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The Substrate Reactivity of μ-Monothiopyrophosphate with Pyrophosphate-Dependent Phosphofructokinase: Evidence for a Dissociative Transition State in Enzymatic Phosphoryl Group Transfer†

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ABSTRACT: μ-Monothiopyrophosphate (MTP), an analogue of pyrophosphate (PP_i) with sulfur in place of oxygen in the bridge position, is a substrate for the enzyme pyrophosphate-dependent phosphofructokinase. At pH 9.4 and 6 °C, the maximal velocity for the phosphorylation of fructose 6-phosphate (F6P) by MgMTP is about 2.8% of that with $MgPP_i$ as the phosphoryl donor. The kinetic mechanism is equilibrium random with rate-limiting transformation of the substrate ternary complex to the product when either MgMTP or $MgPP_i$ is the phosphoryl donor. This is known from independent studies to be the kinetic mechanism at pH 8.0 and 25 °C [Bertagnolli, B. L., & Cook, P. F. (1984) *Biochemistry 23*, 4101-4108]. The dissociation constant of $MgPP_i$ is 14 μM, that of MgMTP is 64 μM, and that of F6P from the enzyme is about 5 mM. The K_m values for $MgPP_i$ and MgMTP are 14.5 and 173 μM, respectively. MgMTP competes with $MgPP_i$ for binding to the enzyme. The values of k_{cat} are 3.4 s^{-1} and 140 s^{-1} for MgMTP and $MgPP_i$, respectively, at pH 9.4 and 6 °C. The estimated rate enhancement factors are 3.6×10^5 and 1.4×10^{14} for the reactions of MgMTP and $MgPP_i$, respectively. Therefore, MgMTP is a reasonably good substrate for PP_i-dependent PFK, on the basis of comparisons of k_{cat}. However, the rate enhancement factors show that the enzyme is a poor catalyst for the reaction of MgMTP. Lesser enzymatic catalysis in the reaction of MgMTP compared with $MgPP_i$ is largely compensated for by the greater intrinsic reactivity of MgMTP. Thus, the larger substrate MgMTP is well accommodated in the active site, and the dissociative reaction of MgMTP is well accommodated in the transition state. The results are interpreted to indicate a dissociative transition state for phosphoryl group transfer by PP_i-dependent PFK. A modified synthesis and purification of MTP are described, in which (trimethylsilyl)trifluoromethanesulfonate and tetra-*N*-butylammonium iodide are used in place of iodotrimethylsilane to dealkylate tetramethyl-MTP.

Phosphoryl group transfers are among the most common and important reactions in biochemistry. Despite this fact, the chemical mechanism of enzymatic phosphoryl group transfer is poorly understood (Knowles, 1980). In particular, the nature of the transition state is not known.

Nonenzymatic phosphoryl group transfer reactions in protic solvents proceed through dissociative transition states, in which there is little bonding between the phosphoryl group in flight and either the leaving group or the attacking nucleophile (Bunton, 1970; Benkovic & Schray, 1973; Williams, 1989;

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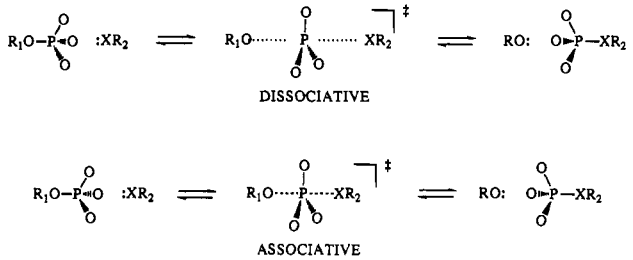
Scheme I



Herschlag & Jencks, 1986, 1989a,b, 1990). A dissociative transition state is illustrated at the top of Scheme I. In an associative transition state, shown at the bottom of Scheme I, there is substantial bonding between the phosphoryl group and both the attacking nucleophile and leaving group. Herschlag and Jencks (1990) summarize the evidence for dissociative transition states in nonenzymatic phosphoryl group transfers.

Knowles has pointed out that it is not obvious how an enzyme could catalyze phosphoryl transfer through a dissociative transition state (Knowles, 1980). This has led from time to time to proposals that enzymatic phosphoryl transfers differ from the nonenzymatic reactions by proceeding through associative transition states (Hassett et al., 1982; Knight & Cleland, 1989; Mildvan & Fry, 1987; Hall & Williams, 1986).

We have undertaken to determine the effect of replacing the central oxygen atom of PP_i[1] with sulfur on enzymatic phosphoryl group transfers. μ-Monothiopyrophosphate (MTP) was originally described by Loewus and Eckstein (1983), who reported some of its properties. We have improved the synthesis and purification of this compound. We have also investigated the mechanism of hydrolysis of MTP (Halkides & Frey, 1991; Lightcap et al., 1991). In nonenzymatic hydrolysis, MTP is about 17–50 million-fold more reactive than PP_i, depending on protonation level; and both compounds react by mechanisms that are dissociative in character (Halkides & Frey, 1991). There is evidence that the hydrolysis of $HMTP^{3-}$ is purely dissociative; that is, solvated metaphosphate monoanion (PO_3^-) may be formed as a discrete intermediate and captured within the solvation sphere.[2] Given this background, the comparative reactivities of MTP and PP_i as phosphoryl group donors in enzymatic reactions should give information about the mechanism of the enzymatic process.

We here compare the reactivities of MgMTP and $MgPP_i$ as phosphoryl donor substrates for pyrophosphate-dependent phosphofructokinase (PP_i-dependent PFK), which catalyzes the reaction of $MgPP_i$ with F6P to form FDP and MgP_i. This enzyme is present in diverse microorganisms that utilize PP_i as a phosphoryl donor in several reactions (O'Brien et al., 1975; Reeves et al., 1974). The kinetic mechanism is one in which there is equilibrium random binding of substrates and products, and phosphoryl group transfer within ternary complexes limits the overall rate of the reaction (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986; Cho & Cook, 1988, 1989; Cho et al., 1988). This kinetic mechanism makes PP_i-dependent PFK a nearly ideal enzyme for this type of study, in which the reactivities of closely related substrates are compared. The simplest interpretation of our results is that the transition state for phosphoryl transfer catalyzed by PP_i-dependent PFK is dissociative in character.

Experimental Procedures

Materials

Chemicals. All chemicals were reagent-grade or better, and the purification procedures of Perrin et al. (1980) were followed. Distilled water was deionized to a resistivity of at least 16 MΩ by passage over a mixed-bed ion-exchange resin, and organic impurities were removed. Methylene chloride and toluene were prepared by shaking the solvent with concentrated H_2SO_4 several times (until the color in the acid layer was minimal), with water, with 5% NaOH or 5% Na_2CO_3, with 5% $NaHCO_3$, and with water and then decanting over Drierite (W. A. Hammond Drierite Chemical Co.) or $CaCl_2$. Finally, each solvent was stirred with CaH_2 and distilled. Hexane was distilled from CaH_2. Diisopropylethylamine was stirred with CaH_2 and distilled. To minimize peroxide formation, 2-propanol was dried with CaO, then refluxed with and distilled from anhydrous $SnCl_2$, and used within several days. Dioxane was poured through dried basic alumina, refluxed with anhydrous $SnCl_2$, distilled, and used within 1 day. Methanol was made anhydrous by distillation from magnesium and iodine (Perrin et al., 1980). It was stirred with sulfanilic acid (Aldrich Gold-Label) and distilled a second time under anhydrous conditions. (Trimethylsilyl)trifluoromethanesulfonate (Aldrich) was distilled at reduced pressure. Tetrabutylammonium iodide (Aldrich) was recrystallized as described (Perrin et al., 1980), with substitution of hexane and toluene for petroleum ether and benzene, respectively. Trimethyl thiophosphate (Alfa) and dimethyl phosphite (Aldrich) were distilled at reduced pressure. SO_2Cl_2 was distilled just prior to use. Dowex AG1-X8 (200–400 mesh) from Bio-Rad was precycled with 1 M NaOH, water, 1 M HCl, and water. Other chemicals used in synthesis were obtained from commercial vendors and used as supplied.

