoethanol at 4 °C for 60 min and then dialyzed against 100 volumes of buffer containing 20 mM Tris-HCl, pH 7.4, and 10 mM β-mercaptoethanol for 10 h. [b] α-Casein or phosvitin was preincubated with (+) or without (−) 5 mM spermine in the same manner. [c] Assays were carried out at 30 °C for 10 min in the standard reaction mixture containing 10 ng of enzyme and 2 mg/mL α-casein or phosvitin.

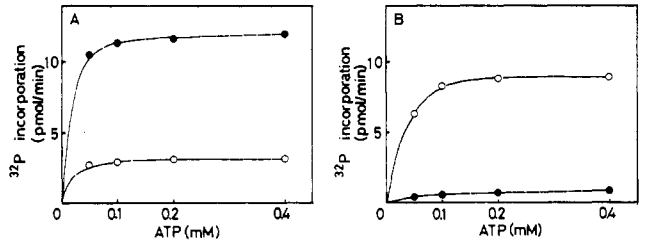

FIGURE 6: Effect of various concentrations of ATP on the stimulation and inhibition of NII activity by spermine. Assays were carried out for 10 min in the standard reaction mixture containing 10 ng of enzyme and 2 mg/mL α-casein (A) or phosvitin (B) except that ATP concentrations were varied from 0.05 to 0.4 mM in the presence (●) or absence (○) of 1 mM spermine.

different sources (Mäenpää, 1977; Ahmed et al., 1978). With phosvitin as a substrate, the optimal Mg^{2+} concentration in the absence of spermine was 2.5 mM, and spermine strongly inhibited the protein kinase activity at any concentration of Mg^{2+} (Figure 5B). Thus, the effects of spermine were not greatly influenced by Mg^{2+} concentrations.

As the activity of the NII enzyme was enhanced with NaCl (Desjardins et al., 1972), changes in the polyamine effects were also studied in the presence of various concentrations of NaCl. The stimulatory effect of spermine gradually decreased with increases in NaCl concentrations, when casein was used as a substrate (Figure 5C). At concentrations over 150 mM NaCl, the stimulatory effect of spermine disappeared. On the other hand, there was a partial release of inhibition around 150 mM NaCl, with phosvitin as a substrate (Figure 5D).

We further investigated the effect of various concentrations of ATP on spermine activation and inhibition of NII activity. As shown in Figure 6, the stimulation and inhibition rates by spermine were not greatly affected by ATP concentrations. Then, changes in spermine effects were studied by varying the concentrations of α-casein and phosvitin, respectively, at different concentrations of spermine. The degree of stimulation was much the same at various concentrations of α-casein (Figure 7A). On the other hand, when the concentration of phosvitin was varied, there was a biphasic reversal of spermine inhibition with increases in phosvitin concentrations (Figure 7B). Since Mg^{2+} binds to phosvitin (Grizzuti & Perlmann, 1973), high concentrations of phosvitin might affect the phosphorylation reaction by changing the Mg^{2+} concentration. However, as Figure 5B shows, no remarkable change was observed in the rate of inhibition, at any concentration of Mg^{2+} tested. Therefore, it seems less probable that the abrupt reversal of spermine inhibition was mainly caused by a change in Mg^{2+} concentration. When the data in Figure 7B were replotted against the spermine:phosvitin ratio as shown in Figure 7C, we found that the abrupt reversal of spermine inhibition began to occur when the spermine:phosvitin ratio was below 0.3. This finding suggested that the phosvitin–polyamine interaction was, at least in part, involved in the polyamine inhibition of NII activity.

To investigate which interactions are more closely involved in the activation and inhibition of NII activity by polyamines, enzyme–polyamine, or substrate protein–polyamine, we studied the effects of the preincubation of enzyme or substrates with spermine (Table II). When preincubated α-casein was Table II used as a substrate, the phosphorylation of α-casein was stimulated 1.9-fold, whereas the phosphorylation of phosvitin was inhibited by 77% when preincubated phosvitin was used. Meanwhile, no significant alteration was observed in the phosphorylation of α-casein and phosvitin by NII, regardless of whether or not the enzyme was preincubated with spermine. Thus, it would appear that substrate protein–polyamine interactions are more closely concerned with the effects of

