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INHIBITION OF PHOSPHORYLASE PHOSPHATASE BY POLYAMINES

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SUMMARY. Phosphorylase phosphatase from rabbit liver or from bovine heart, purified as the M_r 35,000 form, is inhibited by the naturally occurring polyamines, spermine and spermidine. The inhibition is apparently competitive with a K_i of 0.037 mM for spermine and 0.65 mM for spermidine. Further investigation showed that the inhibition is substrate-directed, i.e. due to an interaction of the polyamines with the rabbit muscle phosphorylase a used as the substrate.

The naturally occurring polyamines, spermine and spermidine, are physiologically important compounds of considerable interest. Levels of these effectors increase when growth rate increases; their interactions with nucleic acids and their effects on protein synthesis have been extensively investigated (1,2,3). It has been briefly reported that the polyamines may affect protein phosphorylation reactions. The phosphorylation of protamine by sea urchin spermatozoan adenosine 3':5' - monophosphate dependent protein kinase is inhibited by polyamines (4) while the in vitro phosphorylation of non-histone chromatin proteins from porcine liver was found to be enhanced by polyamines (5). This prompted us to investigate the possible effects of polyamines on the dephosphorylation of proteins, using the conversion of rabbit muscle phosphorylase a into phosphorylase b by phosphorylase phosphatase as a model system. The enzymes used for this study were phosphorylase phosphatases purified from rabbit liver (6) and bovine heart (7) by the procedure of Brandt et al. (6). Both enzymes were isolated as a form of M_r 35,000 which has been termed protein phosphatase C (8).

EXPERIMENTAL

Phosphorylase b was prepared from rabbit skeletal muscle as by Fischer and Krebs (9) and converted into phosphorylase a using phosphorylase kinase and [γ - ^{32}P]-ATP (10). The phosphorylase a was recrystallized and treated

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with Norit as described by Brandt et al. (6). Phosphorylase phosphatase was prepared from rabbit liver up to and including the DEAE-Sephadex stage as reported by Brandt et al. (6). This preparation had a specific activity of 185 units/mg protein. Bovine heart phosphorylase phosphatase was isolated to homogeneity by the same method and had a specific activity of 7,900 units/mg protein (7). Phosphorylase phosphatase activity was assayed essentially as previously reported (6) with the exception that the assays were carried out in a volume of 100 µl and contained 0.1% gelatin in addition. The reactions were stopped by the addition of 20 µl of 50% trichloroacetic acid and the release of radioactivity in the supernatant was determined after removal of the precipitated protein by centrifugation. Activities were expressed as described by Brandt et al. (6), one unit of phosphorylase phosphatase activity being that which converted 0.2 mg phosphorylase a/min.

A crude preparation of the chymotryptic-derived phosphopeptide of [^{32}P]-labelled phosphorylase a was obtained by the general procedure of Nolan et al. (11). When the chymotrypsin-derived [^{32}P]-labelled phosphopeptide was used as the substrate, the incubation mixtures (100 µl) were identical to those used when phosphorylase a was the substrate. The [^{32}P]-labelled phosphopeptide was added to a concentration equivalent to that originating from 0.2 mg/ml phosphorylase a. The incubations were terminated by the addition of 100 µl of 20 mM silicotungstic acid/ 1 mM sodium phosphate/ 1 N sulfuric acid. The precipitated protein was removed by centrifugation. Ammonium molybdate (25 µl of a 7.5% solution) was added to 150 µl of the supernatant and the phosphomolybdate complex was extracted with 250 µl of a 1:1 (v/v) mixture of isobutanol-benzene (12). The radioactivity in the organic layer was determined after separation of the aqueous and organic phases by centrifugation.

Spermine, spermidine, cadaverine and putrescine were obtained as the hydrochlorides from Sigma Chemical Co.; hexanediamine was obtained from Aldrich Chemical Co. [γ-^{32}P]-ATP was obtained from ICN Pharmaceuticals Inc. and diluted to a specific radioactivity of 150-300 µCi/µmole before use.

RESULTS AND DISCUSSION

At 1 mM concentrations the polyamines were found to exert an inhibitory effect on the dephosphorylation of rabbit muscle phosphorylase a by both the rabbit liver and bovine heart phosphorylase phosphatase preparations (Table I). Spermine and spermidine were the most inhibitory. The dibasic alkylamines, putrescine and cadaverine, were less inhibitory and hexanediamine was only slightly inhibitory. The nature of the inhibition of the phosphatase reaction by spermine and spermidine was further investigated. Similar results were obtained with the rabbit liver and bovine heart enzymes, and representative results are presented.

