Investigations of Kinase Substrate Specificity with Aqua Rh(III) Complexes of Adenosine 5′-Triphosphate†

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ABSTRACT: In this paper the substrate activities and binding affinities of the stereoisomers of the β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_3ATP$ complexes toward selected members of the kinase family of enzymes are reported. Hexokinase and glycerokinase were found to be specific for the Λ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer as substrate while adenylate kinase was found to specifically catalyze the reaction of the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer. Pyruvate kinase recognized both the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer and the Δ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomer as substrates in the catalyzed phosphorylation of the alternate substrate, glycolate. ^{31}P NMR analysis of the respective product complexes showed that α-P phosphoryl ligand exchange had not preceded or followed catalysis. Creatine kinase was found to be specific for the Λ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomer. Discrimination of the $Rh(H_2O)_nATP$ isomers via preferential binding of the substrate-active isomer was observed for hexokinase and adenylate kinase but not for glycerokinase, fructose-6-phosphate kinase, creatine kinase, arginine kinase, or acetate kinase.

The use of metal ions having slow ligand exchange rates to construct stable metal–nucleotide complexes for use as probes of enzyme active sites and mechanisms was initiated in the laboratories of Cleland and Mildvan (Foster & Mildvan, 1972; DePamphilis & Cleland, 1973). The Co^{III}- and Cr^{III}nucleotide complexes that have been prepared in recent years have found application in the study of enzyme kinetic mechanisms, substrate structure, substrate and cofactor binding sites, and metal ion catalysis [for reviews, see Cleland and Mildvan (1979), Cleland (1982, 1985), and Dunaway-Mariano (1985)]. Nevertheless, the intrinsic properties of the Cr(III) and Co(III) metal ions have placed certain restrictions on their use as enzyme active site probes. Specifically, within the Co^{III}-ATP[1] series, the full spectrum of γ-monodentate, β,γ-bidentate, and α,β,γ-tridentate isomers is available but only as the corresponding pentaamine, tetraamine, and triamine complexes (Cornelius et al., 1977; Speckhard et al., 1986; Knight, 1984). The Co^{III} amine center of these complexes is a poor mimic for the Mg^{II}aqua center of the natural kinase substrate, $Mg^{II}(H_2O)_nATP$. This is reflected by the observed weak binding of the $Co^{III}(NH_4)_nATP$ complexes to kinases and by the relatively few examples of demonstrated substrate activity. Unlike the aqua $Co^{III}ATP$ complexes, which are redox unstable, stable aqua $Cr^{III}ATP$ complexes can be prepared. The aqua $Cr^{III}ATP$ complexes show, in general, tight binding to kinases and in many instances have displayed substrate activity. Major limitations to the use of the Cr^{III}-ATP complexes derive from the preference of the $Cr^{III}ATP$ complexes to exist as β,γ-bidentate structures as opposed to α,β,γ-tridentate structures and to their paramagnetic properties, which limit structural characterization. Specifically, reaction of $Cr(H_2O)_6^{3+}$ with ATP produces the β,γ-bidentate complex but not the α,β,γ-tridentate complex. Treatment of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex with strong acid produces a new material believed to be α,β,γ-tridentate $Cr(H_2O)_3ATP$, but in the absence of NMR or X-ray crystallographic structural data, the structure assignment is uncertain.

As a result of the limitations surrounding the use of the Co^{III}- and $Cr^{III}ATP$ complexes, the assignment of the β,γ-bidentate $Mg(H_2O)_4ATP$ substrate structure for almost all of the kinases examined to date by the exchange-inert metal–ATP method rests solely on the demonstration of substrate activity of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex and inactivity of the α,β,γ-tridentate $Cr(H_2O)_3ATP$ complex. We do not know whether the observed failure of the α,β,γ-tridentate $Cr(H_2O)_3ATP$ complexes to serve as substrate for these kinases is a reflection of the actual substrate specificities of these enzymes (i.e., for the β,γ-bidentate structure), that the α,β,γ-tridentate $Cr(H_2O)_3ATP$ complex is not a close enough mimic of the α,β,γ-tridentate $Mg(H_2O)_3ATP$ complex to be recognized as substrate, or that the complex assumed to be α,β,γ-tridentate $Cr(H_2O)_3ATP$ has a different structure altogether.

