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EFFECT OF POLYCARBOXYLATES ON PHOSPHORYLASE b

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SUMMARY

Activation of phosphorylase b by AMP is stimulated by certain aliphatic and cyclic polycarboxylates. This stimulation was depended on the number and the position of the carboxyl groups, the stereochemistry and the size of the molecule, and was more pronounced at low AMP concentrations. Kinetic studies indicated that in the presence of polycarboxylates the affinity of the enzyme for AMP was enhanced, the cooperative binding of the nucleotide was removed, and the enzyme was no longer inhibited by glucose-6-phosphate. Although polycarboxylates have no effect on the sedimentation pattern of phosphorylase b in the absence of AMP, the partial association of the enzyme caused by AMP is greatly enhanced in the presence of the acids.

INTRODUCTION

Following the demonstration that rabbit muscle phosphorylase b (EC 2.4.1.1) requires AMP for catalytic activity (1), the nucleotide induced activation was found to be stimulated by several polycationic molecules as protamine (2,3) and polyamines (4), divalent metal ions (5,6), fluoride (7), a phosphopeptide derived from the NH_2-terminal region of the enzyme (8) and phenothiazines (9).

In the present work we have investigated the effect of some polycarboxylic acids on the catalytic and structural properties of glycogen phosphorylase b in order to obtain additional infor-

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mation concerning the relationship among electrostatic interactions, protein conformation, and enzyme catalysis.

MATERIALS AND METHODS

Phosphorylase b was isolated from rabbit skeletal muscle according to the procedure of Fischer and Krebs (10). The enzyme was recrystallized at least four times at 0° C from a buffer of pH 6.8 consisting of 40 mM glycerol-2-phosphate, 30 mM 2-mercaptoethanol, 1 mM EDTA, 1 mM AMP, and 10 mM Mg^{++}. No additional AMP or Mg^{++} was added after the first recrystallization. Before use, crystals were centrifuged, dissolved in the appropriate buffer and freed of AMP by exhaustive filtration on Sephadex G-25 column and by a two times treatment with acid-washed Norit (1 mg/mg of protein).

Oyster glycogen was purchased from BDH and it was freed of AMP by dialysis against 0.8 mM acetate (pH 4.0) containing Dowex-1 (acetate) as described by Helmreich et al (11). Glucose-1-phosphate and AMP were purchased from Fluca.

Phosphorylase b was assayed in the direction of glycogen synthesis (12). For routine assay, phosphorylase b (10-20 µg/ml) was incubated at 30° C with 1 mM AMP, 1% glycogen, 16 mM glucose-1-phosphate, 0.5 mM EDTA, 30 mM 2-mercaptoethanol, and 40 mM glycerol-2-phosphate buffer (pH 6.8) in a final volume of 0.2 ml. P_i released in the reaction was measured by the method of Fiske and Subbarow (13). Activity of phosphorylase b was expressed in µmol product formed min^{-1} mg $enzyme^{-1}$.

When polycarboxylates were present in the assay system, their possible interference on the estimation of phosphate was checked (14). For the maximum concentrations of the polycarboxylates used, a small increase of the blue color in the Fiske and Subbarow procedure was observed only for citrates, oxalates, trimesates, mellitates, and quinolinates. The experimental values were corrected when needed.

Phosphorylase b concentration was measured spectrophotometrically using the extinction coefficient $E_{1\,cm}^{1\%}$ at 280 nm of 13.2 (15). The molecular weight of the dimeric enzyme was taken as 200000 (16).

Sedimentation velocity experiments were carried out using an MSE Centriscan 75 preparative and analytical ultracentrifuge, with a 10 mm single-sector cell at a rotor speed of 60000 rpm. Sedimentation coefficients were obtained from direct measurements of the scanner traces and corrected for viscosity and density of the buffer at 20° C. Phosphorylase b (10 mg/ml) was centrifuged in 0.5 mM EDTA, 30 mM 2-mercaptoethanol, 40 mM glycerol-2-phosphate buffer, pH 6.8 at 20° C, in presence or absence of AMP and polycarboxylates.

RESULTS

Effect of polycarboxylates on AMP-dependent phosphorylase b activity. It was found that certain polycarboxylates greatly enhance AMP activation of phosphorylase b. Table 1 shows the effect of a series of polycarboxylates (65 mM) on the activation of phosphorylase b by AMP at low effector concentration (10 μM). The effect of the various polycarboxylates on the nucleotide-dependent activation of phosphorylase b was either positive (stimulation) or negative (inhibition) and was depended on the number of carboxyl groups of the acid, the nature of the aliphatic chain, the nature of the cyclic hydrocarbon, the position of the carboxyl groups on the ring, and the general stereochemistry of the molecule.

