Evidence for Intermediate Formation in the Mechanism of Potato Starch Phosphorylase from Exchange of the Ester and Phosphoryl Oxygens of α-D-Glucopyranose 1-Phosphate†

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ABSTRACT: We have examined under a variety of conditions the ability of potato starch phosphorylase to cause exchange of the ester and phosphoryl oxygens of α-D-glucopyranose 1-phosphate (Glc-1-P). In the presence of phosphorylase and starch, under conditions where 40–50% of the Glc-1-P is consumed in starch elongation, little if any exchange occurs that cannot be accounted for by accompanying starch phosphorolysis. Nor are the oxygens scrambled in the same or longer times by enzyme only when no release of inorganic phosphate occurs. But when D-maltotriose is used as a primer, or during primerless synthesis, and in the presence of phosphorylase and α- or β-cyclodextrin, a large degree of scrambling does occur. We conclude that under these latter conditions a glucosyl cation or covalent glucosyl-enzyme intermediate is formed. If this same intermediate is formed in the absence of starch or its analogue, then the phosphate counterion is not free to rotate; if the intermediate is formed with starch, then again one must assume that the rotation of phosphate ion is hindered, or that formation of the intermediate is rate determining.

A variety of polysaccharide phosphorylases including potato starch phosphorylase[1] and the disaccharide phosphorylase sucrose phosphorylase catalyze the transfer of glucosyl units between Glc-1-P and a polysaccharide or monosaccharide acceptor. These transfers are known to proceed via C–O bond breaking with the glycogen and sucrose enzymes (Cohn, 1949) and with overall retention of configuration at the anomeric position of the transferred unit. In the investigation of the mechanisms of these transfers a key question is or has been whether the reactions are concerted (SN2-like) displacements of the leaving group by the acceptor or occur via formation of an intermediate, which may be a glucosyl group ionically or covalently bound to the enzyme (Koshland, 1954).

In the case of sucrose phosphorylase, the involvement of such an intermediate has been amply demonstrated by the facts that the enzyme shows "ping-pong" reaction kinetics (Silverstein et al., 1967) and catalyzes the exchange of phosphate groups between Glc-1-P and inorganic phosphate in the absence of fructose or sucrose (Doudoroff et al., 1947), and by the actual capture of the glucosyl enzyme (Voet and Abeles, 1970). In the cases of glycogen and starch phosphorylases, the involvement of such an intermediate remains uncertain. These enzymes follow rapid equilibrium random bi-bi kinetics (Gold et al., 1970, 1971a) that show that the rate-determining step involves the interconversion of phosphorylase·Glc-1-P·polysaccharide and phosphorylase·P_i·polysaccharide ternary complexes, but do not specify how this interconversion occurs. The inhibition of glycogen phosphorylase by 5-gluconolactone (Tu et al., 1971; Gold et al., 1971b), which is considered to be a "transition state analogue" of a glucosyl cation, is suggestive of a reaction mechanism involving a glucosyl cation-like transition state. The relative rates of glycogen synthesis from Glc-1-P and its 1-deuterio derivative, $k_H/k_D = 1.10$, have been interpreted in a similar fashion (Tu et al., 1971), but the accuracy of these results has been challenged (Firsov et al., 1974), and the lack of a secondary isotope effect interpreted in terms of a rate-determining displacement of the leaving group by a group on the enzyme to give a glucosyl-enzyme covalent intermediate. But the isotope effect presumably does not rule out a direct front-side displacement of P_i from Glc-1-P by the hydroxyl group of the primer, since this mechanism also involves concerted bond making and breaking.

As noted by Gold and Osber (1972), a variety of intermolecular exchange experiments have been attempted with the polysaccharide phosphorylases, but none of these efforts have confirmed nor refuted the involvement of an intermediate. Most recently, Gold and Osber reported the results of experiments with the most subtle probe heretofore used to detect intermediate formation, namely, exchange of the ester and phosphoryl oxygens of Glc-1-P. Unlike the earlier exchange experiments, this probe is sensitive to the reversible breaking of the C–O(PO_3) bond of Glc-1-P if rotation of the enzyme-bound orthophosphate ion can occur. Gold and Osber found that glycogen phosphorylase *a* does in fact catalyze such an exchange reaction in the absence of glycogen, but at a rate that is only 0.3% as fast as the rate of glycogen elongation, and they have concluded that the exchange is either a side reaction or else it is limited by the rate of reorientation of orthophosphate in the phosphorylase·glucosyl group·P_i complex. But since glycogen phosphorylase is known to react via a ternary complex, it seemed possible to us that an alternative explanation for the low rate of exchange relative to glycogen synthesis is that only in the ternary complex are the maximal rates of C–O(PO_3) bond cleavage and/or orthophosphate reorientation possible. We have investigated this possibility with potato starch phosphorylase by determining the rate of phosphorylase-catalyzed exchange of the ester and phosphoryl oxygens of Glc-1-P in the presence of α- or β-cyclodextrin. The latter starch-like substances are inhibitors of potato starch phosphorylase (Staerk and Schlenk, 1967), but cannot act as glucosyl group acceptors since they lack the necessary hydroxyl

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[1] Abbreviations used: α-CD, α-cyclodextrin; β-CD, β-cyclodextrin; Glc-1-P, α-D-glucopyranose 1-phosphate; P_i, inorganic phosphate; potato starch phosphorylase, α-1,4-glucan:orthophosphate glucosyltransferase (EC 2.4.1.1) from potato; DEAE, diethylaminoethyl.

groups. We have also determined the extent of exchange in Glc-1-P isolated after phosphorylase-catalyzed polysaccharide synthesis with and without added primers.