CHES, PIPES, and MOPS were obtained from Sigma or Aldrich in the free-acid form. Reduced glutathione and $MgCl_2 \cdot 6H_2O$ were obtained from Aldrich. NADH was obtained from Sigma (grade III) or Pharmacia as the disodium salt. Disodium F6P was obtained from Sigma. Trisodium thiophosphate ($Na_3SPO_3 \cdot 12H_2O$) was a gift from Dr. Radha Iyengar. It was recrystallized from water and ethanol three times (Åkerfeldt, 1960), stirred with dry methanol, and dried in a vacuum oven at about 40 °C for about 2 h. Tetralithium PP_i was prepared as a substrate for comparison with tetralithium MTP by carrying $Na_4P_2O_7$ through the final anion-exchange purification procedure described below for the synthesis of Li_4MTP.

Enzymes. Aldolase and glycerophosphate dehydrogenase/triosephosphate isomerase from rabbit muscle were purchased from Sigma as sulfate-free powders. PP_i-dependent PFK from *Pseudomonas freudenreichii* was purchased frm Sigma as the affinity-purified preparation. BSA was purchased from Sigma.

Synthesis of μ-Monothiopyrophosphate (MTP). Tetramethyl-MTP was synthesized essentially as described before (Michalski et al., 1974) and identified by its proton-decoupled ^{31}P NMR spectrum, which consisted of a singlet with a

[1] Abbreviations: PP_i, inorganic pyrophosphate; MTP, μ-monothiopyrophosphate, in which the bridging O of PP_i is replaced by S; tetramethyl-MTP, the tetramethyl ester of MTP; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NMR, nuclear magnetic resonance; F6P, fructose 6-phosphate; FDP, fructose 1,6-bisphosphate; PP_i-dependent PFK, pyrophosphate-dependent phosphofructokinase; NAD, nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; PE(S)P, the analogue of phosphoenolpyruvate with sulfur bridging the 2-acrylyl and phosphoryl groups; CHES, 2-(cyclohexylamino)ethanesulfonic acid; PIPES, 2,2′-piperazine-1,4-diylbis(2-ethanesulfonic acid); MOPS, 4-morpholinepropanesulfonic acid.

[2] E. S. Lightcap and P. A. Frey, manuscript in preparation.

chemical shift of 19 ppm in acetone-d_6 and 19.45 ppm in $CDCl_3$. Caution whould be exercised in handling this compound to avoid inhalation of or skin contact with this potentially toxic substance.

MTP was synthesized by demethylation of tetramethyl-MTP under flowing nitrogen in a three-neck flask equipped with a magnetic stirrer and a dropping funnel. (All glassware was oven-dried.) The apparatus was enclosed within a loose-fitting plastic bag, through which flowed the nitrogen exhaust from the reaction apparatus. The reaction was carried out in very dim light. A solution of tetramethyl-MTP (304 mg, 1.22 mmol) and tetrabutylammonium iodide (1.798 g, 4.87 mmol) in 4 mL of CH_2Cl_2 was added to the three-neck flask and cooled to −60 °C. Separately, (trimethylsilyl)trifluoromethanesulfonate was fractionally distilled at 40 Torr into a two-neck flask sealed with a septum, and a portion of the liquid (1.19 g, 5.35 mmol) was removed with a syringe and added to 4 mL of CH_2Cl_2. This solution was immediately transferred to the dropping funnel and added over 50 min to the contents of the three-neck flask, which were stirred and maintained at −60 °C with dry ice/2-propanol. The temperature of the reaction mixture was raised to approximately −30 °C for 45 min, using a dry ice/mixed xylenes bath, and then returned to −60 °C. A mixture of 2-propanol (321 mg, 5.35 mmol) and diisopropylethylamine (1.40 g, 10.8 mmol) in 4 mL of CH_2Cl_2 was added dropwise to the reaction mixture over 25 min. After the addition of the first few drops, the reaction mixture, which had been yellow, became colorless. The volatiles were removed by rotary evaporation as quickly as possible, and 6.1 mL of 2.18 M LiOH was added immediately afterward. This solution of crude MTP was frozen by dropwise addition to liquid nitrogen, after a sample had been removed for examination by ^{31}P NMR. The yield of Li_4MTP was about 35%; the side products included thiophosphate and phosphate.

Li_4MTP was purified at 4 °C under nitrogen with minimal time between steps. The crude MTP was diluted to 50 mL and applied to two columns (2.4 × 8.7 cm) of Dowex AG1-X8 (200–400 mesh) anion-exchange resin. The columns had been washed with 250 mL each of 1.0 M HCl, water, and 0.1 M LiCl in 0.01 M LiOH. (All solutions had been prepared from CO_2-free water and stored under N_2.) A solution of 0.1 M LiCl in 0.01 M LiOH was used to elute Li_4MTP at a rate of about 1.2 mL/min. During and after the elution of Li_4MTP from these columns, the fractions (~12.5 mL) were assayed for thiophosphate and total phosphate (see below). Fractions containing MTP were identified by their constant ratio of phosphate to thiophosphate that was slightly greater than 2 and were pooled. MTP emerged between 440 and 580 mL of effluent. Model experiments demonstrated that phosphate emerged ahead of MTP, followed by thiophosphate, and PP_i coeluted with MTP (data not shown). A column of Dowex AG1-X8 (2.4 × 0.5 cm) was converted to the bromide form and equilibrated by passing solutions of 50 mL of 1.0 M HCl, 50 mL of water, 50 mL of 1.0 M LiBr, and two portions (4 mL, followed by 50 mL) of 0.2 M LiBr in 10 mM LiOH. The pooled fractions of MTP from the previous column were diluted 5-fold with water and applied to this column at a flow rate of about 2 mL/min. The column was eluted with 0.2 M LiBr in 0.01 M LiOH at the flow rate of 1.3 mL/min. The column fractions (about 0.6 mL) were assayed for phosphate; the fractions containing the higher concentrations of MTP were pooled, and a more dilute pool was made from side fractions. The concentration of MTP was about 20 mM in the first pool, which consisted of fractions collected between 4 and 10 mL of eluent. The concentration was about 7 mM in the second pool, which emerged between 2–4 and 10–12 mL of eluent. Each pool was divided into portions of 0.2 mL in Eppendorf tubes, which were frozen in liquid nitrogen. The yields of the two pools of MTP, henceforth referred to as concentrated and dilute MTP, were about 115 and 29 μmol, respectively. The primary signal in the ^{31}P NMR spectra of either concentrated or dilute MTP (acquired in 10 mM LiOH in the presence of 20% D_2O) was a singlet at 15.6 ppm (spectra not shown). Very small signals (<2%) could be detected for phosphate, thiophosphate, and PP_i. Enzymatic assay for PP_i indicated that it represented 0.3–0.8% of the total phosphate. Li_4MTP was found to be stable for many months when stored in the manner described here.

Methods

General Methods. ^{31}P NMR spectra were obtained on a Nicolet NTC-200, a Bruker AM-500, or a Bruker AM-400 spectrometer at temperatures which ranged from 9 to 25 °C. Chemical shifts were measured relative to 85% H_3PO_4. All had deuterium lock channels, and deuterium was present in the solvent of all samples, together with 1 mM EDTA or EGTA. The pH values of solutions were determined using a Radiometer Model PHM 26 pH meter. The pH values of kinetic assays were found at the end of each experimental run either at the temperature of the run or at room temperature. In the case of the latter, the pH of the assay solution was calculated using the temperature dependence of the pK_a of the buffer (Ellis & Morrison, 1982).