The number of methylene groups of the saturated aliphatic dicarboxylic acids seems to be critical for stimulation. Thus while oxalate and malonate inhibit AMP-induced activation of phosphorylase b, glutarate shows the highest stimulation. The presence of additional functional groups on the aliphatic chain of the acid, as amino, keto or hydroxyl groups, affect positively or negatively the action of dicarboxylate. In this respect the action of L- and meso-tartarate on enzyme activity is characteristic. Thus, when the two hydroxyl groups of tartarate are in cis position (meso-tartarate), the dicarboxylate acts as inhibitor, in contrast

Table 1
Effect of polycarboxylates on the activation of phosphorylase b by AMP

Polycarboxylate	% Activity	Polycarboxylate	% Activity
oxalate	60	citrate	69
malonate	49	1,3,5 pentane	
succinate	155	tricarboxylate	320
glutarate	200	EDTA	305
adipate	190	cyclohexane 1,2 di-	
pimelate	172	amine tetraacetate	194
maleate	23	o-phthalate	4
L-glutamate	175	diphenate	44
L-aspartate	185	trimellitate	74
D-aspartate	195	trimesate	164
2-ketoglutarate	150	pyromellitate	283
3,3 dimethylglutarate	180	mellitate	255
meso-tartarate	46	2,6 dipicolinate	21
L-tartarate	188	quinolinate	29
		isocinchomeronate	57

The enzyme (10 µg/ml) was assayed at 30° C in 0.5 mM EDTA, 30 mM 2-mercaptoethanol, 40 mM glycerol-2-phosphate buffer pH 6.8, with 16 mM glucose-1-phosphate, 1% glycogen and 10 µM AMP, in presence of 65 mM of each polycarboxylate. The enzyme activity without polycarboxylate was taken as 100%.

when the two hydroxyl groups are in trans position (L-tartarate), the dicarboxylate acts as stimulator (Table 1). The number of methylene groups and the presence of additional functional groups seems to be critical also for the effect of aliphatic tricarboxylic acids. Thus, while citrate inhibits, 1,3,5 pentane tricarboxylate greatly enhances AMP induced activation of phosphorylase b (Table 1).

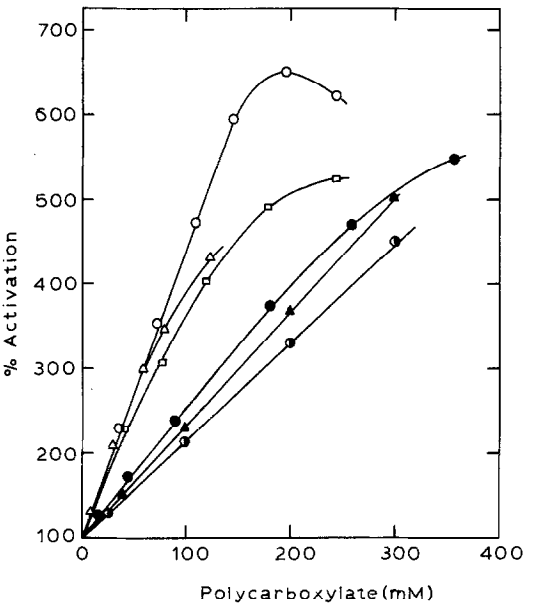

Fig. 1. Effect of polycarboxylates on the activity of phosphorylase b at low AMP concentration. The enzyme (10 µg/ml) was assayed at 30° C in 0.5 mM EDTA, 30 mM 2-mercaptoethanol, 40 mM glycerol-2-phosphate buffer pH 6.8, with 16 mM glucose-1-phosphate, 10 µM AMP, 1% glycogen and various concentrations of polycarboxylates mentioned on the figure. The enzyme activities without polycarboxylates were taken as 100%. (○) 1,3,5 pentane tricarboxylate; (△) EDTA; (□) pyromellitate; (●) glutarate; (▲) aspartate; (◑) glutamate.

In the Fig. 1 is shown the effect of increasing concentrations of polycarboxylate stimulators on the activity of phosphorylase b at low AMP concentration (10 µM). In the range of polycarboxylate concentrations tested, 1,3,5 pentane tricarboxylate was found the most effective activator of the enzyme, while glutamate stimulation was very small. In order to obtain additional information concerning the effect of 1,3,5 pentane tricarboxylate on the allosteric interaction of phosphorylase b, we studied AMP binding on the enzyme at three different concentrations of this polycarboxylate. In Fig. 2, Lineweaver-Burk plots are shown for AMP activation of phos-

