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Sulfuryl Transfer Catalyzed by Phosphokinases†

James A. Peliska‡ and Marion H. O'Leary*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, and Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68583
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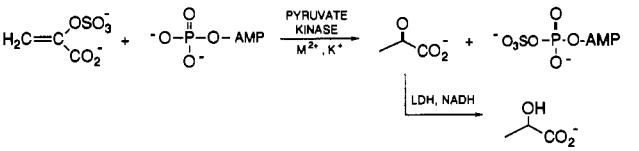
ABSTRACT: Adenosine 5′-sulfatopyrophosphate is a substrate for nucleoside diphosphate kinase. The reaction appears to proceed through a ping-pong mechanism analogous to the physiological reaction involving ATP, presumably by way of a sulfohistidine intermediate. Unlike the phosphoryl transfer reactions, the corresponding sulfuryl transfers catalyzed by nucleoside diphosphate kinase do not have a strict divalent metal requirement. The estimated rate constants for the metal- and nonmetal-catalyzed sulfuryl transfers differ by less than an order of magnitude and are approximately 1000-fold slower than the corresponding phosphate transfers. These results suggest that the role of the metal ion in nucleoside diphosphate kinase is to coordinate the α,β-phosphates of the substrate. Sulfuryl and phosphoryl transfer probably occur through dissociative transition states.

We recently showed that pyruvate kinase catalyzes the reaction between sulfoenolpyruvate, the sulfate analogue of PEP,[1] and ADP to form $ADPSO_3$ and pyruvate (Scheme I) (Peliska & O'Leary, 1989). This represents the first demonstrated case of a sulfuryl transfer catalyzed by a phosphokinase. We were interested in determining whether other phosphokinases can catalyze similar reactions and, if so, what factors might govern sulfuryl transfer mechanisms. For this reason, we have investigated the substrate and inhibition characteristics of $ADPSO_3$ with a variety of other phosphokinases.

The synthesis of $ADPSO_3$ was originally performed by Ikehara et al. (1961). Yount et al. (1966) determined that snake venom phosphodiesterase cleaves $ADPSO_3$ between the α- and β-phosphates. The compound is not a substrate for myosin or alkaline phosphatase. Perhaps these negative results prevented further investigation of $ADPSO_3$ as an analogue of ATP in enzyme-catalyzed reactions.

A large number of other ATP analogues have been studied as substrates for various enzymes (Yount, 1975). Enzymes can often tolerate modifications in the base portion of the nucleotide but not in the transferring phosphate. The thiophosphate analogues, used in studying the stereochemistry and mechanism of phosphoryl transfer (Eckstein, 1983), seem to be the only useful, catalytically active derivatives of the phosphate moiety.

Scheme I



In the present study, we examined the chemistry and enzymatic activity of $ADPSO_3$ with nucleoside diphosphate kinase and the inhibitory properties of this same substrate with various other kinases.

Materials and Methods

Materials. Sulfur trioxide pyridine complex (Aldrich), sodium pyruvate (Sigma), sodium formate (Mallinckrodt), sodium acetate (Mallinckrodt), disodium NADH (Sigma), disodium NAD (Sigma), dicyclohexylammonium ADP (Sigma), dicyclohexylammonium ATP (Sigma), sodium adenosine 5′-phosphosulfate, glucose (MC&B), and disodium fructose

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* Address correspondence to this author at the University of Nebraska.
‡ University of Wisconsin—Madison.

[1] Abbreviations: HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PEP, phosphoenolpyruvate; $ADPSO_3$, ADP sulfate; LDH, lactate dehydrogenase; APS, adenosine phosphosulfate; NDP kinase, nucleoside diphosphate kinase; NDP, nucleoside diphosphate.

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6-phosphate (Sigma) were used as supplied. HEPES (free acid) was purchased from U.S. Biochemical. EDTA (Aldrich Gold Label) was used as supplied, since washing EDTA stock solutions with dithizone in CCl_4 had no noticeable effect on the kinetics. Tris buffer was purchased from Boehringer Mannheim Biochemicals. DEAE-Sephadex A-25 (Cl^-) was purchased from Pharmacia and was cleaned with 1 M HCl and 1 M KCl and then equilibrated with water before use. Water was purified with a Millipore Super Q water purification system.

Pyruvate kinase (rabbit muscle), lactate dehydrogenase (rabbit muscle), hexokinase (yeast), fructose-6-phosphate kinase (rabbit muscle), acetate kinase (*Escherichia coli*), adenylate kinase (chicken muscle), and nucleoside diphosphate kinase (yeast) were purchased from Sigma Chemical Co. Pyruvate phosphate dikinase (*Clostridium symbiosum*) was a gift from Dr. George Reed.

Methods. ^{31}P NMR spectra were recorded at 109.35 MHz on a Bruker WP-270 NMR spectrometer. Chemical shifts are expressed relative to 85% H_3PO_4 as an external standard with the lock signal provided by D_2O; negative values are upfield from the standard.

UV spectra and kinetics were measured on a Cary 118 spectrophotometer with a thermostated cell holder at 25 ± 0.1 °C or by using a Pharmacia FPLC equipped with a Mono Q HR 5/5 column, UV-1 detector (A_{254}), and HP 3390A integrator. Concentrations of nucleotides were calculated from standard curves of peak area versus concentration. All FPLC runs were performed at a flow rate of 1.0 mL/min with a 21 mL, 0.05–0.4 M NaCl gradient in 20 mM Tris-HCl, pH 7.8. FPLC solvents were filtered through 0.2 μM filters and degassed before use.

Concentrations of metal–nucleotide complexes in reaction solutions were calculated on the basis of stability constants of 73 mM^{-1} for ATP (O'Sullivan & Smithers, 1979), 4 mM^{-1} for ADP (O'Sullivan & Smithers, 1979), and 0.042 mM^{-1} for $ADPSO_3$ (Yount et al., 1966), using the procedure described by O'Sullivan and Smithers (1979).

Total phosphate was determined by the method of Ames (1966), using the detection method of Bencini et al. (1983). Periodate oxidations for the detection of vicinal glycols were performed as described by Dixon and Lipkin (1954) by using a molar extinction coefficient of 10^4 at 222.5 nm, pH 6 (Crouthamel et al., 1949).