Chemical Assays. Inorganic phosphate was assayed by complexation of phosphomolybdate with malachite green (Tashima & Yoshimura, 1975), using monopotassium phosphate as the standard. Phosphate esters and alkyl phosphoanhydrides were ashed (Ames, 1966) prior to phosphate analysis. Thiophosphate was assayed by reaction with DTNB (Riddles et al., 1983), with modifications designed to accommodate the reaction of thiophosphate with 2 equiv of DTNB, the second reaction being much slower than the first (Goody & Eckstein, 1971). First, the absorbance at 410 nm of a solution 1.5 mM in DTNB and 0.1 M in phosphate (pH 7.25) was measured. Next, a solution containing thiophosphate was added and the contents were mixed, the volume of the sample solution being such as to bring the final DTNB concentration to 1.0 mM. After 2 min the final absorbance at 410 nm was measured. Samples containing MTP were typically hydrolyzed as follows prior to assay: A 0.1-mL sample was diluted in 0.5 mL of 0.1 M phosphate buffer (pH 7.25) for about 15 min to ensure complete hydrolysis of MTP, and a 0.5-mL aliquot of this mixture was added to 1.0 mL of DTNB solution. The apparent molar extinction coefficient was found to be 18 900 $M^{-1}\cdot cm^{-1}$, based on total phosphate assay of the thiophosphate solution, and this value was used to calculate the concentration of thiophosphate in the unknown.

Assay of PP_i-Dependent PFK. The activity of PP_i-dependent PFK was measured spectrophotometrically, either by coupled assay or by production of thiophosphate, using a Cary 118C recording spectrophotometer with the thermostated cell holder connected to a Forma Model 2160 circulating bath. Kinetic experiments were performed in cuvettes of 1-cm path length by preincubating all components except PP_i or MTP until temperature equilibration had been achieved. The temperature was 6 °C, unless otherwise indicated. The rate of raction was determined after the addition of PP_i or MTP, as described under Data Analysis.

Solutions of substrates and salts were generally used within 1 day of preparation, or they were frozen in small portions

using liquid nitrogen and thawed as needed. The concentrations of substrates and inhibitors of PP_i-dependent PFK were found by phosphate assay as described above. Buffers were brought to the correct pH by addition of KOH, and the concentration of buffer in the assay was 100 mM, unless otherwise noted. When it was desired to make buffer solutions of known and reproducible ionic strength, measured amounts of standardized KOH solutions were used.

In the enzyme-coupled assay, the activity of PP_i-dependent PFK with either PP_i or MTP as the substrate was measured by observing the production of 2 equiv of NAD^+ from NADH (Bertagnolli & Cook, 1984), with the rate being calculated using the value 6220 $M^{-1}\cdot cm^{-1}$ for the molar absorptivity of NADH at 340 nm. The FDP produced was converted to 2 equiv of dihydroxyacetone phosphate by the action of aldolase and triosephosphate isomerase, and dihydroxyacetone phosphate was reduced to glycerol phosphate by glycerophosphate dehydrogenase.

In a few experiments in which MTP was the substrate, the activity of PP_i-dependent PFK was measured in a direct assay by monitoring the production of thiophosphate at 227 nm (Halkides & Frey, 1991). In these experiments the concentration of CHES was 25 mM, and the concentration of KCl was 21.6 mM. The molar absorptivities of thiophosphate and MTP are 3390 and 390 $M^{-1}\cdot cm^{-1}$, respectively, in the presence of 25 mM CHES (pH 9.4), 2 mM $MgCl_2$, at 6 °C, and KCl to an ionic strength of 0.1 M (Halkides & Frey, 1991).

Standard Assay of PP_i-Dependent PFK. One unit of PP_i-dependent PFK activity was defined as that amount of enzyme which converts 1 μmol of F6P to FDP per minute at pH 9.4 (100 mM in potassium CHES) at 6 °C in the presence of 1 mM $Na_4P_2O_7$, 24 mM F6P, 2 mM $MgCl_2$, 0.2 mM NADH, and excess glycerophosphate dehydrogenase, triosephosphate isomerase, and aldolase. The rates of reactions under different conditions were always calculated relative to the standard assays run on the same day. Velocities measured under other than standard assay conditions are reported as micromoles of F6P consumed per minute per unit of enzyme.

Initial Rate Measurements. Aldolase from Sigma was dissolved in 20 mM PIPES or MOPS (pH 7.0–7.2) such that its concentration was about 25 mg of enzyme and salts/mL (330 units/mL). Its concentration in a kinetic assay of PP_i-dependent PFK was at least 6.6 units/mL. Glycerophosphate dehydrogenase/triosephosphate isomerase was dissolved in a 5 mM solution of reduced glutathione (taken to pH 6.0 with KOH) to make the concentration of enzyme 6.5 mg of enzyme and salts/mL (1000 dehydrogenase units/mL). Its concentration in the assay solution was 20–44 units/mL. Experiments with each enzyme had shown such large concentrations to be necessary, due to the decreases in activity of both enzymes at high pH and low temperature.

PP_i-dependent PFK (Sigma) was dissolved in 5 mM PIPES or MOPS (pH 7.0–7.2) to a concentration of 3.5–7 mg of protein and salts/mL, with 1 mg/mL BSA (Cook et al., 1978). This solution was frozen in liquid nitrogen and stored at −60 °C. The concentration of PP_i-dependent PFK was generally 20–240 μg of protein and salts/mL in assays in which MTP was the substrate. When PP_i was the substrate, the stock solution of PP_i-dependent PFK was diluted with buffer to a final concentration of 60–90 μg of protein and salts/mL. In the assay mixture itself the concentration of PP_i-dependent PFK was near 0.5 μg of protein and salts/mL.

The rate of PP_i-dependent PFK-catalyzed FDP production with MgMTP or $MgPP_i$ as the substrate was shown to be linear with respect to enzyme concentration at the beginning and end of each set of experiments. The production of FDP was absolutely dependent on the presence of PP_i-dependent PFK in all experiments.

Inhibition Studies. The conditions of the inhibition studies were 100 mM CHES (pH 9.4), 2 mM $MgCl_2$, and 6 °C. When thiophosphate was tested as a product inhibitor, the solution of Na_3PSO_3 was adjusted to pH 9.4 with HCl 1 day prior to use; the solution was frozen in liquid N_2 and thawed just prior to use.

Data Analysis. The rates of enzymatic processes were determined by taking the tangents at zero time to the graphs of absorbance versus time. When PP_i was the substrate, little or no lag was seen, and the initial slope was taken to be the rate. When MTP was the substrate, the initial rate of NADH consumption sometimes declined quickly to a value that remained nearly constant for some time. Inasmuch as the initial burst is probably due to the presence of a small amount of contaminating PP_i in the MTP (see below), the rates in these cases were found by determining the slope after the burst phase. The production of thiophosphate, as measured by the increase in absorbance at 227 nm, increased to a constant rate after a short lag, which was ignored in determining the rate. Rates were fitted to mechanisms using the nonlinear least-squares fitting programs of Cleland (1979). To fit the initial rate data in the absence of products, the programs SequenO and SequenL were used. SequenO was used to fit data to

$$v = \frac{V_m AB}{K_{ia}K_b + K_a B + K_b A + AB} \tag{1}$$

in which A and B are the concentrations of the two substrates. SequenL was used to fit data to the logarithm of eq 1. To fit the product inhibition data, the programs COMPO and NONCOMPO and their logarithmic equivalents were used. The former program treats the case of competitive inhibition and fits data to

$$v = \frac{V_m A}{K_a(1 + I/K_{is}) + A} \tag{2}$$

The latter program treats noncompetitive inhibition by fitting data to

$$v = \frac{V_m A}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{3}$$

Attempts to fit the data to the rate equation for uncompetitive inhibition gave unsatisfactory results.

