Phosphorylated Thiosugars: Synthesis, Properties, and Reactivity in Enzymatic Reactions†

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ABSTRACT: A number of phosphorylated thiosugars have been prepared and tested as substrates for metabolic reactions. 6-Thioglucose-6-P is readily synthesized by reaction of 6-tosylglucose with trisodium thiophosphate at pH 10 in aqueous solution; the product has only sulfur between carbon and phosphorus. When ethyl glycerate is tosylated and treated similarly with thiophosphate, a 5:1 mixture of 3-thioglycerate-3-P and the 2-isomer is formed. 6-Thioglucose-6-P is converted by glycolytic enzymes to triose phosphates, 3-thioglycerol-3-P and 3-thioglycerate-3-P, and is oxidized by enzymes of the hexose monophosphate shunt to 5-thioribulose-5-P, which can be converted via phosphoribulokinase and ribulose-bis-P carboxylase into 3-P-glycerate and 3-thioglycerate-3-P. For most of the non-phosphoryl-transferring enzymes there are only moderate effects on V_{max} and K_m. Phosphoglucoisomerase, however, is very sensitive to the sulfur for oxygen change, with V_{max} decreasing 60-fold and K_m increasing 15-fold. Surprisingly, phosphoribulokinase has a V/K value for 5-thioribulose-5-P that is over 3 orders of magnitude less than for ribulose-5-P. 6-Thioglucose-6-P was found to be a substrate for several enzymes that transfer the phosphoryl group. It is as good a substrate for alkaline phosphatase as glucose-6-P, and with phosphoglucomutase it is converted to 6-thioglucose-1-P with a rate that is 11% of the rate of reaction of glucose-1-P, with a K_{eq} value of 45.6. The free energy of hydrolysis of the phosphorylated thiol is thus −7.2 kcal/mol at pH 7. In contrast to these facile reactions, 6-thioglucose-6-P is a very poor substrate for yeast hexokinase (rate over 5 orders of magnitude less than the rate with glucose and MgATP) and 6-thioglucose is phosphorylated at a similar rate. The factors that lead thiols to be very slow substrates for kinases, but relatively good ones for phosphoglucomutase and alkaline phosphatase, are not understood. 3-Thioglycerate-3-P is not a substrate for phosphoglyceromutase, but the 2-isomer is converted by enolase into phosphoenolthiopyruvate. While all phosphorylated thiols are acid labile (half-life from 4 to 90 min when monoprotonated), 5-thioribulose-5-P is particularly acid labile with a half-life less than 1 s at pH 4 as the result of a cyclization that produces the cyclic thiosugar as the product. The results of this work show phosphorylated thiols to be generally accepted isosteric analogues of metabolic intermediates except when the phosphoryl group is transferred, in which case some enzymes accept the phosphorylated thiols as substrates, while others do so poorly or not at all.

Many metabolic intermediates are phosphorylated alcohols, and the phosphate group serves as a convenient handle to keep the intermediate within the cell (Davis, 1958) and permits adequate affinity for the enzymes that use the metabolite. Phosphorylated thiols are isosteric analogues of phosphorylated alcohols but are very acid labile, with half-lives in most cases when monoprotonated of 4–90 min at 25 °C. By contrast, the half-life of glucose-6-P when monoprotonated is 12 h at 100 °C (Degani et al., 1966). All of these compounds are stable as dianions.

The reactions of phosphorylated thiols are of interest for two reasons. First, they serve as a sensitive test of the geometric sensitivity of enzyme active sites, since they are similarly charged to normal phosphate esters, but the C–S (1.82 Å) and S–P (1.95 Å) bonds are somewhat longer than C–O (1.43 Å) and O–P (1.57 Å) bonds (Huheey, 1983; Frey & Sammons, 1985; Corbridge, 1966). Unlike thiophosphorylated alcohols, phosphorylated thiols have the same hydrogen-bonding capabilities as phosphate esters, except for the bridging atom. Second, the ability of enzymes to catalyze phosphoryl transfer from phosphorylated thiols may provide clues to the transition-state structure in such reactions. The present paper will report on the properties of 6-thioglucose-6-P, and compounds made enzymatically from it, and also 3-thioglycerate-3-P and 2-thioglycerate-2-P. The following paper in this issue will report on similar compounds with nitrogen in place of sulfur in the bridge. A similar study of thiol and amino analogues as substrates for glycerokinase has been reported by Knight and Cleland (1989).

Materials and Methods

Enzymes, buffers, and reagents from Sigma or Boehringer were used without further purification. NAD and NADP were from Boehringer, and tosyl chloride was from Aldrich. Co$(NH_3)_4$PNP was synthesized according to Haromy et al. (1983).

Methods. ^{31}P and ^{13}C NMR spectra were obtained on either Nicolet NT-200 or Bruker AM500 or AM400 spec-

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trometers, while proton NMR spectra were obtained on the NT-200 or a Bruker WH270 instrument. ^{31}P NMR chemical shifts are referenced to external 200 mM D_3PO_4 with an external lock signal. Proton and ^{13}C chemical shifts are referenced to external TMS.[1] The ^{31}P NMR spectra contained 16000 data points and were smoothed with exponential multiplication using 3-Hz line broadening.

Synthesis of 6-Thioglucose-6-P. 6-Thioglucose-6-P was synthesized by reaction of 6-tosylglucose and thiophosphate in aqueous solution at high pH. Glucose (56 mmol) was dissolved in 100 mL of dry pyridine at 4 °C, and 56 mmol of tosyl chloride in 50 mL of dry pyridine was added dropwise over 1 h. The reaction solution was allowed to warm to room temperature and stand overnight. It was then quenched by addition of 600 mL of 10 mM aqueous NaOH and titrated to pH 8. TLC on silica gel indicated one major species associated with sugar and fluorescence quenching (~50% yield). A less polar species (likely a ditosyl derivative) was also present. The tosylglucose could be either purified on silica gel or used directly. In a typical synthesis, 8 mmol of trisodium thiophosphate (final concentration 190 mM) was added to the crude solution of tosylglucose that originally contained 8 mmol of starting glucose, and the pH was adjusted to 10.3. The solution was kept under N_2 for 40 h at room temperature and monitored by ^{31}P NMR. The product showed a triplet, which collapsed upon proton decoupling into a singlet 17 ppm downfield from 100 mM D_3PO_4. This resonance increased with time until it was 44% of the total phosphate present (this constitutes a nearly quantitative reaction of thiophosphate with the amount of tosylglucose present). Two additional products were observed that showed doublets at 15 ppm (presumably from reaction of thiophosphate with glucose tosylated on a secondary alcohol). The major species had a half-life of 29 min at pH 3, 30 °C, and 330 min at pH 5.94. It was purified on a 2.2 × 90 cm column of QAE Sephadex at pH 10.3, 10 mM ammonia, and eluted with a LiCl gradient. 6-Thioglucose-6-P elutes at 0.15 M salt. Fractions containing 6-thioglucose-6-P were pooled, lyophilized, and dissolved in methanol, followed by addition of 9 parts of acetone to precipitate the compound. This procedure removes LiCl. The lithium salt was converted to the potassium or sodium salts by passage over Dowex-50 resin in the appropriate salt form.