Enzyme rate data were analyzed by using the computer programs of Cleland (1979). Competitive inhibition patterns were fit to eq 1, and Michaelis–Menten kinetics were fit to eq 2.

$$v = VA/[K(1 + (I/K_i)) + A] \quad (1)$$

$$V = VA/(K + A) \quad (2)$$

Synthesis of Adenosine 5'-Sulfatopyrophosphate. The synthesis is a variation on that of Yount et al. (1966). A 6.0-g (0.06 mol) sample of $KHCO_3$ was added to a solution of 0.5 g of dicyclohexylammonium ADP (0.8 mmol) in 25 mL of water. The mixture was heated to 45 °C and then 3.8 g of sulfur trioxide–pyridine complex (0.024 mol) was added over 20 min. Carbon dioxide was rapidly evolved and the solution turned light brown. After an additional 20 min, the reaction mixture was diluted to 200 mL with ice-cold water and loaded onto a 3 × 26 cm column of DEAE-Sephadex A-25 (4 °C) at a flow rate of 1.5 mL/min. The column was rinsed free of pyridine with 200 mL of water followed by a 0–1 M gradient of LiCl (2-L total volume). Fractions were collected in 8-mL volumes. The fractions were assayed for nucleotides by their absorbance at 260 nm. The resulting elution profile showed two major peaks corresponding to ADP (fractions 90–110) and $ADPSO_3$ (fractions 156–173). The fractions containing the desired product were pooled and the volume was reduced under vacuum to approximately 10 mL. A 300-mL portion of 1:4 ethanol–acetone was then added and the white precipitate collected by centrifugation. The solid was washed with acetone and ether. Residual solvent was removed under vacuum to yield 0.10 g of the lithium salt of $ADPSO_3$. The product showed the expected ratio of phosphate, sugar, and adenosine. In addition, the material displayed an identical ^{31}P NMR spectrum and Mono Q elution pattern to that produced by the action of pyruvate kinase on ADP and sulfoenolpyruvate (retention time = 17.0 min) (Peliska & O'Leary, 1989). Acid hydrolysis gave ADP as the sole nucleotide product.

Preparation of ADP-Free Adenosine 5'-Sulfatopyrophosphate. The preparation described above generally resulted in the generation of approximately 10% ADP due to hydrolysis during workup. For some enzymological work, $ADPSO_3$ samples substantially free of ADP were required. A solution of lithium $ADPSO_3$ (3–100 mM) containing contaminating ADP was incubated with 0.4–24 μg of alkaline phosphatase at room temperature in 20 mM glycine buffer, pH 10.5. Samples of the reaction mixture were removed at intervals and the rection progress was monitored by FPLC. After approximately 1 h, there was a marked decrease in the concentration of ADP and a simultaneous increase in adenosine because of the cleavage of the phosphate linkages by alkaline phosphatase. The resulting solution was freed of protein by using a UNISEP Minicent-30 ultrafilter to give a solution showing less than 1% ADP.

Hydrolysis of Adenosine 5'-Sulfatopyrophosphate. The rate of hydrolysis of $ADPSO_3$ was studied at 50 ± 0.1 °C. The buffers utilized were HCl (pH 1.0–2.0), formate (pH 3.0), acetate (pH 4.0–5.0), HEPES (pH 8.2), and K_2CO_3 (pH 10.0). All buffers were adjusted to ionic strength 0.1 M with KCl. Buffer pH was adjusted at 50 °C. All kinetic runs were carried out with 0.8 mM $ADPSO_3$ in sealed Eppendorf microfuge tubes. Portions were removed at intervals and quenched with $NaHCO_3$ at 0 °C, and the reaction was assayed by following the disappearance of $ADPSO_3$ on a Mono Q HR 5/5 ion-exchange column. First-order rate constants were derived from semilog plots of $ADPSO_3$ peak area versus time and by least-squares analysis of the linear relationship between ln $[ADPSO_3]_t$ and time. Infinity points were measured after at least 10 half-lives and were always found to be zero.

Enzyme Studies. All solutions were prepared fresh for each experiment; enzyme solutions were kept on ice and were generally stabilized by the addition of 1–2% bovine serum albumin. All kinetic studies except for those involving nucleoside diphosphate kinase were run in 100-μL total volumes in microfuge tubes. Reactions were stopped by the addition of 10 μL of 1 M $HClO_4$ or a 2-fold excess of EDTA over metal. Quenched solutions were centrifuged and then chromatographed on a Mono Q HR 5/5 column and nucleotide concentrations were determined from peak area calibration curves. In the case of coupled assays containing NADH, the reaction was monitored by the appearance of NAD. All other reactions were monitored by the appearance of ADP. This technique allowed reproducibility within ±1–2%. The effectiveness of each quench technique was determined by allowing a quenched solution to stand at room temperature for at least 40 min, followed by the chromatographic assay. To assure that the rates measured for reactions were initial velocities,

the linearity of the assay was assured by generating progress curves consisting of at least five data points. Accurate initial velocities could generally be determined if the reaction was limited to less than 10% substrate consumption. Enzyme activity was monitored throughout each experiment.

Hexokinase Kinetics. Reactions were carried out in 40 mM HEPES, pH 7.6, containing 200 mM glucose, 1 mM NADH, 60 mM KCl, 11 mM $MgCl_2$, 10 units of LDH, and 10 units of pyruvate kinase. Reactions were initiated by the addition of hexokinase.

For $ADPSO_3$ substrate activity experiments, the solution contained 10 mM $MgCl_2$ or 4 mM $MnCl_2$, 200 mM glucose, 20 mM $ADPSO_3$, and approximately 20 mg/mL hexokinase. At intervals up to 1.5 h, samples were withdrawn and assayed for ADP production by FPLC.

Phosphofructokinase Kinetics. Reactions were carried out in the presence of 50 mM HEPES, pH 7.8, 8 mM $MgCl_2$, 1 mM NADH, 2 mM PEP, 2 mM fructose 6-phosphate, 50 mM KCl, 10 units of pyruvate kinase, and 20 units of LDH. $ADPSO_3$ substrate activity studies were conducted with 3 mM fructose 6-phosphate, 10 mM Mg^{2+} or 4 mM Co^{2+}, and 4 mM $ADPSO_3$. At time intervals up to 1 h, samples were withdrawn and assayed for ADP production by FPLC.

Acetate Kinase Kinetics. Reactions were carried out in the presence of 50 mM HEPES buffer, pH 7.5, 350 mM acetate, 1 mM PEP, 1 mM NADH, 10 mM $MgCl_2$, 20 units of pyruvate kinase, and 20 units of LDH.

Pyruvate Phosphate Dikinase Kinetics. $ADPSO_3$ was tested as a substrate for pyruvate phosphate dikinase in the presence of 100 mM HEPES, pH 7.5, 40 mM NH_4Cl, 5 mM $MgCl_2$, 10 mM pyruvate, and 10 mM $ADPSO_3$. The reaction was initiated by the addition of 10 units of enzyme. At various time intervals, reaction samples were removed and assayed for ADP production by FPLC.

Adenylate Kinase Kinetics. $ADPSO_3$ was tested as a substrate for adenylate kinase in the presence of 100 mM HEPES, pH 7.5, 4 mM $MnCl_2$, 12 mM AMP, 33 mM $ADPSO_3$, and 2.5 mg of enzyme/mL at 37 °C. At various time intervals, reaction samples were removed and assayed for ADP production by FPLC.

Nucleoside Diphosphate Kinase Kinetics. The kinetics of nucleoside diphosphate kinase were monitored at 340 nm by coupling the formation of ADP to pyruvate kinase in the presence of LDH and NADH to give a continuous assay of pyruvate formation. Assay solutions contained 100 mM HEPES, pH 8.0, 1.5 mM PEP, 0.2 mM NADH, 50 mM KCl, 0.47 mM dTDP, 3.13 mM ATP, 5 mM Mg^{2+}, and excess pyruvate kinase and LDH in 1-mL total volume. Reactions were initiated by the addition of enzyme as described below. dTDP does not interfere with the coupled assay since it is a very poor substrate for pyruvate kinase (Agarwal & Parks, 1971).