Dose inhibition curves (Fig. 1) show that significant inhibition was observed at concentrations of spermidine and spermine as low as 10^{-5} M and 10^{-6} M, respectively. The dose inhibition curve in the case of spermine

TABLE I. INHIBITION OF THE PHOSPHORYLASE PHOSPHATASE REACTION BY POLYAMINES

POLYAMINE	PERCENTAGE INHIBITION	
	RABBIT LIVER PHOSPHATASE	BOVINE HEART PHOSPHATASE
Spermine	75	75
Spermidine	63	56
Cadaverine	20	11
Putrescine	14	13
Hexanediamine	7	-

The polyamines were tested at concentrations of 1 mM in the assay of phosphorylase phosphatase as described in Methods, using appropriate dilutions of the rabbit liver and bovine heart phosphorylase phosphatase preparations.

appeared to be biphasic, with an inflection point at about 10^{-4} M spermine. This was of interest since the concentration of phosphorylase a in the assay was 10^{-4} M. The kinetics of the inhibition by both spermine and spermidine was apparently competitive. K_i values of 0.037 mM and 0.65 mM for spermine and spermidine, respectively, were calculated from the data shown in Fig. 2.

Further investigation revealed that the action of the polyamines was substrate-directed. When the effects of the spermine and spermidine on the dephosphorylation of the chymotrypsin-derived phosphopeptide (10) of phosphorylase a were examined, no inhibition was seen, as compared to their action on the native enzyme (Fig. 3). Thus the mechanism of the inhibition was inferred to be due to an interaction of the polyamines with the rabbit muscle phosphorylase a, the latter being converted into a poorer substrate. Such substrate-directed effects on the dephosphorylation of phosphorylase a have been well-established for the inhibitory action of adenosine 5'-monophosphate (11, 13-17). In addition, the polyamines have been previously shown to bind to rabbit muscle phosphorylase (18). Further evidence that the polyamines did not exert their effect by a direct interaction with the phosphatases is shown by the fact that the dephosphorylation of [^{32}P]-labelled lysine-rich histone by bovine heart phosphorylase phosphatase is not affected by polyamines (not shown).

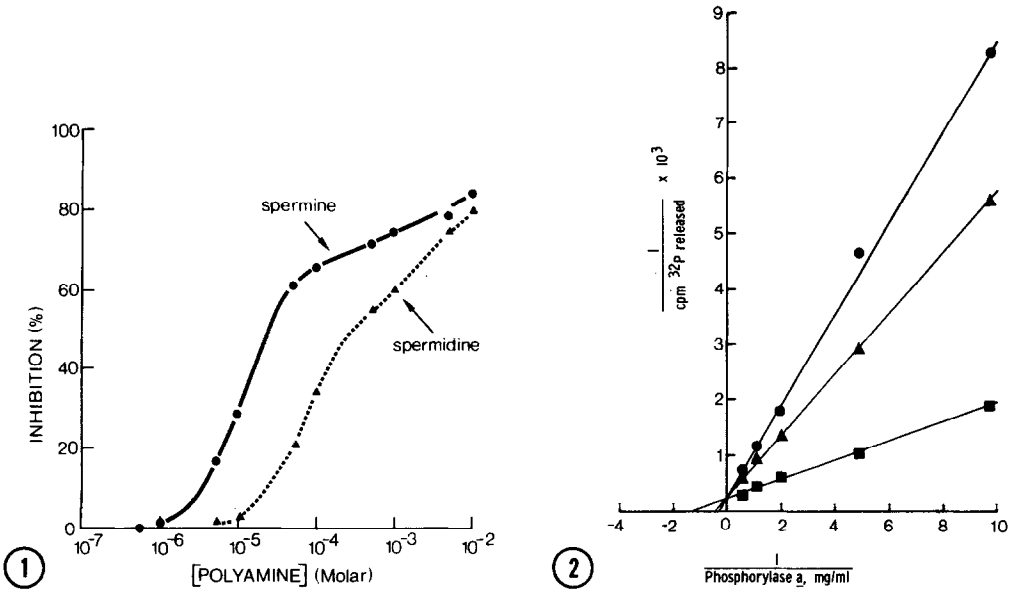

Fig. 1. Concentration Dependence of the Inhibition of the Phosphorylase a into Phosphorylase b Conversion by Polyamines.

