Rose, I. A. (1960) *J. Biol. Chem. 235*, 1170.
Rowan, L. G., Hahn, E. L., & Mims, W. B. (1965) *Phys. Rev. 137*, A61.
Serpersu, E. H., McCracken, J., Peisach, J., & Mildvan, A. S. (1988) *Biochemistry 27*, 8034.
Shimizu, T., Mims, W. B., Peisach, J., & Davis, J. L. (1979) *J. Chem. Phys. 70*, 2249.
Snetsinger, P. A., Cornelius, J. B., Clarkson, R. B., Bowman, M. K., & Belford, R. L. (1988) *J. Chem. Phys. 92*, 3696.
Suelter, C. H. (1970) *Science 168*, 789.
Tietz, A., & Ochoa, S. (1958) *Arch. Biochem. Biophys. 78*, 477.
Villafranca, J. J., & Raushel, F. M. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 2961.

Thiol and Amino Analogues as Alternate Substrates for Glycerokinase from *Candida mycoderma*†

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ABSTRACT: The kinetic and catalytic mechanism of glycerokinase from *Candida mycoderma* was examined with thiol and amino analogues of glycerol and with MgAMPPCP, an analogue of MgATP. (*S*)-1-Aminopropanediol was phosphorylated on nitrogen (V_{max} 0.4% that of glycerol) while the *R* enantiomer was phosphorylated on oxygen (V_{max} 0.7% that of glycerol). (*S*)-1-Mercaptopropanediol was phosphorylated on oxygen (V_{max} 3.5% that of glycerol), while the *R* enantiomer was phosphorylated on sulfur (V_{max} 0.001% that of glycerol). The hydroxyl group at C-2 thus orients the substrate in the active site, while that at the carbon remote from phosphorylation enhances both catalysis and binding of the substrate, presumably because of hydrogen-bonding interactions. The kinetic mechanism is random with a high degree of synergistic binding between the substrates, so that the mechanism appears ordered with glycerol adding first but equilibrium ordered with MgATP binding first with the amino analogues.

Glycerokinase catalyzes the phosphorylation of glycerol by MgATP to produce L-glycerol 3-phosphate (the *R* enantiomer) in a classic example of the ability of an enzyme to distinguish between chemically identical functional groups in a prochiral molecule. From their studies with fluoro analogues of glycerol, Eisenthal et al. (1972) proposed that all three hydroxyls of glycerol are involved in the substrate recognition process. We have examined the roles of the primary hydroxyls in both catalysis and binding by examining the effects of replacement with either thiol or amino groups. On the basis of studies with alternate substrates and dead-end inhibitors, Janson and Cleland (1974) concluded that glycerokinase had an ordered kinetic mechanism with glycerol adding before MgATP. However, these studies would not have distinguished a truly ordered mechanism from a random one with a high degree of synergistic binding between the substrates, and just such a highly synergistic random mechanism has been found for yeast hexokinase by Viola et al. (1982). We have examined the order of addition of substrates to glycerokinase and the degree of synergistic binding by using dead-end inhibitors, by using alternate substrates, and by measuring the MgATPase activity in the presence and absence of a second substrate. Glycerokinase from different sources has been reported to show nonlinear reciprocal plots for MgATP (Thorner & Paulus, 1973), interpreted as either two types of MgATP binding or negative cooperativity. We have explored this possibility with the enzyme from *Candida mycoderma*. We have also examined the pH dependence of the phosphorylation of glycerol and its thiol and amino analogues.

Materials and Methods

Materials. Buffers were titrated to the desired pH with KOH. (*R,S*)-1-Mercapto-2,3-propandiol and (*R,S*)-1-amino-2,3-propanediol from Sigma and Aldrich when used as substrates displayed a burst caused presumably by contaminating glycerol. The thiol from Aldrich and Sigma contained 0.7% and 2%, respectively, while the amine from Aldrich contained 0.5% fast-reacting material. Incubation of these compounds with glycerokinase, MgATP, pyruvate kinase, and phosphoenolpyruvate at pH 7.5 followed by Amicon filtration yielded products free of contamination. (*R,S*)- as well as (*R*)- and (*S*)-2,2-dimethyl-1,3-dioxolane was from Aldrich. AMPPCP, AMPPNP, NAD, and NADH were from Boehringer. Glycerokinase from *Candida mycoderma* or *Escherichia coli* was from Sigma and was dialyzed vs 20 mM Hepes, 5 mM dithiothreitol, and 0.3 mM EDTA, followed by several changes of 20 mM Hepes and 5 mM dithiothreitol, prior to use, and was stored under nitrogen. All other coupling enzymes and high-purity sucrose were from Sigma.

Methods. ^{31}P and ^{13}C NMR spectra were obtained on either Nicolet NT-200 or Bruker AM500 NMR spectrometers while proton NMR spectra were obtained on the NT-200, or on a Bruker WH270 instrument. ^{31}P NMR chemical shifts are referenced to external 200 mM D_3PO_4, while proton and ^{13}C chemical shifts are referenced to external TMS.

Analogue Syntheses. (*R,S*)-1-Mercaptopropanediol 1-phosphate was synthesized from glycerol by tosylation and

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displacement with thiophosphate (Knight et al., 1984). Glycerol (56 mmol) was dissolved in 100 mL of dry pyridine and cooled to 0 °C. *p*-Toluenesulfonyl chloride (56 mmol) in 50 mL of dry pyridine was added dropwise over 1 h. The solution was held at 0 °C for an additional hour and allowed to stand overnight at 25 °C. Cold 15 mM KOH (600 mL) was added, and the pH was adjusted to 8 with KOH. Solvents were removed under reduced pressure, and the resulting oil was dissolved in 140 mL of water and added to 80 mL of 0.49 M $Na_3SPO_3 \cdot 18H_2O$ under nitrogen at 25 °C. The pH was maintained at 11 for 4 h, and then the solution was cooled to 4 °C. The product was purified as the lithium salt by anion-exchange chromatography at pH 10.3 on QAE-Sephadex equilibrated with 10 mM ammonia, with elution by a LiCl gradient. Fractions containing the product were pooled, lyophilized, and extracted with dry acetone/methanol (10:1) to remove LiCl. The lithium salt was obtained in 50% yield and was converted to the potassium salt by cation exchange with potassium Chelex.