Synthesis of 6-Thioglucose. When this compound was synthesized by the published procedure of reaction of thioacetate with 6-tosylglucosetetraacetate (Akagi et al., 1962), the product contained some contaminating glucose (2–3% burst in a hexokinase assay). This compound was thus synthesized prior to use by hydrolysis of 6-thioglucose-6-P at pH 2 for 12 h under N_2. The solution was then passed over a column of Dowex-1-Cl to remove the phosphate. This synthetic route produced no detectable glucose, as judged by the failure to see a burst in the hexokinase reaction.

Synthesis of 3-Thioglycerate-3-P and 2-Thioglycerate-2-P. The calcium hemisalt of DL-glyceric acid (100 mmol) was converted to the free acid by addition of Dowex-50-H^+ resin. After filtration, the filtrate was concentrated in vacuo and dried over P_2O_5. Dowex-50-H^+ resin (100 mL) was dried by washing with ethanol and storage in vacuo over P_2O_5. The glyceric acid and resin were then added to 900 mL of anhydrous ethanol at 4 °C. After several days the mixture was filtered, the resin was washed with ethanol, and the filtrate and washings were flashed to dryness. The resulting oil was dissolved in water, and pH was adjusted to 7, and unreacted glycerate was removed by passage over a Dowex-1-Cl column. The fractions containing ester (Waldi, 1965) were pooled, evaporated to dryness, and dried over P_2O_5 in vacuo (yield, 80 mmol).

Ethyl glycerate (40 mmol) was added to 75 mL of anhydrous pyridine at 4 °C, and 44 mmol of tosyl chloride in 30 mL of pyridine added dropwise over 2 h. The reaction mixture was allowed to warm to room temperature overnight. The next day the solution was cooled to 4 °C, and 44 mmol of NaOH in 100 mL of cold water was added. Pyridine was removed under reduced pressure, and the ester was stirred in 500 mL of water with 50 mL of Dowex-50-H^+ at 4 °C. After several days, the solution was filtered, concentrated to 150 mL, and neutralized, and 40 mmol of trisodium thiophosphate in 50 mL of water was added, and the pH was adjusted to 11. The solution was stirred under nitrogen for 4 h and produced 3-thioglycerate-3-P and 2-thioglycerate-2-P in a 5:1 ratio. These compounds were separated on a 2.2 × 47 cm column of QAE-A25 Sephadex at pH 10.3 by washing with 300 mL of 150 mM LiCl/10 mM ammonia and eluting with a linear gradient of LiCl (150–500 mM, plus 10 mM ammonia). Thiophosphate eluted at 270 mM salt, followed by the 3-isomer (290 mM) and the 2-isomer (300 mM). Fractions containing phosphorylated thioglycerates were pooled, concentrated, and lyophilized after adjustment to pH 9.5. LiCl was removed by extraction with cold dry methanol/acetone (1:10), and the lithium salt was converted into the potassium one by passage through Chelex-100. Some decomposition occurred during lyophilization.

Enzymatic Synthesis of Phosphorylated Thio Analogues. Unless otherwise specified, all reactions were carried out at pH 9 and 22 °C for 15 min. Enzymes were then removed by ultrafiltration through Centricon 10 membranes at 8000 rpm for 30 min at 5 °C.

6-Thiogluconate-6-P. This compound was synthesized from 5 mM 6-thioglucose-6-P with 10 units/mL glucose-6-P dehydrogenase. The reaction mixture contained 50 mM Ches, 1 mM NADP, 10 mM oxidized glutathione, and 4 units/mL glutathione reductase. After the synthesis was complete the enzymes were removed via Amicon filtration, the pH was raised to 9.5, and the product was purified by ion-exchange chromatography on a QAE Sephadex column as above. The yield (based on reaction with alkaline phosphatase and DTNB or on enzymatic assay with 6-P-gluconate dehydrogenase) was quantitative.

6-Thioglucose-1-P. This compound was synthesized from 1 mmol of 6-thioglucose-6-P by incubation with 1000 units of phosphoglucomutase at pH 8.4 with 10 mM Taps/1 μM glucose-1,6-bis-P/7 mM $MgCl_2$ in 10 mL at 25 °C for 24 hr under N_2. The reaction was then Amicon filtered to remove the enzyme, and the filtrate was treated with Chelex and filtered, and the pH was adjusted to 7 with HCl. 6-Thioglucose-1-P was purified on Dowex-1-Cl eluted with a gradient of NaCl at 4 °C. Fractions containing thiol were pooled, concentrated, and stored under N_2.

6-Thiofructose-6-P. 6-Thioglucose-6-P (10 mM) was incubated with 70 units/mL phosphoglucoisomerase and 100 mM Ches.

6-Thiofructose-1,6-bis-P. 6-Thioglucose-6-P (1 mM) was incubated with 300 units/mL phosphoglucoisomerase, 20

[1] Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate; Ches, 2-(*N*-cyclohexylamino)ethanesulfonate; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); 2-PDS, 2,2′-dipyridyl disulfide; 4-PDS, 4,4′-dipyridyl disulfide; GSSG, glutathione disulfide; TMS, tetramethylsilane.

units/mL phosphofructokinase, 100 mM Ches, 3 mM $MgCl_2$, and 2 mM ATP.

The syntheses for stability studies done at pH 4 were carried out at pH 9, but with 100 mM citrate buffer. After the enzymes were filtered off, the pH was lowered to 4.

Endpoint Assays for Stock Solutions and Stability Studies. Free sulfhydryl concentrations were determined with either DTNB (ϵ = 13600 for the color developed at pH 8; Danehy, 1971), 2-PDS (ϵ = 7060 for the colored product; Grassetti & Murray, 1967), or 4-PDS (ϵ = 19800 for the color developed; Grassetti & Murray, 1967). DTNB (2 mM) was dissolved in either 100 mM phosphate, Taps, or Bicine buffer, pH 8.1, and absorbance was recorded at 412 nm. The dipyridyl disulfides were dissolved in ethanol and added to buffered solutions. The presence of disulfides in stock solutions of thiols was determined by the method of Zahler and Cleland (1968).

