Characteristics of polyamine stimulation of cyclic nucleotide-independent protein kinase reactions

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The extent of direct stimulation by spermine of reactions catalysed by nuclear N1 and N2 protein kinases purified from liver and prostate depends critically on the nature of the protein substrate. The chemically inert $\text{Co}(\text{NH}_3)_6^{3+}$ ion exerts effects on protein kinase reactions similar to those of spermidine or spermine. This enhancement of the phosphorylation of various protein substrates by polyamines or $\text{Co}(\text{NH}_3)_6^{3+}$ by purified nuclear protein kinase preparations was studied in relation to effects of temperature, pH and other factors. The results provide further support for our hypothesis [Ahmed, Wilson, Goueli & Williams-Ashman (1978) *Biochem. J.* 176, 739–750] that the enhancement of certain protein kinase reactions by polycations relates primarily to their interaction with the protein substrate, yielding more favourable conformations for phosphorylation by the protein kinase, rather than a direct effect on its catalytic activity.

INTRODUCTION

The polyamines spermine and spermidine directly stimulate the phosphorylation of selected proteins by several types of cyclic nucleotide-independent protein kinases (for references see Ahmed *et al.*, 1978, 1979, 1983*a,b*; Jacob *et al.*, 1981; Cochet & Chambaz, 1983). Our previous studies on nuclear enzyme preparations from rat liver and ventral prostate (Ahmed *et al.*, 1978, 1983*a,b*) suggested that spermine and spermidine enhanced cyclic nucleotide-independent protein kinase activities primarily by affecting the conformational status of appropriate protein substrates. Similar conclusions as to the mechanism of polyamine stimulation of these reactions were documented in other investigations (see, e.g., Farron-Furstenthal & Lightholder, 1978; Yamamoto *et al.*, 1979; Hara & Endo, 1982; DePaoli-Roach & Roach, 1982), although additional actions of polyamines on the catalytic activity of the protein kinases could not be over-ruled (Cochet & Chambaz, 1983; Hathaway & Traugh, 1984*a,b*). Although the chemically inert inorganic trivalent cation $\text{Co}(\text{NH}_3)_6^{3+}$ enhances certain cyclic nucleotide-independent protein kinase reactions in a manner very similar to that of polyamines (Ahmed *et al.*, 1983*b*), it is clear that such effects of spermidine, spermine and $\text{Co}(\text{NH}_3)_6^{3+}$ are not due to non-specific increases in the total ionic strength of the incubation media, or to complete replacement of the Mg^{2+} required for these protein phosphorylations.

The new investigations presented here have utilized highly purified preparations of nuclear cyclic nucleotide-independent protein kinases to provide further support for the notion that polyamines enhance the activity of these enzymes primarily by interaction with the protein substrates, and illustrate the marked dependence of the polyamine stimulation on experimental conditions.

MATERIALS AND METHODS

Chemicals

Spermine hydrochloride, spermidine hydrochloride and casein were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. $\text{Co}(\text{NH}_3)_6^{3+} \cdot 3\text{HCl}$ was purchased from Pfaltz and Bauer (Stamford, CT, U.S.A.). Phosvitin and partially dephosphorylated (approx. 30%) phosvitin (dephosphophosvitin) were prepared as described previously (Ahmed *et al.*, 1978).

Enzyme preparations

Various protein kinase preparations from Sprague-Dawley rat liver and ventral-prostate cell nuclei were extracted as described by Goueli & Ahmed (1983), and purified further by chromatography on phosphocellulose (P11), DEAE-Sephadex A-25, Sephacryl S-200 and phosvitin-Sepharose or casein-Sepharose (S. A. Goueli, A. T. Davis & K. Ahmed, unpublished work). The general characteristics of the protein kinases N1 and N2 used in the experiments described below were as follows. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis on 10% (w/v) gels showed a single band of M_r 35000 for protein kinase N1, and two bands (M_r 42000 and 28000) for the protein kinase N2 preparations. Maximal rates of phosphorylation of casein or phosvitin by both types of kinase preparation in the absence of polyamines or $\text{Co}(\text{NH}_3)_6^{3+}$ were observed in the presence of 150–200 mM-NaCl, 2–5 mM- MgCl_2 and 0.1 mM-ATP (the stimulatory effects of polyamines were studied in the absence of added NaCl). The properties of the N1 and N2 kinase preparations are described elsewhere (Goueli *et al.*, 1985), and are analogous to those reported for similar enzymes isolated from cell nuclei or whole tissues from several other sources (see, e.g.,

Thornburg *et al.*, 1979; Rose *et al.*, 1981; Erdmann *et al.*, 1982; see also Hathaway & Traugh, 1984a,b; Cochet & Chambaz, 1983; Dahmus, 1981).