1-Mercaptopropanediols. 2,2-Dimethyl-1,3-dioxolane (28.8 mmol) was dissolved in 25 mL of cold pyridine, and 35 mmol of tosyl chloride was added. After 2 h at 0 °C and 15 h at room temperature, 30 mL of cold 1 M NaOH was added, and the product was removed by extraction with chloroform. After removal of the solvent, the oil was dissolved in 100 mL of 65% acetic acid and heated in a boiling water bath for 40 min to remove the isopropylidene group. After being cooled and removal of solvent, the tosylglycerol was dissolved in 155 mL of water, the pH was adjusted to 7, and 30 mmol of sodium thiophosphate in 60 mL of water was added. The pH was kept at 11 for 30 min, and then the solution was diluted to 3 L and charged onto the QAE-Sephadex column. The product was isolated as described above. Free mercaptopropanediol was prepared by incubating the phosphate ester at pH 3 for 4 h under nitrogen and extracting the product into ethyl acetate. Trace amounts of phosphate were removed from aqueous solutions by passage through Dowex 1-Cl. This synthesis was carried out with *S*, *R*, and racemic starting material to produce the corresponding thiols, which were stored under nitrogen at 4 °C. Proton spectra showed multiplets at 2.05, 3.0, and 3.13 ppm, while ^{13}C spectra showed singlets at 26.7, 63.8, and 72.8 ppm.

1-Aminopropanediols. The tosyl derivative of 2,2-dimethyl-1,3-dioxolane (31.8 mmol; see above) and sodium azide (310 mmol) were dissolved in 150 mL of dimethyl sulfoxide and heated for 70 min in a boiling water bath. After being cooled, 150 mL of chloroform was added, and the solution was extracted with 7 volumes of water. The chloroform was removed under reduced pressure, and the resulting oil dissolved in 50 mL of methanol and hydrogenated over 10% palladium on carbon (4 g) under 3 atm of H_2. After filtration of the catalyst and removal of solvent, the oil was dissolved in water, titrated to pH 2.3 with HCl, and heated for 50 min at 100 °C to remove the protecting group. The product was purified on Dowex 50-H^+ by elution with 0.5 M ammonia. After removal of ammonia under reduced pressure, the product was stored in solution at pH 7.0 (35% yield). (The low yield results from incomplete hydrogenation of the azide; reduction with dithiothreitol has given yields over 50%.) This procedure was used to synthesize (*R*), (*S*)-, and (*R*,*S*)-aminopropanediols from appropriate precursors. Proton NMR gave multiplets at 1.9, 2.75, and 2.9 ppm, while the proton-decoupled ^{13}C NMR spectrum showed singlets at 43.2, 63.7, and 73.0 ppm.

Kinetic Assays. All assays were at 25 °C in 100 mM buffers in 1-mL volume. All stock solutions were filtered through disposable 40-μm filters (prerinsed to remove any glycerol present as a wetting agent) to limit the introduction of dust and the resulting light scattering. The phosphorylation of glycerol and analogues by MgATP was followed by coupling the production of MgADP to the pyruvate kinase–lactic dehydrogenase system. Reaction mixtures contained 0.2 mM NADH, 1.0 mM phosphoenolpyruvate, 1 mM dithiothreitol, 7 mM free Mg^{2+} (up to 17 mM did not affect the kinetics) as the chloride salt, and 10–60 units each of pyruvate kinase and lactate dehydrogenase. Glycerol or the analogue was added last unless otherwise specified.[1] Velocities were normalized to the rate obtained with 5.4 mM glycerol and 5 mM MgATP, pH 7.5, to control for daily variations in enzymatic activity.

Concentrations of stock glycerol solutions were determined enzymatically with the assay above. ATP concentrations were determined either spectrophotometrically (ϵ_{259} = 15 400 M^{-1} cm^{-1}) or enzymatically with glucose/hexokinase and glucose-6-phosphate dehydrogenase/NADP. Stock solutions of mercaptopropanediol were calibrated with DTNB, 2-PDS, or 4-PDS (Grimshaw et al., 1979). Phosphorylated mercaptopropanediol solutions were titrated to the appropriate pH and calibrated either by acid hydrolysis followed by reaction with 2-PDS or by hydrolysis with alkaline phosphatase in the presence of DTNB (Knight & Cleland, 1985). Stock solutions of aminopropanediols were titrated to the appropriate pH and calibrated by reaction with periodate, glycerol being used as the standard (Dixon & Lipkin, 1954).

NMR Enzyme Assays. The substrate activity of glycerokinase toward the various analogues was examined initially by ^{31}P NMR. Typical reactions contained 3–5.2 mM MgATP, 0.5 mM EDTA, 16 mM Mg^{2+}, 10–200 mM phosphoenolpyruvate, 100 mM TAPS, pH 8–8.5, 10–100 units of pyruvate kinase, and 5–2500 units of glycerokinase. Reaction of 1-mercaptopropanediol 1-phosphate was monitored in 3-mL mixtures containing 180 units of glycerokinase, 5 mM mercaptopropanediol phosphate, 7.5 mM MgADP, 20 units of hexokinase, 4 mM glucose, and 12.5 mM $MgCl_2$.

Data Processing. Kinetic data were fitted with the Fortran programs of Cleland (1979), versions modified to work on IBM-compatible personal computers being used. The equations fitted were

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

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

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

$$v = VA/[K(1 + I/K_{is}) + A] \quad (4)$$

$$\log y = \log [C/(1 + K_2/H)] \quad (5)$$

$$\log y = \log [C/(1 + H/K_1)] \quad (6)$$

$$\log v = \log [V_1A/(K_1 + A) + V_2A/(K_2 + A)] \quad (7)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (8)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (9)$$

$$K_{is\ true} = K_{is\ app}/(1 + [MgATP]/K_{i\ ATP}) \quad (10)$$

$$K_{ii\ true} = K_{ii\ app}/(1 + [MgATP]/K_{m\ ATP}) \quad (11)$$

where $K_{m\ ATP}$ is for the substrate being used.