Stock solutions of 6-thioglucose-6-P were either calibrated via reaction with glucose-6-P dehydrogenase or hydrolyzed with either acid or alkaline phosphatase and assayed for free thiol. 6-Thiogluconate-6-P was assayed either by alkaline phosphatase treatment followed by assay for thiol or with 6-P-gluconate dehydrogenase. 6-Thiofructose-6-P was assayed with phosphoglucoisomerase and glucose-6-P dehydrogenase. 6-Thiofructose-1,6-bis-P was assayed with aldolase and glyceraldehyde-3-P dehydrogenase in the presence of 2.4 mM NAD and 10 mM arsenate. The same assay without aldolase was used for 3-thioglyceraldehyde-3-P. The concentrations of other phosphorylated thiols were determined by the alkaline phosphatase/DTNB method.

Measurement of Enzyme Activity. Hexokinase activity was determined by coupling the glucose-6-P product to the reduction of NADP with glucose-6-P dehydrogenase or by coupling the production of MgADP to the oxidation of NADH with pyruvate kinase and lactate dehydrogenase and monitoring the reactions spectrophotometrically. For direct comparison in the NMR experiments, hexokinase activity with glucose was also measured by ^{31}P NMR with 5.8 mM ATP/5 mM glucose/10 mM $MgCl_2$/100 mM Taps, pH 8.4/1 mM EDTA.

Alkaline phosphatase (*Escherichia coli* from Sigma) activity was assayed with *o*-carboxyphenol (ϵ = 3500 at 300 nm). Assays were contained in 1 mL of 0.8–1.6 μg of alkaline phosphatase/5 mM $MgCl_2$/0.5 mM $ZnCl_2$/100 mM Taps, pH 8.4/20 mM NaCl in both the presence (1% ethanol) and absence of 0.5 mM 2-PDS (PDS had no effect on the kinetics of the reaction). Activity of 6-thioglucose-6-P as substrate was determined under identical conditions in the presence of 2-PDS. In addition, activity with the two substrates was determined at pH 5.9 (100 mM Mes) with 5 μg of enzyme. Alkaline phosphatase activity vs glucose-6-P and 6-thioglucose-6-P was also determined by ^{31}P NMR, with 100 mM Taps, pH 8.4/10 mM $MgCl_2$/1 mM each EDTA and $ZnCl_2$/5.13 mM glucose-6-P or 6-thioglucose-6-P in 3 mL with 10 μg of alkaline phosphatase.

Phosphoglucomutase activity was assayed with glucose-1-P as substrate in 100 mM buffer/20 mM NaCl/5.5 mM $MgCl_2$/0.5 mM EDTA/0.4 μM glucose-1,6-bis-P/1 mM NAD/10–50 units of glucose-6-P dehydrogenase with and without 0.5 mM 2-PDS. PDS had no effect on the kinetics. Activity with 6-thioglucose-6-P was determined similarly in the presence of PDS but without glucose-6-P dehydrogenase or NAD, which had no effect on the reaction. From 1 to 20 μg of phosphoglucomutase was used in 1 mL. For direct comparison in the NMR studies, phosphoglucomutase activity was measured by ^{31}P NMR with 100 mM Taps, pH 8.4/10 mM $MgCl_2$/1 mM EDTA/80 μM glucose-1,6-bis-P/5.1 mM glucose-1-P. The reaction of phosphoglucomutase with 6-thioglucose-6-P was also demonstrated with ^{31}P NMR. The reaction mixture (4.15 mL) contained 77 mM 6-thioglucose-6-P, 72 mM Taps, pH 8.4, 7.2 mM $MgCl_2$, 1 μM glucose-1,6-bis-P, 0.7 mM EDTA and 400 units phosphoglucomutase. 6-Thioglucose-1-P was tested as an inhibitor vs glucose-1-P with either 100 mM Taps, pH 8.4, or 100 mM Mes, pH 6.4, together with 20 mM NaCl, 5.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM NAD, 0.4 μM glucose-1,6-bis-P, 20 units of glucose-6-P dehydrogenase, and 2 μg of phosphoglucomutase in 1 mL. 6-Thioglucose-1-P was tested as a substrate for phosphoglucomutase at pH 8.5 with similar conditions except with 60 μM enzyme and 74 μM glucose-1,6-bis-P. A small amount of 6-thioglucose-6-P present initially was allowed to react with the coupling system prior to the addition of phosphoglucomutase.

Phosphorylase A activity was determined at pH 8.4 by coupling the glucose-1-P produced from the phosphorolysis of glycogen by phosphate to the reduction of NADP via a phosphoglucomutase and glucose-6-P dehydrogenase couple. The reaction of phosphorylase with thiophosphate was monitored by ^{31}P NMR, with a typical reaction mixture containing 50 μM AMP, 100 mM Taps, pH 8.4, 5 mg/mL glycogen, 12 mM thiophosphate, and 0.3 mM EDTA in 4 mL with 166 units of phosphorylase A.

Glucose-6-P dehydrogenase (*Leuconostoc mesenteroides*) activity was measured with 35–70 μg of enzyme in a 3-mL volume with 1.5 mM NADP, 100 mM Taps, pH 8.4, and 50 mM KCl. The concentrations of either glucose-6-P or 6-thioglucose-6-P (both as potassium salts) were varied. The reaction of the enzyme with 5 mM disodium 6-thioglucose-6-P was also examined by ^{31}P NMR with 21 mM NAD and 100 mM Taps, pH 8.4.

6-P-Gluconate dehydrogenase activity was determined spectrophotometrically with 6–38 μg of enzyme in 1 mL of 100 mM Taps, pH 8.4/1.15 mM NADP. Reaction of 6-P-gluconate dehydrogenase with enzymatically synthesized 6-thiogluconate-6-P was monitored originally by ^{31}P NMR in the presence of 500 units of glucose-6-P dehydrogenase (to oxidize 6-thioglucose-6-P)/100 mM Taps, pH 8.0/25 mM GSSG/0.52 mM NADP/0.5 mM EDTA/60 units of glutathione reductase/10–100 units of 6-P-gluconate dehydrogenase in 4 mL.

The activities of phosphoribulokinase and ribulose-bis-P carboxylase were determined by ^{31}P NMR in 100 mM Taps, pH 8.4/330 units of kinase/0.5 mM NADP/0.5 mM EDTA/25 mM $NaHCO_3$/40 mM GSSG/40 mM ATP/60 mM $MgCl_2$/90 units of glutathione reductase/40 units of the carboxylase/20 mM either glucose-6-P or 6-thioglucose-6-P in 4 mL.