Experimental Section

Materials. The following enzymes were purchased from Sigma: amyloglucosidase (glucoamylase); α-amylase, type III-A, from *Bacillus subtilus*; alkaline phosphatase, type I, from calf intestinal mucosa. D-Maltotriose was obtained from ICN Pharmaceuticals, Inc. α- and β-cyclodextrins (Schardinger dextrins) were purchased from Aldrich and recrystallized from 1-propanol-water (French et al., 1949). Dowex ion exchange resins were Bio-Rad analytical grade. Carbon dioxide was Matheson "Bone Dry" 99.8% minimum purity. Glc-1-P labeled with oxygen-18 in the phosphoryl oxygen positions was prepared by hydrolysis in oxygen-18 enriched water of cyclic α-D-glucopyranose 1,2-phosphate using as a catalyst an extract of *Saccharomyces fragilis* (Zmudzka and Shugar, 1966). Details of this synthesis and that of ester-labeled Glc-1-P are described by Kokesh and Kakuda (1977).

Potato Starch Phosphorylase. Starch phosphorylase was isolated from potatoes[2] by the method of Kamogawa et al. (1968) except that a 0.5% suspension of freshly prepared amylose (Whelan, 1955) was used in place of waxy rice starch for adsorption of the enzyme; centrifugation (5000*g*, 10 min) was necessary to collect the alcohol-precipitated amylose–phosphorylase complex, which was then immediately dissolved in 5 mM citrate buffer, pH 6.3. Attempts to crystallize the enzyme obtained by subsequent DEAE-Sephadex chromatography were unsuccessful, so that prior to and following further treatment the enzyme was stored at 4 °C as a suspension in 50% saturated ammonium sulfate, pH 7. From 5 kg of potatoes we thus obtained 60 mg of protein (determined by the Lowry procedure (1951) with a bovine serum albumin standard). When assayed under Kamogawa's (Kamogawa et al., 1968) conditions (except for a halving of the citrate concentration) in the direction of starch synthesis by appearance of inorganic phosphate by the Lowry–Lopez method (1946), this material had a specific activity of 25 μmol min^{-1} mg^{-1}, which is equal to that for Kamogawa's best enzyme. The enzyme at this stage, however, when assayed using purified Glc-1-P was nearly as active without added starch as it was with it, and half of it was treated further to eliminate "primer."

To further separate the phosphorylase from "primer," i.e., material capable of causing phosphorylase-catalyzed polysaccharide synthesis from Glc-1-P only, the enzyme was simultaneously treated with glucoamylase (Kamogawa et al., 1968) and α-amylase. Half of the phosphorylase suspension was dialyzed overnight at 5 °C against 5 mM citrate buffer, pH 6.3, and combined with 7.2 mg of glucoamylase and 0.13 mg of α-amylase, and the solution diluted to 6 mL with additional buffer and incubated at 30 °C for 6.5 h. (The amylase concentrations are based on Folin and Ciocalteu (1927) analyses of stock solutions.) The phosphorylase was then reisolated by DEAE-cellulose chromatography (as in the earlier part of the preparation) and again assayed for primerless starch synthesis using Glc-1-P that had been treated with α-amylase, glucoamylase, and charcoal according to Kamogawa's procedure. There was no detectable release of P_i until 2.5 h, when a rapid release began. We found that we could increase the lag time for P_i release to 9 h if the phosphorylase was given a second 10-min heat treatment at 55–56 °C (followed by removal of the precipitate). The phosphorylase thus obtained was used for the exchange experiment described in Table II. We subsequently found that this preparation contained an α-amylase impurity (which was partially destroyed in the second heat treatment).

Some 15 months after the original enzyme preparation, we retested the non-amylase-treated phosphorylase and found that the lag times for primerless synthesis were then quite long. Following rechromatography on DEAE-Sephadex, the enzyme still had a specific activity of 20 μmol mg^{-1} min^{-1} and when assayed using purified Glc-1-P without added starch showed P_i release only after about 18 h. This phosphorylase was used in the experiments described in Table III.

Exchange Experiments. A stock solution of phosphoryl-labeled Glc-1-P, about 0.1 M, was adjusted to pH 6.0 with HCl. At time = 0 (within an hour of preparation of the Glc-1-P solution), the Glc-1-P, water, and other components were combined to prepare 5-mL aliquots of each sample and the samples incubated at 30 °C. The extent of P_i release was followed using the Lowry–Lopez method. For samples that did not contain enzyme, the reaction was stopped by cooling to 4 °C; for those that contained enzyme, the samples were heated to 85 °C for 5 min and then cooled. In control experiments the heating was shown to cause complete inactivation of the phosphorylase with a negligible increase in the P_i concentration. The samples were stored at 4 °C for 24 h or less until the alkaline phosphatase hydrolyses were begun. Control experiments had shown that in the samples that were buffered by the Glc-1-P only, 37% release of P_i caused the pH to increase by only 0.2 pH unit.

