The Stereochnolcal Course of the Rubulose-5-phosphate Kinase-catalyzed Reaction*

Henry M. Miziorko† and Fritz Eckstein
From the Max-Planck-Institut fur Experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Gottingen, West Germany

Spinach-leaf ribulose-5-phosphate kinase catalyzes the reaction of (R)-[β,γ-180]adenosine 5'-3-thiotriphosphate) with ribulose 5-phosphate to form ribulose 1-[180]phosphorothioate 5-phosphate. This product is incubated with CO2, Mg, and ribulose-bisphosphate carboxykinase to form the [180]phosphorothioate of D-glycerate. Reduction of this material using phosphoglycerate kinase/ATP, glyceraldehyde-3-phosphate dehydrogenase/NAD, triose-phosphate isomerase, and glyceraldehyde-phosphate dehydrogenase/NADH produces glycerol 3-[180]phosphorothioate, which is subjected to ring closure using diethylphosphorochloridate. This in-line reaction produces a diastereoisomeric mixture of glycerol 2,3-cyclic phosphorothioate. 31P NMR spectroscopy was used to analyze the 180 content of the products. The anti-diastereoisomer, which is the major isomer formed and corresponds to the downfield 31P NMR signal (Pliura et al. (80)) of configuration at phosphorus. The reaction is, therefore, unlikely to involve the participation of a covalent phosphoryl-enzyme intermediate.

Ribulose-5-phosphate kinase catalyzes the formation of ribulose 1,5-biphosphate (cf. Equation 1) in a reaction unique to the reductive pentose phosphate cycle (where M represents divalent cation).

M

Ribulose 5-phosphate + ATP → Ribulose 1,5-biphosphate + ADP (1)

Recent work has identified Ru5P as a site at which control of CO2 fixation can be exerted. Activity of the plant enzyme is modulated by a thioredoxin-mediated light-activation process (1, 2) as well as by interactions between Ru5P kinase and stromal metabolites (3, 4). The enzyme from bacterial sources appears to be allosterically regulated (5-7).

While substantial information concerning physiological control of Ru5P kinase activity is available, work on the characterization of the purified enzyme is limited to a few reports focused primarily on the bacterial enzymes (6, 7). Purification and characterization of the spinach-leaf enzyme has been initiated as a prelude to physical and chemical studies. In the course of these experiments, ATP-S was tested and found to serve as an alternate substrate for Ru5P kinase. The utility of phosphorothioate-containing nucleotide analogs as tools for elucidating the chemical mechanisms involved in phosphoryl-transfer reactions has been well established (8, 9). Availability of highly-purified spinach-leaf Ru5P kinase has facilitated application of this methodology to the study of the mechanism involved in RuBP formation. This report describes the initial results of this approach. [γ-180]ATP-S which is chiral by virtue of the stereospecific enrichment of the thioinositol moiety with 180 has been used in experiments designed to determine the stereochnolcal course of the Ru5P kinase reaction.

A preliminary account of this work has appeared (10).

EXPERIMENTAL PROCEDURES

Materials — Ru5P kinase was extracted from spinach leaves and purified to a specific activity of 350 units/mg using ion-exchange and affinity-chromatography procedures similar to those reported for purification of the enzyme from Alcaligenes eutrophus (6). RuBP carboxylase was prepared as described by Paulsen and Lane (11). Phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triose-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, and glyceraldehyde were obtained from Boehringer Mannheim as suspensions in ammonium sulfate solutions. 31P NMR spectroscopy indicated 180 enrichment ≥90%. The 2,3-cyclic phosphorothioate of D-glyceric acid was prepared as described by Pliura et al. (14). The 3-phosphorothioate of glyceraldehyde was prepared from the cycitonic ester by the procedure outlined by Orr et al. (15).

Methods — The 3-phosphorothioate of D-glycerate was assayed as described by Bergmeyer (16) for D-3-phosphoglycerate.

31P NMR spectra were recorded using a Bruker WP200SY spectrometer operating at 81.01 MHz with 1H broadband decoupling. Samples were measured in 5-mm tubes; a concentric capillary containing 85% H3PO4 was used as an external standard. Chemical shifts are given in parts/million and are positive when downfield from the reference.