The inhibition of the phosphatase reaction was tested at varying concentrations of spermine (●) and spermidine (▲) in the assay system as described in Methods. The data shown above were obtained with rabbit liver phosphorylase phosphatase, in assays containing 0.04 units enzyme/ml.

Fig. 2. Kinetics of the Inhibition of Bovine Heart Phosphorylase Phosphatase by Polyamines.

The inhibition of bovine heart phosphorylase phosphatase was examined over a range of phosphorylase a concentrations in the presence of 1 μM spermine (●), 1 mM spermidine (▲) or no added polyamine (■). Data were analysed by the method of Bliss and James (20) using the computer program of Hanson et al. (21) adapted for the Univac 1106 system by Dr. J. F. Woessner of the University of Miami. The K_m obtained was 0.75 (0.48-1.13) mg/phosphorylase a/ml, and the K_p values in the presence of spermine and spermidine were 2.76 (1.52-5.78) and 1.9 (1.41-2.58) mg/ml respectively. Values in parentheses represent the 95% confidence units of the data.

Polyamines have been shown to increase the affinity of phosphorylase b for adenosine 5'-monophosphate (18) and presumably might also increase the affinity of phosphorylase a for this nucleotide. Thus the effects of the polyamines could be ascribed to the potentiation of an inhibition by trace

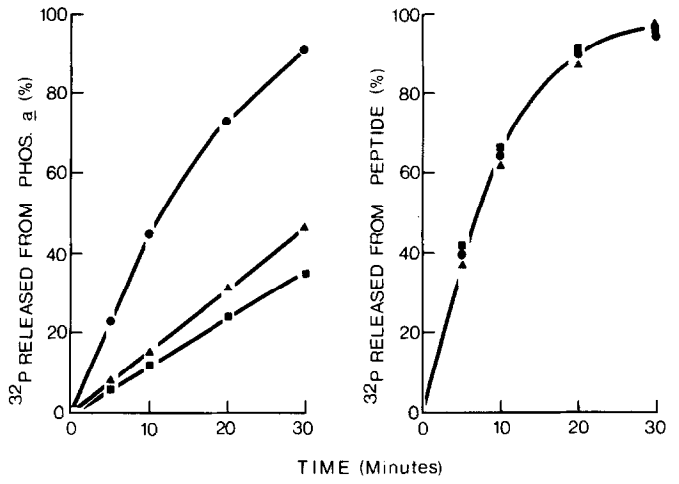

Fig. 3. The Effects of Polyamines on the Dephosphorylation of Phosphorylase and its Chymotryptic-Phosphopeptide.

Rabbit liver phosphorylase phosphatase was assayed in the presence of 1 mM spermine (■), 1 mM spermidine (▲) or with no added polyamine (●) using rabbit muscle phosphorylase a (left hand panel) or the chymotrypsin-derived phosphopeptide of phosphorylase a (right hand panel) as the substrate.

amounts of adenosine 5'-monophosphate in the phosphorylase a preparations. However, treatment of the phosphorylase a with Norit to remove endogenous nucleotides did not affect the inhibition of the phosphatase reaction by polyamines. Also, glucose (30 mM), theophylline (5 mM) or a mixture of the two, both of which have been shown to reverse the adenosine 5'-monophosphate inhibition of phosphorylase phosphatase (6,15,16) did not affect the inhibition caused by polyamines.

During the progress of this work Nakai and Glinsmann also reported that polyamines inhibited the dephosphorylation of rabbit muscle phosphorylase a by a rabbit skeletal muscle phosphorylase phosphatase preparation (19). In contrast to the studies reported here, spermine alone was found to be inhibitory, the kinetics of inhibition being non-competitive with a K_i (0.3 mM) higher than that which we observed. It may be noted, however, that the phosphorylase phosphatase preparation which they used may have contained a

different enzyme form than that used in the present study.

These results indicate that the free polyamines, in particular spermine, can exert a profound effect on the dephosphorylation (and inactivation) of rabbit muscle phosphorylase a in vitro. It is not possible to state whether this effect has any physiological significance; while the tissue concentrations of the polyamines may reach the millimolar range (3), they may be present in a form bound to other macromolecules and their subcellular location may be different from that of phosphorylase a. However, the present studies may be a model for the modulation of the susceptibility of other macromolecules to enzyme modification via interaction with polyamines.

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