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INHIBITION OF POTATO STARCH PHOSPHORYLASE BY α-D-GLUCOPYRANOSE-1,2-CYCLIC PHOSPHATE

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Summary

α-D-Glucopyranose-1,2-cyclic phosphate is a potent inhibitor of potato starch phosphorylase-catalyzed (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) starch elongation. The inhibition is competitive with respect to α-D-glucopyranose 1-phosphate (Glc-1-*P*) with $K_i \sim 0.07$ mM at pH 6.3 and 30°C in 25 mM citrate buffer. The affinity of the phosphorylase · starch complex for the cyclic ester is therefore nearly 30 times as large as for Glc-1-*P*. Under conditions where α-D-glucopyranose-1,2-cyclic phosphate slows starch elongation by a factor of 3, UDPglucose, ADPglucose, D-glucose 6-phosphate, and D-glucose 2-phosphate cause rate reductions of less than 10%. The origin of the relatively strong binding of the cyclic ester to the phosphorylase, and its possible biological significance are discussed.

Introduction

Compounds known to be good inhibitors of various α-1,4-glucan phosphorylases (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) include α-D-5-thio-glucopyranose 1-phosphate [1], nucleoside diphosphoglucoses [2,3], glucose [4,5], and related compounds [6,7]. Some of these are believed to function in vivo as regulators of phosphorylase activity. We wish to report a new class of inhibitor; α-D-glucopyranose-1,2-cyclic phosphate ** is a potent inhibitor of potato starch phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1, from potato). We originally tested the cyclic ester because of its structural similarity to Glc-1-*P*. In particular, we were interested in the possibility that if the mechanism of phosphorylase catalysis involves a covalent glucosyl-enzyme intermediate, then the formation of the 2-phospho

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** Non-standard abbreviations used: Glc-1,2-*P*, α-D-glucopyranose-1,2-cyclic phosphate.

analogue of the intermediate might label an active site group. As described below, Glc-1,2-*P* binds to the phosphorylase · starch binary complex more strongly than Glc-1-*P* does, but the binding is reversible and the cyclic ester is apparently unchanged.

Materials and Methods

Glc-1-*P*, Glc-6-*P*, and ADPGlc were purchased from the Sigma Chemical Co., and UDPGlc from Calbiochem. "Soluble starch" was used as the phosphorylase primer. The barium salt of Glc-1,2-*P* was prepared by the procedure of Zmudzka and Shugar [8]. The potassium salt was obtained by ion exchange of the barium salt and was treated with acid-washed charcoal to remove ultraviolet-absorbing impurities. Glc-2-*P* was prepared by hydrolysis of Glc-1,2-*P* in 0.25 M HCl at 100°C for 5—10 min, and isolated as the potassium salt using the general procedure of Piras [9]. Potato starch phosphorylase was prepared by the method of Kamogawa et al. [10], except for the changes previously noted [11]. (The enzyme used in these experiments was not amylase treated and was stored as a suspension in 2 M $(NH_4)_2SO_4$.) The concentrations of enzyme stock solutions prepared by diluting aliquots of the suspension with 50 mM citrate buffer were determined spectrophotometrically using $\epsilon_{280} = 1.17$ ml · $mg^{-1} \cdot cm^{-1}$ [12].

The initial velocity of phosphorylase-catalyzed release of inorganic phosphate ion from Glc-1-*P* was measured at 30°C in reaction mixtures that contained 25 mM citrate buffer, 1% starch, and various concentrations of Glc-1-*P* and the compounds tested as inhibitors. The reaction was started by adding enzyme (final concentration ~ 10 μg/ml) to the other components that had been equilibrated at 30°C. At various times aliquots (usually 100 μl) of the reaction solution were added to water (final volume 1.00 ml) that had been heated to 85°C. The mixture was kept at 85°C for 5 min and then in ice until the concentration of inorganic phosphate was determined by the Lowry-Lopez method [13]. For each "run" 5—7 points were taken over a total time interval of 8—30 min, depending on the concentration of Glc-1-*P*, and the slope of a plot of $[P_i]$ vs. time determined by a weighted least squares procedure. In a given run the standard deviation of the slope was typically 1—3% of the value of the slope. The concentrations of phosphate esters are based on total-phosphate determinations; aliquots of stock solutions were hydrolyzed in 1 M HCl for 3 h at 100°C, and the concentration of inorganic phosphate determined by the Ames method [14].

pH-stat experiments were done using a Radiometer Model 26 pH meter and No. GK2321C combination electrode. A Fisher pH 7.410 standard buffer was used to standardize and check the stability of the meter. Carbonate-free 0.1 M KOH was added from a 5-μl Hamilton syringe or a 2-ml Gilmont micrometer buret.