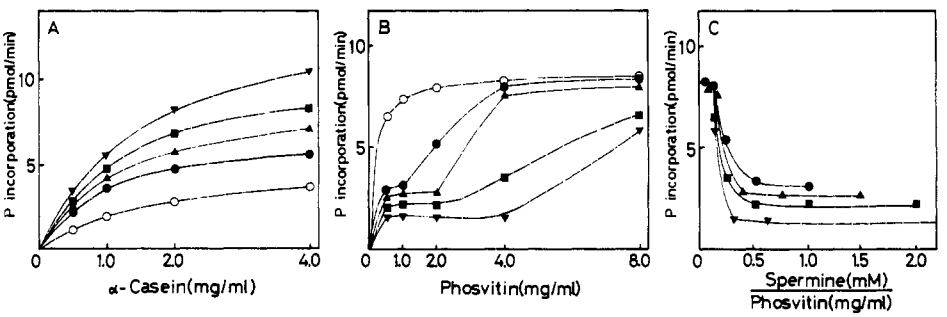

FIGURE 7: Effect of various concentrations of α-casein and phosvitin on the stimulation and inhibition of NII activity at different concentrations of spermine. (A) Assays were carried out for 10 min in the standard reaction mixture containing 10 ng of enzyme and 1 mg/mL bovine serum albumin. α-Casein concentrations were varied from 0.5 to 4.0 mg/mL. Spermine concentrations were 0 (○), 0.2 (●), 0.4 (▲), 0.6 (■), and 0.8 mM (▼). (B) Assays were performed in the same way except that phosvitin concentrations were varied from 0.5 to 8.0 mg/mL in the presence of 0 (○), 0.5 (●), 0.75 (▲), 1.0 (■), and 1.25 mM (▼) spermine. (C) Data in part B were replotted against the spermine:phosvitin ratio.

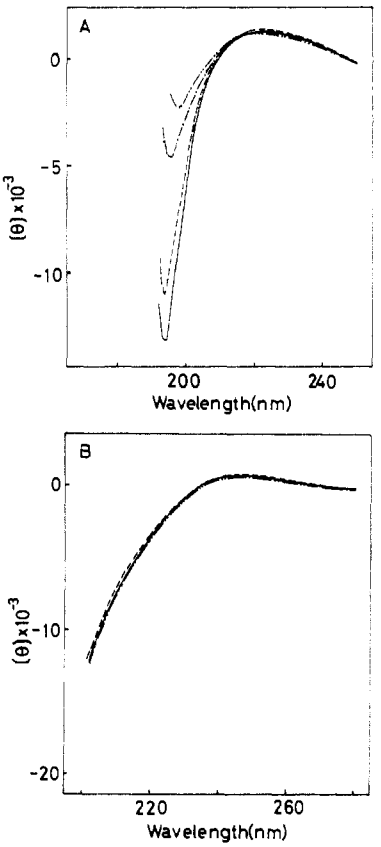

FIGURE 8: Circular dichroism of phosvitin and casein at various concentrations of spermine. Circular dichroism was measured in the assay mixture containing 0.2 mg/mL phosvitin (A) or casein (B) at various concentrations of spermine as described under Materials and Methods. Spermine concentrations were 0 (—), 0.05 (- - -), 0.2 (–·–), and 1.0 mM (–···–). The pH of all solutions was 7.4.

polyamines on NII activity than enzyme–polyamine interactions. Further support that phosvitin and casein interact with polyamines was evidenced by the fact that these compounds bound to a spermidine column and were eluted at 0.32 and 0.27 M NaCl, respectively, with a linear gradient ranging from 0.1 to 1.0 M NaCl (data not shown).

As there have been reports that phosvitin and casein may undergo conformational changes by cation binding (Taborsky, 1974, 1980), circular dichroism measurements were carried out in the presence of various concentrations of spermine. These measurements revealed that the negative band intensity of phosvitin at 195 nm was definitely diminished with increases in spermine concentration (Figure 8A), while putrescine did not induce the changes observed with spermine (data not shown). Thus, the polyamine inhibition of phosvitin phosphorylation is probably due to conformational changes of phosvitin by polyamines. With regard to the polyamine stimulation of casein phosphorylation, the involvement of casein–polyamine interaction was suggested from the results in Table II; however, evidence for the conformational change of casein molecules was not forthcoming in the circular dichroism studies (Figure 8B).

Discussion

Phosvitin, containing about 60% serine residues, serves as a better phosphate acceptor protein for NII than does casein in the absence of polyamines. In the presence of spermine and spermidine, however, the casein phosphorylation by NII was markedly stimulated, while the phosphorylation of phosvitin was strongly inhibited. Polyamine exerts its effects on various enzymes through enzyme–polyamine (Jakubowski, 1980) or substrate–polyamine interactions (Tabor & Tabor, 1976). Here we have shown that substrate protein–polyamine interactions may play an important role in the effects of polyamines on NII activity. Ahmed et al. (1978) reported that the polyamine stimulation of prostatic nuclear protein kinases might be due to substrate protein–polyamine interactions because chemical methylation of phosvitin altered both its activity as a substrate and its response to polyamines. There are several other studies that suggest the involvement of substrate protein–polyamine interaction in the activation of cAMP-independent protein kinase activity (Yamamoto et al., 1979; Daniels et al., 1981).