The activity of 6-thioglucose-6-P as a substrate for phosphoglucoisomerase was determined by fixed-time assays with 100 mM Ches and 63 mM KCl. At timed intervals, aliquots were taken for glucose-6-P dehydrogenase assay. The activity of 6-thiofructose-6-P as a substrate for phosphofructokinase was determined with a pyruvate kinase, lactate dehydrogenase assay. The activity of 6-thiofructose-bis-P with aldolase was measured by coupling with α-glycerophosphate dehydrogenase.

For comparative kinetic studies, oxygen-bridged substrates were assayed similarly.

Data Processing. Values for V_{max}, K_m, and their ratio V/K were obtained by fitting rate data to eq 1 with the least-squares

$$v = VA/(K + A) \quad (1)$$

method, assuming equal variance for the velocities (Cleland,

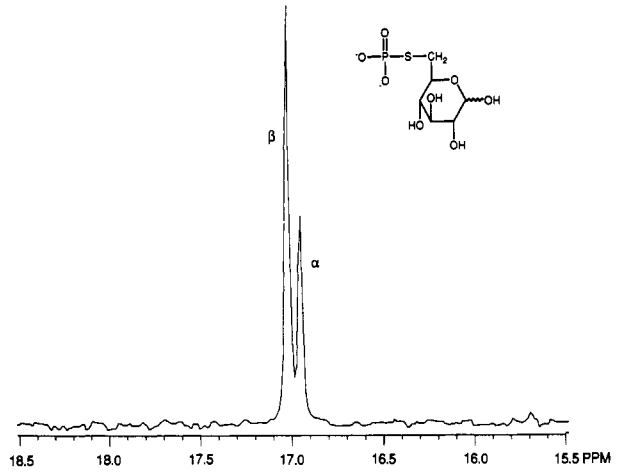


FIGURE 1: Proton-decoupled ^{31}P NMR spectrum of 6-thioglucose-6-P.

Table I: Equilibrium Aldose and Ketose Compositions at pH 8.4[a]

starting sugar	aldose α	aldose β	ketose
6-thioglucose-6-P	0.36	0.64	
glucose-6-P	0.35	0.65	
glucose-6-P[b]	0.38	0.62	
plus phosphoglucoisomerase			
6-thioglucose-6-P	0.28	0.56	0.16
glucose-6-P	0.27	0.52	0.21
glucose-6-P[c]	0.27	0.53	0.20

[a] Compositions based on integrations of ^{31}P NMR spectra. The identification of α and β anomers of 6-thioglucose-6-P is based on analogy with glucose-6-P. [b] Benkovic and Schray (1976). [c] Balaban and Ferretti (1983).

1979). In eq 1, V is the maximum velocity, K is the Michaelis constant, v is the experimental initial velocity, and A is the substrate concentration.

Data corresponding to competitive or noncompetitive inhibitions were fitted to eqs 2 and 3, respectively. Data for the

$$v = VA/[K(1 + I/K_{is}) + A] \quad (2)$$

$$v = VA/[K(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (3)$$

pK_i profile of 6-thioglucose as an inhibitor vs glucose were fitted to eq 4.

$$pK_i = pK_{io} - \log (1 + H/K_1 + K_2/H) \quad (4)$$

Results

Synthesis of 6-Thioglucose-6-P. The overall yield of 6-thioglucose-6-P was 44% based on glucose, with most of the loss being due to incomplete tosylation of glucose in the first step. The proton NMR spectrum of purified 6-thioglucose-6-P was consistent with the expected structure. In particular, the multiplet from the C-6 protons was shifted to 2.2 ppm from the value of 3.4 ppm in glucose-6-P. The ^{13}C NMR spectrum was also consistent with the expected structure, with C-6 shifting upfield from 64 ppm in glucose-6-P to 31 ppm in 6-thioglucose-6-P. A similar spectral change was also observed by Knight and Cleland (1989) between 3-thioglycerol-3-P and glycerol. 6-Thioglucose-6-P did not react with DTNB unless treated with either acid or alkaline phosphatase. The ^{31}P NMR spectrum displayed two resonances due to the α and β anomers of the sugar (Figure 1), and the ratio between the anomers is similar to that seen with glucose-6-P (Table I). The pK was found to be 5.20 ± 0.04 by titration of an 18 mM solution in 100 mM KCl with HCl and monitoring the ^{31}P NMR chemical shift.

Table II: ^{31}P NMR Chemical Shifts of Phosphorylated Thiols at pH 8.4

	δ (ppm)	coupling (Hz)	
6-thioglucose-6-P	17.0	10.7	triplet[a]
6-thioglucose-6-P[b]	18.1		c
6-thiofructose-6-P	16.1		c
6-thiofructose-1,6-bis-P	3.83, 16.1		c
6-thioglucose-1-P	2.1	5.7	doublet
glucose-1-thiophosphate	42.9	10	doublet
6-thiogluconate-6-P	17.1	11	triplet
5-thioribulose-5-P	16.8	10.5	triplet
3-thioglycerate-3-P	16.6	10.3	triplet
2-thioglycerate-3-P	15.8	9.2	doublet
phosphoenolthiophosphate	12.8		singlet

[a] In a proton-decoupled spectrum, the peaks for α and β anomers have a 0.071-ppm separation. [b] Value for monoanion. The chemical shift is still further downfield for the neutral species (the δ is 19.0 at pH 0.95). [c] Not determined (spectra all proton-decoupled).

Table III: Stability of Phosphorylated Thiosugars at 22 °C

	pH	half-life	relative stability[a]
5-thioribulose-5-P	4.0	0.3–0.6 s	1
	8.6	24 min	1
6-thiofructose-1,6-bis-P	4.0	4 min	530
	9.0	59 h	150
6-thiofructose-6-P	3.9	4 min	530
	9.0	818 h	2000
6-thioglucose-6-P	4.0	30 min	4000
	9.0	1490 h	3700
6-thiogluconate-6-P	4.1	91 min	12000
	9.0	2690 h	6700

[a] Relative to the half-life for 5-thioribulose-5-P of 0.45 s for monoanions at pH ~4 or 24 min for dianions at pH ~9.

Several thiosugars were synthesized enzymatically, starting with 6-thioglucose-6-P. Syntheses were at pH 9, since the phosphorylated thiols are more stable at high pH. Enzymes were removed by filtration and the resulting compounds used for stability and comparative kinetic studies without further purification in most cases. The ^{31}P NMR chemical shifts for these intermediates are in Table II. Chemical shifts fall in the 16–17-ppm range for phosphorylated thiols at pH values above 8.5 but shift downfield as the phosphate group becomes protonated (to 18.1 ppm for the monoanion of 6-thioglucose-6-P, for example).

Stability of Phosphorylated Thiols. Half-lives were determined both at pH 4, where the phosphoryl group is a monoanion, and at pH 9, where it is a dianion (Table III). In all cases the monoanion is less stable, but the half-lives vary over a considerable range.