Formation of 3-[180]Phosphorothioate of D-Glycerate — Ru5P kinase (180 units) and RuBP carboxylase (95 units) were incubated in a mixture (5.0 ml) containing 100 mM Tris, pH 8.2, 20 mM MgCl2, 5 mM dithiothreitol, 25 mM KHCO3, 4.7 mM (R)-[β,γ-180]ATP, and 31P NMR spectra (10 μCi, 0.2 nmol). The reaction was initiated by addition of Ru5P (24 mM). Aliquots of the mixture were assayed for 3-phosphoglycerate and the 3-phosphorothioate of glyceric acid. Thin-layer chromatography of aliquots of the reaction mixture on polyethyleneimine-cellulose (0.75 M KH2PO4, pF 3.5, used for development) was performed to verify depletion of the phosphorothioate.
nucleotide substrate; product formation was virtually complete after 5 h. The product was purified by chromatography of the reaction mixture using a DEAE Sephadex column (1.6 x 25 cm) equilibrated with 10 mM triethylammonium bicarbonate, pH 7.5; elution was performed with a linear gradient of 1.5 liters each of 10 mM and 0.5 M triethylammonium bicarbonate. Fractions were monitored for 35S radioactivity and glycerate-3-phosphorothioate content (enzymatically determined). The product was eluted at about 0.3 M buffer. Fractions containing product were pooled and evaporated to dryness, and buffer salts were removed by repeated evaporations with methanol. 5.2 pmol (based on 35S radioactivity) of the 3-[35S]phosphorothioate of D-glycerate was isolated. 31P NMR (D2O) singlet was at 49.20 ppm with contaminants at 1.1, -3.6, and -10.7 ppm.

Formation of Glycerol 3-[35S]Phosphorothioate-3-[35S]Phospho-

phorothioate of D-glycerate (5.2 pmol) was reduced in a solution (total volume 5.0 ml) containing 100 mM Tris, pH 8.2, 10 mM MgCl2, 4 mM NADH, 5 mM ATP, 670 units of phosphoglycerate kinase, 750 units of phosphoglycerate-3-phosphate dehydrogenase, 1500 units of triosephosphate isomerase, and 770 units of glycerol-phosphate dehydrogenase. All enzymes had been exhaustively dialyzed prior to use. 25 mM Tris, pH 7.8, and 0.5 mM dithiothreitol. After incubation at 37°C for 45 min, UV spectrophotometry showed the reaction to be complete. The reaction mixture was purified on a DEAE-

Sephadex column (1.6 x 25 cm) using a linear gradient of 0.7 liter each of 10 and 500 mM triethylammonium bicarbonate, pH 7.5. The product was identified by measuring the 35S radioactivity. It was eluted at about 150 mM buffer. Fractions containing product were pooled and evaporated to dryness, and buffer salts were removed by repeated evaporations with methanol. Glycerol 3-[35S]phosphorothioate was isolated in 79% yield (4.1 pmol, as determined by 31P NMR (D2O) singlet was at 51.11 ppm with contaminants at 0.47 ppm.

Ring Closure to Glycerol 2,3-Cyclic [35S]Phosphorothioate—The ring-closure reaction was performed as described by Flura et al. (14). The triethylammonium salt of glycerol 3-[35S]phosphorothioate (4.1 pmol) was converted to the pyridinium salt by passage through a column (1 x 12 cm) of Merck I ion-exchange resin (pyridinium form). The sample was taken to dryness repeatedly from methanol, using an oil paraffin bath. This material was dissolved in methanol (1 ml) and tri-n-

octylamine (11 pmol) was added. After dissolving the tri-n-octylamine by gently warming the reaction mixture, the solution was evaporated to dryness. The residue was dissolved in dry dioxane (0.5 ml), and the solution was evaporated to dryness. Addition of dry dioxane and solvent evaporation was repeated three times. Glycerol 3-[35S]phosphorothioate (4.1 pmol) was dissolved in dry pyridine (250 µl) and acetonitrile (150 µl). Tri-n-octylamine (11.6 µmol) and two beads of molecular sieves (4 Å) were added to this solution. After incubation at room temperature for 40 min, a solution of diethyl phosphoro- 

chloridate (8.2 pmol) in CH2Cl2 (10 µl) was added. After 1 h, 31P NMR spectroscopy of the reaction mixture showed that all the starting material, resonating at 51 ppm, had disappeared. The product was not isolated but characterized, after addition of some CD3CN, directly from the 31P NMR spectrum of the reaction mixture. The 31P NMR spectrum showed only a doublet accompanied by two small satellite peaks in the phosphorothioate region between 73 and 74 ppm (see Fig. 3), accounting for about 25% yield in the ring-closure reaction. In addition, there were signals at about 17.6 ppm (corresponding to glycerol 2,3-cyclic phosphate), -0.6 ppm (corresponding to diethyl phosphate), and -12.6 ppm (presumably to tetraethyl pyrophosphate).