The limited utility of the Co^{III}- and Cr^{III}nucleotide complexes prompted us to develop a new class of exchange inert metal–nucleotide complexes based on a metal ion having properties superior to Cr(III) and Co(III). Rh(III) was chosen because it forms stable aqua complexes, it is diamagnetic, and the aqua $Rh^{III}ATP$ that it forms exists (Lin et al., 1984), as does $Mg^{II}ATP$ (Huang & Tsai, 1982; Pecoraro et al., 1984), as an equilibrating mixture of β,γ-bidentate and α,β,γ-tridentate isomers. Moreover, unlike the rapid equilibration which occurs among the $Mg^{II}ATP$ isomers (Pecoraro et al., 1984), we found the isomerization of the $Rh^{III}ATP$ isomers to be slow enough to allow the individual isomers to be isolated and utilized as enzyme active site probes (Lu et al., 1988).

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; PEP, phosphoenolpyruvate; NADH, dihydronicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); Mes, 2-(*N*-morpholino)ethanesulfonate; Pipes, 1,4-piperazinebis(ethanesulfonate); EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance, *K*, Michaelis constant; *V*, maximum velocity.

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In this paper we report on the binding affinities and substrate activities of the bidentate and tridentate isomers of $Rh^{III}ATP$ toward selected members of the kinase family of enzymes and on how these affinities and activities compare with those observed previously with the $Co^{III}ATP$ and $Cr^{III}ATP$ complexes.

MATERIALS AND METHODS

General Procedures. α,β,γ-Tridentate $Co(NH_3)_3ATP$ (Cornelius et al., 1977) and β,γ-bidentate $Cr(H_2O)_4ATP$ (Dunaway-Mariano & Cleland, 1980a) were prepared according to published procedures. Pure isomers of β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_3ATP$ were prepared by using the method of Lu et al. (1988). Prior to use, the HPLC-purified $Rh(H_2O)_4ATP$ and $Rh(H_2O)_3ATP$ stereoisomers were "desalted" by passing them individually through (0.6 × 20 cm) Sephadex G-10 columns (4 °C, deionized water as eluant). [^{14}C]Glucose, [^{14}C]glycerol, [^{14}C]-glycolate, [^{14}C]creatine, and [^{14}C]AMP were purchased from ICN or NEN. All enzymes, nucleotides, buffers, and chromatography resins were purchased from Sigma Chemical Co.

Kinase Substrate Activity Experiments. The substrate activities of the $Rh^{III}ATP$, $Co^{III}ATP$, and $Cr^{III}ATP$ complexes were tested by using radioisotopic assays similar to those described by Dunaway-Mariano and Cleland (1980b). Reaction mixtures (0.25–0.50 mL) containing the $Co^{III}ATP$, $Rh^{III}ATP$, or $Cr^{III}ATP$ complex, ^{14}C-labeled cosubstrate, buffer, and required cofactors were incubated for a specified time at either 0 or 25 °C. The reactions were terminated by the addition of 20 μL of 70% $HClO_4$ and two drops of CCl_4 to the vortexting mixture. The adenylate kinase, hexokinase, glycerokinase, and pyruvate kinase reaction solutions were diluted to 1 mL with deionized water, filtered through a glass wool plug in a disposable pipet, and then absorbed onto 0.7 × 25 cm Dowex-50-X2-H^+ columns at 4 °C. For the hexokinase, glycerokinase, and pyruvate kinase reactions, the unconsumed cosubstrates were eluted from the columns with 50 mL of 10 mM HCl and then the M(ADP)(RP) product complexes were eluted with 50 mL of 1 M HCl. For the adenylate kinase reaction, the unconsumed AMP and the $M(ADP)_2$ product complex were separated on the column by elution with 200 mL of 10 mM HCl/25 mM KCl followed by 50 mL of 1 M HCl. In the case of the creatine kinase reaction, the quenched reaction solution was adjusted to pH 5 with KOH following removal of the protein and then chromatographed on a 0.7 × 30 cm Dowex-1×2-100 (Cl^-) column. The unconsumed creatine present in the reaction was eluted first from the column with 10 mM K^+Mes (pH 6) and then the M(ADP)(CP) complex was eluted with 50 mL of 1 M HCl. The amount of radioisotope present in the column fractions was determined by using liquid scintillation counting techniques.