Alkaline Phosphatase Catalyzed Hydrolysis of Glc-1-P. The method described below is based on that described by Cohn (1957). To remove any inorganic phosphate, each 5-mL sample was made 80–90 mM in Mg^{2+} by the addition of $MgCl_2$, and the pH adjusted to 9.0 with concentrated NH_4OH (about 40 μL necessary). Any precipitate was removed by centrifugation, 0.1 mL of a suspension of alkaline phosphatase (100 mg/mL) was added, and the mixtures were incubated at 30 °C for 90 min, during which time the pH was occassionally readjusted. Then an additional 0.05 mL of the alkaline phosphatase suspension was added and the incubation continued for an additional 90 min. The solutions were then stored overnight at 4 °C.

The samples were centrifuged, and the precipitate ($MgNH_4PO_4$ and protein) was suspended in 1 mL of 5% Cl_3CCOOH. The suspension was allowed to stand for 10 min; then the precipitate was collected and treated as above with a fresh 1-mL aliquot of 5% Cl_3CCOOH. The Cl_3CCOOH supernatants were combined and adjusted to pH 9 with concentrated NH_4OH, and the precipitate was collected and purified further by thrice dissolving it in 0.5 mL of 1 M HCl and reprecipitating by the addition of concentrated NH_4OH. The $MgNH_4PO_4$ was finally redissolved either by addition of HCl (Table II) or Dowex 50, H^+ form (Tables I and III), to a slurry of the salt in 1 mL of water. This solution was passed through a small column containing about 1.5 mL of Dowex 50, H^+ form, and the eluate was adjusted to pH 4.4 with 1 M KOH and evaporated to dryness. In the case of samples described in Tables I and III, the KH_2PO_4 also was recrystallized by dissolving it in 1 mL of water, adding 3 mL of 95% ethanol, and allowing the resultant mixture to stand for 3 h at room temperature and then overnight at 4 °C. The KH_2PO_4 was collected by centrifugation and then washed twice with 2 mL of ethanol and twice with 2 mL of ether.

Our method for transfer of the KH_2PO_4 into pyrolysis tubes

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[2] Identified as having been grown on Prince Edward Island, but not identified by variety.

TABLE I: Glc-1-P Control Samples.[a]

Treatment	Av atom % excess O-18 in P_i[b]
No buffer	
Heated 5 min, 85 °C	0.85 ± 0.02
No treatment	0.85 ± 0.03
14 h, 30 °C	0.82 ± 0.02
24 h, 30 °C	0.82 ± 0.04
0.05 M citrate buffer	
No treatment	0.84 ± 0.01
14 h, 30 °C	0.85 ± 0.01
24 h, 30 °C	0.81 ± 0.01

[a] Five-milliliter samples of 49.2 mM phosphoryl-labeled Glc-1-P, pH 6.0, treated as indicated. The concentration of inorganic phosphate, 0.3 mM, was not affected by any of the above treatments. [b] Average and standard deviation of duplicate determinations on P_i obtained by alkaline phosphatase catalyzed hydrolysis of Glc-1-P.

differs from that described by Cohn (1957). Each KH_2PO_4 sample was dissolved in 4.1 mL of water and an aliquot taken for P_i analysis (Ames, 1966). One-milliliter aliquots of each solution were pipetted into the bulbs of two Y tubes (described below) and evaporated on a rotary evaporator. Then a second 1 mL of solution was added to each tube and evaporated to dryness. The KH_2PO_4 appeared to be deposited in a crystalline form. Each tube was then placed on a vacuum line for 30 min or more, and finally in a vacuum oven at 100 °C for 10 h. (At the end of the last operation dry air was admitted to the cooled oven and the tubes were each stoppered until filled with CO_2.) On a vacuum line, 90–100 μmol of CO_2 (accurately measured) was added to each tube and the tube sealed. The sealed tubes were heated in a muffle furnace at 260 °C for 2 h and then left at room temperature for 6 days. The CO_2 was then collected, dried by four successive trap-to-trap distillations, and sealed in break-seal tubes. The exterior surfaces of the Y tubes were cleaned and the bulbs of the tubes (which contained the pyrolyzed KH_2PO_4) cut off and placed in 50-mL Erlenmeyer flasks with 40 mL of 1 N H_2SO_4. These solutions were boiled for 30 min and transferred with careful washing to 250-mL volumetric flasks, and the P_i concentration of each final solution was determined by the Ames method. These results agreed to within 5% with the earlier analyses.

The Y tubes used above were fabricated from 10-mm OD Pyrex tubing. A 21-cm length of tube had at one end a 16–17-mm OD bulb; about 4.5 cm above the bottom of the bulb, a second 10-cm length of 10-mm tube that contained a break-seal was joined to and bent parallel to the main tube. The Y tubes were attached to a vacuum line by use of a Kontes O-ring type vacuum connector (No. K-179910) which eliminated the need to make glass-to-glass seals.