[1] Despite all of the precautions used, there was still ~10 μM contaminant that behaved like glycerol in the assay solutions. Reactions were initiated after this material had reacted.

Table I: Kinetic Parameters for (*R*)-Thiol Analogue in 100 mM Taps, pH 8.4

substrates	relative velocity	K_m or K_i for glycerol or analogue (mM)
glycerol, MgATP	100[a]	0.020 ± 0.001
(*R*)-mercaptopropanediol, MgATP	0.00030 ± 0.00002[b] 0.0010 ± 0.00015[d]	19 ± 2[c]
L-glycerol-3-P, MgADP	1.5 ± 0.2[e]	0.30 ± 0.08
(*R,S*)-1-mercaptopropandiol-1-P, MgADP	0.0040 ± 0.0003[f]	22 ± 4[g]

[a] Rate at 5.4 mM glycerol and 5 mM MgATP; K_m for glycerol determined at 5 mM MgATP. [b] Rate at 5 mM MgATP and 12 mM (*R*)-mercaptopropanediol. [c] From competitive inhibition vs glycerol at 5 mM MgATP. [d] Rate at 5 mM MgATP and 234 mM (*R*)-mercaptopropanediol. [e] Apparent V_{max} and K_m at 1.5 mM MgADP. [f] Rate at 5 mM (*R,S*)-1-mercaptopropanediol 1-phosphate and 7.5 mM MgADP. The reaction was followed by coupling MgATP with glucose and hexokinase (see Methods). [g] From competitive inhibition vs glycerol 3-phosphate at 1.5 mM MgADP.

Results

Syntheses. It was evident that the commercially available racemic thiol and amino analogues of glycerol contained contaminating glycerol. The syntheses used here gave products free of glycerol (the time courses for reaction with MgATP were linear to over 7% conversion, with a lower detection limit of 0.1% impurity), and when chiral precursors were used, products of >99% enantiomeric purity were obtained. Individual pairs of enantiomers gave equal but opposite specific optical rotations ($[\alpha]^{22}_D$ (deg) values: (*S*)-aminopropanediol, −0.871; *R* enantiomer, +0.861; (*S*)-mercaptopropanediol, +5.7; *R* enantiomer, −5.7). The p*K* values were 9.6 for the thiols and 9.1 for the amines at 0.1 M ionic strength. The synthetic mercaptopropanediols and aminopropanediols displayed identical proton and ^{13}C NMR spectra with those of commercially available compounds. The ^{31}P NMR spectrum of 1-mercaptopropanediol 1-phosphate at pH 9.2 displayed a triplet (J_{H-P} = 11.4 Hz) which collapsed to a singlet at 16.8 ppm upon proton decoupling. This chemical shift demonstrates S-phosphorylation (Knight et al., 1984). This compound did not react with DTNB until after acid hydrolysis, which released phosphate. Storage of the thiol analogues as their phosphate esters until use prevented air oxidation.

^{31}P NMR Analysis of Products from Glycerokinase-Catalyzed Phosphorylation of Mercaptopropanediols and Aminopropanediols. Reaction of (*R,S*)-mercaptopropanediol with MgATP in the presence of glycerokinase at pH 8.2 produced a species with a chemical shift at 4.1 ppm with a rate 0.7% that shown by glycerol under the same conditions. This product (1-mercaptopropanediol 3-phosphate) was produced in only 50% yield, however. Phosphate (2.6 ppm) was also produced, but there was no evidence for S-phosphorylation. When (*R,S*)-1-mercaptopropanediol 1-phosphate was incubated with glycerokinase, MgADP, glucose, and hexokinase, there was a decrease in the starting material and the production of glucose 6-phosphate (4.4 ppm). The phosphate was transferred from sulfur in this reaction at 0.27% the rate of the reverse reaction of L-glycerol 3-phosphate under similar conditions (this is 0.004% of the rate of glycerol phosphorylation). When the protein was filtered off with an Amicon filter after 12 h, the filtrate contained 1.4 mM glucose 6-phosphate (28% reaction of starting material).

The glycerokinase-catalyzed phosphorylation of (*S*)-mercaptopropanediol by MgATP produced a species with a ^{31}P chemical shift of 4.2 ppm. This enantiomer was phosphorylated on the terminal hydroxyl (a triplet was observed in the proton-coupled spectrum) at 3% the rate of glycerol. When the *R* enantiomer was used, a species with a chemical shift at 16.7 ppm (pH 8.4) was produced. This phosphorylation on sulfur occurred at 3×10^{-4}% of the glycerol rate. The predominant product, however, was phosphate (Figure 1A), which was produced at 0.007% the rate of glycerol phosphorylation. When the concentration of (*R*)-mercaptopropanediol was increased to 234 mM, however, both phosphate and (*R*)-1-mercaptopropanediol 1-phosphate were produced at similar rates (0.0012% and 0.001% of the glycerol rate; see Figure 1B). These data are summarized in Table I.