In control experiments, ring closure of nonchiral glycerol 3-phos-

phorothioate produced a mixture of diastereoisomers which displayed singlets at 73.77 and 73.25 ppm in a ratio of 1:0.82 in the 31P NMR spectrum (Fig. 14) when measured in pyridine/acetonitrile. In aqueous solutions, the peak assignments remain the same (Fig. 1B), but the singlets are separated by only 0.15 ppm. This observation confirms the earlier report by Flura et al. (14). Slight changes in chemical shifts (Fig. 1, A versus B) are accounted for by the differences in pH and solvent composition.

RESULTS AND DISCUSSION

In order to determine the stereochemical course of the Ru5P kinase-catalyzed transfer of a thio phosphoryl group from chiral [γ-35S]ATP to Ru5P, the thio phosphorylated product must be amenable to configurational analysis. The method devised for this analysis here consists in coupling the Ru5P kinase reaction to the RuBP carboxylase reaction (Fig. 2). In this way, the product of the first reaction, [35S]RuBP(S), is immediately transformed by addition of CO2 and subsequent cleavage to 3-[35S]phosphorothioate of D-glycerate and D-3-phosphoglycerate. These can be separated chromatographi-
A sequence of reactions for the configurational analysis of the former has been developed (14). For this analysis, it is enzymatically reduced to glycerol 3-[18O]phosphorothioate, a reaction sequence which proceeds without perturbing the configuration of the phosphorothioate group. Glycerol 3-[18O] phosphorothioate can be cyclized chemically to the mixture of the two diastereomers of glycerol 2,3-cyclic [18O]phosphorothioate. The diastereomers can be distinguished inter alia by 31P NMR spectroscopy. Their 31P NMR signals have been assigned by comparing their retention times in gas chromatography with material which has been derived from the sn-cyclic phosphorothioate of D-glycerate whose structure has been determined by x-ray structural analysis. Thus, it has been established that the anti-isomer of glycerol 2,3-cyclic phosphorothioate which has the longer retention time in gas chromatography has the more downfield chemical shift in the 31P NMR spectrum (14). The localization of 18O in either the syn- or anti-isomer can be determined by observation of the 18O-induced upfield chemical shift on the phosphorus resonance (Re1s. 17–19, and for review see Refs. 20 and 21). The rationale involved in the 31P NMR analysis of the 18O content of the diastereomeric mixture of glycerol 2,3-cyclic phosphorothioate and the consequences of retention or inversion of configuration in the Ru5P kinase reaction are outlined in Fig. 3.

A trace amount of [35S]ATPγS was added to the (Rp)-[(β,γ-18O,γ-18O)ATPγS used in the Ru5P kinase-dependent reaction mixture in order to facilitate the detection of phosphorothioate-containing products in all subsequent chromatography steps. The first reaction was coupled to the RuBP carboxylase reaction by addition of this enzyme and CO₂. This was designed to maximize recovery of the 3-[18O]phosphorothioate of D-glyceric acid. It has been established (22, 23) that RuBP is converted, at mildly alkaline pH, to a species which no longer functions as a substrate but, rather, inhibits RuBP carboxylase. Continuous trapping of RuBP(S) by conversion to the relatively stable 3-phosphorothioate of glyceric acid should minimize accumulation of RuBP(S) with subsequent formation of inhibitor at the expense of product. Nevertheless, after depletion of [γ-18O]ATPγS, only 30% of the theoretically predicted 3-phosphorothioate of D-glyceric acid was accounted for, suggesting that hydrolysis or other side reactions had reduced yields of product. No detailed kinetic experiments were carried out to compare the rates of phosphorylation and thiophosphorylation of Ru5P by the kinase. However, a rough estimate would suggest that the latter reaction is 2–3 orders of magnitude slower than the former.