Calculations of Oxygen-18 Enrichments. The analysis of the CO_2 on a Nier-type mass spectrometer yielded the peak intensity ratios P^{45}/P^{44} and $P^{46}/(P^{44} + P^{45})$ (where P^m indicates the peak intensity at m mass units) relative to those for a CO_2 standard with stated abundances of ^{13}C, ^{17}O, and ^{18}O of 1.09115×10^{-2}, 3.7995×10^{-4}, and 2.021×10^{-3}, respectively (C. E. Rees, personal communication, April 30, 1974). Calculation of the abundances of these three isotopes in our CO_2 samples obviously requires an assumption in that we must calculate three abundances from two pieces of data. We have assumed that the relative ***differences*** in the abundances of ^{13}C and ^{17}O between our tank CO_2 and the standard are small. Also, in the absence of the necessary numbers we have assumed that at equilibrium the ratio $[^{13}C^{16}O^jO]/[^{12}C^{16}O^jO]$ is independent of whether j is 16, 17, or 18,[3] and that the equilibrium constant $[C^{16}O^jO]^2/([C^{16}O_2][C^jO_2]) = 4$, the statistical value (Urey, 1947; Dostrovsky and Klein, 1952). Results for ^{13}C, ^{17}O, and ^{18}O were 1.0731×10^{-2}, 3.7995×10^{-4}, and 1.997×10^{-3}, respectively. Such measurements on tank CO_2 were repeated with each batch of water-equilibrated CO_2 samples and showed that the mass spectrometer measurements have very high precision. For CO_2 that had been equilibrated with $H_2{}^{18}O$, the atom % excess ^{18}O in the water (based on a normal value of 0.204 atom %) was calculated using equations derived along the lines of those of Dostrovsky and Klein (1952) but with explicit consideration of all of the above isotopes. (No additional assumptions are necessary to obtain these equations.) For these calculations we used values of $K = [H_2{}^{16}O][C^{16}O^jO]/([H_2{}^jO][C^{16}O_2])$ of 2.088 (Cohn, 1957) and 2.000 (the statistical value) for j = 18 and 17, respectively. Molar amounts of CO_2 were calculated from the temperature, volume, and pressure of the CO_2 using the Beattie Bridgman equation.

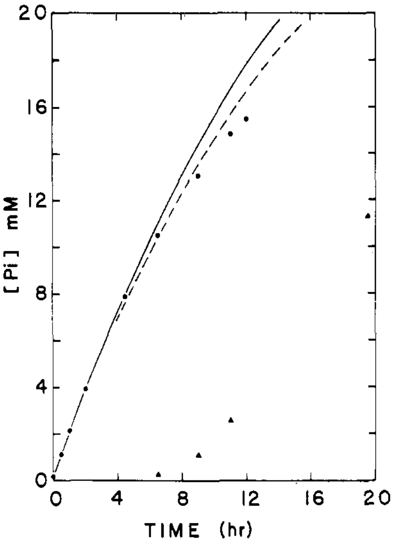

FIGURE 1: Plots of rate of P_i formation from Glc-1-P for experiments described in Table II. (●) In presence of phosphorylase and starch; (▲) in presence of phosphorylase only. Lines are calculated for P_i formation in presence of starch primer as described in the Appendix: (—) using Gold's (Gold et al., 1971a) constants; (- - -) using Lee's (1960) constants.

Other equipment and methods were as described by Kokesh and Kakuda (1977).

Results

With Glc-1-P in which the phosphoryl oxygen positions were enriched in ^{18}O we have determined the extent of exchange of the phosphoryl and ester oxygens under a variety of conditions. Experimentally, the degree of exchange is determined by hydrolysis of the (remaining) Glc-1-P with alkaline phosphatase, which causes O–P cleavage. The P_i obtained therefore contains the three phosphoryl oxygen atoms of the Glc-1-P and the fourth oxygen is derived from the solvent. The P_i is pyrolyzed to release 1 mol of H_2O per mol of P_i, and the ^{18}O content of the water is determined by equilibration of the water with CO_2 and then analysis of the CO_2 on a mass-ratio mass spectrom-

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[3] This is a reasonable assumption on statistical mechanical grounds: Dr. M. Zerner, personal communication (1975).

TABLE II: First Exchange Experiment.[a]

Conditions	Time (h) at 30 °C	% P_i released[b]	Av atom % excess O-18 in P_i[c]	% exchange Found[d]	% exchange Calcd
No treatment	0	0	0.84 ± 0.01	3	
Buffer only	19.5	0	0.78 ± 0.04	32	
Enz + α-CD	19.5	0.3	0.67 ± 0.08	83	71[e]
Enz + β-CD	19.5	3.3	0.70 ± 0.09	69	71[e]
Enz only	19.5	36.4	0.68 ± 0.04	79	2 ($x = \infty$)[f]
Enz + starch	12.0	50.7	0.81 ± 0.02	17	7 ($x = \infty$)[f] 15 ($x = 10$) 63 ($x = 1$)

[a] Five-milliliter samples of 31.0 mM phosphoryl-oxygen labeled Glc-1-P in 0.05 M citrate buffer, pH 6.0. Initial [P_i] = 0.175 mM. Where applicable, concentrations of other components are phosphorylase, $\nu_0 = 2.0$ µmol mL^{-1} h^{-1}; cyclodextrin, 0.54 mM; starch, 10 mg/mL. [b] As % of initial Glc-1-P concentration. [c] Average and standard deviation of duplicate determinations on P_i obtained by alkaline phosphatase catalyzed hydrolysis of Glc-1-P. [d] Calculated using initial enrichment of 0.847 atom %. Exchange (%) = 100 (0.847 − atom % excess observed)/(0.847 − 0.635). Based on results from Table I (cf. Results), these values have a standard deviation of 11%. [e] Calculated using eq 3 with initial conditions given in footnote *a*. [f] Calculated using methods described in the Appendix; x is the probability that the intermediate of eq 4 leads to P_i compared with a probability of 1.0 for formation of Glc-1-P. A value of $x = \infty$ yields the degree of exchange expected from starch phosphorolysis.