Data from the inhibition of the reaction of PP_i by MTP were fitted to competitive, noncompetitive, and uncompetitive models. In addition, a model of inhibition treating MTP as a competitive (alternate) substrate was derived under the assumption that the binding of substrates was an equilibrium random process (Segel, 1975). The program CSUBO was kindly written for us by Dr. W. Wallace Cleland to fit data to this model. The program fits data to

$$v = \frac{VA + (\mathrm{CNC})C}{\mathrm{CON} + (\mathrm{CDC})C + A} \tag{4}$$

$$V = \frac{V_a}{1 + K_b/B} \qquad \mathrm{CDC} = \frac{K_a K_{ib} K_c + K_a K_{ic} B}{K_c K_{ic}(B + K_b)}$$

$$\mathrm{CNC} = \frac{V_c K_a}{K_c(1 + K_b/B)} \qquad \mathrm{CON} = \frac{K_a(B + K_{ib})}{B + K_b}$$

In these equations A and C are the concentrations of $MgPP_i$

and MgMTP (the competitive substrates) and B is that of F6P (which is not varied). V_a and V_c are the maximal velocities of substrates A and C in the absence of the other. To calculate the kinetic parameters from the fitting parameters, the values of K_b and K_{ib} were taken from the complete determination of the kinetic parameters in the presence of PP_i and F6P (Table II). To discriminate among fits, we selected the lowest values of variance. The values from different fits were occasionally close; in addition, the values for variance between a logarithmic fit and a nonlogarithmic fit cannot be compared (Cleland, 1979). In such cases we examined the randomness of the residuals and the magnitudes of standard errors of the parameters (Mannervik, 1982).

Results

Synthesis of MTP. A major barrier to the investigation of the chemical and enzymatic properties of MTP is the synthesis, purification, and storage of this labile compound. In the original description of MTP, Loewus and Eckstein (1983) produced samples that were contaminated with high concentrations of lithium carbonate, and the compound was found to be very labile to hydrolysis, with a half-life of only a few minutes in the physiological pH range. The data of Loewus and Eckstein indicated that the tetraanionic form of MTP would be stable enough to be purified and stored for lengthy periods in frozen solutions. The properties of MTP make it an interesting compound as a potential substrate analogue of PP_i in mechanistic analysis; therefore, we devised a modified synthetic procedure that leads reproducibly to concentrated solutions of Li_4MTP that can be frozen in liquid N_2 and stored for months at low temperatures.

Loewus and Eckstein used iodotrimethylsilane to dealkylate tetramethyl-MTP. This reagent is labile to decomposition by light, oxygen, or water (Schmidt, 1981). We reproduced the results of Loewus and Eckstein with iodotrimethylsilane in one experiment; however, we observed highly variable and mainly low yields in numerous additional attempts, apparently as a result of the lability of this reagent. Efforts to purify iodotrimethylsilane by distillation from copper and to use it immediately or to synthesize it did not yield consistent results; therefore, we decided to replace iodotrimethylsilane with another reagent.

Other dealkylation methodologies have employed alkali metal iodides and chlorotrimethylsilane in place of iodotrimethylsilane. Adopting this principle, we substituted the more highly reactive (trimethylsilyl)trifluoromethanesulfonate for chlorotrimethylsilane and tetrabutylammonium iodide for the alkali metal iodide. We presumed that tetrabutylammonium iodide would have good solubility in CH_2Cl_2, which we expected to be advantageous for procedures carried out at temperatures as low as −60 °C.

We have purified MTP by anion-exchange chromatography in the presence of 0.01 M LiOH to yield a dilute solution of Li_4MTP in 0.1 M LiCl at pH 12. Loewus and Eckstein (1983) concentrated purified MTP by precipitation with 2-propanol and observed partial cleavage to thiophosphate and phosphate. We were able to concentrate Li_4MTP by use of a second small anion-exchange column, which was eluted with 0.2 M LiBr at pH 12 to give up to 20 mM solutions of Li_4MTP. These solutions could be aliquoted, frozen in liquid N_2, and stored at −77 °C for months with virtually no hydrolysis.

Preliminary Enzymatic Experiments. The conditions of our kinetic experiments differed from those in previous studies of PP_i-dependent PFK; therefore, we assessed the effects of these changes on the stability of the enzyme and the dependence of the enzymatic rate on added salts. At pH 9.4 and 6 °C and in the presence of F6P, the enzyme activity is stable for over 1 h, much longer than the time required for the kinetic assay. Experiments with up to 1 mM MTP as the phosphoryl group donor in the presence of 2 mM $MgCl_2$ showed that the enzymatic rate did not vary with the concentration of $MgCl_2$ in this range; and MgMTP did not inactivate the enzyme.

Table I: Kinetic Parameters for MTP and PP_i as Substrates for PP_i-Dependent PFK[a]

MgMTP as phosphoryl donor	$MgPP_i$ as phosphoryl donor
$V_m = 0.033 \pm 0.003$	$V_m = 1.27 \pm 0.06$
$K_{m\,MTP} = 173 \pm 41$ μM	$K_{m\,PP} = 14.5 \pm 2.3$ μM (4.6 μM)[b]
$K_{i\,MTP} = 64 \pm 11$ μM	$K_{i\,PP} = 14.0 \pm 4.5$ μM (23 μM)[b]
$K_{m\,F6P} = 14.8 \pm 3.7$ mM	$K_{m\,F6P} = 5.0 \pm 0.7$ mM (127 μM)[b]
$K_{i\,F6P} = 5.5 \pm 0.9$ mM	$K_{i\,F6P} = 4.8 \pm 1.8$ mM (620 μM)[b]

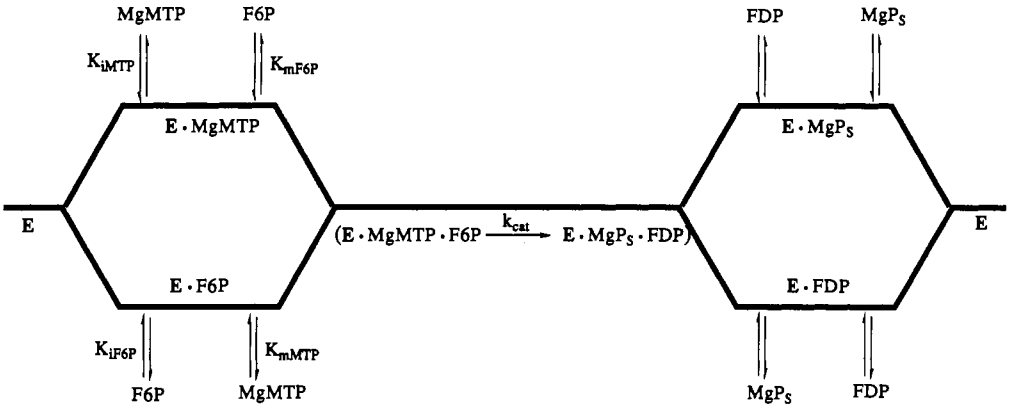
[a] Values obtained at pH 9.4 and 6 °C in 100 mM CHES buffer containing 2 mM $MgCl_2$. [b] Values for PP_i reported by Bertagnolli and Cook (1984) at pH 8 and 25 °C.