Protein kinase assays

Protein kinases N1 and N2 from rat ventral prostate or liver were tested for activity towards different protein substrates such as phosvitin, partially dephosphorylated phosvitin (dephosphophosvitin) and casein in a reaction medium consisting of 4 mM-MgCl₂, 0.1–3.0 mM-[γ -³²P]ATP (3000–7000 d.p.m./nmol of ATP), 1 mM-dithiothreitol, 30 mM-Tris/HCl, pH 7.45 (at 37 °C), 2–5 mg of protein substrate and 0.1–1.0 μ g of enzyme protein, in a final volume of 0.5 ml. In all measurements of protein kinase activities, the reactions were started by the addition of the enzyme under conditions where the reaction rates were linear with respect to time at 37 °C. In general, the various amines were preincubated with the protein substrates for a period of about 10–15 min. The radioactivity of ³²P incorporated in the various protein substrates was determined as described previously (Ahmed *et al.*, 1978, 1983a,b). In most experiments 8–16 mM-NaCl was added to the controls to compensate for the ionic contribution from added polyamines or Co(NH₃)₆³⁺. Maximal stimulation of the various protein kinase reactions by spermine was observed at concentrations of 0.5–1.0 mM (Ahmed *et al.*, 1978, 1979, 1983a,b; Goueli *et al.*, 1985).

Other procedures

All pH values refer to the temperature indicated and were measured with a pH-meter model 26 (Radiometer, Copenhagen, Denmark), with a calomel combination

electrode (A. H. Thomas Co., Philadelphia, PA, U.S.A.) standardized at the appropriate temperature. Protein was assayed by the procedure of Bradford (1976), with bovine serum albumin as standard.

RESULTS AND DISCUSSION

Effects of polyamines and cobalt(III) hexa-ammine on phosphorylation of different protein substrates by purified nuclear protein kinases

Our previous studies (Ahmed *et al.*, 1978) disclosed that spermine, and to a lesser extent spermidine, at concentrations around 1 mM enhanced the rate and extent of phosphorylation of certain protein substrates catalysed by the cyclic AMP-independent protein kinases associated with chromatin or non-histone protein preparations isolated from rat ventral-prostate cell nuclei. The effects of the polyamines were mimicked by Co(NH₃)₆³⁺ at 1 mM (Ahmed *et al.*, 1983b). These stimulatory effects of polyamines were not a consequence of inhibitory effects on any associated protein phosphatase activity endogenous to these preparations (K. Ahmed, S. A. Goueli & H. G. Williams-Ashman, unpublished work). By contrast, phosphorylation of lysine-rich histones by the same enzyme preparations or by purified cytosolic cyclic AMP-dependent protein kinase was essentially unaffected by spermine, spermidine or Co(NH₃)₆³⁺ (Reddi *et al.*, 1971; Ahmed *et al.*, 1978, 1983a,b). All of these effects of polycations on protein kinase reactions promoted by relatively crude nuclear preparations were observed at optimal Mg²⁺ concentrations for a given protein kinase reaction. The requirement for Mg²⁺ could not be replaced

Table 1. Effect of temperature on spermine and Co(NH₃)₆³⁺ stimulation of reactions catalysed by purified liver protein kinase N2 with different protein substrates and various concentrations of ATP

Protein substrates were added at a concentration of 2 mg/ml, and [γ -³²P]ATP at 3 mM or 0.1 mM as shown. The amount of enzyme protein in the reaction was 0.01 μ g. Numbers in parentheses are percentage changes from the corresponding control in the absence of spermine or Co(NH₃)₆³⁺.