TABLE III: Second Exchange Experiment.[a]

Conditions	Time (h) at 30 °C	% P_i released[b]	Av atom % excess O-18 in P_i[c]	% exchange Found[d]	% exchange Calcd
No treatment	0	0	0.87 ± 0.09	<0	
Heated 5 min, 85 °C	0	0	0.86 ± 0.01	<0	
No additions	4	0.5	0.82 ± 0.03	13	
No additions	9	0.6	0.82 ± 0.01	13	
Enz only	9	1.5	0.80 ± 0.01	22	
Enz only	24	4.0	0.82	13	
Enz + β-CD	9	0.8	0.72 ± 0.04	60	65[e]
Enz + maltotriose	4	40.2	0.75	46	2 ($x = \infty$)[f]
Enz + starch	4	38.2	0.75 ± 0.10	46 (13)[g]	2 ($x = \infty$)[f]

[a] Five-milliliter samples of 59.0 mM phosphoryl-oxygen labeled Glc-1-P (with no added buffer), pH 6.0. Initial [P_i] = 0.45 mM. Where applicable, concentrations of other components are phosphorylase, $\nu_0 = 6.95$ µmol mL^{-1} h^{-1}; cyclodextrin, 0.53 mM; D-maltotriose, 5 mg/mL; starch, 1 mg/mL.[b-f] See Table II. [g] Based on the single result of 0.819 atom % excess ^{18}O in the P_i.

eter. Based on an average of the eight analyses of "untreated" Glc-1-P in Tables I–III, the P_i obtained from unexchanged Glc-1-P contained 0.847 ± 0.039 atom % excess ^{18}O. This corresponds to 0.847 × 4/3 = 1.130 atom % excess per phosphoryl oxygen of the Glc-1-P. After complete exchange of the ester and phosphoryl oxygens, the P_i obtained will have an enrichment of 0.847 × 3/4 = 0.635 atom % per oxygen.

Controls. Table I gives the results of various control experiments, and additional controls are listed in Tables II and III. One can conclude that the 85 °C heat treatment used to denature the phosphorylase in the experiments described below, or incubation at 30 °C for up to 24 h, causes no significant exchange. That is, the apparent exchange for incubation of Glc-1-P at 30 °C is greater after 14 h than after 24 h, and we therefore assume that it represents our experimental error. The lack of exchange in the control samples is not unexpected. But the controls also provide data on the reliability of the method of isotope analysis (in our hands). Assuming that all of the P_i analyses for the control samples in Table I should give identical results, then the observed standard deviation of 0.024 atom % is equivalent to a standard deviation of 11% for the observed degree of exchange.

Exchange Experiments. Tables II and III and Figure 1 include results of experiments in which labeled Glc-1-P was incubated without phosphorylase (controls like Table I), with phosphorylase only, with phosphorylase and cyclodextrins, and with phosphorylase and maltotriose or starch.

In the case of the phosphorylase only experiment listed in Table III, very little if any primerless starch synthesis occurred and little if any exchange was observed, the apparent exchange being greater after 9 h than after 24 h. With α- or β-cyclodextrins included (see Tables II and III), again very little release of P_i occurred but the observed degree of exchange is about that calculated on the basis that starch synthesis and Glc-1-P exchange in the presence of cyclodextrin have the same rate-determining step. On this basis (see Appendix for details) one would expect 71 and 65% exchange for the cyclodextrin experiments of Tables II and III, respectively. The observed values are 83 and 69% for α- and β-CD (Table II) and 60% for β-CD (Table III).

Large degrees of exchange were also noted in the phosphorylase only experiment of Table II where the incubation was continued long enough that primerless synthesis occurred (see Figure 1), and the exchange reaction had proceeded about twice as far as P_i release. When the primer was maltotriose, which is the shortest α-1,4-glucan that will act as a primer for potato phosphorylase (Swanson and Cori, 1948), P_i release and exchange occurred to about the same extent (but this result is for a single sample). But when starch was the primer, three of the four pieces of data indicate that exchange is slight—and within one standard deviation of that which would result from resynthesis of Glc-1-P from starch and P_i. These calculated degrees of exchange are included in Tables II and III; details of the calculation are given in the Appendix.