MTP was shown to be a true substrate of PP_i-dependent PFK as follows: (1) The enzymatic reaction produced thiophosphate and FDP at the same rates. (2) Total FDP production could not be explained by the presence of traces of PP_i in the MTP. To determine whether thiophosphate was produced enzymatically, the rate of FDP production measured in the coupled assay was compared with the rate of thiophosphate production measured by UV spectrophotometry. The similarity of the rates showed that MTP is a substrate for PP_i-dependent PFK, with thiophosphate and FDP being the products. To examine whether the observed rate with MTP could be attributed to a trace of contamination by PP_i, the extent of MTP consumption and FDP production by PP_i-dependent PFK was followed by coupled assay. In one experiment the rate declined only slightly from its initial value after 20% of the theoretical yield of FDP had been produced. To determine the level of PP_i contamination, a sample of MTP was hydrolyzed under conditions expected to produce complete hydrolysis of MTP but no hydrolysis of PP_i (Lightcap et al., 1991). The hydrolysate, in contrast to MTP, supported only a trace reaction with PP_i-dependent PFK; and this reaction, when treated as an assay for PP_i, indicated a maximum contamination of 0.3% PP_i in the MTP. This amount was consistent with that indicated by the ^{31}P NMR spectrum of MTP (data not shown).

Initial Velocity Studies. The initial velocity of the enzymatic reaction was measured as a function of [F6P] and either [MgMTP] or [$MgPP_i$] at pH 9.4 and 6 °C. Double-reciprocal plots of initial rates versus substrate concentrations at several fixed concentrations of cosubstrates gave families of lines converging at the left of the ordinate, and replots of the slopes or intercepts versus the concentration of the fixed cosubstrate were linear. The patterns were consistent with either a steady-state ordered kinetic mechanism or an equilibrium random mechanism, and they ruled out equilibrium ordered binding and ping pong mechanisms. The data were fitted to eq 1, the rate law for the allowed mechanisms.

The kinetic parameters for the phosphorylation of F6P by MgMTP catalyzed by PP_i-dependent PFK at pH 9.4 with 100 mM potassium–CHES buffer at 6 °C are given in Table I, together with the kinetic parameters for the phosphorylation of F6P by $MgPP_i$ under the same conditions. The values of $K_{m\,MTP}$ and $K_{i\,MTP}$ are 4.5- and 11-fold larger than $K_{m\,PP}$ and $K_{i\,PP}$, respectively. The value of $K_{m\,F6P}$ is slightly larger (3-fold) with MTP than with PP_i as the phosphoryl donor. The value of $K_{i\,F6P}$ is the same regardless of whether the phosphoryl donor is PP_i or MTP; and the values of $K_{i\,PP}$ and $K_{m\,PP}$ are identical, as are $K_{m\,F6P}$ and $K_{i\,F6P}$, when PP_i is the phosphoryl donor.

Scheme II



These latter relationships are most compatible with the equilibrium random kinetic mechanism, shown in Scheme II for MgMTP as the phosphoryl donor, which has been proposed by Cook and co-workers with PP_i as the phosphoryl donor at pH 8 and 25 °C (Bertagnolli & Cook, 1984). The kinetic parameters in the reverse direction are not defined in Scheme II because they have not been determined. It is probably not possible to study the reverse reaction because MgMTP would not be produced. Instead, the product would be unsymmetrical monothiopyrophosphate (O_2SPOPO_3), which is more stable than MTP owing to the greater strength of the P–O bond relative to the P–S bond.

The kinetic parameters reported by Bertagnolli and Cook at the lower pH and higher temperature are included in Table I for comparison with those at pH 9.4. The values of $K_{m\,PP}$ and $K_{i\,PP}$ are similar at the two pH's, whereas the values for $K_{m\,F6P}$ and $K_{i\,F6P}$ are significantly smaller at pH 8.0. This indicates that F6P is subject to pH-dependent binding interactions, in confirmation of the findings of Cho and Cook (1988), whereas the binding of $MgPP_i$ is insensitive to pH.

The value of V_m for $MgPP_i$ is 38.5-fold larger than that for MgMTP at pH 9.4 and 6 °C. This difference qualifies MgMTP as a slow but good substrate for PP_i-dependent PFK. To calculate k_{cat} at pH 9.4 and 6 °C, the rate of the reaction under our standard conditions was compared with the rate at pH 8.0, 25 °C, with 1 mM $Na_4P_2O_7$, 2 mM $MgCl_2$, and 2 mM F6P, the standard assay conditions of Bertagnolli and Cook (1984), who evaluated k_{cat} (890 s^{-1}) at pH 8.0 and 25 °C. The ratio of velocities under the two conditions was found to be 7.2; using this ratio and the kinetic parameters under each set of conditions, we calculated the k_{cat} at pH 9.4 and 6 °C to be 140 s^{-1} with $MgPP_i$ and 3.64 s^{-1} with MgMTP as the phosphoryl donor.

Inhibition Studies. To verify that MgMTP reacts at the active site, inhibition of the MgMTP-dependent phosphorylation of F6P by $MgPP_i$ was examined. With the concentration of F6P held at 5.6 mM, near its K_m, the concentrations of $MgPP_i$ and MgMTP were varied in the rate measurements.

Unlike competitive inhibition, for which eq 2 is the rate law, parallel reacting substrates bind and react at the active site, and each competitively inhibits the reaction of the other. Equation 4 is the rate law for parallel reacting substrates in an equilibrium random bi bi mechanism. The kinetic parameters determined in this experiment (Table II) are in general agreement with those obtained in the other initial velocity studies (Table I). In particular, the values for V_a and K_a (Table II) are very close to those for V_m and $K_{m\,PP}$ in the reaction of $MgPP_i$. The values obtained for K_c and $K_{i\,c}$ are within error of $K_{m\,MTP}$ and $K_{i\,MTP}$, respectively. V_c in Table II corresponds to the maximal velocity for MTP, but it is poorly determined and inaccurate. The data indicate that PP_i and MTP are parallel reacting phosphoryl donor substrates.

Table II: Kinetic Parameters for MgMTP and $MgPP_i$ Acting as Competitive Substrates[a]

parameter[b]	fitted value ± SE	parameter[b]	fitted value ± SE
V	0.583 ± 0.021	V_c	0.0018 (estimated)
CON	13.33 ± 1.48	K_a	13.6 ± 2.9 μM
CDC	0.133 ± 0.025	K_c	102 ± 29 μM
CNC	$1.25 \times 10^{-4} \pm 2.3 \times 10^{-3}$	$K_{i\,c}$	98.5 ± 23 μM
V_a	1.10 ± 0.085		

[a] The rates were measured in 100 mM CHES buffer at pH 9.4 and 6 °C in the presence of 5.6 mM F6P and 2.0 mM $MgCl_2$. [b] The parameters are those defined in eq 4 under Experimental Procedures and were evaluated as described there. Substrate A is $MgPP_i$ and C is MgMTP.

Table III: Product Inhibition by Thiophosphate

inhibition versus F6P[a]	inhibition versus MgMTP[b]	
noncompetitive	noncompetitive[c]	competitive[d]
$K_{m\,F6P}$ = 13.5 ± 5.0 mM	$K_{m\,MTP}$ = 183 ± 37 μM	$K_{m\,MTP}$ = 151 ± 31 μM
K_{is} = 8.0 ± 2.7 mM	K_{is} = 6.2 ± 1.5 mM	K_{is} = 3.63 ± 0.43 mM
K_{ii} = 4.6 ± 1.8 mM	K_{ii} = 7.0 ± 2.9 mM	

[a] Rates measured at 6 °C in 0.1 M CHES buffer at pH 9.4 and 198 μM MgMTP. Data were fitted to eq 3. [b] Rates measured under the conditions in footnote except with 15.0 mM F6P. [c] The data were fitted to eq 3. [d] The data were fitted to eq 2.