The observation that the thiophosphorylated analog of RuBP can be enzymatically converted to the phosphorothioate of D-glycerate is not surprising on chemical grounds, since the perturbation in structure is of a type that should not affect any of the bond formation or cleavage events during the reaction. Moreover, RuBP carboxylase is not particularly selective in binding phosphorylated metabolites (24), so recognition of RuBP(S) at the substrate-binding site is not unexpected. The enzyme binds many phosphorylated metabolites, it is very restricted in its ability to carboxylate a sugar phosphate; RuBP(S) is the first compound that has been found to serve as an alternate substrate. Future experiments will further test the utility of RuBP(S) as a mechanistic probe in studies on the RuBP carboxylase and oxygenase reactions.

Reduction of the 3-[18O]phosphorothioate of D-glycerate to glycerol 3-phosphorothioate has been reported before (14). We found it necessary to dialyze all enzymes required for this reaction since the presence of ammonium sulfate interfered strongly with the subsequent cyclization reaction.

The cyclization of glycerol 3-phosphorothioate to a mixture of diastereomers of glycerol 2,3-cyclic phosphorothioate was performed according to the procedure reported previously (14). This reaction consists of the activation of the phosphorothioate by diethyl phosphorochloridate and subsequent intramolecular substitution of the 2-OH group and follows an in-line mechanism (20). The yield of glycerol 2,3-cyclic phosphorothioate observed in this reaction was low and varied between 25 and 40%. Variable yields had also been noticed by Piura et al. (14) and were attributed to the technical problems involved in scaling down the reaction to work with micromole quantities of material. The major side product we observed was glycerol 2,3-cyclic-phosphate as identified by its resonance in the 31P NMR spectrum at 17.6 ppm. It is not clear at present whether it is formed by a direct nucleophilic attack of the sulfur of glycerol 3-phosphorothioate on the activating agent or whether the starting material is desulfurized by as yet unknown mechanisms. The former pathway seems to be unlikely since, on similar activations of phosphorothioates, no or only little desulfurization has been observed (25–27). Loss of sulfur subsequent to ring closure may be ruled out since 31P NMR indicates that two thirds of the glycerol 2,3-cyclic phosphate retains an 18O label. This is the result predicted for cyclization of nonchiral glycerol 3-[18O] phosphate.

In the ring-closure reaction performed with the chiral glycerol 3-[18O]phosphorothioate, the yield of glycerol 2,3-cyclic phosphorothioate was around 25%, and the ratio to glycerol 2,3-cyclic phosphate was approximately 1:3 as determined from the 31P NMR spectrum. As the chemical shifts of phos-

![Fig. 3. Retention versus inversion of configuration at phosphorus in the Ru5P kinase reaction: consequences on the 18O content of 2,3-cyclic phosphorothioates of glycerol derived from the reaction product.](image-url)
phorothioate esters are far downfield and thus well separated from phosphorothioate esters which in turn is the product of a Ru5P kinase reaction which has proceeded with inversion of configuration at phosphorus.

Enzyme-catalyzed nucelophilic substitutions at phosphorothioate esters are far downfield and thus well separated from phosphorothioate esters, it was not necessary to work up the reaction mixture of this cyclization reaction to analyze for the 180-induced upfield shift. Evaluation of the NMR data indicates, considering that the [180-34O]ATPγS was only 93% labeled, that the ring-closure reaction proceeded with 75% stereospecificity. A comparison with Fig. 3 demonstrates that a spectrum with such a line pattern has arisen from ring closure of (S)-glycerol 3-[180]phosphorothioate which in turn is the product of a Ru5P kinase reaction which has proceeded with inversion of configuration at phosphorus.

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REFERENCES


FIG. 4. 31P NMR spectrum of the diastereoisomeric mixture of 2,3-cyclic phosphorothioates of sn-glycerol formed upon ring closure of the enzymatically reduced reaction product of the coupled Ru5P kinase and RuBP carboxylase reactions. Resonances at 73.73 and 73.75 ppm are assigned to the 180- and 34O-containing anti-diastereoisomers; resonances at 73.25 and 73.21 ppm are due to the 180- and 34O-containing syn-diastereoisomers. Parameters were: offset, 7390 Hz; sweep width, 900 Hz; pulse width, 7.2 μs; data collection, 16 K; acquisition time, 9.09 s; line broadening, 0.3 Hz; number of transients, 6778.