Free Energy of Hydrolysis of 6-Thioglucose-6-P. The equilibrium constant for the conversion of 6-thioglucose-6-P to 6-thioglucose-1-P was determined by allowing the phosphoglucomutase-catalyzed conversion to go to completion and integrating the ^{31}P NMR resonances for the two species. Nitrogen was bubbled through the sample prior to addition of enzyme and the NMR tube capped to prevent thiol oxidation. After 8 h, the reaction was Amicon filtered to remove the enzyme and assayed for disulfides (none were present). As an aid to integration, 0.5 mM $Co(NH_3)_4PNP$ was added (δ 11.4 ppm; Haromy et al., 1983). The final concentrations were 1.6 mM 6-thioglucose-6-P and 71.4 mM 6-thioglucose-1-P, so K_{eq} was 45.6 (ΔG of −2.2 kcal/mol). If the free energy of hydrolysis of 6-thioglucose-6-P is the same as that of glucose-1-P (5.0 kcal/mol; Atkinson et al., 1961), the free energy of hydrolysis of the phosphorylated thiol group at pH 7 is −7.2 kcal/mol, or a value close to that for MgATP. At other pH's, however, these values are considerably different, with the value for MgATP being more negative at high pH and that for

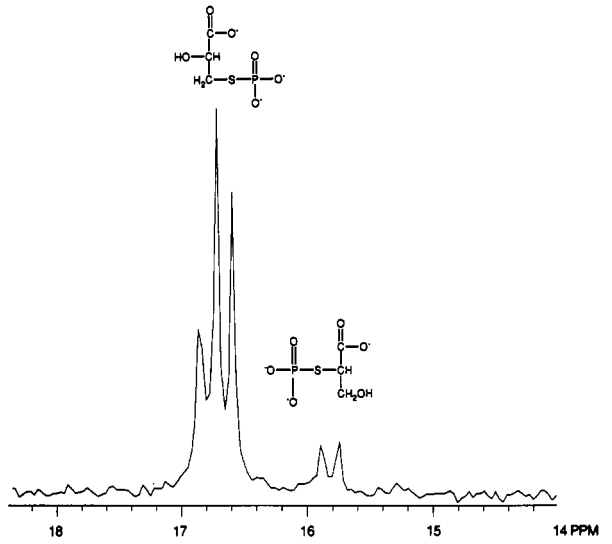


FIGURE 2: Proton-coupled ^{31}P NMR spectrum of a mixture of 3-thioglycerate-3-P and 2-thioglycerate-2-P.

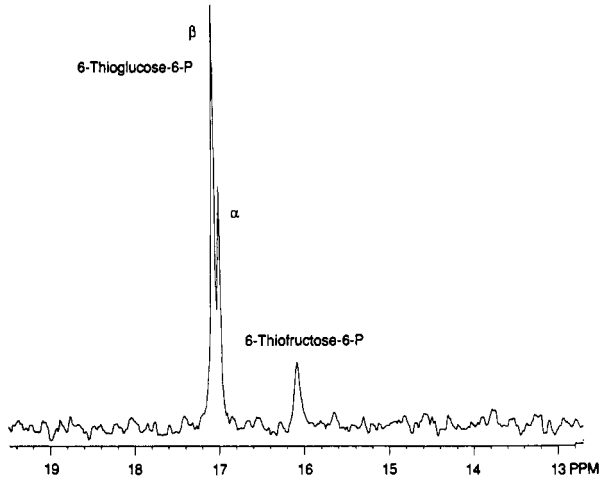


FIGURE 3: Proton-decoupled ^{31}P NMR spectrum of an equilibrium mixture of 6-thioglucose-6-P and 6-thiofructose-6-P in the presence of phosphoglucoisomerase.

6-thioglucose-6-P more negative at lower pH. The calculated pH profiles for these free energies of hydrolysis are shown in Figure 4 of the following paper.

Synthesis of Phosphorylated Thioglycerates. The overall yield of phosphorylated thioglycerates from ethyl glycerate was 25%, with the major loss occurring during the ester-deblocking step, since the resin was gummy and likely contained insoluble starting material. Chromatography did not completely resolve the 2- and 3-isomers, although rechromatography with a shallow gradient and monitoring of fractions by ^{31}P NMR did produce small amounts of both species free from the other. The ^{31}P NMR spectrum of 3-thioglycerate-3-P was a triplet centered at 16.7 ppm at pH 10.3 while that of the 2-isomer was a doublet at 15.8 ppm (Figure 2).

Metabolism of 6-Thioglucose-6-P by Glycolytic Enzymes. The reaction of 6-thioglucose-6-P with phosphoglucoisomerase was examined by ^{31}P NMR. When this enzyme (300 units/mL) was incubated for 1 h at pH 8.4 with 5 mM 6-thioglucose-6-P, a new broad (3.9-Hz line width) resonance at 16.1 ppm was produced due to 6-thiofructose-6-P (Figure 3). The individual anomers of 6-thioglucose-6-P gave line widths of 1.5 Hz (β) and 1.7 Hz (α). Equilibrium was reached in 4 h, and the equilibrium concentrations are in Table I.

The conversion of 6-thioglucose-6-P to 6-thiofructose-1,6-bis-P via 6-thiofructose-6-P was monitored by a coupled assay

Table IV: Kinetic Parameters for Hydrolysis by Alkaline Phosphatase

substrate	V_{max} (units)	K_m (μM)	V/K (units)
pH 8.4			
o-carboxyphenyl-P[a]	20 ± 1	39 ± 5	0.51 ● 0.05
o-carboxyphenyl-P	19 ± 1	34 ● 4	0.56 ● 0.04
6-thioglucose-6-P	23 ± 1	11 ± 1	2.1 ± 0.2
pH 5.9			
o-carboxyphenyl-P	1.54 ± 0.1	3 ± 0.5	0.51 ± 0.09
6-thioglucose-6-P	2.1 ± 0.1	5.1 ± 0.6	0.41 ± 0.03

[a] Without PDS present; all other data were obtained in the presence of 0.5 mM 2-PDS.

