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α-GLUCAN PHOSPHORYLASES CATALYZE THE GLUCOSYL TRANSFER FROM α-D-GLUCOSYL FLUORIDE TO OLIGOSACCHARIDES

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SUMMARY. Regulated and nonregulated phosphorylases were found to catalyze in a slow, orthophosphate dependent reaction the direct transfer of the glucosyl residue from α-D-glucosyl fluoride to an oligosaccharide primer. The enzyme catalyzed formation of the glucosyl residue requires stereospecific protonation of α-D-glucosyl fluoride by a Brønstedt acid. The results are interpreted by a mechanism whereby phosphate acts as a proton shuttle and the cofactor pyridoxal 5'-phosphate is required to promote the acid-base function of phosphate.

The catalysis of α-glucan phosphorylases has long been considered to be restricted to a reversible glucosyl transfer between saccharides and glucose-1-P (1). Our recent findings, that D-glucal was utilized via a phosphorylase catalyzed protonation reaction (2) not only presented the first example of a new class of phosphorylase substrates but also provided the first direct evidence that Brønstedt acid catalysis is an obligatory step in the phosphorylase reaction. Since the utilization of D-glucal, like that of the natural substrate glucose-1-P, proved dependent on pyridoxal 5'-phosphate or other protonatable derivatives of the cofactor and in addition required orthophosphate, the further study of the reaction with D-glucal and related substrates appeared to hold considerable promise as a means of gaining a more complete understanding of the catalytic mechanism of phosphorylases.

D-glucal and glucosyl fluorides have been classified as glycosylic enzyme substrates, which can form glycosidic products on protonation. This was convincingly demonstrated by Hehre et al. in the case of carbohydrases (3, 4). We therefore decided to reinvestigate the substrate capabilities of glucosyl fluorides with respect to α-glucan phosphorylases, although earlier studies have failed to demonstrate their utilization by rabbit muscle phosphorylase (5, 6) and potato phosphorylase (6).

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MATERIALS AND METHODS

Maltodextrinphosphorylase from E. coli, potato phosphorylase, glycogen phosphorylase from rabbit skeletal muscle and phosphorylase b derivatives were prepared as previously reported (2). Phosphorylase concentrations refer to the subunit molecular weight. In order to remove primer, potato phosphorylase was treated repeatedly with Sepharose-bound α-amyloglucosidase (2).

Maltotetraose and homologous linear oligosaccharides were kindly provided by Boehringer, Mannheim. Acarbose and acarbose homologs (7) were a generous gift of Dr. Truscheit, Bayer AG, Leverkusen, F.R.G. Limit dextrin and glucose-1,2-P were prepared and purified as described previously (2). α-Cyclodextrin was from Sigma Chemicals. Silica gel 60, 200-400 mesh, and siliga gel 60 thin layer plates were from Merck, Darmstadt. Radiochemicals were from Amersham-Buchler, Braunschweig.

α-D-Glucosyl fluoride was prepared on a millimolar scale from radioactive glucose by established procedures (4, 8) with the following modifications. The solution of crude tetraacetyl-α-D-glucopyranosyl fluoride (8) in methylene chloride was neutralized by washing with a $NaHCO_3$ solution and concentrated to 2 ml. The solution was immediately poured onto a column (1x 15 cm) of silica gel 60, previously equilibrated with ether: petroleum ether (60-70°) (1:2) and chromatographed under pressure. Pure tetraacetyl-α-D-glucopyranosyl fluoride (Rf 0.4 in ether: petroleum ether (1:2)) was crystallized by evaporation of the solvent. Tetraacetyl-α-D-glucopyranosyl fluoride (0.1 mmol) was deacetylated in 0.1 ml 40 mM sodium methoxide in dry methanol. Flash chromatography on a column (1x10 cm) of silica gel 60, using ethanol: ethyl acetate (1:5) as solvent, yielded pure α-D-glucosyl fluoride (Rf 0.67 in solvent A (see below)). The product was stored at -20° either in dry methanol or after lyophilization. β-D-Glucopyranosyl fluoride was synthesized according to (8) and chromatographically purified according to (4).