The kinetics of the sulfation of free enzyme with $ADPSO_3$ were studied by varying $ADPSO_3$ concentration at various metal concentrations by using a fixed-time assay. Reaction mixtures containing metal, enzyme, and $ADPSO_3$ were injected into assay mixtures containing ATP and dTDP at various intervals and the $[ENZ]_{free}/[ENZ]_{tot}$ was calculated on the basis of eq 4. The observed first-order rate constants, k_{obs}, were derived by least-squares analysis of the linear relationship between ln $([ENZ]_{free}/[ENZ]_{tot})$ and time. Reaction mixtures without added metals included 5 mM EDTA.

Isolation of Sulfoenzyme. A reaction mixture containing 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 2 mM $ADPSO_3$, and approximately 0.1 mg of NDP kinase in 0.1-mL total volume was incubated for 30 min at room temperature after which 1 μL was removed, diluted 100-fold, and assayed as above. The remaining sample was injected onto a Pharmacia Sephadex superfine, high-resolution (Fast Desalting) column and eluted with 20 mM Tris-HCl, pH 7.8, at a flow rate of 1 mL/min. Absorbance at 280 nm was monitored and 2-mL fractions were collected. Fractions containing enzyme activity were pooled and the volume was reduced to approximately 50 μL by using a Bio-Rad Unisep Ultracent-30 cartridge. This sample was reinjected onto the same column and eluted as before. The resolved peak containing enzyme was collected for kinetic analysis.

Derivation of Rate Equations. Incubation of nucleoside diphosphate kinase with a slow substrate, such as $ADPSO_3$, should result in the generation of an enzyme–SO_3 intermediate if the second substrate (dTDP) is absent and the enzyme follows ping-pong kinetics. If this completely sulfated enzyme ($[ENZ]_{tot} = [ENZ\text{–}SO_3]_0$) is mixed with ATP and dTDP, the following scheme will result:

first turnover:

$$ENZ\text{–}SO_3 + dTDP \xrightarrow[k_1]{} dTDPSO_3 + ENZ$$

subsequent turnovers:

$$ATP + dTDP \xrightarrow[k_2]{ENZ} ADP + dTTP$$

where k_1 and k_2 represent the first-order enzymatic rate constants for each step. The rate law for free enzyme (ENZ) formation will be

$$d[ENZ\text{–}SO_3]/dt = -k_1[ENZ\text{–}SO_3]$$

The integrated rate equation takes the form

$$[ENZ\text{–}SO_3]_t/[ENZ\text{–}SO_3]_0 = e^{-k_1 t}$$

Since $[ENZ\text{–}SO_3]_{tot} = [ENZ\text{–}SO_3]_0$ and $[ENZ\text{–}SO_3]_t = [ENZ]_{tot} - [ENZ]_t$

$$[ENZ]_t = [ENZ]_{tot}\{1 - e^{-k_1 t}\}$$

where $[ENZ\text{–}SO_3]_t$, $[ENZ\text{–}SO_3]_0$, and $[ENZ]_{tot}$ represent the concentrations of sulfated enzyme at any given time, t, the initial concentration of sulfated enzyme, and the concentration of free enzyme, respectively.

The rate of ADP formation therefore becomes

$$d[ADP]/dt = k_2[ENZ]_t = k_2([ENZ]_{tot}\{1 - e^{-k_1 t}\})$$

Integration of this expression gives the rate equation describing the concentration of ADP at any given time, t:

$$[ADP]_t = k_2[ENZ]_{tot}\{t + 1/k_1 e^{-k_1 t} - 1/k_1\} \quad (3)$$

Note that $k_2[ENZ]_{tot}$ is V_{max} of the enzymatic reaction of ATP and dTDP in the absence of $ADPSO_3$.

Under the situation when all the initial enzyme is not sulfated, $[ENZ]_{tot} \neq [ENZ\text{–}SO_3]_0$, and the following rate equation can be derived in a similar fashion:

$$[ADP]_t = k_2[ENZ]_{tot}t + k_2[ENZ\text{–}SO_3]_0\{1/k_1 e^{-k_1 t} - 1/k_1\} \quad (4)$$

Determination of the Equilibrium Constant of NDP Kinase Sulfation by $ADPSO_3$. The equilibrium constant for sulfation of NDP kinase by $ADPSO_3$ was determined at three different concentrations of ADP. A reaction mixture containing 100 mM HEPES, pH 7.6, 0.25 mM $ADPSO_3$, 5 mM $MgCl_2$, 5 μg/mL NDP kinase, and 0, 0.05, 0.125, or 0.250 mM ADP was incubated for 30 min at 24 °C. Each mixture was assayed by the coupled pyruvate kinase assay. The resulting absorbance curve was fit to eq 4, from which $[ENZ\text{–}SO_3]/[ENZ]$,

and thus the equilibrium constant could be calculated:

$$K_{eq} = [ADP][ENZ\text{–}SO_3]/[ADPSO_3][ENZ] \quad (5)$$

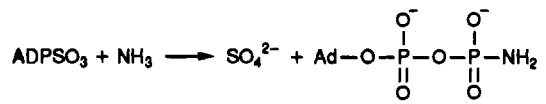
RESULTS

Synthesis of $ADPSO_3$. $ADPSO_3$ was synthesized in one step by the reaction of ADP with SO_3–pyridine in an aqueous bicarbonate solution according to the method of Yount et al. (1966). The sample was identical by ^{31}P NMR and by HPLC with material generated by the action of pyruvate kinase on sulfoenolpyruvate and ADP (Peliska & O'Leary, 1989). The final product contained approximately 1% of what is presumably $AMPSO_3$ resulting from the sulfation of AMP present in the reaction mixture due to ADP hydrolysis. Most of the $AMPSO_3$ present in the reaction mixture could be eliminated by DEAE-Sephadex chromatography, but peak tailing prevented its complete removal.

Yount et al. (1966) reported that this synthetic procedure produced a mixture of α-ADP sulfate, with the sulfate linkage at the α-phosphate of ADP, and β-ADP sulfate, with the sulfate linkage at the β-phosphate of ADP. We found no evidence of two isomeric forms using this procedure. ^{31}P NMR showed only β-$ADPSO_3$ (Peliska & O'Leary, 1989). In syntheses in which significant amounts of contaminating AMP were present in the ADP, $AMPSO_3$ was detected by both high-resolution anion-exchange chromatography and ^{31}P NMR. This could be the actual structure of the second component observed by Yount et al. (1966).

The $ADPSO_3$ prepared as above generally contained approximately 5–10% ADP due to sample hydrolysis. For determination of equilibrium constants, it was necessary to have a sample of $ADPSO_3$ free of contaminating ADP. This was achieved by converting the ADP to adenosine by using alkaline phosphatase, which produced material containing less than 1% ADP. Consistent with earlier findings (Yount et al., 1966), it was determined that $ADPSO_3$ is not a substrate for alkaline phosphatase, while the phosphate groups of ADP are easily cleaved to give adenosine.