Table V: Kinetic Parameters for Catalysis by Phosphoglucomutase

substrate	V_{max} (μM/min)	K_m (mM)	V/K (min^{-1})
pH 8.4			
glucose-1-P	48 ● 3	0.023 ± 0.002	2.1 ± 0.1
6-thioglucose-6-P	11.1 ± 0.2	1.22 ± 0.06	0.0091 ± 0.0002
pH 6.0			
glucose-1-P	22	0.028 ± 0.003	0.77 ± 0.04
6-thioglucose-6-P	0.98 ± 0.04	0.41 ± 0.04	0.0024 ± 0.0002

for the ADP produced by phosphorylation of 6-thiofructose-6-P. The addition of 0.13 mM 6-thioglucose-6-P to a solution containing 250 μM NADH, 1 mM phosphoenolpyruvate, 11 units of pyruvate kinase, 7 units of lactate dehydrogenase, 100 mM Taps, pH 8.4, 10 mM $MgCl_2$, 1 mM ATP, 3 units of phosphoglucoisomerase, and 2.1 units of phosphofructokinase in 1 mL resulted in a decrease in absorbance at 340 nm at a rate several percent of that observed when the same concentration of glucose-6-P was used as the substrate.

The reaction with aldolase, triose-P isomerase, and 3-P-glycerol dehydrogenase was assayed in 100 mM Taps, pH 8.4/10 mM $MgCl_2$/0.3 mM NADH/75 units of phosphoglucoisomerase/104 units of phosphofructokinase/5.2 units of aldolase, 10 units of triose-P isomerase/10 units of 3-P-glycerol dehydrogenase in 1 mL. The addition of 0.133 mM 6-thioglucose-6-P resulted in a decrease in absorbance at 340 nm. When a similar reaction was conducted with 20 μM 6-thioglucose-6-P but no triose-P isomerase, one equivalent of NADH was consumed but the addition of triose-P isomerase resulted in consumption of a second equivalent of NADH. These results indicate that 6-thioglucose-6-P can be metabolized to trioses by glycolytic enzymes.

The instability of 3-thioglyceraldehyde-3-P made it impractical to isolate it. It was synthesized enzymatically in a system containing glyceraldehyde-3-P dehydrogenase to oxidize it as fast as it was formed. The reaction mixture contained 100 mM Ches, pH 9, 10 mM $MgCl_2$, 1 mM ATP, 0.9 mM NAD, 2 mM arsenate, 50 units/mL phosphofructokinase, 50 units/mL aldolase, 16 units/mL glyceraldehyde-3-P dehydrogenase, and 0.05 mM 6-thioglucose-6-P. Reaction was initiated with 500 units/mL phosphoglucoisomerase. After the initial reaction stopped, 50 units/mL triose-P isomerase was added and the reaction was again allowed to reach an endpoint (0.97 and 0.92 equiv for the two phases). The dehydrogenase reaction was the rate-limiting step for the first reaction in the absence of triose-P isomerase. The initial rate with 6-thioglucose-6-P was 2% of that with the same concentration of glucose-6-P, while the second endpoints were reached at the same rate, since dihydroxyacetone-P was the reactant in both cases.

Enzyme Kinetic Studies. Values for V_{max}, K_m, and their ratio V/K were determined for the phosphorylated thiols and the corresponding oxygen-bridged metabolites. Values from fits of the data to eq 1 are shown in Tables IV–VI.

Table VI: Kinetic Parameters for Oxygen- and Sulfur-Bridged Substrates for Selected Enzymes

		oxygen-bridged substrate		sulfur-bridged substrate		
enzyme	substrate	pH	K_m (μM)	V_{max}[a]	K_m (μM)	V/K[a]
G6PDH[b]	glucose-6-P	8.4	150 ± 30	121 ± 7	1900 ± 200	96 ± 7
6PGDH[b]	6-P-gluconate	8.4	100 ± 30	4.3 ± 0.2	20 ± 2	22 ± 1.3
PGI[b]	glucose-6-P	9.0	270 ± 20	1.7 ± 0.1	4000 ± 500	0.117 ± 0.005
PFK[b]	fructose-6-P	8.9	44 ± 6	43 ± 2	80 ± 6	23 ± 3
aldolase	fructose-bis-P	8.8	8.0 ± 0.9	114 ± 2	10.9 ± 0.6	83 ± 3

[a] Values relative to 100 for the oxygen-bridged substrate. [b] Enzymes: G6PDH, glucose-6-P dehydrogenase; 6PGDH, 6-P-gluconate dehydrogenase; PGI, phosphoglucoisomerase; PFK, phosphofructokinase. Cosubstrates were saturating.

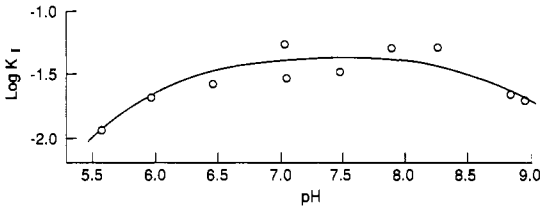


FIGURE 4: pK_i profile of 6-thioglucose as a competitive inhibitor of glucose in the hexokinase reaction. MgATP = 3 mM; citrate = 1 mM. The fitted pK values were 6.1 and 8.9.

Reaction with Hexokinase. The reaction of yeast hexokinase with 6-thioglucose-6-P was monitored by NMR. The reaction mixture (4 mL) contained 4220 units of hexokinase, 94 mM Taps, pH 8.3, 4.7 mM ADP, 9.4 mM $MgCl_2$, 4.8 mM 6-thioglucose-6-P, 6.25 mM glycerol, 0.94 mM EDTA, and 10 units of glycerokinase (to remove the ATP formed). Hexokinase showed only 0.0005% the activity toward 6-thioglucose-6-P as it did toward saturating glucose in the forward direction. When the reaction was conducted with 1 mM $ZnCl_2$ in place of $MgCl_2$, the rate was one-third that with $MgCl_2$.

The reaction of 4220 units of hexokinase with 5.2 mM 6-thioglucose was examined at 100 mM Taps, pH 8.4/10 mM $MgCl_2$/5 mM ATP/1 mM EDTA/10 mM phosphoenolpyruvate/20 units of pyruvate kinase in 4 mL. Only phosphate was produced at 0.006% the rate of the phosphorylation of glucose in a comparable system. In an attempt to slow down the inherent ATPase activity of hexokinase, the reaction was repeated with 0.5 M 6-thioglucose and 2500 units of hexokinase in 100 mM Hepes, pH 7.7/2.5 mM dithiothreitol. With the ATPase suppressed by saturation of the sugar binding site with the thiosugar, it was phosphorylated at 0.0002% the rate expected for glucose and a new resonance at 17 ppm appeared in the ^{31}P NMR spectrum.

6-Thioglucose was also tested as an inhibitor vs glucose in the hexokinase reaction, using the pyruvate kinase, lactate dehydrogenase coupled assay in the presence of 1 mM citrate. At pH 7, 6-thioglucose was a linear competitive inhibitor vs glucose with a K_i of 18.8 ± 1.3 mM. The pH dependence of the inhibition constant is shown in Figure 4. Binding decreases at both low and high pH with pK's of 6.1 ± 0.2 and 8.9 ± 0.2.