Aliquots of a stock solution of α-D-glucosyl fluoride in dry methanol were pipetted into 1 ml plastic vials and evaporated to dryness. Reaction mixes (25-100 µl) were prepared by adding standard solutions of maleate-Tris buffer, pH 6.5, oligosaccharides, and P_i or effectors as indicated. At zero time and after incubation at 30° for the time stated, 1-25 µl were withdrawn and applied to silica gel 60 thin layer plates followed by an equal volume of dry methanol, and developed in (A) 1-butanol:acetic acid:water (5:4:3) or (B) 2-propanol:water:NH_3(aq) (7:2:1). Spots were visualized by the sulfuric acid-char method. Radioactivity was traced using the THIN-LAYER SCANNER II of Berthold (Wildbad, F.R.G.) or measured after scraping the spots from the thin layer plates. Paper chromatograms were run in ascending mode in 1-butanol: pyridin:water (6:4:3). The separated compounds were collected by elution from paper strips with water. Individual eluates containing radioactive oligosaccharides were further analyzed by digestion either with amyloglucosidase (30 U/ml in 50 mM acetate buffer, pH 4.5) or with maltodextrin phosphorylase (0.06 mM in 66 mM arsenate buffer, pH 6.5). Glucose formed on treatment of radioactive products by alkaline phosphatase was thought to arise from glucose-1-P.

RESULTS

α-D-glucosyl fluoride cannot simply replace glucose-1-P in a standard phosphorylase assay in the direction of polysaccharide synthesis, containing an oligosaccharide primer and a glucosyl donor (6). If, however, conditions are employed similar to those used for the phosphorylase dependent utilization of D-glucal (2), i.e. high enzyme concentrations, 1-10 mM P_i and 1-10 mM non-radioactive primer together with radioactive α-D-glucosyl fluoride, incorporation of radioactivity into oligosaccharides could be detected. Fig. 1

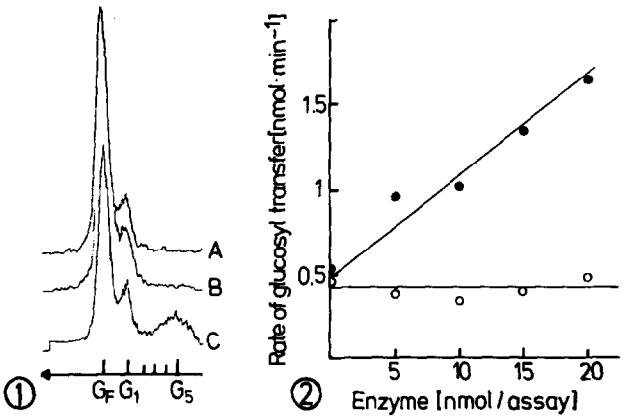

Figure 1. Radiochromatogram scans of products formed from α-D-glucosyl fluoride at 30° for 10 h. Conditions were 40 mM maleate-Tris buffer, pH 6,5, 10 mM maltotetraose, 3.3 mM α-D-[^{14}C]glucosyl fluoride; (A) without enzyme, (B) 0.02 mM potato phosphorylase, (C) 0.02 mM potato phosphorylase, 10 mM P_i. G_F, α-D-glucosyl fluoride; G_1-G_5, glucose-maltopentaose.

Figure 2. Dependence of rate of glucosyl transfer from α-D-glucosyl fluoride on enzyme concentration. For conditions see Fig. 1 (C). (o) Rate calculated from the fraction of free radioactive glucose found in the products. (●) Rate calculated from the radioactive glucose incorporated in the sum of all products (see Methods).

illustrates an experiment with potato phosphorylase: Spontaneous hydrolysis of α-D-glucosyl fluoride in the absence or presence of enzyme accounts for a measurable production of free glucose ($t_{1/2} \sim 62$ h at 30°; see also (4)). Only in the presence of P_i new radioactive products were formed. Under these conditions the rate of glucosyl transfer to oligosaccharides was proportional to the enzyme concentration (Fig. 2).