Originally, the DEAE-Sephadex elution was performed by using an aqueous NH_4HCO_3 gradient. Fractions were collected and the NH_4HCO_3 removed under reduced pressure to afford the ammonium salt of $ADPSO_3$. This procedure repeatedly produced ADP due to hydrolysis, and a significant amount (10–20%) of a decomposition product with elution characteristics markedly different from those of $ADPSO_3$ on a Mono-Q anion-exchange resin (RT = 4.9 min). On the basis of similar studies with the synthesis of adenosine 5′-phosphosulfate (Cooper et al., 1979), this product is tentatively identified as the β-phosphoramidate of ADP resulting from the reaction of $ADPSO_3$ with ammonia:



Use of a LiCl gradient followed by precipitation of the lithium salt of $ADPSO_3$ eliminated this side reaction.

Hydrolysis of $ADPSO_3$. The hydrolysis of $ADPSO_3$ at 50 °C was examined as a function of pH. The results are shown in Figure 1. The reactions followed pseudo-first-order kinetics for at least three half-lives. The data were fit to the rate law

$$k_{obs} = k_{H^+}[H^+] + k_0 \quad (6)$$

The rate constants were found to be $k_{H^+} = 0.15\ s^{-1}\ M^{-1}$ and $k_0 = 7 \times 10^{-7}\ s^{-1}$.

Kinase Inhibition by $ADPSO_3$. $ADPSO_3$ was found to be a competitive inhibitor against ATP for the enzymatic reactions catalyzed by hexokinase, phosphofructokinase, and acetate kinase with Mg^{2+} as the metal cofactor. The inhibition constants are given in Table I. Two different binding constants were calculated: one based on the total $ADPSO_3$ present and one based on the concentration of $MgADPSO_3$. Each value represents an upper limit value due to the ambiguity in the inhibitory substrate form, as shown below.

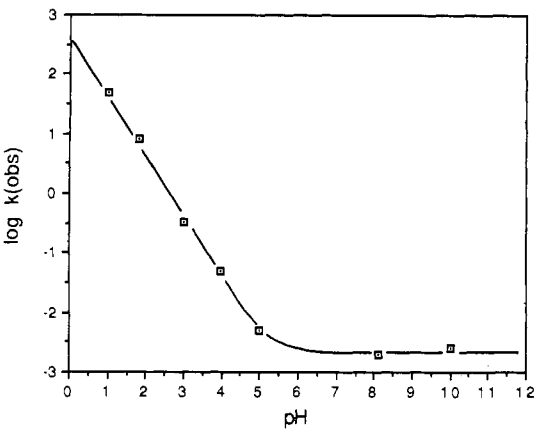


FIGURE 1: pH–rate profile for the first-order hydrolysis of $ADPSO_3$ at 50 °C, ionic strength 0.1. The rate constants (k_{obs}) have units of min^{-1}. The data were fit to eq 6, where $k_{H^+} = 0.15\ s^{-1}\ M^{-1}$ and $k_0 = 7 \times 10^{-7}\ s^{-1}$. No general acid/base catalysis was observed.

Table I: Inhibition of Kinase Reactions by $ADPSO_3$ in the Presence of Mg^{2+} [a]

enzyme	K_m MgATP	K_i $ADPSO_3$	K_i $ADPSO_3$·Mg
hexokinase[b]	0.17 ± 0.02	0.41 ± 0.04	0.15 ± 0.01
F-6-P kinase[c]	0.025 ± 0.004	0.31 ± 0.04	0.073 ± 0.011
acetate kinase[d]	0.30 ± 0.03	0.58 ± 0.06	0.15 ± 0.02

[a] Values of K_i for $ADPSO_3$ were calculated on the basis of the concentrations of both total $ADPSO_3$ and the metal complex $ADPSO_3$·Mg. Reactions were coupled to pyruvate kinase and lactate dehydrogenase at 24 °C and assayed by FPLC. [b] At pH 7.6 in the presence of 11 mM Mg^{2+} and 200 mM glucose. [c] At pH 7.8 in the presence of 8 mM Mg^{2+} and 2 mM fructose 6-phosphate. [d] At pH 7.5 in the presence of 350 mM acetate and 10 mM Mg^{2+}.

The metal dependency of enzyme inhibition by $ADPSO_3$ with hexokinase was examined to determine whether free $ADPSO_3$ or the metal complex $ADPSO_3$·Mg is the inhibiting species. In these experiments, the total concentration of $MgCl_2$ was varied at constant $ADPSO_3$ concentration. After correcting for the inhibitory effects of Mg^{2+}, the results show that both free $ADPSO_3$ and its metal complex inhibit, with a strong dependence on metal concentration (Figure 2). The plot begins to break at high metal concentration as the dissociation constant of the complex (25 mM; Yount et al., 1966) is approached. These data indicate that the metal complex binds approximately 6.5 times as tightly to the enzyme as does the free nucleotide.

Substrate Activity of $ADPSO_3$. $ADPSO_3$ shows no detectable substrate activity for hexokinase, phosphofructokinase, acetate kinase, or pyruvate phosphate dikinase. Pyruvate phosphate dikinase follows a ping-pong mechanism via an enzyme–pyrophosphate intermediate. The possibility that $ADPSO_3$ undergoes the first half-reaction, forming an enzyme–phosphosulfate intermediate, but not the second half-reaction was examined by looking for time-dependent inactivation of the enzyme due to the formation of an enzyme–phosphosulfate adduct. No inactivation was observed, suggesting that $ADPSO_3$ does not undergo this reaction.

When a large amount of adenylate kinase was incubated in the presence of $ADPSO_3$, AMP, and $MnCl_2$ at 37 °C, a stoichiometric amount of ADP and a second nucleotide product

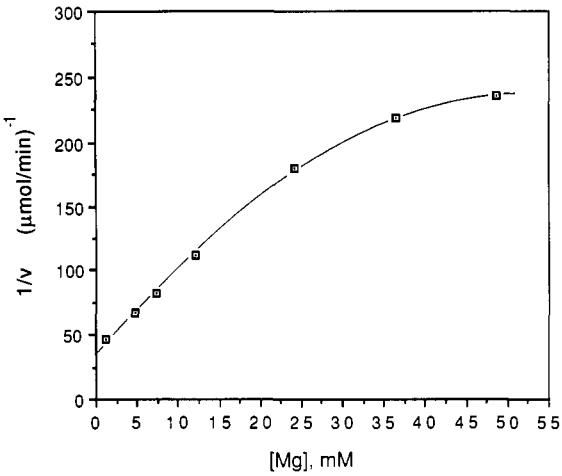


FIGURE 2: Mg^{2+} concentration dependence of hexokinase inhibition by $ADPSO_3$ in the presence of the indicated concentrations of $MgCl_2$. The data are corrected for inhibition by high concentrations of Mg^{2+} by subtracting the control rate in the absence of $ADPSO_3$ from the rate in the presence of 1 mM $ADPSO_3$.

were detected on high-resolution anion-exchange chromatography. The second product coelutes with authentic adenosine 5′-phosphosulfate. This result suggests that adenylate kinase can catalyze the transfer of a sulfuryl group from $ADPSO_3$ to AMP to form ADP and adenosine 5′-phosphosulfate:

$$ADPSO_3 + AMP \rightleftharpoons ADP + AMP\text{-}SO_3$$

The reaction rate was estimated to be at least 10^5–10^6-fold slower than the corresponding reaction between ATP and AMP under similar conditions. No further kinetics were performed due to the very slow reaction rates observed.