Control reactions, not containing enzyme, were carried out along side the catalyzed reactions and analyzed in the same manner described above. In some instances the reaction solutions containing the α,β,γ-tridentate $Rh(H_2O)_3ATP$ complex were preincubated with nucleotide pyrophosphatase (5 units/mL) and alkaline phosphatase (5 units/mL) to selectively hydrolyze any contaminating β,γ-bidentate $Rh(H_2O)_4ATP$ complex (Lin & Dunaway-Mariano, 1988) and uncomplexed ATP prior to the addition of the kinase. In the case of the adenylate kinase reaction, inorganic phosphate (10 mM in the reaction) was added to inhibit the alkaline phosphatase prior to the addition of the AMP cosubstrate. The exchange-inert metal–ATP complexes used in the creatine kinase reaction were pretreated with 20 units/mL alkaline phosphatase (for the bidentate complexes) or 50 units/mL hexokinase in the presence of 50 μM glucose (for the tridentate complex) to remove traces of contaminating ATP.

^{31}P NMR Analysis of the Pyruvate Kinase Reaction. A 10-mL reaction solution (25 °C) initially containing 250 μM Δ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ or Δ β,γ-bidentate $Rh(H_2O)_4ATP$, 2500 units/mL pyruvate kinase, 1 mM $MgCl_2$, 25 mM KCl, 2.5 mM glycolate, and 50 mM K^+Mes (pH 5.9) was quenched after 30 min of incubation with 1 mL of 70% $HClO_4$. The resulting solution was diluted to 20 mL with deionized H_2O, filtered through a glass wool plug in a pipet, and adjusted to pH 4 with KOH solution. After the solid $KClO_4$ was removed by filtration, the solution was concentrated in vacuo to a ~1-mL volume and then chromatographed on a Sephadex G-10 column (1.4 × 30 cm) with deionized water (4 °C). The column fractions containing the adenine chromophore (based on A^{260}) were pooled, concentrated, and then analyzed with a Bruker AM 400 NMR spectrometer operating at 162 MHz (40% D_2O, 14 mM EDTA, pH 4, 20 °C, H_3PO_4 external standard).