With regard to other enzymes, it has been reported that polyamines stimulate the activity of a ribonuclease with poly(cytidylic acid) as a substrate but inhibited it with poly(uridylic acid) and that the effects of polyamines would be due to RNA–polyamine interactions (Igarashi et al., 1975). In fact, there is ample evidence that polyamines interact with nucleic acids and alter their conformations (Tabor & Tabor, 1976). As phosvitin and casein also interact with polyamines, it is conceivable that polyamines may induce conformational changes in substrate proteins to alter the accessibility of NII to its proper recognition sites (Tuazon & Traugh, 1978; Mercier, 1981), thereby resulting in the activation or inhibition of NII activity. Although the substrates used in this study were nonphysiological, our results do suggest that polyamines alter the phosphorylation of nuclear proteins by NII through interactions with substrate proteins. A selective regulation of phosphorylation would be feasible if polyamines interacted with respective phosphate acceptor proteins. Since the millimolar concentrations used in our present work were within physiological ranges, the evidence suggests that polyamines might play a key role in the control of NII activity in the cell nuclei.

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Stereochemical Studies on the Hydration of Monofluorofumarate and 2,3-Difluorofumarate by Fumarase†

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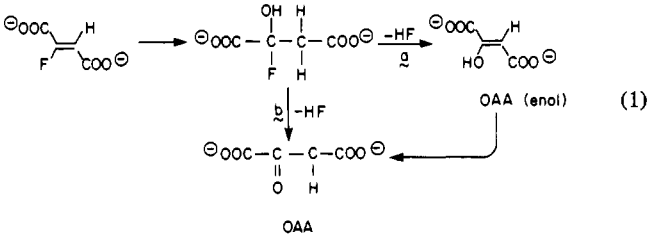
ABSTRACT: Stereochemical and product analyses have been studied in our continuing work on the bioprocessing of fluorinated substrate analogues. The hydration pathways of the fumarase-catalyzed reaction on fluorofumarate lead to a product distribution of L-*threo*-β-fluoromalate to oxalacetate of 1 to 16. The β-fluoromalate product has not been previously reported. Oxalacetate formation from the initial product, α-fluoromalate, an α-fluorohydrin, proceeds by way of a direct nonenzymic decomposition path (as opposed to collapse to the enol of oxalacetate with subsequent tautomerization). Difluorofumarate is hydrated to an α-fluorohydrin, α,β-difluoromalate, which decomposes to 3(*S*)-fluorooxalacetate trapped by in situ malate dehydrogenase mediated reduction to L-*threo*-β-fluoromalate (2*R*,3*S*). L-*threo*-Fluoro[2-^{3}H]-malate is a slow substrate for the reverse reaction as measured by labilization of ^{3}H while the erythro isomer is barely detectable. The pathways responsible for this volatilization are discussed. Acetylenedicarboxylate hydration stereochemistry was also determined where the initial product of the reaction, the enol of oxalacetate, tautomerized and was trapped by enzymic reduction to L-malate.

The enzyme fumarase (EC 4.2.1.2) is a ubiquitous cellular enzyme, catalyzing the reversible hydration of fumarate to malate during the action of the citrate cycle. It has been the object of many structural and mechanistic studies (Alberty et al., 1957; Alberty & Bender, 1959), including the stereochemistry of hydration (Gawron et al., 1961; Gawron & Fondy, 1959; Englard, 1958) and the nature of the catalyzed reaction (carbonium ion, carbanion, or concerted) (Nigh & Richards, 1969; Hansen et al., 1969; Alberty et al., 1957; Schmidt et al., 1969; Blanchard & Cleland, 1980; Porter & Bright, 1980; Jones et al., 1980). In addition to fumarate and malate, the enzyme will process other dicarboxylic acids including several halofumarates, acetylenedicarboxylate, and D-tartrate (Hill & Teipel, 1971). Hill and colleagues noted that the chloro-, bromo-, and iodofumarates are hydrated to the β-halo-*threo*-L-malate derivatives but that fluorofumarate yields oxalacetate (OAA)[1] at 400% the V_{max} of fumarate, presumably by initial hydration in the opposite sense to yield α-fluoromalate (Teipel et al., 1968). Hill and Teipel suggested that this initial α-fluoromalate product could lose H–F to yield the enol of OAA, which by tautomerization would yield OAA (path a of eq 1). Since the presumed α-fluoromalate product



(1)

is an α-fluorohydrin, we felt that a more facile breakdown route would be simple collapse of this initial product to OAA directly (path b of eq 1). α-Fluoro alcohols are extremely unstable as evidenced by trifluoromethanol, which undergoes exothermic loss of HF at temperatures above −20 °C (Seppelt, 1977). Recent studies point to the in situ generation of α-

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[1] Abbreviations: OAA, oxalacetate; HPLC, high-performance liquid chromatography; MDH, malate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase; Fmal, fluoromalate; PK, pyruvate kinase; PLP, pyridoxal phosphate; Tris, tris(hydroxymethyl)aminomethane.

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