Nucleoside Diphosphate Kinase. When NDP kinase was incubated with $ADPSO_3$ in the presence of GDP, ADP and $GDPSO_3$ were detected by high-resolution anion-exchange chromatography. It is interesting that phosphosulfates such as $ADPSO_3$ (RT = 17.0 min) and $GDPSO_3$ (RT = 17.9 min) elute well after ATP (RT = 11.3 min) and GTP (RT = 11.8 min) on anion exchangers even though they have one less negative charge. Similar results have been seen with other sulfuryl adenylates such as adenosine 5′-phosphosulfate, which elutes well after AMP on a Dowex-1 anion-exchange resin (Robbins, 1963).

Authentic $GDPSO_3$ was synthesized by the same method as $ADPSO_3$ and was identified by its ^{31}P NMR spectrum. The phosphorus chemical shifts and coupling constants of $GDPSO_3$ are quite similar to those of $ADPSO_3$, and the α- and β-phosphates were assigned by analogy. A summary of ^{31}P NMR chemical shifts and coupling constants is given in Table II. These data indicate that NDP kinase catalyzes the transfer of a sulfuryl group from $ADPSO_3$ to nucleoside diphosphates.

Desulfation of Nucleoside Diphosphate Kinase. NDP kinase normally transfers a phosphoryl group via a ping-pong mechanism:

$$ATP + ENZ \rightleftharpoons ADP + ENZ\text{-}PO_3$$

$$NDP + ENZ\text{-}PO_3 \rightleftharpoons NTP + ENZ$$

Initial experiments with $ADPSO_3$ indicated that sulfuryl transfer was at least 1000 times slower than phosphoryl transfer. If NDP kinase catalyzes transfers of a sulfuryl group of $ADPSO_3$ via the same mechanism an enzyme–SO_3 intermediate should be easily detected by using kinetic techniques. NDP kinase was incubated with $ADPSO_3$ and Mg^{2+} in the absence of NDP, and this solution was then injected into an assay solution containing ATP, dTDP, and Mg^{2+}. The ADP formed was coupled to pyruvate kinase and lactate dehydrogenase. The resulting plot of absorbance versus time showed a pronounced lag followed by a steady-state rate identical with the rate obtained with native enzyme (Figure 3). The lag represents the first-order desulfation of the enzyme–sulfuryl group to generate free enzyme, which then undergoes reaction with ATP and dTDP (see the Materials and Methods section for the derivation of rate equations). The initial slope of the curve is essentially zero, indicating that originally all the enzyme is present in its sulfated form. The sulfation rate constant k_1 was derived by fitting the experi-

Table II: ^{31}P NMR Chemical Shifts and Coupling Constants for Nucleotide Phosphates and Sulfates in D_2O[a]

nucleotide	phosphate	chemical shift (ppm)	J_{P-P} (Hz)
ADP[b]	α	−9.9	
	β	−5.4	21.9
ATP[b]	α	−10.2	
	β	−20.6	19.1[d]
	γ	−4.9	
$ADPSO_3$[b]	α	−10.8	
	β	−21.8	20.0
GDP[c]	α	−9.4	
	β	−4.9	20.0
$GDPSO_3$[c]	α	−10.5	
	β	−21.3	18.1

[a] All chemical shifts are expressed relative to 85% H_3PO_4 as an external standard with the lock signal provided by D_2O; negative values are upfield from the standard. [b] Determined at 202.4 MHz in 0.05 M HEPES buffer, pH 8, in 10% D_2O–H_2O. [c] Determined in D_2O–HCO_3^-, pH 7.5, at 109.4 MHz. [d] $J_{\alpha\beta} = J_{\beta\gamma}$.

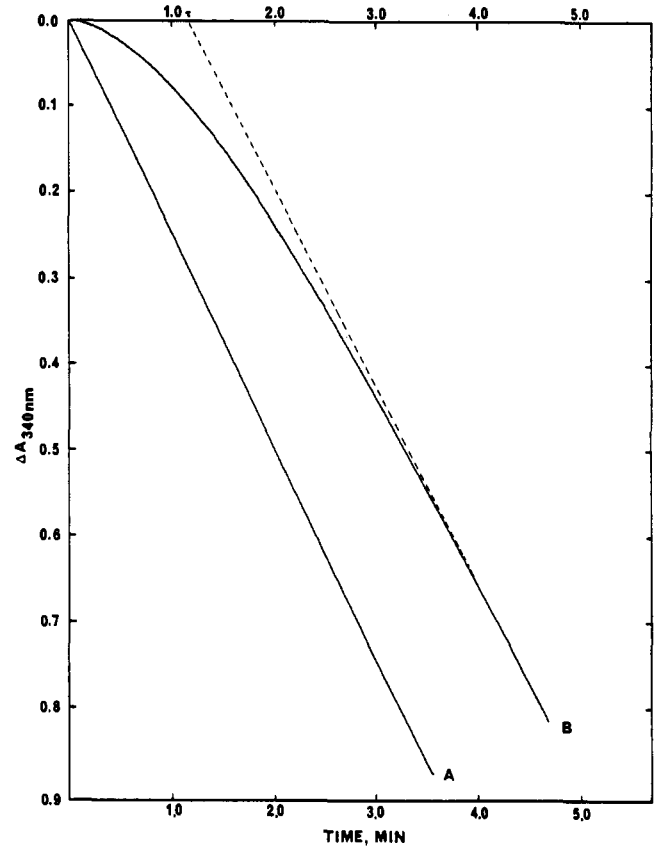


FIGURE 3: Kinetic evidence for the intermediacy of a sulfoenzyme intermediate in the reaction of $ADPSO_3$ and nucleoside diphosphate kinase. A 0.1-mL reaction mixture containing 100 mM HEPES, pH 8.0, 2 mM $ADPSO_3$, 10 mM $MgCl_2$, and 8 units of NDP kinase was equilibrated for 15 min at room temperature, after which a 5-μL sample was injected into an assay mixture containing 0.2 mM NADH, 1.5 mM PEP, 50 mM KCl, 0.47 mM dTDP, 3.13 mM ATP, 20 units pyruvate kinase, and 20 units LDH at 25 °C. The reaction mixture in curve A contained no $ADPSO_3$ and represents the steady-state rate in the absence of a sulfoenzyme intermediate.

Table III: Kinetic Parameters for the Interaction of Nucleoside Diphosphate Kinase with $ADPSO_3$[a]

half-reaction	metal	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
sulfation	none	0.45	4.1	9.1
	Mg^{2+}[c]	0.25	20.0	80.0
desulfation[b]	Mg^{2+}	ND	0.83	ND

[a] At pH 8.0, 25 °C. Reactions are coupled to pyruvate kinase and lactate dehydrogenase in the presence of dTDP, PEP, K^+, and NADH. ND, not determined. [b] This should be considered a lower limit since it was calculated that the assay contained 13% of nonmetal-complexed dTDP, which should also be a substrate for the reaction. [c] Estimated values.

mental data to eq 3. Resulting rate constants are given in Table III.

If the enzyme is not completely sulfated at the time it is added to the ATP/dTDP assay mixture, the initial slope will not be zero and the lag will be smaller (though the rate constant will be the same), to a degree depending on the extent of sulfation, as shown by eq 4.