The utilization of α-D-glucosyl fluoride by phosphorylases from rabbit skeletal muscle, potato tuber and E. coli is summarized in Table I. The reaction of phosphorylases with α-D-glucosyl fluoride is primer dependent and requires orthophosphate. This is particularly clear in the case of potato phosphorylase, which was exhaustively treated to remove primer. Traces of primer and/or P_i carried over from the reactants are responsible for a low basic rate of glucosyl transfer. Like the glucose-1-P dependent synthesis of polysaccharides, the α-D-glucosyl fluoride dependent glucosyl transfer was inhibited by glucose-1,2-P (9) and α-cyclodextrin (10). A muscle phosphorylase b derivative reconstituted with pyridoxal 5'-phosphate monomethylester was inactive. Also shown in Table I are Michaelis constants for α-D-glucosyl

Table I. Relative rates and kinetic constants of glucosaccharide synthesis catalyzed by α-glucan phosphorylases with α-D-glucosyl fluoride

additions[a]	α-glucan phosphorylases: potato	E.coli	rabbit muscle[b]	phos b-PLP-Me[b,c]
		(μmol·24 h^{-1}·mg^{-1})		
none	0	0.03	0.05	0
10 mM P_i	1.55	0.36	0.15	0
10 mM P_i; 2.5 mM glucose-1,2-P	0.85	0.04	0.08	-
10 mM P_i; 10 mM α-cyclodextrin	0.15	-	-	-
kinetic constants		(mM)		
K_m(α-D-glucosyl fluoride)	9	12.5	n.d.	
K_i[d] (α-D-glucosyl fluoride)	20[e]	5.6	0.65[e]	

Rates represent μmol radioactive glucose transferred to glucosaccharides larger than maltotriose. (a) The reactions were performed at 30° in 50 mM maleate-Tris buffer, pH 6.5, 10 mM radioactive α-D-glucosyl fluoride and 10 mM maltotetraose or (b) 1% limit dextrin and 1 mM AMP. (c) Phosphorylase b, reconstituted with pyridoxal 5'-phosphate monomethylester. (d) Competitive to glucose-1-P. (e) Taken from Ariki and Fukui (6)

fluoride and a comparison of competitive inhibition constants (6). Moreover, the K_i value obtained with maltodextrin phosphorylase is given.

The fate of the glucosyl moiety derived from α-D-glucosyl fluoride was determined by using a radioactive substrate. When maltotetraose was used as a primer in the reaction of potato phosphorylase or E. coli maltodextrin phosphorylase radioactive glucose was found in glucosaccharides (and glucose-1-P; see below) comigrating with linear maltooligosaccharides (Fig. 1). After prolonged incubation (24 h) a small fraction of the radioactive glucose was also found in the position of maltose and maltotriose. The disproportionation of the primer in the presence of high enzyme concentrations and degradation of maltotetraose to maltotriose and maltose was observed before (11). If non-

Table II. Enzymic digestion of [^{3}H]glucosaccharides obtained from maltodextrin phosphorylase with α-D-[^{3}H]glucosyl fluoride in the presence of maltotetraose[a]

enzyme lysate from	radioactivity found in: G_1[b]	G_2	G_3	$\geq G_4$
		(%)		
amyloglucosidase	63	11	7	19
phosphorylase/arsenate	82	4	3	11

(a) For conditions see Table I. (b) G_1, G_2..., number of glucosyl residues in oligosaccharides.

degradable maltosyl acarbose (ref.: compound K 5 in (7)), which can serve as a primer with glucose-1-P, was used as the glucosyl acceptor, the major product arising from glucosyl transfer of α-D-glucosyl fluoride was the maltotriose derivative of acarbose. When limit dextrin was used as primer in the reaction with rabbit muscle phosphorylase, the primary radioactive product formed was a polysaccharide.