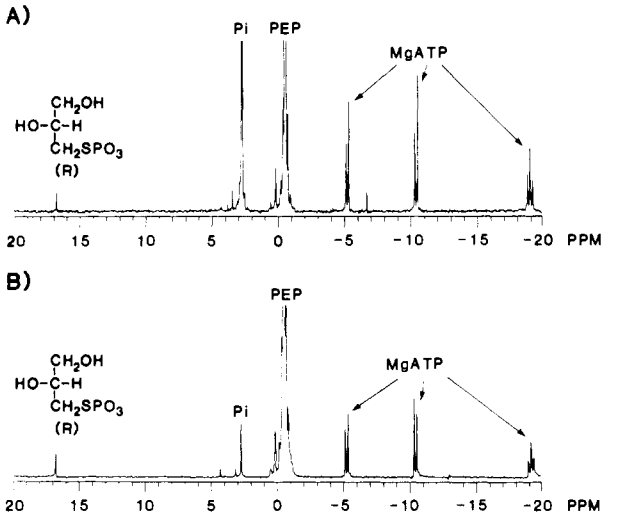

FIGURE 1: ^{31}P NMR spectra of reactions of (*R*)-mercaptopropanediol with glycerokinase. Reaction mixtures contained 100 mM Taps, pH 8.4, 20 mM Mg^{2+}, 5.2 mM ATP, 0.5 mM EDTA, 52 units of pyruvate kinase, 480 units of glycerokinase, and (A) 121 mM PEP and 11.7 mM thiol or (B) 200 mM PEP and 234 mM (*R*)-mercaptopropanediol.

The ^{31}P NMR spectrum of the reaction of (*R,S*)-1-aminopropanediol with MgATP in the presence of glycerokinase showed two new resonances at 5.7 and 4.2 ppm, with the one at 5.7 ppm appearing 13 times faster than the upfield resonance (0.3% the rate of glycerol at pH 9.2 and 0.08% at pH 8.2). When the reaction was allowed to go to completion, both species were present in equal amounts, but after the enzymes were filtered off and the pH was lowered to 3.0 for 3 h and then raised to 8.4, only the resonance at 4.1 ppm remained, along with an increase in the amount of phosphate present corresponding to the decomposition of the 5.7 ppm peak. When (*R*)-aminopropanediol was phosphorylated by glycerokinase, the product showed a ^{31}P NMR resonance at 4.1 ppm, while the same reaction with (*S*)-aminopropanediol produced a species with a resonance at 5.7 ppm.

Initial Velocity Studies with Thiol and Amino Analogues. (*S*)-1-Mercaptopropanediol and (*R*)- and (*S*)-1-aminopropanediol displayed sufficient substrate reactivity to be studied by initial velocity methods. Table II contains the kinetic parameters of these analogues at pH 8.5 and 9.5 with 5.1 mM MgATP. (*R*)-1-Mercaptopropanediol was a competitive inhibitor vs glycerol with a K_i of 19 mM but was too inactive for initial velocity studies. Figure 2 shows the initial velocity pattern for (*S*)-1-aminopropanediol and MgATP, and a similar pattern was seen for the *R* enantiomer. These patterns correspond to equilibrium-ordered addition of MgATP prior to the amino analogues. The initial velocity patterns for the phosphorylation of (*S*)-1-mercaptopropanediol (data not

Table II: Kinetic Parameters for Amino and Thiol Analogues with 5 mM MgATP

substrate	V_{max} (mM/min)	K_m (mM)	V/K (min^{-1})
100 mM Taps, pH 8.5			
glycerol	4.9 ± 0.1	0.030 ± 0.002	163 ± 8
(*S*)-mercaptopropanediol	0.173 ± 0.005	60 ± 3	0.029 ± 0.001
(*S*)-aminopropanediol	0.0059 ± 0.0012	0.31 ± 0.02	0.019 ± 0.006
(*R*)-aminopropanediol	0.0055 ± 0.0009	6 ± 2	0.0010 ± 0.0002
100 mM Ches, pH 9.5			
glycerol	3.4 ± 0.08	0.060 ± 0.004	57 ± 2
(*S*)-mercaptopropanediol	0.060 ± 0.001	66 ± 2	0.00090 ± 0.00002
(*S*)-aminopropanediol	0.015 ± 0.001	0.31 ± 0.06	0.042 ± 0.006
(*R*)-aminopropanediol	0.024 ± 0.004	10 ± 2	0.0024 ± 0.0002

Table III: Kinetic Parameters from Initial Velocity Patterns (mM)

substrate	$K_{m\ substrate}$	$K_{i\ substrate}$	K_{MgATP}	$K_{i\ MgATP}$	$K_{i\ MgATP}/K_{MgATP}$
glycerol, pH 8.2	0.020 ± 0.002		0.015 ± 0.001	0.54 ± 0.04[a]	36
glycerol, pH 9.4	0.060 ± 0.005		0.24 ± 0.02	2.5 ± 0.03[a]	10
(*S*)-aminopropanediol, pH 9.4	0.28 ± 0.02		*b*	2.0 ± 0.2	>10
(*R*)-aminopropanediol, pH 9.4	8.0 ± 0.7		*b*	2.2 ± 0.2	>5
(*S*)-mercaptopropanediol, pH 8.2	46 ± 5	38 ± 11	0.37 ± 0.06	0.30 ± 0.07	0.9
(*S*)-mercaptopropanediol, pH 9.4	64 ± 10	169 ± 45	0.9 ± 0.2	2.4 ± 0.4	2.7

[a] K_m for MgATPase activity in the absence of phosphoryl acceptor. [b] Equilibrium-ordered initial velocity pattern. K_m for MgATP taken at aminopropanediol concentration of $4K_m$, so $K_{i\ MgATP}/K_{MgATP}$ ratio is a minimal value.

Table IV: Inhibition Patterns for Dead-End Inhibitors

inhibitor	varied substrate	fixed substrate, concn (mM)	pH	K_{is} (mM)	K_{ii} (mM)	inhibition type[a]
MgAMPPCP	glycerol	MgATP, 0.332	9.4		1.2 ± 0.2	UC
MgAMPPCP	glycerol	MgATP, 0.026	8.1		0.6 ± 0.07	UC
MgAMPPCP	glycerol	MgATP, 0.026	7.0	1.3 ± 0.3	1.1 ± 0.5	NC
MgAMPPCP	MgATP	glycerol, 2.2	8.1	0.3 ± 0.04		C
MgAMPPCP	(*S*)-mercaptopropanediol	MgATP, 0.241	8.1	3.8 ± 0.4	5.4 ± 0.8	NC
(*R*)-mercaptopropanediol	MgATP	(*S*)-mercaptopropanediol, 25	8.1	50 ± 16	36 ± 5	NC
(*S*)-aminopropanediol	glycerol	MgATP, 2.5	7.2	0.9 ± 0.1		C
(*R*)-aminopropanediol	glycerol	MgATP, 2.5	7.2	23 ± 1.4		C

[a] C, competitive; UC, uncompetitive; NC, noncompetitive.

shown) were normal intersecting patterns. At pH 8.2 the patterns intersected on the horizontal axis, while at pH 9.37 they intersected slightly above the axis, showing slight synergism between the substrates. The kinetic parameters are in Table III.