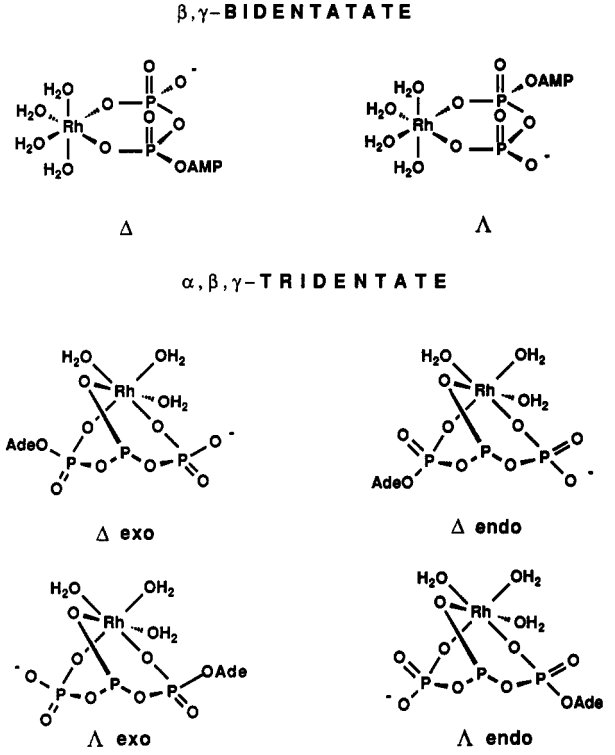
Inhibition Studies. The kinase inhibition studies were carried out at 25 °C and at pH 6.0 or 6.5 (75 mM K^+Mes, 10 mM $MgCl_2$). The pyruvate kinase/lactate dehydrogenase couple (1 mM PEP, 0.4 mM NADH, 10 units/mL pyruvate kinase, and 10 units/mL lactate dehydrogenase) was used to monitor the *Candida mycoderma* glycerokinase (2 mM glycerol, 200–600 μM ATP), rabbit muscle creatine kinase (10 mM creatine, 0.3–2.0 mM ATP), *Escherichia coli* acetate kinase (5 mM acetate, 100–600 μM ATP), chicken muscle adenylate kinase (2 mM AMP, 0.15–1.5 mM ATP), and lobster tail muscle arginine kinase (250 mM arginine, 3–10 mM ATP) catalyzed reactions. The glucose-6-phosphate dehydrogenase coupled assay (10 units/mL glucose-6-phosphate dehydrogenase, 1 mM NADP) was used to monitor the yeast hexokinase catalyzed reaction (0.5 mM citrate, 1 mM glucose, 30–300 μM ATP). The fructose-1,6-diphosphate aldolase (10 units/mL)/3-phosphoglyceraldehyde dehydrogenase (10 units/mL)/AsO_4^{3-} (10 mM)/NAD^+ (1 mM) coupled assay was used to monitor the rabbit muscle fructose-6-phosphate kinase catalyzed reaction of fructose 6-phosphate (1 mM) and MgATP (40–200 μM). The K_i value was calculated, with the aid of the computer programs of Cleland (1979), from the initial velocity data using the equation $V_0 = VA/[K(1 + I/K_i) + A]$ for competitive inhibition or the equation $V_0 = VA/[K + A(1 + I/K_i)]$ for uncompetitive inhibition where V_0 is the initial velocity, V the maximum velocity, K the Michaelis constant, K_i the inhibition constant, A the substrate concentration, and I the inhibitor concentration.

RESULTS

In this study the substrate activities and binding affinities of β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers (whose structures are shown in Chart I) were tested with several kinases, including hexokinase, glycerokinase, adenylate kinase, pyruvate kinase, and creatine kinase.

Kinase Substrate Activities. The substrate activities of the $Rh(H_2O)_nATP$ complexes were tested using high concentrations of enzyme (usually >10 μM).[2] Previous studies with

Chart I: Structural Representation of the Two β,γ-Bidentate $Rh(H_2O)_4ATP$ Stereoisomers and Four α,β,γ-Tridentate $Rh(H_2O)_3ATP$ Stereoisomers[a]



[a] The stereochemical nomenclature used is according to that suggested by Cornelius and Cleland (1978).

the β,γ-bidentate $Cr(H_2O)_4ATP$ complexes had shown that product release following turnover in the kinase active site is slow[3] (Dunaway-Mariano & Cleland, 1980b). Thus, in order to minimize the reaction periods for the sake of avoiding isomerization of the $Rh(H_2O)_nATP$ complex, the product from a single turnover was analyzed. Likewise, to minimize the amount of contaminating isomers present in the $Rh(H_2O)_nATP$ sample relative to the amount of enzyme present, the $Rh(H_2O)_nATP$ complex was used in less than a 10:1 ratio to the enzyme. The substrate activities of the $Rh(H_2O)_nATP$ complexes were measured at pH 6 and at 0 or 25 °C, under which conditions minimal isomerization takes place within the 10-min reaction periods used (Lu et al., 1988) and under which conditions these kinases are known to be active (Dunaway-Mariano & Cleland, 1980b). The substrate activities of the complexes were tested several times with each kinase. The results from typical experiments are presented below, and they are summarized in Table I.