Discussion

We have found that potato starch phosphorylase catalysis of the synthesis of starch from Glc-1-P and starch primer is considerably greater than any catalysis of the exchange of the ester and phosphoryl oxygens of Glc-1-P in the absence of primer. This result is similar to that obtained by Gold and Osber (1972) with phosphorylase *a*, where the exchange reaction was found to be only 0.3% as fast as glycogen synthesis, and also can be compared with results of earlier intermolecular exchange experiments. Cohn and Cori (1948) showed that in the absence of polysaccharide primer neither glycogen nor potato phosphorylase will catalyze the exchange of P_i with the phosphate group of Glc-1-P. Similarly, they showed that in the presence of arsenate ion potato phosphorylase does not yield free glucose from Glc-1-P in the absence of starch, although glucose formation (presumably via glucose 1-arsenate) occurs in the presence of starch (Katz and Hassid, 1951).

Incubation of ^{18}O-labeled Glc-1-P with phosphorylase and α- or β-CD was carried out to see if binding of a starch-like molecule to the enzyme could effect exchange. The cyclodextrins are competitive inhibitors (with respect to starch) of starch phosphorylase (Green and Stumpf, 1942; Staerk and Schlenk, 1967) but cannot act as primers for polysaccharide synthesis. We have found that, in the presence of the cyclodextrins, but absence of any added polysaccharide primer, that starch phosphorylase catalyzes the loss of oxygen-18 from the phosphoryl oxygens of Glc-1-P. We assume that this loss is due to an exchange of the phosphoryl and ester oxygens of Glc-1-P and not from exchange of the phosphoryl oxygens with those of water. This assumption, which can and must be checked, is based on the findings of Cohn (1949) and Gold and Osber (1972) that glycogen phosphorylase does not catalyze such an exchange in the presence of glycogen and is consistent with our finding that none of our observed exchanges exceeded 100%. In contrast to the effect of the cyclodextrins on the intramolecular exchange, Cori (1961) has found that in the presence of arsenate ion and β-CD (but absence of glycogen) that glycogen phosphorylase does not catalyze the formation of glucose from Glc-1-P. The observed exchange is consistent with and is the first evidence for the formation of an intermediate via reversible breaking of the C–OPO$_3$ bond of Glc-1-P in the presence of a cyclodextrin. This reaction can be described as

$$\text{phosphorylase} \cdot \text{Glc-1-P} \cdot \text{CD} \rightleftharpoons \text{phosphorylase} \cdot \text{glucosyl group} \cdot \text{P}_i \cdot \text{CD}$$

where the enzyme-bound P_i is free to rotate. Furthermore, the rate of the exchange reaction has been found to be consistent with the rate of phosphorylase-catalyzed starch synthesis.

In the cases where the primer is maltotriose or that of primerless synthesis, exchange also accompanies polysaccharide synthesis. The latter reactions, therefore, may also occur by way of an intermediate that may be described as a phosphorylase·glucosyl group·P_i·primer quaternary complex. If the C–O(PO$_3$) bond of Glc-1-P is reversibly broken during the course of polysaccharide synthesis from starch primer, though our results do not require it, then the lack of exchange in the remaining Glc-1-P requires that capture of the glucosyl unit by the 4-OH group of the starch must occur considerably faster than the phosphate ion can rotate and capture the glucosyl unit to reform Glc-1-P. Otherwise exchange would be observed because equilibration of Glc-1-P with the phosphorylase·Glc-1-P·starch complex is rapid. For example, if we assume that during starch elongation an intermediate is formed and the accompanying P_i is totally free to rotate, and if the ratio of the rates of C–O(C) and C–O(PO$_3$) bond formation is 1.0, then for the experiment in Table II the Glc-1-P remaining after 12 h would be 63% exchanged. If the ratio of the rates is 10.0, then the exchange after this period would be only 15%. (Details of these calculations are given in the Appendix.)

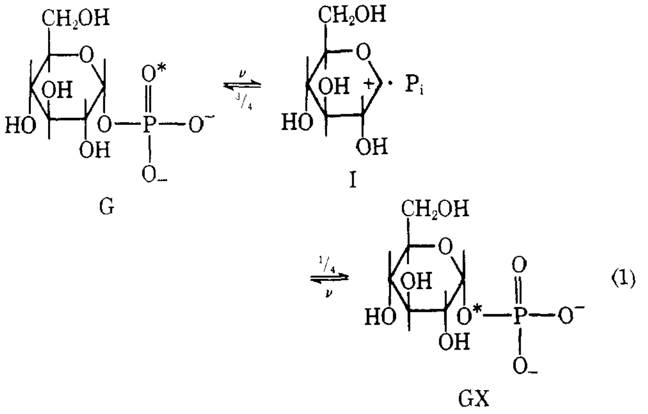
In summary, then, the exchange results are consistent with formation of an intermediate in the presence of phosphorylase and cyclodextrins, and during phosphorylase-catalyzed elongation of maltotriose or the primer of primerless synthesis. They do not require, but are not inconsistent with, intermediate formation also occurring during elongation and degradation of starch primer. If we assume the operation of a common mechanism for primer elongation, then the simplest explanation of all of the exchange data is that phosphorylase causes reversible breakage of the C–O(PO$_3$) of Glc-1-P, but at least under some conditions the P_i counterion is not free to rotate. If the rate of the cleavage of the C–O(PO$_3$) bond of Glc-1-P is independent of whether starch, cyclodextrin, etc. are also bound to the phosphorylase, and if C–O(PO$_3$) bond reformation is much faster than C–O(C) bond formation, then the pattern of exchange that we have observed would be explained if the relative ease of P_i rotation in the phosphorylase·glucosyl group·P_i·X complex were in the order: $X = \text{no starch} \simeq \text{starch} \ll \text{cyclodextrin} < \text{maltotriose} \simeq \text{primer of primerless synthesis}$. However, we emphasize that this is not a unique explanation of the results.