When the sulfoenzyme was incubated with AMP in the presence of Mg^{2+}, no formation of free enzyme was observed, indicating that this half-reaction is specific for nucleoside diphosphates.

Enzyme Sulfation Kinetics and Metal Dependency. The enzyme sulfation half-reaction was studied by varying the $ADPSO_3$ concentration at fixed concentrations of $MgCl_2$ and assaying the reaction mixture at intervals to determine the degree of enzyme sulfation by means of eq 4. First-order kinetics were observed in all cases and the reciprocal plots of these first-order rate constants, $1/k_{obs}$, verses $1/[ADPSO_3]$ are linear. The reaction rate varied with Mg^{2+} concentration, but reaction was observed even in the absence of divalent metal. Thus, the sulfate transfer must occur by both metal-ion-dependent and metal-ion-independent pathways.

The following scheme is proposed to explain these results:

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_s} E\text{–}SO_3$$

$$E + S_m \overset{K_{sm}}{\rightleftharpoons} E{\cdot}S_m \xrightarrow{k_{sm}} E\text{–}SO_3$$

where S = $ADPSO_3$, S_m = $ADPSO_3{\cdot}Mg$ and E = enzyme.

The rate equation for this mechanism takes the form

$$v = \frac{k_s[S]/K_s + k_{sm}[S_m]/K_{sm}}{1 + [S]/K_s + [S_m]/K_{sm}} \quad (7)$$

Under the conditions used, $[Mg^{2+}] \gg [S]$; thus, $[S_m] = c[S]$, and eq 7 becomes

$$v = \frac{k_s/K_s + k_{sm}c/K_{sm}}{1/[S] + 1/K_s + c/K_{sm}} \quad (8)$$

Taking the reciprocal gives a linear equation in $1/[S]$:

$$1/v = 1/[S]\{1/(k_s/K_s + k_{sm}c/K_{sm})\} + \{(1/K_s + c/K_{sm})/(k_s/K_s + k_{sm}c/K_{sm})\} \quad (9)$$

Therefore, the slope of each line fits the equation

$$1/(\text{slope}) = c(k_{sm}/K_{sm}) + (k_s/K_s) \quad (10)$$

and if 1/slope is plotted against c = $[ADPSO_3{\cdot}Mg]/[ADPSO_3]$ (calculated from a stability constant of 0.04 mM^{-1}), the resulting slope represents k_{sm}/K_{sm} for the metal-activated sulfuryl transfer. The experimental data were plotted in this way, and the resulting curve was linear as expected. It is important to note that the slope replot does not pass through the origin, as would be required if Mg^{2+} were required for the reaction. Therefore, the lack of a metal dependence appears not to be due to the presence of a small amount of contaminating metal in the reaction mixture but to be a true characteristic of the reaction.

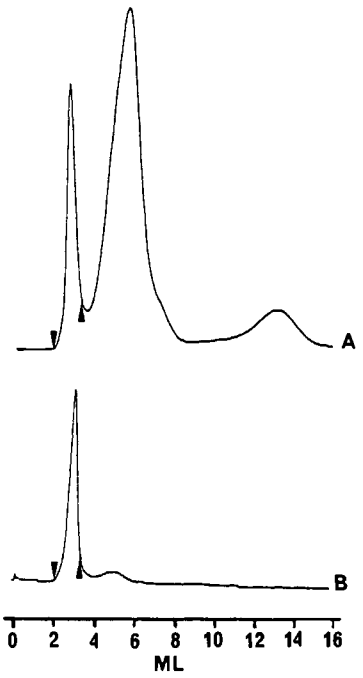


FIGURE 4: Separation of sulfated nucleoside diphosphate kinase from $ADPSO_3$ on high-resolution Sephadex superfine. A 0.1-mL reaction mixture containing 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 2 mM $ADPSO_3$, and approximately 0.1 mg of enzyme was incubated for 30 min at room temperature, then loaded onto a Pharmacia fast desalting column, and eluted with 20 mM Tris-HCl, pH 7.8, at a flow rate of 1 mL/min. (A) Elution profile after the first desalting column. The enzyme collected (indicated by arrows) was reduced by ultrafiltration and rechromatographed on the same column (B). The final enzyme fraction collected is shown by the arrows in graph B. Ordinate scales (A_{254}) are not the same in the two chromatographics.

The addition of ATP to the reaction mixture completely protected the enzyme from sulfation by $ADPSO_3$, indicating that enzyme sulfation occurs at the same active site as phosphorylation. Rate constants for the various sulfate transfer reactions are summarized in Table III.

Determination of the Sulfation Equilibrium Constant. The equilibrium constant for the sulfation of NDP kinase by $ADPSO_3$ was determined at three different ADP concentrations. The kinetic results were fitted to eq 4, from which the initial ratio of $[ENZ\text{–}SO_3]/[ENZ]$ could be determined. The calculated equilibrium constants for metal-independent and metal-catalyzed reactions are

$$K_{eq} = [ADP][ENZ\text{–}SO_3]/[ADPSO_3][ENZ] = 0.116 \pm 0.005$$

$$K_{eq} = [MgADP][ENZ\text{–}SO_3]/[MgADPSO_3][ENZ] = 11.5 \pm 0.6$$

Isolation of Sulfated Enzyme. It has been shown that the phosphoenzyme intermediate resulting from the phosphorylation of NDP kinase by ATP can be isolated and characterized (Parks & Agarwal, 1973). A similar technique was used to isolate the sulfoenzyme intermediate generated by the action of NDP kinase on $ADPSO_3$. Sulfated enzyme was applied to a high-resolution Sephadex column, collected, and reapplied to the same column. The results are shown in Figure 4. The first peak shows enzyme activity. The second peak is $ADPSO_3$. After two successive runs, sulfated enzyme free of $ADPSO_3$ was obtained (Figure 4B). The desulfurylation kinetics of the isolated sample were identical with those of the

initial enzyme, showing that the sulfoenzyme is both a chemically and kinetically competent intermediate.

Discussion

When $ADPSO_3$ is treated with nucleoside diphosphate kinase in the presence of GDP, a new nucleotide, $GDPSO_3$, is formed stoichiometrically with ADP:

$$ADPSO_3 + GDP \underset{\text{kinase}}{\overset{\text{NDP}}{\rightleftharpoons}} ADP + GDPSO_3$$

The products of the reaction, ADP and $GDPSO_3$, were identified by their chromatographic properties. $GDPSO_3$ was also identified by synthesis and by its NMR properties (Table II).

Sulfuryl transfer is likely to proceed by a mechanism analogous to that of the physiological reaction, which proceeds through a ping-pong mechanism involving a phosphoenzyme intermediate. If so, then it should be possible to form the sulfoenzyme by incubation of NDP kinase and $ADPSO_3$ in the absence of NDP. The addition of NDP will regenerate free enzyme by accepting the sulfuryl group of the sulfoenzyme. The following discussion describes evidence supporting this mechanism.