Thiophosphate was tested as a product inhibitor with respect to F6P at 198 μM MgMTP and with respect to MgMTP at 15.0 mM F6P, with the results shown in Table III. Thiophosphate was found to be a noncompetitive product inhibitor with respect to F6P. With thiophosphate acting as an inhibitor with respect to MgMTP, the data fitted eq 3 (competitive inhibition) slightly better than eq 2 (noncompetitive) as judged by the errors associated with the inhibition constants in Table III.

Noncompetitive inhibition by thiophosphate with respect to F6P is analogous to noncompetitive inhibition by phosphate reported by Bertagnolli and Cook (1984), who explained this on the basis that phosphate inhibits by forming the ternary product complex E·FDP·P_i and also the dead-end complex E·$MgPP_i$·P_i. Thiophosphate presumably inhibits through the formation of analogous complexes. Competition between thiophosphate and MgMTP for binding to the free enzyme

accounts for competitive product inhibition.

Discussion

Kinetic Mechanism with MTP as the Phosphoryl Group Donor. The kinetic mechanism for PP_i-dependent PFK has been extensively characterized by Cook and co-workers, who showed, through initial rate and product inhibition studies and rates of isotope exchange at equilibrium, that the reaction proceeds through equilibrium random binding of substrates and rate-limiting conversion of the substrate ternary complex E·$MgPP_i$·F6P to the product ternary complex E·MgP_i·FDP (Bertagnolli & Cook, 1984; Cho et al., 1988; Cho & Cook, 1989). For the purpose of determining whether the kinetic mechanism is altered by the substitution of bridging O in PP_i by S in MTP, and to evaluate k_{cat} and K_m for MgMTP, we have here investigated the kinetics of the reaction at a higher pH with both $MgPP_i$ and MgMTP.

The initial rates and inhibition patterns are consistent with expectations. That is, the kinetic patterns with both $MgPP_i$ and MgMTP are as found by Cook and co-workers at a lower pH and higher temperature. With MgMTP as the phosphoryl donor substrate, the kinetic mechanism remains equilibrium random binding, with rate-limiting interconversion of ternary complexes. Retention of equilibrium binding kinetics can be expected for the reaction of a slow substrate at a higher pH, and it is verified by the kinetic parameters and inhibition patterns.

At pH 9.4 with $MgPP_i$ as the phosphoryl donor, the values of K_m and K_i are the same for both substrates; however, with MgMTP as the phosphoryl donor $K_{m\,MTP} > K_{i\,MTP}$ and $K_{m\,F6P} > K_{i\,F6P}$. The simplest interpretation of these relationships is that substrates bind independently with $MgPP_i$ as the phosphoryl donor, but there may be a slight steric interference between substrates in forming the ternary complex E·MgMTP·F6P. This could be due to the larger size of MTP compared with PP_i. The differences between the values of K_m and K_i with MgMTP as the phosphoryl donor are small, corresponding to only about 0.5 kcal/mol in free energy terms.

The dissociation constants for complexes of PP_i-dependent PFK and F6P or PP_i have been determined as functions of pH by analyzing substrate protection against inactivation by pyridoxal phosphate (Cho & Cook, 1988). The variation of the dissociation constants with pH corresponds to pK_a values of 8.7 for binding F6P and 8.8 for binding $MgPP_i$. The pH dependence of the dissociation constants are in reasonable agreement with the values of K_i and K_m we determined kinetically at pH 9.4 and 6 °C, especially when considered in light of the fact that the temperatures and buffers differed in the two sets of experiments.

Rate Enhancement Factors for PP_i-Dependent PFK. When MgMTP is the substrate, the V_m is 2.6% of the value when $MgPP_i$ is the substrate (Table I). The values of k_{cat} for PP_i-dependent PFK at pH 9.4 and 6 °C are 140 s^{-1} with $MgPP_i$ and 3.6 s^{-1} with MgMTP. An estimate of the rate enhancement by this enzyme may be made by comparing the values of k_{cat} with the rate constant for hydrolysis of the phosphoryl donor substrate. This comparison depends on the approximation that the 6-hydroxyl group of F6P is comparable in chemical reactivity to the water molecule. The rate constant for the hydrolysis of MgMTP is estimated to be 1.0×10^{-5} s^{-1} from the activation energy for hydrolysis and the measured values between 15 and 35 °C (Lightcap et al., 1991). The ratio of the k_{cat} for enzymatic transfer of a phosphoryl group to F6P from MgMTP to the rate constant for uncatalyzed transfer to water is about 3.6×10^5. Therefore, the enzyme actively catalyzes phosphoryl transfer from MgMTP to F6P.

The rate enhancement factor for the reaction of PP_i is more difficult to estimate because the rate at which $MgPP_i$ undergoes hydrolysis is not known. A provisional estimate can be based on the relative hydrolytic reactivities of MTP^{4-} and PP_i^{4-} reported elsewhere (Halkides & Frey, 1991) and the fact that complexation of these species with divalent metal cations has little effect on their hydrolytic reactivities (Green, 1950; Lightcap et al., 1991). MTP^{4-} undergoes hydrolysis faster than PP_i^{4-} by a factor that lies between 6×10^6 and 2.7×10^7 (Halkides & Frey, 1991). Adopting the factor 1×10^7 for this difference, we estimate the hydrolytic rate constant for $MgPP_i$ at 6 °C to be about 1.0×10^{-12} s^{-1}. The value of k_{cat} for the enzymatic phosphorylation of F6P by $MgPP_i$ at 6 °C is 140 s^{-1}. Therefore, the rate enhancement factor for the reaction of $MgPP_i$ is about 1.4×10^{14}.

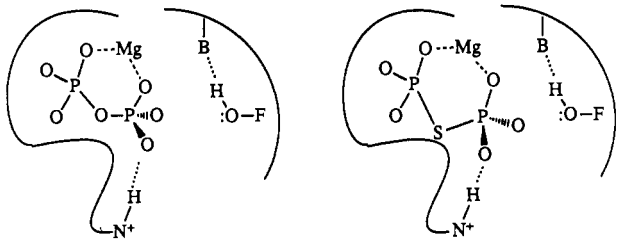
We regard MgMTP as a reasonably good substrate for PP_i-dependent PFK. Although it reacts with a k_{cat} of 2.6% of the k_{cat} for reaction of $MgPP_i$, the 38-fold difference between these values is small when compared with the overall rate enhancement factors. Nevertheless, PP_i-dependent PFK is not nearly as good a catalyst with MgMTP as it is with $MgPP_i$ because the rate enhancement factor with MgMTP is only about one four hundred millionth that with $MgPP_i$. That is, catalysis is 3.9×10^8 greater with $MgPP_i$ than with MgMTP as the phosphoryl donor.

The apparent paradox that MgMTP is a good phosphoryl donor substrate for PP_i-dependent PFK, but the enzyme is a poor catalyst for its reaction, is resolved by the intrinsic reactivity of MgMTP. The approximately 10^7-fold greater intrinsic reactivity of MgMTP relative to $MgPP_i$ nearly compensates for the (3.9×10^8)-fold deficit in enzymatic catalysis at the active site, allowing MgMTP to react almost as fast as $MgPP_i$. However, the interactions of the active site with MgMTP do not catalyze phosphoryl transfer to nearly the same degree as those between $MgPP_i$ and the active site.