Hexokinase. The reaction of 500 μM β,γ-bidentate $Rh(H_2O)_4ATP$ with ~100 μM yeast hexokinase (1000 units/mL) and 1 mM [^{14}C]glucose in 100 mM K^+Mes (pH 6) was carried out at 0 °C. Within 8 min, 120 μM $Rh(H_2O)_4$(ADP)-(glucose-6-P) product had formed, corresponding (approximately) to a single turnover of the $Rh(H_2O)_4ATP$ complex in the enzyme active site.

Table I: Substrate Activities of the $Rh(H_2O)_nATP$, $Co(NH_3)_nATP$, and $Cr(H_2O)_nATP$ Complexes with Kinases

kinase	substrate[a] inactive complexes	substrate active complexes	active stereoisomer
hexokinase	m-CoATP[b]	b-CoATP[c]	Λ
	t-CrATP[d]	b-CrATP[d]	Λ ≫ Δ
	t-CoATP	b-RhATP	Λ
	t-RhATP		
glycerokinase	b-CoATP[d]	b-CrATP[d]	Λ ≫ Δ
	t-CoATP	b-RhATP	Λ
	t-CrATP[d]		
	t-RhATP		
adenylate kinase	b-CoATP[d]	b-CrATP[d]	Δ
	t-CrATP[d]	b-RhATP	Δ
	t-RhATP		
pyruvate kinase	b-CoATP[d]	b-CrATP[d]	Δ
	t-CoATP	b-RhATP	Δ
	t-CrATP[d]	t-RhATP	β-Δ, α-exo
creatine kinase	b-CoATP[d]	b-CrATP[d]	Λ
	t-CrATP[d]	t-RhATP	β-Λ, α-exo
	b-RhATP		

[a] m-CoATP, γ-monodentate $Co(NH_3)_5ATP$; b-CoATP, β,γ-bidentate $Co(NH_3)_4ATP$; t-CoATP, α,β,γ-tridentate $Co(NH_3)_3ATP$; b-CrATP, β,γ-bidentate $Cr(H_2O)_4ATP$; t-CrATP, α,β,γ-tridentate $Cr(H_2O)_3ATP$; b-Rh-ATP, β,γ-bidentate $Rh(H_2O)_4ATP$; t-RhATP, α,β,γ-tridentate $Rh(H_2O)_3ATP$. [b] Taken from Knight (1984). [c] Taken from Cornelius and Cleland (1978). [d] Taken from Dunaway-Mariano and Cleland (1980b).

The stereospecificity of hexokinase (1000 units/mL; ~100 μM) toward the purified Δ and Λ β,γ-bidentate $Rh(H_2O)_4ATP$ isomers (100 μM) was tested in the presence of 1 mM [^{14}C]glucose in 100 mM K^+Mes (pH 6) at 0 °C. The Λ isomer formed 45 μM $Rh(H_2O)_4$(ADP)(glucose-6-P) product within 10 min while the Δ isomer produced none. Reaction of the Δ isomer at 25 °C for 10 min also failed to produce product.

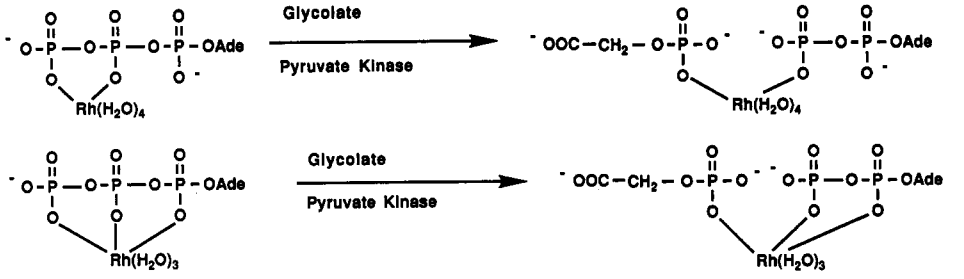
The substrate activity of the α,β,γ-tridentate $Rh(H_2O)_3ATP$ complex (500 μM) as a mixture of stereoisomers (Chart I) was tested by incubation with ~100 μM hexokinase (1000 units/mL) and 1 mM [^{14}C]glucose in 100 mM K^+Mes (pH 6) at 0 °C for 10 min. None of the expected product, $Rh(H_2O)_3$(ADP)(glucose-6-P) was formed from this reaction or from an identical reaction carried out at 25 °C. Likewise, reaction of the α,β,γ-tridentate $Co(NH_3)_3ATP$ complex (200 μM) as a mixture of stereoisomers, with ~100 μM hexokinase and 1 mM [^{14}C]glucose in 80 mM of K^+Pipes (pH 7) at 25 °C for 30 min failed to produce the expected product, $Co(NH_3)_3$(ADP)(glucose-6-P).