To further delineate the kinetic mechanism, dead-end inhibition studies were conducted. MgAMPPCP was tested as an inhibitor vs MgATP, glycerol, and (*S*)-1-mercaptopropanediol, while (*R*)-mercaptopropanediol was tested vs glycerol for the phosphorylation of (*S*)-mercaptopropanediol by MgATP. The kinetic parameters are in Table IV. MgAMPPCP was uncompetitive vs glycerol at pH 9.4 and 8.1 but appeared to be noncompetitive at pH 7.0. When the K_{ii} values at pH 8.1 and 9.4 were corrected with eq 11, the dissociation constant of MgAMPPCP from its complex with (*E*)-glycerol was 0.3 mM, in agreement with the value for competitive inhibition vs MgATP. The use of eq 10 and 11 to correct the observed K_{is} and K_{ii} values for the noncompetitive inhibition vs (*S*)-mercaptopropanediol gave dissociation constants of MgAMPPCP from complexes with free enzyme and (*E*)-(*S*)-mercaptopropanediol of 2.1 and 3.3 mM, respectively.

MgATPase Activity. The ability of glycerokinase to catalyze transfer of the γ-phosphate of MgATP to water in the absence of an accepter was measured both by ^{31}P NMR and by monitoring the production of MgADP with the coupled assay (Figure 3). The kinetic parameters were determined at pH 8.2 (K_m = 0.54 ± 0.04 mM, V_{max} 0.006% that of glycerol) and 9.35 (K_m = 2.5 ± 0.3 mM, V_{max} 0.005% that of glycerol).

Linearity of Reciprocal Plots for MgATP. Figure 4 shows reciprocal plots for MgATP in the presence of saturating (5.4 mM) glycerol. The plot is linear at pH 7.4 and 8.5 (not shown) but shows negative cooperativity at pH 5.7. A fit to eq 7 gave 3.4 ± 0.8 and 40 ± 10 μM as the Michaelis constants of the two phases. Reciprocal plots were linear for glycerol at all pH values from 6.5 to 9.5.

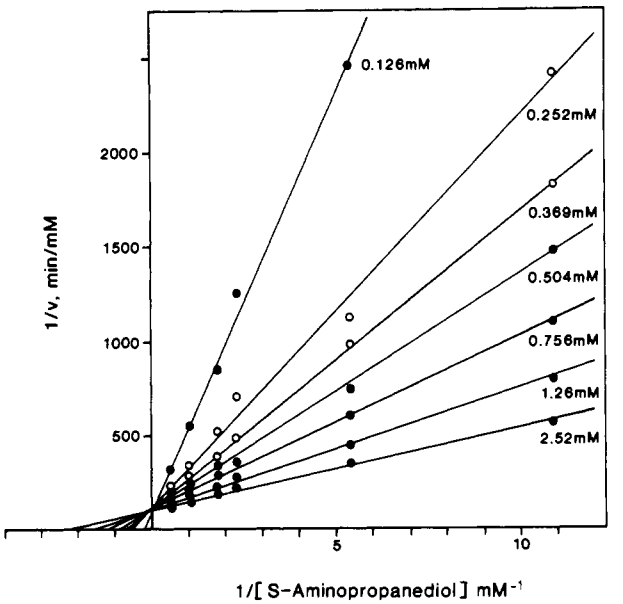

FIGURE 2: Equilibrium-ordered addition of MgATP (levels shown on lines) and (*S*)-1-aminopropanediol in 100 mM Ches, pH 9.4.

pH Profiles. The pH dependence of the phosphorylation of glycerol at 5 mM MgATP and as a function of MgATP at 5.4 mM glycerol was determined. A group that must be

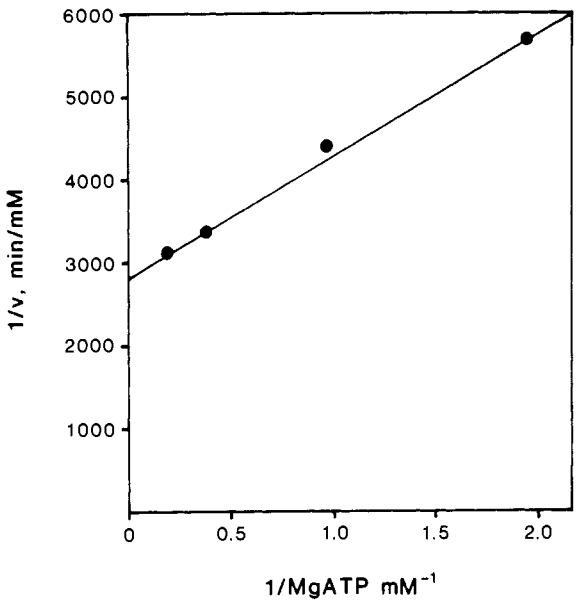

FIGURE 3: MgATPase activity of glycerokinase in the absence of an acceptor at pH 8.2 (100 mM Taps).