Reaction with Alkaline Phosphatase. When the activities of 5.1 mM glucose-6-P and 6-thioglucose-6-P with alkaline phosphatase were examined by ^{31}P NMR, phosphate was produced in both cases, with the latter reacting at 109% the rate of the former. The kinetic parameters for the hydrolysis of 6-thioglucose-6-P and *o*-carboxyphenyl-P are compared in Table IV.

Reaction with Phosphoglucomutase. When 800 units of phosphoglucomutase in 3 mL was incubated with 5 mM 6-thioglucose-6-P, the reaction was over when first examined after 44 min, and a new resonance was observed at −2.1 ppm (proton decoupled). In the proton-coupled spectrum this peak was a doublet (coupling constant 5.7 Hz). When the reaction was repeated with 0.05 unit/mL of enzyme, the rate of production of 6-thioglucose-1-P was 0.0055 mM/min, which was 11% the expected rate for conversion of glucose-1-P to glucose-6-P. The kinetic constants for these reactions are compared in Table V.

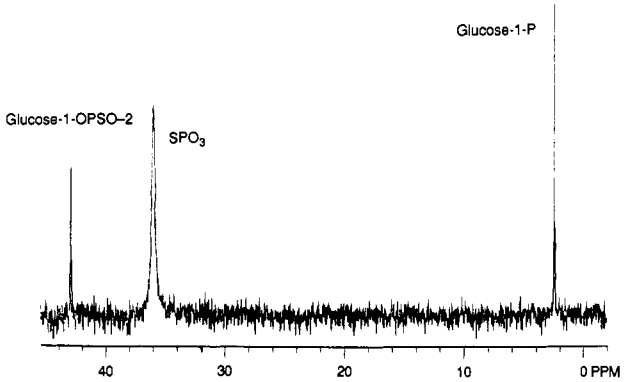


FIGURE 5: Proton-decoupled ^{31}P NMR spectrum of a reaction mixture containing phosphorylase, glycogen, and thiophosphate. The peaks at 2, 36, and 43 ppm are glucose-1-P, thiophosphate, and glucose-1-thiophosphate.

6-Thioglucose-1-P was a noncompetitive inhibitor vs glucose-1-P at pH 8.4, and the inclusion of 0.5 mM dithiothreitol did not affect the results. The slope inhibition constant (2.0 ± 0.8 mM) was much larger than the intercept one (0.15 ± 0.02 mM). At pH 6.4, the inhibition was competitive (K_{is} = 0.7 ± 0.1 mM). The reverse reaction of 6-thioglucose-1-P with phosphoglucomutase was slow, and the kinetics showed substrate inhibition. At 1 mM substrates, the ratio of forward to reverse reaction rates is $\sim 10^4$.

Reaction with Phosphorylase. When phosphorylase was reacted with thiophosphate in the presence of glycogen, we observed the production of glucose-1-P from contaminating phosphate (or hydrolysis of thiophosphate during the experiment) and also a new resonance at 43 ppm that is likely due to glucose-1-thiophosphate (containing non-bridge sulfur). This compound was formed at only 0.003% the rate of glucose-1-P, however (Figure 5). In a proton-coupled spectrum, this peak was a doublet.

Metabolism of 6-Thioglucose-6-P by Enzymes of the Hexose Monophosphate Shunt. Oxidation of 6-thioglucose-6-P in the presence of glucose-6-P dehydrogenase produced a species with a ^{31}P NMR resonance at 17.1 ppm. The kinetic parameters for the production of 6-thio-6-P-gluconate determined spectrophotometrically are in Table VI.

Addition of 6-P-gluconate dehydrogenase to a reaction mixture containing enzymatically synthesized 6-thio-6-P-gluconate produced a new compound with a resonance at 16.8 ppm (presumably 5-thioribulose-5-P), which decomposed to phosphate. With 500 units of 6-P-gluconate dehydrogenase and 0.52 mM NADP in 4 mL, 80% of 6-thio-6-P-gluconate reacted in 5 min and 90% in 7 min, while a control reaction without enzyme showed no change in 2 h. After 2 h in the presence of the dehydrogenase, only phosphate was evident.

To investigate the instability of 5-thioribulose-5-P, it was generated from 6-thio-6-P-gluconate under conditions similar to those used above, but at either pH 7.5 (100 mM Hepes) or pH 8.6 (100 mM Taps) and 25 °C. The half-life was 1–2 min at pH 7.5 and ~24 min at pH 8.6. With only 2 mM Taps at pH 8.6, the half-life was 49 min. In the absence of a NADP-regenerating system (100 mM Taps, pH 8.6/7.54 mM 6-thioglucose-6-P/19 mM NADP) the half-life was ~30 min. The decomposition of 5-thioribulose-5-P did not produce free thiol, since an aliquot of the reaction mixture did not react rapidly with DTNB or 4-PDS (less than 2% of the expected reaction). Prolonged incubation with DTNB did give a slow increase in absorbance at 412 nm, showing that the product of the decomposition of 5-thioribulose-5-P was the cyclic thiosugar, which has a very slow rate of ring opening (Grimshaw et al., 1979).

The reaction of 5-thioribulose-5-P (generated in situ) with P-ribulokinase and ribulose-bis-P carboxylase produced several new peaks in the ^{31}P NMR spectrum, including those associated with ADP, one at 16.63 ppm (3-thioglycerate-3-P and/or the bisphosphate of 5-thioribulose), and ones at 4.3 ppm (3-P-glycerate) and 4.05 ppm (the 1-phosphate of 5-thioribulose-1,5-bis-P). The resonance at 4.3 ppm continued to increase after the disappearance of 5-thioribulose-5-P, while the resonance at 16.63 ppm remained unchanged as the one at 4.05 decreased. These results suggest that 5-thioribulose-bis-P is produced and then converted into 3-thioglycerate-3-P and 3-P-glycerate, but an individual resonance for the thiophosphoryl group of the bis-P was not resolved and presumably overlaps either the 16.63- or 16.8-ppm peaks. The rate of these reactions is limited by the level of the kinase; with only 50 units of kinase and no carboxylase present, we did not observe a new thiphosphorylated species and estimate that the kinetic reaction with 5-thioribulose-5-P was less than 3×10^{-4}% the rate with ribulose-5-P.

Reactions of Thioglycerate Phosphates. When a mixture of 3-thioglycerate-3-P and 2-thioglycerate-2-P (1.6:1, 6 mM total concentration; 2.3 mM of the 2-isomer) was reacted with 20 mg enolase at pH 9 (100 mM Ches) and 4 mM $MgCl_2$, a new resonance (a singlet) was observed after 50 min at 12.8 ppm due to phosphoenolthiopyruvate. The amount formed was 0.3 mM, and the level of 2-thioglycerate-2-P had decreased by this amount, corresponding to an equilibrium constant of 0.15. We thus confirm the report of Sikkema and O'Leary (1988) that 2-thioglycerate-2-P is a substrate for enolase.