Protein substrate	Addition	10 ⁻³ × ³² P incorporation (nmol/h per mg of enzyme protein)			
		0.1 mM-ATP 37 °C	3 mM-ATP		
			27 °C	37 °C	47 °C
Dephosphophosvitin	—	206	46	80	299
	1 mM-Spermine	318 (54%)	194 (322%)	443 (454%)	958 (220%)
	1 mM-Co(NH ₃) ₆ ³⁺	262 (27%)	178 (287%)	396 (395%)	852 (185%)
	—	111	43	116	281
Phosvitin	1 mM-Spermine	133 (20%)	77 (79%)	209 (80%)	533 (90%)
	1 mM-Co(NH ₃) ₆ ³⁺	87 (-22%)	69 (61%)	197 (70%)	495 (76%)
	—	89	45	102	200
	1 mM-Spermine	340* (282%)	151* (236%)	328* (222%)	598* (199%)
Casein	1 mM-Co(NH ₃) ₆ ³⁺	383* (330%)	149* (231%)	359* (252%)	705* (252%)

* Visible precipitate.

Table 2. Effect of spermine and $\text{Co}(\text{NH}_3)_6^{3+}$ on the phosphorylation of different protein substrates mediated by purified rat liver protein kinase N1 in the presence of low or high ATP concentration

Rat liver purified protein kinase N1 (1 $\mu\text{g}/\text{reaction}$) was used. Values in parentheses represent the percentage stimulation as compared with the corresponding controls in the absence of spermine or $\text{Co}(\text{NH}_3)_6^{3+}$. The reaction pH was 6.8 at 37 °C with phosvitin as substrate, and 7.2 with casein or dephosphophosvitin as substrate.

Protein substrate	Addition	^{32}P incorporated (nmol per mg/h of enzyme protein) in the presence of:	
		0.1 mM-ATP	3.0 mM-ATP
Dephosphophosvitin (2 mg/ml)	—	2334	1304
	1 mM-Spermine	2900* (24%)	5862 (350%)
	1 mM- $\text{Co}(\text{NH}_3)_6^{3+}$	2500* (7%)	5058 (288%)
Phosvitin (2 mg/ml)	—	1429	1874
	1 mM-Spermine	1262 (–12%)	2433 (30%)
	1 mM- $\text{Co}(\text{NH}_3)_6^{3+}$	1239 (–13%)	2509 (34%)
Casein (5 mg/ml)	—	1184	1646
	1 mM-Spermine	2639* (123%)	3341* (105%)
	1 mM- $\text{Co}(\text{NH}_3)_6^{3+}$	2735* (131%)	3408* (107%)

* Visible precipitate in the complete reaction mixture.

by spermine (cf. Ahmed *et al.*, 1978). The stimulatory effects of $\text{Co}(\text{NH}_3)_6^{3+}$ described (Ahmed *et al.*, 1983b) may be attributable to the charge properties of this chemically inert cobalt(III) hexa-ammine (see, e.g., Widom & Baldwin, 1980).

It was desirable to extend the above-mentioned studies on the stimulatory influences of spermine and other polycations on impure preparations of nuclear protein kinases by using much more purified preparations of these enzymes. These protein kinase preparations are not activated by cyclic AMP or other cyclic nucleotides, or by Ca^{2+} or $\text{Ca}^{2+}/\text{calmodulin}$, or by Ca^{2+} and anionic phospholipids.

Table 1 depicts a representative experiment on effects of 1 mM-spermine or $\text{Co}(\text{NH}_3)_6^{3+}$ on initial rates of phosphorylation of nearly saturating concentrations of phosvitin, partially dephosphorylated phosvitin and casein at three different temperatures. With this purified liver nuclear protein kinase N2, it is evident that the stimulatory influences of the polycations depend on the nature of the protein substrate. Other studies (results not shown) showed that the effects of spermine and $\text{Co}(\text{NH}_3)_6^{3+}$ on the phosphorylation of the three proteins, shown in Table 1, with a ventral-prostate kinase-N2 preparation were qualitatively similar to those seen with the corresponding purified liver kinase.