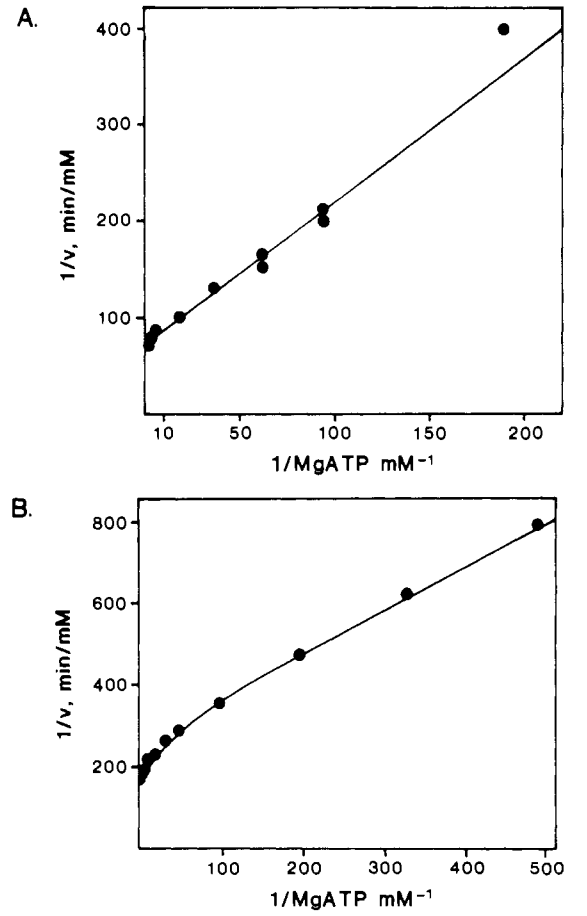

FIGURE 4: Glycerokinase displays normal Michaelis–Menton kinetics in 100 mM Hepes, pH 7.4 (A), but negative cooperativity in 100 mM Mes, pH 5.6 (B). Glycerol, 5.4 mM.

protonated for activity is seen in the V/K profiles for both substrates (pK 8.3 for V/K_{MgATP} and pK 9.1 for $V/K_{glycerol}$), but the V_{max} is pH independent. The pH profiles for the phosphorylation of (*S*)-mercaptopropanediol at 20 mM MgATP are in Figure 5 (similar results were obtained at 5 mM MgATP). In this case the pK of 9.1 of the group that must be protonated is seen in both V/K and V profiles. Below pH 6, V/K for mercaptopropanediol appears to drop to a new

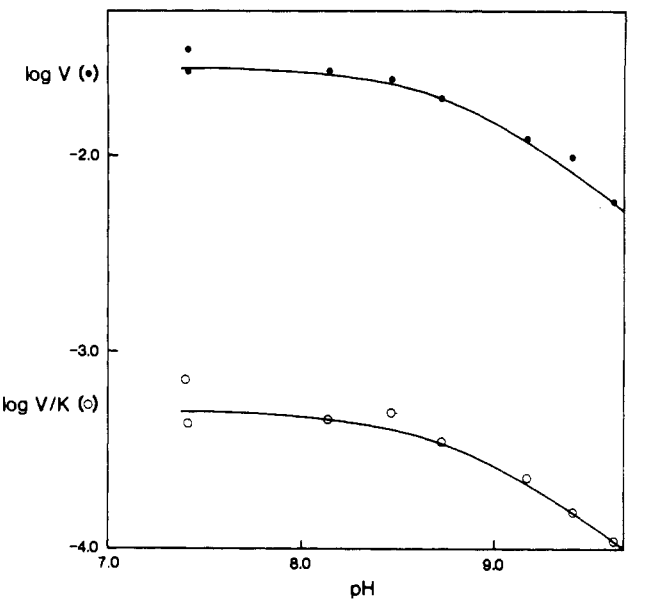

FIGURE 5: pH variation of V_{max} and V/K for the phosphorylation of (*S*)-mercaptopropanediol by 20 mM MgATP.

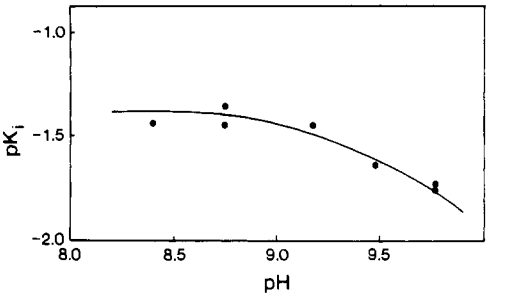

FIGURE 6: pK_i profile for (*R*)-mercaptopropanediol as a competitive inhibitor vs glycerol at 5 mM MgATP.

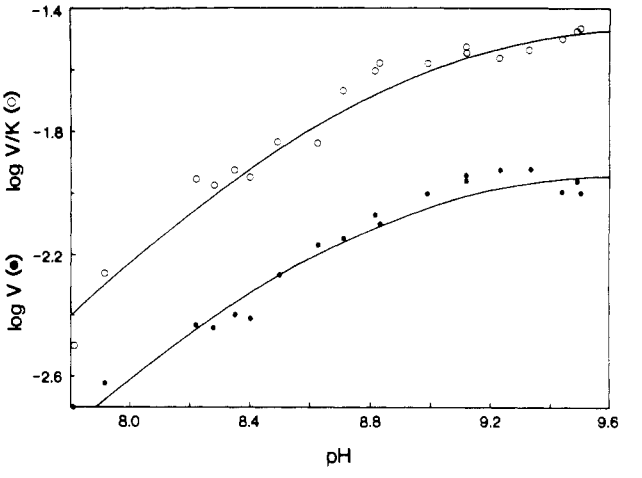

FIGURE 7: pH variation of V_{max} and V/K for the phosphorylation of (*S*)-aminopropanediol by 5 mM MgATP. Both profiles continue to drop below pH 7 (data not shown).

plateau level, and the pK did not appear to change appreciably in the presence of 30% dimethyl sulfoxide, although there was a decrease in the V/K value in the presence of solvent (data not shown).