Enzymatic degradation of the radioactive oligosaccharide fraction by either amyloglucosidase or by arsenolysis with phosphorylase preferentially yields radioactive glucose (and maltose) (Table II). The distribution of label was like that in oligosaccharides synthesized from [^{14}C]glucose-1-P under similar conditions. Treatment of the radioactive products with alkaline phosphatase liberated ~17% of the radioactivity as free glucose. This was taken as evidence that glucose-1-P was also formed in the course of the reaction as a consequence of a phosphorolytic degradation of the primary radioactive oligosaccharide.

DISCUSSION

Competitive inhibition of polysaccharide synthesis from glucose-1-P by α-D-glucosyl fluoride was interpreted as a consequence of nonproductive binding of this substrate analog to the glucose-1-phosphate binding site at the active center of phosphorylases (6, 12). The data presented here clearly demonstrate that in the presence of a suitable primer and orthophosphate, the ternary phosphorylase-α-D-glucosyl fluoride-primer complex decomposes and the glucosyl moiety of glucosyl fluoride is transferred to the oligosaccharide primer.

The further fate of the glucosyl moiety, though not studied in detail, is keeping in the specificity of the phosphorylase reaction. Glucosaccharides isolated from the reaction of radioactive α-D-glucosyl fluoride are susceptible to digestion by phosphorylase or amyloglucosidase to free radioactive glucose as one would expect if the glucosyl moiety were transferred to the terminal 4-OH group forming an α-1,4-glucosidic linkage. The fraction of radioactivity found in glucose-1-P was less than expected from the glucose-1-

P/P_i equilibrium (1) in the presence of excess orthophosphate if only the newly attached glucose residues were susceptible to phosphorolysis. This could be due either to dilution by other terminal nonradioactive glucose residues or to a slower equilibration as a consequence of inhibition of the reaction by α-D-glucosyl fluoride (6).

The phosphorylase reaction seems to be specific for the α-anomeric form of glucosyl fluorides in correspondence with the known specificity of phosphorylases for α-D-glucose-1-P (1). No glucosyl transfer was observed with the ß-anomer; however, exceedingly slow utilization of ß-D-glucosyl fluoride would have escaped detection, since it was rapidly hydrolyzed nonenzymatically. Specific binding of α-D-glucosyl fluoride at the active center of phosphorylases is also supported by its inhibitory action towards the natural substrate and the inhibition of its utilization caused by the active-site-directed inhibitor glucose-1,2-P (9).

In spite of the slowness of the α-D-glucosyl fluoride reaction, the rate compares favorably with the rate of decomposition of a recently introduced transition state analog, pyridoxal 5'-diphosphate-α-D-glucose (13, 14). From the data in Table I and preliminary experiments terminated after 15 min incubation an apparent $t_{1/2}$ of decomposition of the enzyme-glucosyl fluoride-primer complex in the presence of orthophosphate of less than 5 min was calculated for the potato phosphorylase catalyzed reaction. Radioactive oligosaccharides accounted for > 93% of the early enzymatically synthesized products. The fraction of radioactivity comigrating with the region of glucose-1-P was ~7%. This makes it rather unlikely that glucose-1-P was the primary product formed on glucosyltransfer.

In describing a plausible mechanism for the phosphorylase catalyzed reaction with α-D-glucosyl fluoride or D-glucal we have taken into consideration the essential attack of glycosylic substrates by protonation (3, 4) and the dependence of the reaction on phosphate and a protonatable 5'-phosphate group of pyridoxal phosphate. In the case of the D-glucal reaction, the evidence indicated a protonation of the substrate from below the plane of the glucopyran-

ose ring (2). In the case of the glucosyl fluoride reaction, protonation by the same source apparently requires the α-anomeric configuration of the C-F bond. The corresponding position of a phosphate binding site next to the glucose binding site and to the site for pyridoxal 5'-phosphate in phosphorylases (15, 16) makes it attractive to suggest that the conjugated acid of a phosphate dianion provides the proton for the protonation of the substrate whereas the pyridoxal 5'-phosphate dianion is required to make an acid-base function of the phosphate group (P_i or glucose-1-P) possible. In this model (2), the phosphate group is only a proton shuttle but whether or not the proton actually originates from pyridoxal 5'-phosphate can not be decided.

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