Concerning the nature of the glucosyl group in the intermediate formed from Glc-1-P by C–O(PO$_3$) bond cleavage, our results do not distinguish between a glucosyl cation and a glucosyl group that is covalently bonded to the phosphorylase. Again assuming that we can extrapolate from data on glycogen phosphorylase, the best information on this question is from kinetic isotope effect studies with glycogen phosphorylase *b* by Firsov et al. (1974). Their results require concerted bond making and breaking during starch elongation and degradation and are consistent with a covalent glucosyl-enzyme intermediate. In this regard it is also of interest that Aramovic-Zikic et al. (1974) have suggested that a carboxyl group is essential for the activity of glycogen phosphorylase *b*, and DeToma and Abeles (1970) have evidence that in the covalent glucosyl-sucrose phosphorylase intermediate the linkage is to a carboxyl group.

The exchange method employed here has yielded much new information about the mechanism of potato starch phosphorylase. Further studies using the method are planned.

Appendix: Kinetic Equations

Exchange in the Presence of Cyclodextrins. A possible scheme for exchange via reversible intermediate formation in the presence of cyclodextrins is



(1)

where G represents free and enzyme bound Glc-1-P that is labeled with oxygen-18 in the phosphoryl positions, and GX is the ester labeled Glc-1-P.[4] I represents an intermediate phosphorylase•glucosyl group•P$_i$•cyclodextrin complex, in which the glucosyl group may or may not be covalently linked to the enzyme, and the P$_i$ is assumed to be free to rotate. The maximum rate of C–O(PO$_3$) bond cleavage is ν,[5] and the factors ¾ and ¼ are the probabilities that I will yield G and GX, respectively. The rate equation for this mechanism based on the steady-state assumption for all enzyme complexes is

$$\frac{d[G]}{dt} = -\frac{d[GX]}{dt} = -\frac{1}{4}\nu f_{EG} + \frac{3}{4}\nu f_{EGX} \tag{2}$$

where

$$f_{EG} \simeq \frac{[E \cdot CD \cdot G]}{[E \cdot CD \cdot G] + [E \cdot CD \cdot GX]} = \frac{[G]}{[G] + [GX]} = \frac{[G]}{[G]_0}$$

in which E•CD•G is the phosphorylase•cyclodextrin•G ternary complex, and the concentrations of free enzyme, binary enzyme complexes, and the enzyme•cyclodextrin•P$_i$ ternary complex are assumed to be negligible, and therefore $f_{EGX} = 1 - f_{EG}$. The initial concentration of phosphoryl labeled Glc-1-P is $[G]_0 = [G] + [GX]$, since little release of P$_i$ occurs. Substitution of f_{EG} and f_{EGX} into eq 2, integration, and use of the initial condition $[G] = [G]_0$ at $t = 0$ yields[6]

$$[G] = 0.25[G]_0(3 + e^{-at}) \tag{3}$$

where $a = \nu/[G]_0$. If ν is assumed to be the initial rate of P$_i$ release in the presence of starch primer, then the degrees of exchange calculated and observed in the presence of cyclodextrin are in good agreement. Cf. the Results section and Tables II and III.

Exchange in the Presence of Starch due to Intermediate Formation and Starch Phosphorolysis. Mechanism 1 can be expanded to take account of the possibility that the intermediate phosphorylase•glucosyl group•P$_i$•starch complex can lead to starch elongation.

$$\begin{tikzcd} G \arrow[r, shift left, "\nu"] & I \arrow[l, shift left, "3/4"] \arrow[r, shift left, "1/4"] \arrow[d, shift left, "x"] & GX \arrow[l, shift left, "\nu"] \\ & P_i \arrow[u, shift left, "\nu'"] & \end{tikzcd} \tag{4}$$

In eq 4, G, GX, and I have the same meaning as in eq 1, P$_i$ represents free and enzyme bound inorganic phosphate, and x is the probability that the intermediate will lead to starch elongation relative to a probability of 1 for Glc-1-P formation. In the intermediate, free P$_i$ rotation is again assumed. According to this mechanism, exchange of phosphoryl and ester oxygens of Glc-1-P can result from the reactions G → I → GX, i.e., as a result of intermediate formation, or from P$_i$ → I → GX, i.e., as a result of starch phosphorolysis. For this mechanism the differential rate expressions are:

$$\begin{aligned} \frac{d[G]}{dt} &= -\nu f_{EG}\frac{(x + 1/4)}{(x + 1)} + \nu f_{EGX}\frac{3/4}{(x + 1)} + \nu' f_{EP}\frac{3/4}{(x + 1)} \\ \frac{d[GX]}{dt} &= -\nu f_{EGX}\frac{(x + 3/4)}{(x + 1)} + \nu f_{EG}\frac{1/4}{(x + 1)} + \nu' f_{EP}\frac{1/4}{(x + 1)} \\ \frac{d[P_i]}{dt} &= \nu' f_{EP}\frac{1}{(x + 1)} + \nu f_{EG}\frac{x}{(x + 1)} + \nu f_{EGX}\frac{x}{(x + 1)} \end{aligned} \tag{5}$$