Mechanism of Enzymatic Phosphoryl Transfer. The mechanism of phosphoryl group transfer by PP_i-dependent PFK will account for three principal findings in this paper: First, MgMTP is a reasonably good substrate for PP_i-dependent PFK, reacting with a k_{cat} of 1/38th that for $MgPP_i$. Second, MgMTP is reasonably well bound at the active site, with slightly less affinity than $MgPP_i$, presumably owing to its larger size. Third, PP_i-dependent PFK is not an efficient catalyst of phosphoryl transfer from MgMTP. Thus, MgMTP is reasonably well tolerated in both the ground state of the ternary complex and in the transition state for phosphoryl transfer; however, PP_i-dependent PFK is a poor catalyst of phosphoryl transfer from MgMTP.

These facts are most consistent with a dissociative transition state for phosphoryl transfer by PP_i-dependent PFK. If the transition state were associative, the active site would compress $MgPP_i$ and F6P in the ternary Michaelis complex, and the associative transition state would be smaller than the bound substrates (Jones et al., 1991). Such an active site should exhibit poor binding properties for MgMTP in the ternary complex, in the transition state, or in both, owing to the larger size of MTP compared with PP_i. However, the kinetic parameters indicate that MgMTP is reasonably well accommodated in both the ternary complex and the transition state. If the transition state is dissociative, the substrates will not be compressed by enzymic binding in the ternary complex, and the transition state will not be smaller than the bound substrates. In this case, the active site may well accommodate a slightly larger phosphoryl donor, as it does.

Why does this enzyme not catalyze the reaction of MgMTP, a highly reactive phosphoryl donor, with greater efficiency? This question cannot be answered with certainty at the present time. However, two factors that are undoubtedly important in catalysis are the geometry of the transition state and the chemical mechanism of the reaction. There is reason to believe that the geometry of MTP is inappropriate for catalysis by an enzyme that catalyzes phosphoryl transfer from PP_i. There is also the possibility that the mechanisms through which PP_i and MTP react in phosphoryl group transfer are not the same. Consider first the geometry of MTP. The P–S–P bond angle and P–S bond lengths in MTP are likely to be similar to those in tetramethyl-MTP, as the P–O–P angles and P–O bond lengths are similar in PP_i and its esters (Halkides et al., 1991). In tetramethyl-MTP, the P–S–P angle is 105° and the P–S bond lengths are 2.09 Å (Halkides et al., 1991). Another tetraester of MTP has similar structural features (Bukowska–Strzyzewska et al., 1976). The P–O–P angle in a series of pyrophosphates ranges from 127° to 130°, and the P–O bond lengths are about 1.61 Å (Halkides, et al., 1991). These differences cause the phosphorus atoms to be about 0.3–0.4 Å further apart in MTP compared to PP_i. This greater distance might be compatible with catalysis through a dissociative transition state; however, the 105° P–S–P angle is very different from the P–O–P angle in PP_i. Therefore, if one phosphoryl group of MgMTP is anchored at a particular subsite in the ternary complex, the other phosphoryl group will be projected at a different angle from that of $MgPP_i$ bound at the same site. This is illustrated in the following geometric conceptualizations of the ternary complexes.

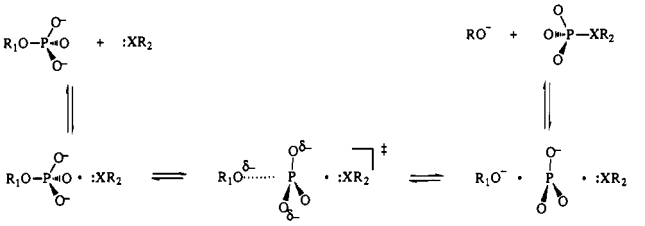


E·$MgPP_i$·F6P E·MgMTP·F6P

If $MgPP_i$ in the ternary complex projects a phosphoryl group *in line* toward the 6-hydroxyl group of F6P, it is very likely that the corresponding phosphoryl group of MgMTP at the same site will not be in line but be misaligned by about 20–25° for phosphoryl transfer to F6P. Such a poor alignment would retard the reaction significantly.

It is also possible that MgMTP and $MgPP_i$ undergo phosphoryl transfer by different mechanisms. Available evidence indicates that phosphoryl transfer with cleavage of bonds between phosphorus and nitrogen or oxygen follow the upper mechanism in Scheme I, and this presumbly is the mechanism by which PP_i reacts nonenzymatically. However, recent evidence from competitive trapping experiments indicates that MTP undergoes nonenzymatic hydrolysis through the preassociation dissociative mechanism in Scheme III,[2] which differs from Scheme I by the formation of metaphosphate monoanion as a discrete intermediate. In the reaction of MTP, a P–S bond is cleaved rather than a P–O bond as in PP_i, and the P–S bond is much weaker (Thain, 1957). Cleavage of a P–S bond should be much easier than cleavage of a P–O bond, and MTP may undergo cleavage without nucleophilic participation by an acceptor. If MgMTP and $MgPP_i$ react by different chemical mechanisms, the active site interactions required to catalyze their reactions will differ

Scheme III



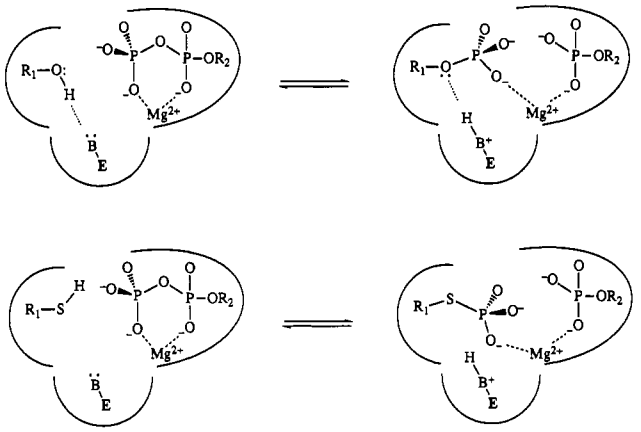
correspondingly. In this case, an active site suited to catalyzing the reaction of $MgPP_i$ will be much less effective in catalyzing the reaction of MgMTP.

Enzymatic Reactivities of Other S-Phosphates. Phosphorothiolates, S-phosphomonoesters, are substrates for a number of other enzymes. Several phosphorothiolates are substrates of alkaline phosphatase from *Escherichia coli* and intestine. Neumann and co-workers showed that this enzyme catalyzes the hydrolysis of cysteamine *S*-phosphate at about 70% of the rate of 4-nitrophenyl phosphate with a similar value of K_m (Neumann et al., 1967; Neumann, 1968). The authors suggested that alkaline phosphatase does not interact with the bridge atom of the phosphate ester (Neumann et al., 1967). 6-Deoxy-6-thioglucose 6-phosphate is a good substrate for alkaline phosphatase, reacting at a rate equal to that of glucose 6-phosphate (Knight et al., 1991). 5′-Deoxy-5′-thioadenosine 5′-monophosphate and 5′-deoxy-5′-thioinosine 5′-monophosphate are good substrates for intestinal alkaline phosphatase (Rossomando et al., 1983). Alkaline phosphatase is an unusual enzyme in that it lacks virtually any specificity for the R group of a phosphomonoester. Catalysis by alkaline phosphatase is generally limited by the rate of dephosphorylation of the phosphoenzyme; therefore, if the phosphorylation step were moderately slowed by the presence of sulfur in the leaving position, the overall rate might not be affected. 6-Deoxy-6-thioglucose 6-phosphate is a very good substrate for phosphoglucomutase, with a V_m comparable to that of the natural substrates (Knight et al., 1991). The fact that the bridging-sulfur analogues are good substrates for alkaline phosphatase and phosphoglucomutase is consistent with dissociative transition states for these reactions.