Comparable experiments were carried out to establish the effects of polyamines and $\text{Co}(\text{NH}_3)_6^{3+}$ on the phosphorylation of different substrates by purified protein kinase N1. Table 2 clearly shows that again the polycation stimulation differed markedly with the nature of the substrate. It is noteworthy that enhancement by spermine of casein phosphorylation catalysed by the nuclear kinase N1 was not as great as with the kinase-N2 preparations (cf. Tables 1 and 2). Yutani *et al.* (1982) reported that casein phosphorylation by mammalian nuclear protein kinase N1 was not stimulated by polyamines. But we have consistently observed that our kinase-N1 preparations always catalysed casein phosphorylation that was enhanced by spermine and

$\text{Co}(\text{NH}_3)_6^{3+}$ under our experimental conditions, which were somewhat different from those of Yutani *et al.* (1982).

Influence of the reaction conditions on the stimulatory effects of polycations

The results in Tables 1 and 2 also indicate that several other factors can modify the stimulatory effects of polycations on the protein kinase reactions described above. Thus, with respect to protein kinase N2, in the presence of 3 mM-ATP at 37 °C as compared with 0.1 mM-ATP at 37 °C, marked differences in the stimulation of phosphorylation evoked by spermine or $\text{Co}(\text{NH}_3)_6^{3+}$ were apparent. Thus, at 3 mM-ATP, the stimulation of phosphorylation of dephosphophosvitin, casein and phosvitin was enhanced by roughly 4-fold, more than 2-fold, and about 70%, respectively. By comparison, the respective stimulations at 0.1 mM-ATP (at 37 °C) were 54%, 282% and 20%. Maximal enhancement of the reactions by these cations occurred at 37 °C with 3 mM-ATP present; the effects of changes in temperature on the polycation stimulations were not very dramatic at this high ATP concentration. (The rise in the control rates of phosphorylation with a rise in temperature from 27° to 37 °C was considerably less than that observed on increasing the temperature from 37 ° to 47 °C.) Comparable experiments were carried out to compare the effects of high and low ATP concentrations on stimulation by spermine or $\text{Co}(\text{NH}_3)_6^{3+}$ of the phosphorylation of phosvitin, dephosphophosvitin and casein by purified liver nuclear kinase N1. The typical results in Table 2 show that the polycation stimulation varied markedly not only with the nature of the protein substrates but also with the ATP concentration. It should be pointed out that, with nearly saturating concentrations of dephosphophosvitin as substrate at 37 °C and pH 7, the apparent K_m for ATP for these nuclear protein kinase reactions is well below 0.1 mM when the MgCl_2 concentration is held at 4 mM (Goueli & Ahmed, 1983).

Table 3. Effect of pH on stimulation of protein kinase reaction by spermine and $\text{Co}(\text{NH}_3)_6^{3+}$

Rat liver purified protein kinase N2 (0.01 $\mu\text{g}/\text{tube}$) was used. Actual reaction pH values were as follows: with 0.1 mM-ATP, the Tris/HCl buffer of pH 7.45 gave a value of 6.8, whereas with Tris/HCl buffer of pH 7.87 the reaction pH was 7.4. In the presence of 3 mM-ATP, the corresponding reaction pH values were 6.74 and 7.28 respectively for the pH 7.87 buffers. The concentration of the phosvitin substrate was 2 mg/ml. All pH values refer to 37 °C. Values in parentheses are percentage changes from the corresponding control.

Concn. of ATP in the reaction	Addition	Reaction pH...	10 ⁻³ × ³² P incorporated into phosvitin (nmol/h per mg of enzyme protein) in the presence of:	
			6.8	7.4
0.1 mM	—		111	91
	1 mM-Spermine		133 (20%)	96 (7%)
	1 mM- $\text{Co}(\text{NH}_3)_6^{3+}$		87 (-22%)	49 (-46%)
3.0 mM	—		116	114
	1 mM-Spermine		209 (80%)	201 (76%)
	1 mM- $\text{Co}(\text{NH}_3)_6^{3+}$		197 (70%)	189 (66%)