where f_{EG}, f_{EGX}, and f_{EP} represent the fraction of the phosphorylase present as phosphorylase•starch•G, phosphorylase•starch•GX, and phosphorylase•starch•P$_i$ ternary complexes, respectively. These fractions can be written as functions of the concentrations of G, GX, and P$_i$, and the Michaelis constants $K_G = [E{\cdot}S][P_i]/[E{\cdot}S{\cdot}P_i]$, where S is starch. If ν_f is the maximum rate of starch synthesis and ν_r is the maximum rate of starch phosphorolysis, then mechanism 4 requires that

$$\begin{aligned} \nu &= \frac{(x + 1)}{x}\nu_f \\ \nu' &= (x + 1)\nu_r \end{aligned} \tag{6}$$

Under the "initial conditions", $[G] = [G]_0$, $[GX] = [P_i] = 0$, it follows from eq 5 (and one's intuition) that $(d[P_i]/dt)_0/(d[GX]/dt)_0 = 4x$. In general the solution of eq 5 is more complex. As discussed below, the values of the constants ν_f, ν_r, K_G, and K_P are available, so that the set of differential equations obtained by substitutions of eq 6 into eq 5 can be used to calculate the concentrations of G, GX, and P$_i$ as a function of x and time. This can be done quite simply by expressing the integrals of eq 5 as Riemann sums (Johnson and Kiokemeister, 1960). Thus at $t = 0$, $[G] = [G]_0$, $[GX] = 0$, and $[P_i] = [P_i]_0$, and the instantaneous velocities $d[G]/dt$, $d[GX]/dt$, and $d[P_i]/dt$ can be evaluated for a given x. After a small time interval Δt, $\Delta G = (d[G]/dt)\Delta t$, etc., and $[G] = [G]_0 + \Delta G$, etc. The instantaneous velocities are then reevaluated and the process repeated for a second Δt interval, and so on. The results of such calculations are internally consistent—i.e., the sum of $[G] + [GX] + [P_i]$ at any time equals $[G]_0 + [P_i]_0$, and results are unchanged by reducing Δt by a factor of ten. The extent of exchange at any time was calculated from $100[GX]/(\frac{1}{4}([GX] + [G]))$. The results of such calculations have been included in the Discussion and Tables II and III.

Concerning the values of the constants used, ν_f is obtained by an initial rate assay of the starch containing samples. According to Gold et al. (1971a), at pH 6.3 and 30 °C, $K_G = 2.0 \pm 0.3$ mM, $K_P = 5.9 \pm 1.2$ mM, and $\nu_f/\nu_r = 2.61$, corresponding to an overall equilibrium constant of $[P_i]/[\text{Glc-1-P}] = 7.7$. This overall equilibrium constant is pH dependent, but 7.7 is close to the value for pH 6.0 reported by Hanes and Maskell (1942) and to that which we have determined (Kokesh and Kakuda, unpublished experiments). We therefore have assumed that K_P, K_G, and ν_f/ν_r are the same at pH 6.0 as at pH 6.3.

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[4] In eq 1, G is represented as labeled in a single phosphoryl oxygen, although the three external oxygens, of course, are equivalent. This representation is meant to stress the fact that, at low levels of oxygen-18 enrichment, there is (at most) a single oxygen-18 atom per Glc-1-P molecule, so that at equilibrium the probability of G is three times that of GX.

[5] Throughout this section, one can consider that ν and ν' are zero-order *rate constants.*

[6] The derivation used to obtain eq 3 is essentially the same as that of Midelfort and Rose (1976) for the rate equation for an enzyme-catalyzed isotopic exchange between two parts of the same substrate molecule.

Exchange in the Presence of Starch due to Starch Phosphorolysis. Even if no intermediate is formed during starch elongation, the exchange reaction G → GX can result from the synthesis of Glc-1-P from P_i and starch. This case is equivalent to exchange by the reactions of eq 4 when $x = \infty$. If the condition $x = \infty$ is substituted into the set of differential equations obtained by substitution of eq 5 into eq 6, the result is eq 7.

$$\begin{aligned}\frac{d[G]}{dt} &= -\nu_f f_{EG} + {}^3\!/_4 \nu_r f_{EP} \\ \frac{d[GX]}{dt} &= -\nu_f f_{EGX} + {}^1\!/_4 \nu_r f_{EP} \\ \frac{d[P_i]}{dt} &= \nu_f(f_{EG} + f_{EGX}) - \nu_r f_{EP}\end{aligned} \tag{7}$$

where f_{EG}, etc. were defined previously. Using the methods discussed above we calculated the concentrations of G, GX, and P_i as a function of time using Gold's (Gold et al., 1971a) values of K_P, K_G, and ν_f/ν_r. Some of these results are shown in Figure 1. The data were also used to calculate the extent of exchange of the Glc-1-P, as noted in Tables II and III. Also shown in Figure 1 are the results of calculations using K_G = 3.5 mM and K_P = 7.5 mM as determined by Lee (1960) at pH 6.3, and ν_f/ν_r = 3.60 as calculated using K_{eq} = 7.7. Use of these constants leads to lower estimates of exchange.

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