Since the desulfation half-reaction is much slower than the corresponding dephosphorylation (vide infra), the sulfoenzyme intermediate can be detected kinetically as shown in Figure 3. When the sulfoenzyme generated by incubation with $ADPSO_3$ is injected into a mixture of ATP and dTDP, the sulfoenzyme first transfers its sulfuryl group to dTDP. The free enzyme thus produced reacts with ATP and dTDP, eventually reaching a steady-state rate that is identical with the rate observed with native enzyme. The sulfoenzyme could be chromatographed on high-resolution Sephadex (Figure 4). After two successive runs, enzyme free of nucleotide was obtained. The kinetic characteristics of the isolated enzyme are identical with those of the original enzyme.

Every NDP kinase examined has been shown to go through a phosphoenzyme intermediate, and the phosphorylated enzyme residue has been identified as a histidine in several cases (Garces & Cleland, 1969). If $ADPSO_3$ proceeds by the same mechanism, a sulfohistidine enzyme intermediate is expected. Mayers and Kaiser (1968) have synthesized imidazole-*N*-sulfonic acid and showed it to be an excellent, stable sulfating agent for a wide variety of reagents, thus lending credence to the proposed sulfohistidine intermediate.

Sulfation of the enzyme is slow relative to phosphorylation, with a half-life of 2 s at high substrate concentration. Enzyme sulfation follows saturation kinetics. The most interesting characteristic of this reaction is its apparent lack of a divalent metal requirement. Although Mg^{2+} stimulates the sulfation reaction, it is not absolutely required for activity. The corresponding phosphate transfer, like all known kinase reactions, shows a strict requirement for divalent metals, making these two substrates uniquely different. To assure that this kinetic phenomenon is not due to contaminating metals, the sulfation kinetics were examined at three different Mg^{2+} concentrations as well as with EDTA and no added metal. Even with EDTA present, the reaction follows saturation kinetics.

The rate equation for a reaction supported by both metal- and nonmetal-activated enzymes is derived under Results. A detailed analysis of the sulfation kinetics again suggests that Mg^{2+} is not necessary for catalysis. The fact that divalent metal stimulates the reaction indicates that metals can participate in the reaction sequence either by lowering the K_m for $ADPSO_3$ or by accelerating sulfuryl transfer. Qualitatively, the data suggest that both modes might be involved. Quantitative analysis is more difficult. The low substrate concentrations used and the limited range of experimentally feasible metal concentrations limits the accurate calculation of V_m and K_m for the metal-catalyzed reaction. Still, a clear increase in V/K is observed as the metal concentration is increased, suggesting that the metal plays an important role in catalysis.

The conversion of sulfoenolpyruvate to $ADPSO_3$ catalyzed by pyruvate kinase was found to be dependent on divalent metals (Peliska & O'Leary, 1989). The interpretation in this case is made difficult since pyruvate kinase requires two different divalent metals for catalysis: one enzyme-bound metal that coordinates to the bridging oxygen of PEP and one metal associated with the nucleotide substrate (Doughterty & Cleland, 1985). The possibility remains that the actual sulfuryl transfer requires only one metal and the nucleotide-bound metal is not absolutely required for catalysis.

Kinetic Comparison of Phosphate and Sulfate Transfer. The kinetic mechanism of nucleoside diphosphate kinase from brewers' yeast has been studied in detail by Garces and Cleland (1969). Using initial velocity, product inhibition, and isotope exchange studies, they demonstrated ping-pong kinetics and determined the complete set of kinetic constants for the forward and reverse reactions.

In addition, the kinetics for phosphorylation and dephosphorylation of NDP kinase from bovine liver and pea seed have been studied by using rapid mixing techniques (Walinder et al., 1969; Edlund & Walinder, 1974). These studies showed that the individual rate constants for both the phosphorylation and dephosphorylation reactions were faster than the overall enzyme turnover. For example, the rate of phosphorylation of bovine liver NDP kinase was calculated to be 2700 min^{-1}, while the rate of phosphoenzyme dephosphorylation by dGDP was found to be 24 000 min^{-1}. The overall turnover number is 1300 min^{-1}, well below the determined individual rate constants.

While these rate constants were determined with enzyme from a different source than studied here (Table III), they do allow a qualitative analysis of the differences between sulfuryl and phosphoryl transfer. As might be expected, substitution of sulfate for phosphate decreases the rate constants by 2–3 orders of magnitude.

The Equilibrium Characteristics of NDP Kinase with Sulfate and Phosphate Esters. Garces and Cleland (1969) calculated partial equilibrium constants for the two half-reactions:

$$MgATP + E \rightleftharpoons E\text{–}PO_3 + MgADP \qquad K_{eq1} = 0.118$$

$$MgUDP + E\text{–}PO_3 \rightleftharpoons MgUTP + E \qquad K_{eq2} = 6.76$$

The phosphoenzyme has a free energy of hydrolysis approximately 1 kcal/mol more negative than that of the terminal phosphate in MgATP.

These values can be compared to those determined for the sulfation of NDP kinase by $ADPSO_3$. Both metal and nonmetal complex forms of $ADPSO_3$ are substrates for this reaction, each with its corresponding equilibrium constant. If the overall equilibrium constant for the sulfation and desulfation reactions with $ADPSO_3$ and dTDP is assumed to be unity, the reaction equilibria become

overall:

$$ADPSO_3 + dTDP \rightleftharpoons ADP + dTDPSO_3 \qquad K_{eq} = 1$$

with Mg^{2+}:

$$MgADPSO_3 + E \rightleftharpoons E\text{–}SO_3 + MgADP \qquad K_{eq} = 11.5$$

$$MgdTDP + E\text{–}SO_3 \rightleftharpoons E + MgdTDPSO_3 \qquad K_{eq} = 0.087$$

without Mg^{2+}:

$$ADPSO_3 + E \rightleftharpoons E\text{-}SO_3 + ADP \qquad K_{eq} = 0.12$$

$$dTDP + E\text{-}SO_3 \rightleftharpoons E + dTDPSO_3 \qquad K_{eq} = 8.3$$

The interesting feature is the apparent reversal of the thermodynamic properties of the two systems. In the case of phosphoryl transfer, the phosphoenzyme intermediate has a free energy of hydrolysis about 1 kcal more negative than that of the terminal phosphate bond in MgATP. In contrast, with $MgADPSO_3$ as the substrate, the corresponding sulfoenzyme intermediate appears to be in a thermodynamic and kinetic well. This is a direct consequence of the poor metal binding characteristics of $ADPSO_3$. The formation constant for MgADP is 2 orders of magnitude smaller than that for $ADPSO_3$ (0.25 mM versus 24 mM), indicating that the driving force for enzyme sulfation is formation of the ADP–metal complex.

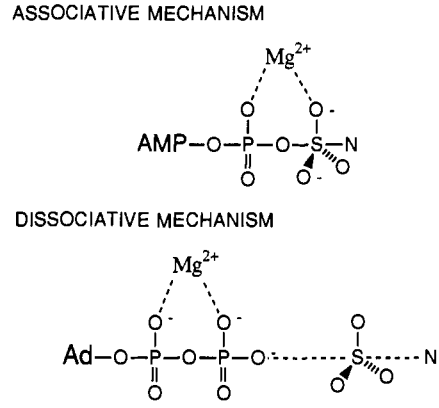
On the other hand, if the metal-independent reaction is considered, the thermodynamic properties of the system switch, and now free enzyme is favored over sulfoenzyme. This is analogous to the reaction catalyzed by ATP. In this case, metal coordination is not a factor and the resulting equilibrium illustrates the true free energy difference between the sulfate linkage of $ADPSO_3$ and the sulfoenzyme. On the basis of an equilibrium constant of 0.12 for enzyme sulfation, this indicates that the sulfoenzyme has a free energy of hydrolysis about 1.2 kcal/mol more negative than $ADPSO_3$.