Glycerokinase. The substrate activities of the purified Λ and Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomers and a mixture of the four α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers were evaluated by measuring product formation in reaction solutions containing 100 μM of the $Rh(H_2O)_nATP$ complex, 1 mM [^{14}C]glycerol, and 1000 units/mL (~60 μM) *E. coli* glycerokinase in 100 mM K^+Mes (pH 6) at 0 °C. After 10 min the Λ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer produced 40 μM of the $Rh(H_2O)_4$(ADP)(glycerol-P) product (approximately one turnover in the enzyme active site), while the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer and the mixture of α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers produced a negligible amount of product. Reaction of 200 μM α,β,γ-tridentate $Co(NH_3)_3ATP$ (as a mixture of isomers) with 0.5 mM [^{14}C]glycerol and 200 units/mL (~10 μM) glycerokinase in

[2] The enzyme concentrations reported in this paper were calculated from the weight of the enzyme (supplied by Sigma Chemical Co.) and the reported subunit weight. Since the purity of these enzymes is unknown and activity loss owing to aggregation at high enzyme concentration likely, the enzyme concentrations reported are only approximations.

[3] The release of the $Rh(H_2O)_n$(ADP)(substrate-P) product complex is slow because this complex resembles the intermediate $Mg(H_2O)_n$(ADP)-(substrate-P) formed in the kinase-catalyzed reaction of the natural substrate $Mg(H_2O)_nATP$ rather than the final products $Mg(H_2O)_nADP$ and substrate-P. Since the $Rh(H_2O)_n$(ADP)(substrate-P) complex does not readily convert to $Rh(H_2O)_nADP$ plus substrate-P the enzyme active site remains in a closed conformation, thus impairing dissociation (Dunaway-Mariano & Cleland, 1980b).

Scheme I: Reaction Products Formed from the Pyruvate Kinase Catalyzed Phosphorylation of Glycolate with Δ β-P β,γ-Bidentate $Rh(H_2O)_4ATP$ and Δ β-P, exo α-P α,β,γ-Tridentate $Rh(H_2O)_3ATP$



80 mM K^+Pipes (pH 7) failed to produce any of the product, $Co(NH_3)_3(ADP)$(glycerol-P).

Adenylate Kinase. The reactions of the Λ and Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomers (100 μM) and a mixture of the four α,β,γ-tridentate $Rh(H_2O)_3ATP$ (100 μM) isomers with 1 mM [^{14}C]AMP and 2000 units/mL (~65 μM) chicken muscle adenylate kinase in 100 mM K^+Mes (pH 6) at 25 °C were carried out for 8 min. A total of 60 μM $Rh(H_2O)_4(ADP)_2$ product complex was formed from the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer while negligible product formation was observed for the Λ β,γ-bidentate $Rh(H_2O)_4ATP$ or the α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers.