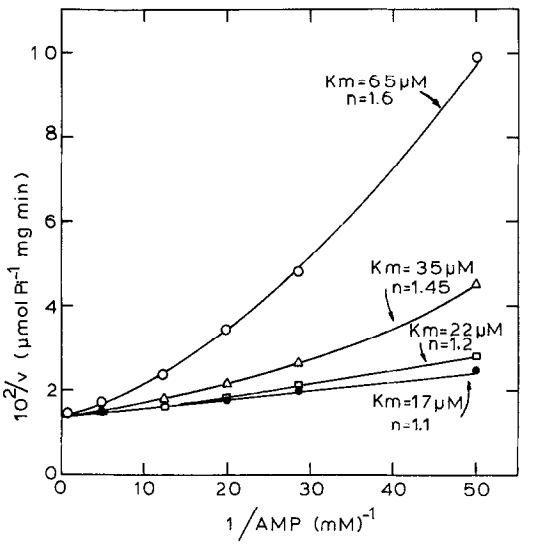

Fig. 2. Effect of 1,3,5 pentane tricarboxylate on the kinetics of activation of phosphorylase b by AMP. The assay mixture contained in addition to AMP, 10 μg/ml of phosphorylase b, 16 mM glucose-1-phosphate, 1% glycogen and (○) 0; (△) 37; (□) 90; or (●) 146 mM 1,3,5 pentane tricarboxylate. Reactions were carried out at 30° C and buffered with 0.5 mM EDTA, 30 mM 2-mercaptoethanol, 40 mM glycerol-2-phosphate buffer, pH 6.8 . n=Hill coefficient.

phorylase b. In the absence of polycarboxylate the enzyme shows the expected cooperative binding of AMP with a Hill coefficient n=1.6 . When 1,3,5 pentane tricarboxylate is added, the cooperative binding of AMP is abolished and the binding of the nucleotide to the enzyme is highly enhanced. In contrast to its effect on K_m value of AMP, 1,3,5 pentane tricarboxylate has no effect on the affinity of the enzyme for the substrates glucose-1-phosphate and glycogen. At 1 mM AMP and 1% glycogen or 16 mM glucose-1-phosphate the presence of 150 mM of 1,3,5 pentane tricarboxylate does not affect the K_m values for glucose-1-phosphate and glycogen. Further studies on the subject presented in Table 2, show that the polycarboxylates are able to reverse the inhibition of phosphorylase b

Table 2
Influence of 1,3,5 pentane tricarboxylate on the inhibition of phosphorylase b by glucose-6-phosphate

Additions to reaction	% Activity
none	100
glucose-6-phosphate (10 mM)	50
1,3,5 pentane tricarboxylate (245 mM)	83
glucose-6-phosphate (10 mM) + 1,3,5 pentane tricarboxylate (245 mM)	77

Reaction mixtures at 30° C contained 18 µg/ml of phosphorylase b, 16 mM glucose-1-phosphate, 1 mM AMP, and 1% glycogen in 0.5 mM EDTA, 30 mM 2-mercaptoethanol, 40 mM glycerol-2-phosphate buffer pH 6.8 . Other components were added as indicated.

caused by glucose-6-phosphate. At a polycarboxylate concentration of 245 mM the inhibition induced by glucose-6-phosphate is almost completely reversed.

Effect of polycarboxylates on the structure of phosphorylase b.

In order to obtain information concerning the relationship between the observed changes in catalytic properties of the enzyme by poly carboxylates and its structure, the effect of 1,3,5 pentane tricarboxylate on the association of phosphorylase b has been examined by analytical ultracentrifugation. The following results were obtained: (i) The native enzyme sediments at 20° as a dimer with $s_{20,w}$=8.2, while the enzyme in the presence of 0.5 mM AMP has a sedimentation coefficient $s_{20,w}$=9.5, which corresponds mainly to the dimeric species (17). (ii) Although 1,3,5 pentane tricarboxylate at the concentration used in our ultracentrifugation experiments (146 mM) does not cause any alteration on the sedimentation pattern of phosphorylase b, experiments performed in presence of both polycarboxylate (146 mM) and AMP (0.5 mM) resulted in the

complete conversion of the enzyme into the 13.1 S material, which corresponds to the tetrameric form of the enzyme.

DISCUSSION

Interaction of phosphorylase b with polycarboxylates leads to (a) an enhancement of the enzyme affinity for AMP (Fig. 2), and (b) the desensitization of allosteric interactions toward AMP and glucose-6-phosphate (Fig. 2, Table 2). The observed effect of polycarboxylates on the activity of phosphorylase b is similar in many respects to the effect of the phosphopeptide of phosphorylase a or of spermine observed previously (8).

Polycarboxylates are highly charged molecules and it is expected that the interaction of these effectors with the enzyme, -like polycations- to be electrostatic in nature. However, the involved interactions were found very specific, as was demonstrated from the behaviour of the various polycarboxylate isomers (Table 1). Furthermore, ultracentrifugal determinations of structural changes in the enzyme caused by these electrostatic interactions showed that while polycarboxylates alone do not affect the sedimentation pattern of phosphorylase b, the combination of AMP and polycarboxylates enhances the partial aggregation caused by AMP. It may be postulated that binding of polycarboxylates to phosphorylase b results in an enzyme form which is intermediate between phosphorylase a and b (4).

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