Results and Discussion

The initial velocity of potato starch phosphorylase-catalyzed release of P_i from Glc-1-*P* has been determined as a function of the concentrations of Glc-

1-*P* and Glc-1,2-*P* at a fixed starch concentration that is 10 times as large as the dissociation constant for the phosphorylase · starch binary complex [15]. The results shown in Fig. 1 indicate that Glc-1,2-*P* is a competitive inhibitor with respect to Glc-1-*P*. Extrapolations of the lines of Fig. 1 to $1/v = 0$ yield K_m(Glc-1-*P*) = 1.8 mM, and $K'_m = K_m(1 + [I]/K_i)$ = 33 mM at [Glc-1,2-*P*] = 1.15 mM and 57 mM at [Glc-1,2-*P*] = 2.16 mM. The latter two values yield K_i = 0.066 and 0.070 mM, respectively. The inhibition is reversible in the sense that when the phosphorylase was preincubated in a buffered solution of Glc-1,2-*P* or Glc-1,2-*P* plus starch for 30 min or more and then combined with the remaining reactants the velocity of P_i release was the same as for a control sample in which the phosphorylase was preincubated with buffer only.

To determine if Glc-1,2-*P* is a substrate for potato starch phosphorylase we used a pH-stat method to look for ring opening. A solution containing 25 mM Glc-1,2-*P* and 10 mM citrate buffer was incubated at pH 7 and room temperature. Over periods of 1—2 h no significant pH change (>0.010 unit/h) was observed; nor were significant changes observed when phosphorylase (5 μg/ml) or phosphorylase and starch (0.5%) were added. Under our conditions the formation of 0.05 mmol/ml of phosphate monoester would cause a pH change of 0.025 pH unit.

In order to compare the effectiveness of Glc-1,2-*P* with other possible inhibitors of the potato enzyme the initial velocity of phosphorylase-catalyzed P_i release from Glc-1-*P* was determined at a single concentration of Glc-1-*P* (10 mM) and a single concentration of the potential inhibitor (1 mM). The compounds tested were UDPGlc, ADPGlc, and Glc-6-*P*, which are all known inhibitors of specific phosphorylases, Glc-2-*P*, which is a product of Glc-1,2-*P* hydrolysis, and KCl and $(NH_4)_2SO_4$ (the last at 10 mM), which were included to assess general salt effects. At these concentrations none of these compounds caused significant (±10%) changes in the rate of P_i release but Glc-1,2-*P* reduced the rate by a factor of 3.

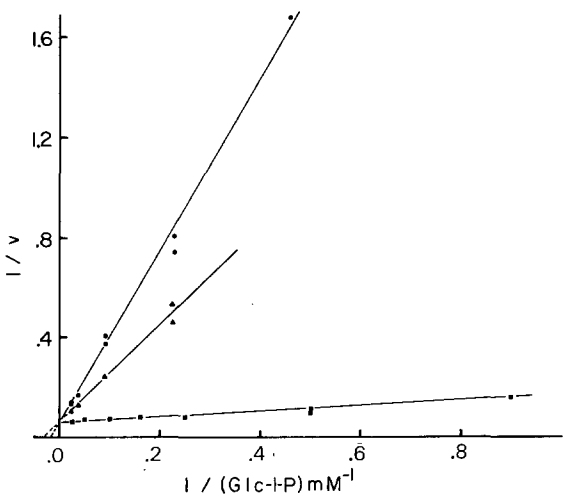

Fig. 1. Lineweaver-Burk plot for release of P_i from Glc-1-*P* in the presence of Glc-1,2-*P* at 30° C in 25 mM citrate buffer, pH 6.3. Units of velocity are μmol/min per mg protein. Glc-1,2-*P* concentrations are: ■, 0; ▲, 1.15 mM; ●, 2.16 mM. (Several points on this plot are the average of two or more experimental points.)