It should be noted that $ADPSO_3$ is expected to be thermodynamically unstable. The free energy of hydrolysis of the sulfate group of adenosine 5′-phosphosulfate is 10–11 kcal/mol higher than the free energy of the phosphate linkage of ATP (Robbins & Lipmann, 1958). The corresponding free energy of hydrolysis of $ADPSO_3$ is expected to be quite similar to that of APS. This places phosphosulfates at a very different thermodynamic crossroad than ATP. The unique properties of ATP place it at an intermediate position in the thermodynamic scale of phosphate compounds, allowing the "funneling" of high-energy phosphate groups through ATP to form low-energy phosphate compounds (Lehninger, 1971). The extreme thermodynamic instability of phosphosulfates suggests that they function primarily as irreversible sulfating agents.

Implications for Sulfokinase and Phosphokinase Reaction Mechanisms. Sulfokinases typically use either adenosine 5′-phosphosulfate or 3′-phosphoadenosine 5′-phosphosulfate as the "high-energy" sulfate source (Robbins, 1962a,b). Possibly the most striking difference between the reactions catalyzed by phosphokinases and sulfokinases is their metal ion requirements. Enzymes that catalyze phosphate transfers (kinases, phosphatases) require divalent metal ions for activity. On the other hand, many sulfokinases and sulfurylases are metal-independent (Roy, 1971; Robbins, 1962a,b; Banerjee & Roy, 1968), probably because of the weak association of metals with these substrates. Sulfate esters hydrolyze via an A-1 mechanism involving proton transfer followed by rate-determining unimolecular elimination of SO_3 (Benkovic, 1972) with S–O bond cleavage (Benkovic & Hevey, 1970). Evidence from solvent isotope effects, methanol–water trapping experiments, and free energy relationships suggests that S–O bond cleavage precedes solvent–S bond formation. The pH rate profile for the hydrolysis of $ADPSO_3$ (Figure 1) is consistent with this same mechanism.

It is reasonable to assume that pyruvate kinase and NDP kinase catalyze sulfuryl transfer through a largely dissociative transition state (Herschlag & Jencks, 1990). How then is the metal ion involved in catalysis and what does this imply about phosphoryl transfer mechanisms? In general, metal ions catalyze sulfate hydrolysis only when chelation occurs at the leaving group, thereby lowering its pK_a. By this rationale, the active metal–nucleotide form involved in sulfuryl transfer is probably the α,β-phosphate chelated form. If the metal were required to stabilize an associative-type transition state by coordinating to the sulfuryl oxygens (Chart I), a large difference between the metal- and nonmetal-catalyzed reaction rates would be expected, and this is not observed.

Chart I



The metal-catalyzed reaction is estimated to be only 5–10 times faster than the metal-independent reaction. The results presented here indicate that the absolute metal dependence of phosphokinases (at least in the case of NDP kinase) is a function of substrate and that the metal may not be directly coordinated to the phosphoryl group during transfer. This is consistent with the results of Herschlag and Jencks (1987), who have shown that Mg^{2+} and Ca^{2+} have no effect on the amount of nucleophilic participation in phosphoryl transfers between *p*-nitrophenyl phosphate and substituted pyridines, with the transition state remaining dissociative and metaphosphate-like. The fact that sulfuryl transfer is 3–4 orders of magnitude slower than phosphoryl transfer may simply indicate that the principal catalytic effects present in sulfokinases are not optimized in phosphokinases.

Even though $ADPSO_3$ has one less charge than ATP, it still binds tightly to various phosphokinases, with dissociation constants approximating the K_m values observed for ATP. Thus, the loss of a negative charge in the terminal position is not severely detrimental to binding. This is consistent with a low level of enzyme–substrate interaction in the terminal position of ATP and a dissociative transition state. This is not unexpected, since strong enzyme coordination at this position could impede group transfer by preventing longitudinal motion. The coordination scheme of $ADPSO_3$ with Mg^{2+} is not known, so the actual role of the metal in enzyme binding is open to speculation.

It is striking that, of the several kinases tested, only two showed significant reactivity with $ADPSO_3$. Studies of coordinationally stable CrATP complexes (Cleland & Mildvan, 1979) indicate that all the enzymes tested utilized only bidentate CrATP as a substrate, although recent evidence suggests that pyruvate kinase can also use the tridentate metal–nucleotide complex in catalysis (Ladato & Reed, 1987; Buchbinder & Reed, 1990). Possibly, only enzymes that utilize this tridentate complex can facilitate sulfuryl transfer, which in turn could imply that $ADPSO_3$ complexes metals in an α,β-bidentate or a tridentate fashion. It would be interesting to determine the ATP–metal complex structure utilized by NDP kinase. Also, since creatine kinase also appears to utilize a tridentate metal complex (Leyh et al., 1985), it would be

interesting to determine the substrate activity of $ADPSO_3$ with this enzyme.

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Site-Directed Alteration of Four Active-Site Residues of a Pyruvoyl-Dependent Histidine Decarboxylase†

Claire Mazow Gelfman, William C. Copeland, and Jon D. Robertus*

Clayton Foundation Biochemical Institute, Department of Chemistry and Biochemistry, University of Texas, Austin, Texas 78712

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ABSTRACT: Site-directed mutagenesis has been used to examine the chemical roles of four active-site residues in histidine decarboxylase (HDC) from *Lactobacillus* 30a. This protein is known to undergo an autoactivation in which chain cleavage between serines-81 and -82 leads to cofactor (pyruvoyl) formation at position 82. Conversion of Ser-81 to Ala virtually eliminates productive cleavage. It is proposed that the residue plays a key role in stabilizing the transition state of the chain cleavage reaction. Conversion of Phe-83 to Met renders the proenzyme thermally less stable than wild type and appears to slightly increase the rate of autoactivation. The K_m value for histidine is increased about 8-fold, confirming crystallographic evidence that Phe-83 is involved in substrate binding. Both wild-type and F83M enzymes show constant K_m and steadily increasing k_{cat} values as a function of temperature. Lys-155 and Tyr-262, by virtue of their positions in the active site of HDC, have been proposed to possibly play specific roles in either autoactivation or catalysis by active HDC. Conversion to Gln and Phe respectively suggests that these residues have real but minor roles in those processes.

Lactobacillus 30a, isolated from equine stomach, can be induced by histidine to express the gene for prohistidine decarboxylase (proHDC). The 310-residue proenzyme, or π chain, is then activated by chain cleavage between Ser-81 and Ser-82, creating an 81-residue β chain and the larger α chain. This process involves attack on the carbonyl of Ser-81 by the side chain of Ser-82. The intermediate undergoes β elimination to form a Schiff base which is ultimately hydrolyzed to produce a pyruvoyl moiety at the amino terminus of the α chain (Recsei

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* To whom correspondence should be addressed.

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