Changes in pH of the reaction mixture as a function of temperature may complicate interpretation of the aforementioned experiments. The added Tris/HCl, ATP/Tris and various protein or phosphoprotein substrates would all contribute to the buffering power of the assay media used to determine protein kinase activities. The pH of Tris is well known to be especially sensitive to temperature. It must also be remembered that the stimulatory effects of polyamines (Ahmed *et al.*, 1978) and $\text{Co}(\text{NH}_3)_6^{3+}$ (Ahmed *et al.*, 1983a) on the protein kinase reactions considered here are seen only at suboptimal ionic strengths, and are no longer apparent at optimal salt concentrations as effected by addition of NaCl at 200 mM. Table 3 shows that the pH of the reaction mixture with phosvitin as substrate did not correspond to the pH of the added Tris buffer at 37 °C in experiments in which the volumes of the reaction mixtures (without enzyme) were scaled up sufficiently to enable measurements to be made with a glass electrode, and the ATP and phosvitin were previously adjusted to the pH of the added Tris/HCl buffer. The corresponding pH changes were not as pronounced with casein or dephosphophosvitin as substrate. Changes in pH over the range 6.8–7.4 had little influence on the stimulatory effects of spermine or $\text{Co}(\text{NH}_3)_6^{3+}$ when the initial concentration of ATP was 3 mM. This accords with previous findings that such nuclear protein kinase reactions are hardly affected by alteration of the pH over the range 6.8–7.5 (Goueli *et al.*, 1985). Tables 1 and 3 also indicate that, at low (0.1 mM) ATP concentrations, spermine enhanced the phosphorylation of phosvitin to a lesser extent than at 3 mM-ATP, whereas $\text{Co}(\text{NH}_3)_6^{3+}$ was actually inhibitory at the low ATP concentrations.

It has been proposed that the stimulatory effects of polyamines may result from primary interactions of the polycations with the protein substrates, so as to alter their conformational status in ways that facilitate access of phosphorylatable sites on the substrates to the active sites on the protein kinases (Ahmed *et al.*, 1978, 1979, 1983a,b; Farron-Furstenthal & Lightholder, 1978; Yamamoto *et al.*, 1979; Jacob *et al.*, 1981; Hara & Endo, 1982; DePaoli-Roach & Roach, 1982). Alternatively, stimula-

tion of protein kinase reactions by spermidine and spermine has been envisaged as involving interactions with catalytic sites of the enzymes (Cochet & Chambaz, 1983; Hathaway & Traugh, 1984a,b).

Hathaway & Traugh (1984a,b) have suggested that casein kinase II has two binding sites for Mg^{2+} , and that the site with low affinity for the bivalent metal cation can also bind spermine at low ionic strengths. However, we have found that the stimulatory effects of polyamines depend markedly on the nature of the protein substrate (tested at nearly saturating concentrations) in the presence of optimal Mg^{2+} concentrations. This is strikingly illustrated by our previous findings that chemically *NN*-dimethylated dephosphophosvitin was a less effective substrate for protein kinases associated with prostate chromatin preparations than was unmodified dephosphophosvitin, whereas 1 mM-spermine stimulated the phosphorylation of the *NN*-dimethylated protein substrate to a relatively much greater extent (Ahmed *et al.*, 1978). It may be recalled that polyamines cannot substitute for Mg^{2+} in these reactions. Moreover, the requirements for Mg^{2+} in the absence of polyamines vary with both the nature of the protein substrate and the total ionic strength of the assay systems (S. A. Goueli, A. T. Davis & K. Ahmed, unpublished work). Noteworthy in this context is the unusually large stimulation of phosphorylation of rat ventral-prostate spermine-binding protein by both chromatin-associated and purified nuclear N2 protein kinase preparations (Goueli *et al.*, 1985), and also the inability of spermine or spermidine to enhance the clear-cut phosphorylation of lysine-rich histones by these enzymes (Ahmed *et al.*, 1978, 1983a,b). More incisive studies are needed to settle the precise mechanisms by which polyamines stimulate these reactions, which obviously might involve interactions with both the protein substrates and the kinase enzymes under certain circumstances. Hathaway & Traugh (1984a) reported that the apparent K_m for casein as a substrate for casein kinase II was decreased by 39% in the presence of spermine, whereas Hara & Endo (1982) found that the protein substrate affinity was uninfluenced by polyamines, which is in accord with our observations (Goueli *et al.*, 1985).

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