Figure 6 shows the pK_i profile for (*R*)-mercaptopropanediol as a competitive inhibitor vs glycerol. The pK of 9.6 is presumably that of the thiol, showing that only the neutral molecule will bind. The pH profiles for phosphorylation of (*S*)-aminopropanediol are in Figure 7. The profiles for the *R* enantiomer are similar (not shown). The decrease in both

Table V: Effect of Viscosity on the Phosphorylation of Glycerol and (S)-Mercaptopropanediol[a]

fixed substrate	viscosogenic agent	V/K ratio[b]	V ratio[b]
75.5 mM (R)-mercaptopropanediol	24% sucrose	3.5	3.3
0.04 mM glycerol	24% sucrose	7.8	1.2
5.4 mM glycerol	24% sucrose	3.0	1.2
2.7 M glycerol	25% glycerol	4.7	0.9

[a] MgATP was varied from 0.12 to 1.2 mM with the thiol and from 0.005 to 0.2 mM with glycerol as acceptor. [b] Ratio of parameter at low viscosity to value at high viscosity.

profiles as the amine is protonated shows that while the protonated amine will bind, only the neutral amine permits phosphorylation.

Isotope Partitioning. These studies were conducted according to the procedure used by Viola et al. (1982) for yeast hexokinase. The concentration of glycerokinase was determined by the extent of CrATP binding (Danenberg & Cleland, 1975). We were able to trap 0.54 equiv of glycerol with 5 mM MgATP when the starting glycerol concentration was 0.33 mM. This is a minimal value, as this glycerol level was not sufficient to convert all of the enzyme into a binary complex.

Viscosity Dependence of Kinetic Parameters. The effects of viscosity with MgATP as the variable substrate are in Table V. Effects were observed on both V and V/K with (S)-mercaptopropanediol as substrate but only on V/K with glycerol.

Activity of E. coli Glycerokinase. At pH 8.2 with 5 mM MgATP and 12 mM $MgCl_2$, the K_m values were 10 ± 1.5 μM for glycerol and 46 ± 8 mM for (S)-mercaptopropanediol, while the relative V_{max} for the latter was 0.5% that of glycerol and the relative V/K value was $(1.15 \pm 0.1) \times 10^{-4}$%.

Discussion

Product analysis by ^{31}P NMR indicated that the thiol and amino analogues of glycerol were substrates for glycerokinase. (S)-Mercaptopropanediol was a relatively good substrate, being phosphorylated on the primary hydroxyl. The R enantiomer was phosphorylated on sulfur so slowly that reaction could be demonstrated only at very high levels of the analogue where its presence in the active site suppressed the MgATPase activity of the enzyme. Glycerokinase catalyzed the transfer of the phosphoryl group from (R)-1-mercaptopropanediol 1-phosphate to MgADP, but this also was a very slow reaction. Crans and Whitesides (1985) reported that (R,S)-mercaptopropanediol was a very good substrate for both the *C. myoderma* and *E. coli* enzymes. They reported that V_{max} was approximately the same as that for glycerol and the K_m was ~1 mM for both systems. This apparent discrepancy is probably the result of contamination by glycerol in the commercial preparations used without purification by these workers. For example, because of the low affinity for the thiol analogue, a 1% contaminant of glycerol would give an apparent K_m of 1.5 mM and the same V_{max} as for glycerol.

(R)-Aminopropanediol (which has the same configuration as the S enantiomer of the thiol analogue) was phosphorylated on oxygen, while (S)-aminopropanediol was phosphorylated on nitrogen to give an acid-labile phosphoramidate. Crans and Whitesides (1985) also reported much higher substrate activity for (R,S)-aminopropanediol than we observed, again presumably as the result of contaminating glycerol.

It is clear from the products produced that the secondary hydroxyl at C-2 controls the orientation of the substrate in the active site, so that the product has the L configuration at C-2 with the phosphate at C-3. Replacement of oxygen with nitrogen or sulfur at C-3 leads to great decreases in the rate of catalysis (250-fold for nitrogen; 33 000-fold for sulfur). Similar decreases in catalytic rate have been reported for amino (9000-fold; Sem et al., 1988) and thiol analogues of glucose with yeast hexokinase (5×10^5-fold; Knight et al., 1984) and for thioglycolate vs glycolate with pyruvate kinase (10^4-fold; Ash et al., 1984). This unwillingness to accept nitrogen or sulfur as the attacking nucleophile appears to be characteristic of kinases but not of all enzymes catalyzing phosphoryl transfer, since alkaline phosphatase hydrolyzes phosphorylated amines and thiols at the same or faster rates as phosphorylated alcohols and phosphoglucomutase catalyzes the interconversion of 6-mercaptoglucose 6-phosphate and 6-mercaptoglucose 1-phosphate at rates only slightly less than those of the natural substrates (Knight & Cleland, 1984). A basic difference in mechanism is probably involved (associative for kinases, dissociative for alkaline phosphatase and phosphoglucomutase?).

The hydroxyl group at C-1 is important both for substrate recognition and for catalysis, and its replacement with an amino or thiol group lowers both the rate of phosphorylation (140- or 30-fold, respectively) and the affinity. In fact, these substitutions cause a greater effect on affinity than do replacements at C-3. Thus the affinity of glycerokinase for the (R)-thiol analogue (hydroxyl at C-1) is 3-fold greater than for the S enantiomer (SH at C-1), and the affinity for the (S)-amino analogue (OH at C-1) is 30-fold greater than for the R isomer (NH_2 at C-1). Specific hydrogen-bond interactions appear to be responsible for these effects.

Kinetic Mechanism of Glycerokinase. Janson and Cleland (1974) concluded that the mechanism was ordered, with glycerol adding before MgATP. The uncompetitive inhibition of MgAMPPCP vs glycerol supports such a mechanism. However, the existence of a MgATPase activity which is suppressed by high levels of the very slowly phosphorylated (R)-mercaptopropanediol shows that glycerokinase, as does yeast hexokinase (Kaji & Colowick, 1965), forms a binary E–MgATP complex and catalyzes phosphoryl transfer to water, although at a very slow rate. In fact, the initial velocity patterns for the aminopropanediols appear equilibrium ordered with MgATP adding first. The initial velocity pattern for (S)-mercaptopropanediol is intersecting, however, and not equilibrium ordered. These data can all be reconciled by assuming a random mechanism for glycerokinase in which there is strong synergism in the binding of MgATP and either glycerol or aminopropanediol but less synergism with the thiol analogues. Thus the inhibitions of (R)-mercaptopropanediol vs MgATP at fixed glycerol and of MgAMPPCP vs (S)-mercaptopropanediol at fixed MgATP were noncompetitive, as expected in a random mechanism without strong synergism in the binding of reactants.