The potassium salt of 3-thioglycerate-3-P (free of the 2-isomer) was tested as a substrate for phosphoglycerate mutase (5 mM 3-thioglycerate-3-P/100 mM Taps, pH 8.2/184 μM 2,3-bis-P-glycerate/0.4 mM EDTA/300 units of phosphoglycerate mutase in 4 mL). Under identical conditions but with 50 units of enzyme, 3-P-glycerate reacted to reach equilibrium in less than 30 min. After incubation of 3-thioglycerate-3-P for 4 h at 25 °C, there was no sign in the ^{31}P NMR spectrum of any reaction. The addition of 4 mM $MgCl_2$ and incubation for 12 h had no effect.

Discussion

The data presented in this paper show that phosphorylated thiols readily undergo most of the reactions of glycolysis that do not involve phosphoryl transfer and the reactions of the hexose monophosphate shunt, including the carboxylation of 5-thioribulose-bis-P to 3-P-glycerate and 3-thioglycerate-3-P. This latter reaction is of interest because the two products are no longer identical, as is the case with the normal reaction, so that individual atoms may be isolated for isotope effect studies. 6-Thiofructose-6-P and the corresponding 1,6-bisphosphate are excellent substrates for phosphofructokinase and aldolase, respectively, with the oxygen to sulfur substitution having little effect on V or K_m values. Similarly, 6-thioglucose-6-P and 6-thiogluconate-6-P are reasonably good substrates for glucose-6-P and 6-P-gluconate dehydrogenases. This is not surprising, since the charge on the phosphoryl group is the same at physiological pH, the same three oxygens are presented to the enzyme, and the only differences lie in slightly longer bonds from sulfur to carbon and phosphorus. With phosphoglucoisomerase, however, the K_m for 6-thioglucose-6-P is 15-fold higher and the V only 1.7% that for glucose-6-P. The most extreme case is phosphoribulokinase, where the V/K value is over 3 orders of magnitude less than for ribulose-5-P. The reason for this low rate is not known, but this enzyme is clearly highly sensitive to the small geometry changes caused by sulfur substitution.

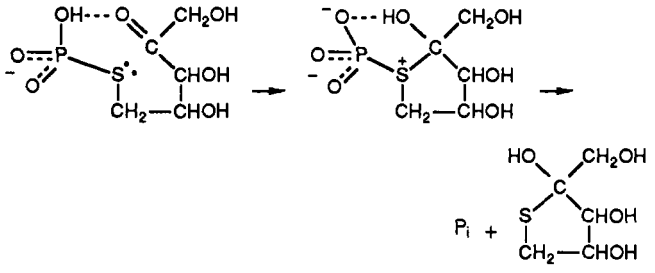
In reactions involving phosphoryl transfer, hexokinase, like glycerokinase with thioglycerol (Knight & Cleland, 1989) and pyruvate kinase using thioglycolate as an analogue of glycolate (Ash et al., 1984), shows a very low rate with 6-thioglucose or its 6-phosphate. Despite the fact that the equilibrium constant is now very close to unity, since the free energy of hydrolysis of 6-thioglucose-6-P is −7.2 kcal/mol vs −7 kcal/mol for MgATP (Guynn & Veech, 1973), the rates are 5 orders of magnitude slower with the thiol analogues. We originally thought this might suggest an associative reaction for hexokinase, but recent measurements by Jones et al. (1991) of inverse secondary ^{18}O isotope effects on the hexokinase reaction show clearly that the transition state is dissociative, not associative. As a result, we do not know why kinases do not readily phosphorylate thiols. Possibly the failure of the sulfur to form a strong hydrogen bond with the catalytic aspartate (Anderson et al., 1978; Viola & Cleland, 1978) prevents the conformation change that sets the stage for catalysis by inducing tighter binding of the nucleotide and bringing the two substrates close enough together to react.

By contrast with hexokinase, both alkaline phosphatase and phosphoglucomutase accept 6-thioglucose-6-P as a good substrate, although the K_m is elevated for the latter (alkaline phosphatase seems to care only about the phosphoryl group and not about the structure of the leaving group). Both of these enzymes catalyze ping-pong mechanisms with a phosphorylated serine as an intermediate, and the evidence from secondary ^{18}O isotope effects on alkaline phosphatase (Weiss & Cleland, 1989) is that the transition state for the transfer to this serine is a dissociative one. Apparently neither enzyme undergoes a conformation change that requires a strong hydrogen bond to the bridging oxygen or sulfur atom. The ability of mutases to accept phosphorylated thiols is not general, however, since phosphoglyceromutase does not accept 3-thioglycerate-3-P as a substrate.

It is of interest that phosphorylase catalyzes reaction of glycogen with thiophosphate only to give glucose-1-thiophosphate and not the phosphorylated thiol. In the framework of the mechanism proposed by Palm et al. (1990), these data suggest that the mechanism involves a four-center reaction of an OH group on phosphate and the atoms of the C–O bond to be cleaved.

An interesting instability was seen with 5-thioribulose-5-P, which was far more acid labile than other phosphorylated thiols, with an extrapolated half-life for the monoprotonated species (assuming the pK to be the same as that of 6-thioglucose-6-P) of 0.3–0.6 s. Further, the product of the decomposition was not the free thiol but the cyclic sugar, which

has a low ring-opening rate (Grimshaw et al., 1979). These data suggest the following mechanism, in which the phosphate group acts as a general acid to facilitate reaction of sulfur with the carbonyl carbon to give a very labile phosphorylated sulfonium intermediate.



Similar modes of decomposition may be involved to some extent for the other ketoses, 6-thiofructose-6-P and the bisphosphate, since they are less stable than the aldoses. The ring would be a 6-membered one in these cases, but the open-chain form of the sugar is only 2% of the anomeric mixture (Benkovic & Schray, 1976), so the rate is less than with the pentose, which can only exist in a noncyclic form.

In conclusion, this study has shown that it is easy to synthesize phosphorylated thiols by reaction of thiophosphate with tosylated compounds in aqueous solution at high pH. The reaction proceeds cleanly with no oxygen-bridged product, presumably because the oxygens of thiophosphate are strongly hydrated in water, while the sulfur is not. The resulting phosphorylated thiols are high-energy compounds but are stable if kept at high pH and can readily be used as analogues of metabolic intermediates. Some enzymes will catalyze phosphoryl transfer from the thiol while others will not, or do so at only very slow rates.

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