Several kinases do not accept these analogues as substrates very well. PE(S)P is a substrate for pyruvate kinase with an apparent V_m of 0.02% that of phosphoenolpyruvate in the presence of Mg^{2+}. When Mn^{2+} replaces Mg^{2+}, the apparent K_m of PE(S)P is lowered by 3-fold, with V_m increased by 20-fold (Sikkema & O'Leary, 1988). The reported kinetic parameters are apparent values because only PE(S)P was varied in concentration for the rate measurements. Thioglycolate is a slow substrate and thiolactate is a very slow substrate for pyruvate kinase (Ash et al., 1984). 6-Deoxy-6-thioglucose 6-phosphate is a very slow substrate for hexokinase, being utilized at 0.02% of the rate of glucose 6-phosphate (Knight et al., 1991). (*R*)-1-Mercaptopropanediol is a very slow substrate for glycerokinase, being phosphorylated on sulfur at 1/33000th the rate for glycerol (Knight & Cleland, 1989). A(S)MP and I(S)MP are not substrates for 5′-nucleotidase (Rossomando et al., 1983).

The slow reactions of several kinases with phosphorothiolates have led to the proposal that kinases catalyze phosphoryl group transfer by an associative mechanism, whereas phosphatases react through a dissociative mechanism (Knight & Cleland, 1989). We here argue for a dissociative transition state for PP_i-dependent PFK. An important difference between our study and the earlier ones is that we have employed a sulfur bridging thiophosphoanhydride, MTP, rather than a sulfur

Scheme IV



bridging ester, a phosphorothiolate, as a substrate. Phosphoryl transfer to an alcohol such as the 6-hydroxyl group of glucose requires general base catalysis (Hershlag & Jencks, 1990), as illustrated at the top of Scheme IV. Therefore, the hydroxyl group will be hydrogen bonded to a general base of the enzyme in the substrate Michaelis complex, and the phosphoester will be hydrogen bonded to the enzymic general acid in the product Michaelis complex. With sulfur in place of oxygen, the hydrogen bonds in the Michaelis complexes will be weakened owing to the large size of sulfur and its poor hydrogen-bonding properties, and nonproductive complexes such as those illustrated at the bottom of Scheme IV can be expected to exist in equilibrium with productive complexes. To the extent that sulfur is mobile or misplaced in the active site, the reaction is slowed. In the case of $MgPP_i$, however, the leaving group is good and probably does not require protonation to react; therefore, hydrogen bonding to this atom in the ground state may not be required. In this case, replacement with sulfur would not interfere with hydrogen bonding.[3] The mechanisms of the hexokinase and alkaline phosphatase reactions have recently been examined by measurements of secondary ^{18}O isotope effects, and the isotope effects have been interpreted to be consistent with dissociative transition states (Weiss & Cleland, 1989; Cleland, 1990; Jones et al., 1990).

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[3] Another possible interaction of the bridging oxygen of PP_i is with a coordinating metal ion. Substitution of this oxygen by sulfur should perturb any such interaction, owing to the differences in metal binding properties of sulfur and oxygen. We have no evidence that metal bonding with bridging sulfur is important in either the enzymatic or nonenzymatic cleavage of metal–MTP complexes.

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Identification of the ATP Binding Sites of the Carbamyl Phosphate Synthetase Domain of the Syrian Hamster Multifunctional Protein CAD by Affinity Labeling with 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

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ABSTRACT: The ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA) was used to chemically modify the ATP binding sites of the carbamyl phosphate synthetase domain of CAD, the multifunctional protein that catalyzes the first steps in mammalian pyrimidine biosynthesis. Reaction of CAD with FSBA resulted in the inactivation of the ammonia- and glutamine-dependent CPSase activities but had no effect on its glutaminase, aspartate transcarbamylase, or dihydroorotase activities. ATP protected CAD against inactivation by FSBA whereas the presence of the allosteric effectors UTP and PRPP afforded little protection, which suggests that the ATP binding sites were specifically labeled. The inactivation exhibited saturation behavior with respect to FSBA with a K_I of 0.93 mM. Of the two ATP-dependent partial activities of carbamyl phosphate synthetase, bicarbonate-dependent ATPase was inactivated more rapidly than the carbamyl phosphate dependent ATP synthetase, which indicates that these partial reactions occur at distinct ATP binding sites. The stoichiometry of [^{14}C]FSBA labeling showed that only 0.4–0.5 mol of FSBA/mol of protein was required for complete inactivation. Incorporation of radiolabeled FSBA into CAD and subsequent proteolysis, gel electrophoresis, and fluorography demonstrated that only the carbamyl phosphate synthetase domain of CAD is labeled. Amino acid sequencing of the principal peaks resulting from tryptic digests of FSBA-modified CAD located the sites of FSBA modification in regions that exhibit high homology to ATP binding sites of other known proteins. Thus CAD has two ATP binding sites, one in each of the two highly homologous halves of the carbamyl phosphate domain which catalyze distinct ATP-dependent partial reactions in carbamyl phosphate synthesis.

In mammalian cells, glutamine-dependent carbamyl phosphate synthetase (CPSase;[1] EC 6.3.5.5), the first and rate-limiting step in de novo pyrimidine biosynthesis, is associated with the multifunctional enzyme CAD (Jones, 1980); each 240-kDa subunit of this oligomeric protein (Shoaf & Jones, 1973; Mori et al., 1975; Coleman et al., 1977; Lee et al., 1985) also catalyzes the second and third reactions in pyrimidine biosynthesis, aspartate transcarbamylase (ATCase; EC 2.1.3.2) and dihydroorotase (DHOase; EC 3.5.2.3). Controlled proteolysis studies indicate that CAD consists of several domains, each with a distinct function (Mally et al., 1981; Davidson et al., 1981; Grayson et al., 1985); both the ATCase and DHOase domains of CAD have been isolated and sequenced (Grayson & Evans, 1983; Kelly et al., 1986; Shigesada et al., 1985; Major et al., 1989; Simmer et al., 1989, 1990a). The primary sequence and structural organization of the CAD CPSase domain are very similar to those of other monofunctional CPSases (Simmer et al., 1990b). The 120-kDa synthetase domain is comprised of two highly homologous subdomains which we have designated CPS.A and CPS.B.

Carbamyl phosphate synthetase catalyzes the ATP-dependent formation of carbamyl phosphate, a key intermediate in pyrimidine and arginine biosynthesis (Jones, 1980). The observation that *Escherichia coli* CPSase catalyzes two partial reactions, a bicarbonate-dependent ATPase and carbamyl phosphate dependent ATP synthesis, in conjunction with other kinetic and mechanistic studies (Anderson & Meister, 1965, 1966; Powers & Meister, 1976, 1978a,b) showed that carbamyl phosphate synthesis occurs in four steps with the formation of two intermediates, carboxyl phosphate and carbamate. The physiological substrate is glutamine, although ammonia can also directly serve as a nitrogen donor:

$$\text{glutamine} \rightarrow \text{glutamate} + NH_3$$
$$ATP + HCO_3^- \rightarrow ADP + \text{carboxy phosphate}$$
$$\text{carboxy phosphate} + NH_3 \rightarrow \text{carbamate}$$
$$\text{carbamate} + ATP \rightarrow \text{carbamyl phosphate}$$

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[1] Abbreviations: ATCase, aspartate transcarbamylase; CAD, the protein having glutamine-dependent carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase activities; CPSase, carbamyl phosphate synthetase; DHOase, dihydroorotase; EDTA, ethylenediaminetetraacetic acid; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; GLN, glutamine; Me_2SO, dimethyl sulfoxide; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TPCK, L-1-(tosylamino)-2-phenylethyl chloromethyl ketone.

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