The ratio of $K_{i\,MgATP}/K_{MgATP}$ is a measure of synergism, since for slow substrates K_i is the dissociation constant from free enzyme and K_m is the value from the (E)-glycerol analogue complex. The values in Table III show that glycerol and the amino analogues are highly synergistic but that the thiol analogue is not. The fact that MgATP appears to bind before the amino analogues in equilibrium-ordered fashion is the result of tighter binding of MgATP (0.3 mM at pH 8.2) than of amino analogue to free enzyme (>3 mM), plus the highly synergistic binding. Glycerol, on the other hand, forms a tighter complex with free enzyme (<0.02 mM) than MgATP, so that it appears to add first in ordered fashion. The initial velocity pattern is intersecting, rather than equilibrium ordered,

because of the stickiness of glycerol (that is, its rate of release from its binary complex is not rapid with respect to V_{max}, as is the case with the slowly reacting analogues). The synergism occurs between the binding of glycerol and MgAMPPCP, as shown by the uncompetitive inhibition of the latter [that is, it appears to bind only to (*E*)-glycerol]. This type of random mechanism with synergism in binding has also been found for yeast hexokinase (Viola et al., 1982), and many ordered mechanisms may be of this type.

Stickiness of Glycerol. As noted above, glycerol is a sticky substrate. This was confirmed by an isotope trapping experiment in which 0.54 equiv of glycerol was trapped by MgATP at a glycerol concentration of 0.33 mM. Attempts to test the stickiness of MgATP from the ternary complex by examining the effect of viscosity on V_{max} and V/K for MgATP failed because of apparent effects of the viscosogenic agents on the enzyme (Table V). Since large effects were seen with the slow (*S*)-mercaptopropanediol analogue, one cannot use this method to determine the stickiness of MgATP with the fast substrate, glycerol.

pH Profiles. A group on the enzyme with a pK of 8.3 in the (*E*)-glycerol complex or 9.1 in the E–MgATP complex must be protonated for activity. The pK is perturbed out of the accessible pH range in the ternary complex, and thus, the V_{max} is pH independent for reaction of glycerol and MgATP. This group can be seen in the V_{max} profile for (*S*)-mercaptopropanediol, since its pK is not perturbed as dramatically in the ternary complex with this analogue. The pK is 9.1 in the V/K profile, since this profile determines the pK in the E–MgATP complex. This group appears to play a role in catalysis, since the binding of (*R*)-mercaptopropanediol is not sensitive to its ionization, and one sees only the pK of 9.6 of the thiol in the pK_i profile for this analogue. The lack of binding of the ionized thiol suggests that a group is present that accepts a hydrogen bond from the hydroxyl of glycerol in this position. If this group were a carboxyl, as in yeast hexokinase (Viola & Cleland, 1978), the failure of a negatively charged thiolate to bind is readily understood. 6-Mercaptoglucose does not bind to yeast hexokinase when it is ionized (Knight et al., 1984).

If the group that accepts the proton from the hydroxyl group during phosphorylation were a carboxyl, as in yeast hexokinase and liver fructokinase (Raushel & Cleland, 1977), one might expect a drop at low pH in the profiles as this group became protonated. An ionization was observed at low pH, but the effect was only partial, and the pK ($\sim$6.3) was not perturbed by organic solvents. Thus if a carboxylate is present in the active site, its pK is lower than the range examined in the present work.

The observation that both V/K and V_{max} decrease with pH when the amino analogues were the substrates shows that the free amine is the active form of the substrate but that the protonated amine will bind nonproductively. This is not surprising for the *S* enantiomer, where clearly only the neutral amine could be phosphorylated, but is more of a surprise for the *R* isomer, where the amino group is at C-1. Possibly the group at C-1 has to accept a hydrogen bond from a group on the enzyme in order for the conformation change to occur which permits catalysis, and this could not occur with a protonated amine. The group whose pK is 9.1 in E–MgATP and which has to be protonated for activity may be playing this role.

References

Ash, D. E., Goodhart, P. J., & Reed, G. H. (1984) *Arch. Biochem. Biophsy. 228*, 31.

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103.

Crans, D. C., & Whitesides, G. M. (1985) *J. Am. Chem. Soc. 107*, 7008.

Danenberg, K. D., & Cleland, W. W. (1975) *Biochemistry 14*, 28.

Dixon, J. S., & Lipkin, D. A. (1954) *Anal. Chem.* 1092.

Eisenthal, R., Harrison, R., Lloyd, W. J., & Taylor, N. F. (1972) *Biochem. J. 130*, 199.

Grimshaw, C. E., Whistler, R. L., & Cleland, W. W. (1979) *J. Am. Chem. Soc. 101*, 1521.

Janson, C. A., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2562.

Kaji, A., & Colowick, S. P. (1965) *J. Biol. Chem. 240*, 4454.

Knight, W. B., & Cleland, W. W. (1984) *Biochemistry 23*, 3347.

Knight, W. B., & Cleland, W. W. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 44*, 1056.

Knight, W. B., Rendina, A. R., & Cleland, W. W. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2011.

Raushel, F. M., & Cleland, W. W. (1977) *Biochemistry 16*, 2176.

Sem, D., Knight, W. B., & Cleland, W. W. (1988) *FASEB J. 2*, A997.

Thorner, J. W., & Paulus, H. (1973) *Enzymes (3rd Ed.) 8*, 487.

Viola, R. E., & Cleland, W. W. (1978) *Biochemistry 17*, 4111.

Viola, R. E., Raushel, F. M., Rendina, A. R., & Cleland, W. W. (1982) *Biochemistry 21*, 1295.