Since potato starch phosphorylase has been shown to use a rapid equilibrium random bi bi mechanism [15], the ratio K_m/K_i = 26 represents the relative affinity of the phosphorylase · starch binary complex for Glc-1,2-*P* compared to Glc-1-*P*. The binding of Glc-1,2-*P* to potato phosphorylase is remarkably strong, especially if the cyclic ester is considered as a mere model of Glc-1-*P*. The K_i for Glc-1,2-*P* is of the same order of magnitude as that for inhibition of phosphorylase *a* by gluconolactone [6], which is a transition state analogue, and for GDPGlc inhibition of slime mold glycogen phosphorylase [3], and about one-tenth as large as K_i reported for inhibition of potato starch phosphorylase by α-D-5-thio-glucopyranose 1-phosphate [1].

The ratio K_m/K_i = 26 if purely an enthalpy effect corresponds to a $\Delta\Delta H$ of binding of $-RT\ln 26 = -2.0$ kcal/mol (−8.4 kJ/mol) at 30°C, which is about the size of a single hydrogen-bond interaction. The stronger binding of Glc-1,2-*P* vs. Glc-1-*P* cannot be totally a charge effect since at pH 6.3 Glc-1,2-*P* is essentially 100% monoanion [16], while based on a pK_{a2} of 6.51 at 30°C [17], Glc-1-*P* is about 62% monoanion. And in the pH range 5.5—8.2 K_m is nearly constant [18]. Therefore, either the charge on Glc-1-*P* plays no physical role in the binding to the enzyme, or if it does play a role, dissociation of Glc-1-P^{-1} → Glc-1-P^{-2} is compensated by loss of a positive charge at the binding site of the enzyme.

If the ratio reflects a purely entropic effect, $\Delta\Delta S = RT\ln 26$ = 6.5 cal · mol^{-1} · $degree^{-1}$ (27 J · mol^{-1} · $degree^{-1}$). It seems reasonable that if Glc-1-*P* is more or less rigid when held to the enzyme-active site then it must sacrifice conformational freedom when going from solution onto the enzyme. In particular, rotation about the C-OPO_3 and CO-PO_3 bonds would be lost. The phosphate group of Glc-1,2-*P*, on the other hand, has no comparable degrees of freedom. Therefore, if the locked conformations about the C-OPO_3 and CO-PO_3 bonds of Glc-1,2-*P* resemble the conformations about these bonds in phosphorylase-bound Glc-1-*P*, binding of the cyclic ester should have an entropic advantage. Page and Jencks [19], have concluded that the average value of ΔS for complete and uncompensated loss of one internal rotation in hydrocarbons is 4.5 cal · mol^{-1} · $degree^{-1}$ (19 J · mol^{-1} · $degree^{-1}$). If we use this same figure for loss of rotations in the phosphate group of Glc-1-*P*, then we would expect an entropy advantage of up to 9.0 cal · mol^{-1} · $degree^{-1}$ (38 J · mol^{-1} · $degree^{-1}$) for Glc-1,2-*P* vs. Glc-1-*P* binding, other factors involved in binding being equal. We are currently attempting to measure the temperature dependence of the ratio K_m/K_i in order to specify the reason for the strong binding of the cyclic ester.

Since potato starch phosphorylase (and plant phosphorylases in general) is not known to exist in active and inactive forms [20], and is not strongly inhibited by compounds that are good inhibitors of phosphorylases from other sources, the possibility that Glc-1,2-*P* might act as an in vivo regulator of potato phosphorylase activity is being considered. The cyclic ester has been shown to be a product of the basic cleavage of UDPGlc [16]: UDPGlc → Glc-1,2-*P* + UMP + H^+. The same reaction has been shown to be metal ion catalyzed and quite rapid near neutral pH even at 37°C [21]. Thus, the rapid enzyme-catalyzed formation of Glc-1,2-*P* from UDPGlc or any other nucleoside diphosphoglucose under neutral conditions should be possible. It is also of interest

that Glc-1,2-*P* is hydrolyzed (to Glc-1-*P*) by extracts of a variety of tissues [22], with the highest activity in plants, although potato tubers were not active.

If it is true that the conformations of enzyme-bound Glc-1-*P* and Glc-1,2-*P* are similar, then the cyclic ester should be a very useful substrate analog for defining the exact locations of active site residues in phosphorylase · substrate complexes. For phosphorylase *a* and *b* the X-ray structures are currently known at low resolution [23,24].

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