Pyruvate Kinase. The reactions of the $Rh(H_2O)_nATP$ complexes (100 μM) with rabbit muscle pyruvate kinase (1000 units/mL, ~15 μM) and [^{14}C]glycolate (1 mM) were carried out at 25 °C in 100 mM K^+Mes (pH 6) containing 4 mM $MnCl_2$. Under these conditions the β,γ-bidentate $Rh(H_2O)_4ATP$ complex, as a mixture of isomers, gave rise to 10 μM of the expected product, $Rh(H_2O)_4$(ADP)(glycolate-P), within 5 min while 14 μM product was produced from the tridentate complex. In a separate experiment, the purified Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer gave rise to 4 μM product while the Λ isomer did not form product. The four α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers were also tested individually for substrate activity under the same reaction conditions. The Δ β-P, exo α-P, α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomer formed 10 μM product within 10 min while the three other tridentate isomers formed less than 1 μM product or productlike material. Reaction of α,β,γ-tridentate $Co(NH_3)_3ATP$ (200 μM; mixture of isomers) under the same reaction conditions failed to produce product within a 30-min reaction period.

The products of the pyruvate kinase catalyzed reaction of glycolate with the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer and with the Δ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomer were characterized on the basis of their proton-decoupled ^{31}P NMR spectrum (see Materials and Methods). The product formed from the tridentate isomer gave rise to a singlet at +14.98 ppm, a doublet at +6.06 ppm (J = 21 Hz), and a doublet at +2.89 ppm (J = 21 Hz) in a 1:1:1 ratio of peak area. These resonances are assigned respectively to the glycolate phosphoryl, the β-ADP phosphoryl, and the α-ADP phosphoryl groups of the $Rh(H_2O)_3$(ADP)(glycolate-P) complex shown in Scheme I. The product formed from the reaction of the bidentate isomer gave rise to a singlet at +15.05 ppm, a doublet at +6.29 ppm (J = 21 Hz), and a doublet at −10.39 ppm (J = 21 Hz) in a 1:1:1 ratio of peak area. These resonances are assigned respectively to the glycolate phosphoryl, the β-ADP phosphoryl, and the α-ADP phosphoryl groups of the $Rh(H_2O)_4$(ADP)(glycolate-P) complex shown in Scheme I. On the basis of the structures of the products generated from the α,β,γ-tridentate- and β,γ-bidentate $Rh(H_2O)_nATP$ complexes isomerization of the complex does not appear to proceed or follow turnover on the enzyme.

Creatine Kinase. A single-turnover reaction of ATP and creatine catalyzed by rabbit muscle creatine kinase was carried out to test the conditions to be used for the exchange of inert complexes. Accordingly, ATP (1 mM) and [^{14}C]creatine (1 mM) were incubated at 25 °C in 100 mM K^+Mes (pH 6) containing $MgCl_2$ (5 mM) and creatine kinase (6000 units/mL, ~0.4 mM). After 5 min the reaction was terminated and analyzed for [^{14}C]creatine phosphate by using Dowex-1-X2-Cl anion exchange column chromatography to separate the mixture. The reaction was found to contain 12 μM [^{14}C]-creatine phosphate.

Reaction (10 min, 25 °C) of β,γ-bidentate $Cr(H_2O)_4ATP$ (100 μM) with [^{14}C]creatine (0.5 mM) in 100 mM K^+Mes (pH 6) containing creatine kinase (3000 units/mL, ~0.2 mM) generated 4 μM of the expected product, $Cr(H_2O)_4$(ADP)-(creatine-P). Individual β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_nATP$ stereoisomers (100 μM) were reacted (10 min, 25 °C) with 1 mM [^{14}C]creatine and creatine kinase (8000 units/mL, ~0.5 mM) in 100 mM K^+Mes (pH 6). The Λ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ complex produced 11 μM of the expected product, $Rh(H_2O)_3$-(ADP)(creatine-P) while the three remaining tridentate stereoisomers and the two bidentate stereoisomers generated less than 1 μM ^{14}C-labeled product.

Determination of the Inhibition Constants for β,γ-Bidentate $Rh(H_2O)_4ATP$ and α,β,γ-Tridentate $Rh(H_2O)_3ATP$ Isomers with Kinases. In order to evaluate how tightly the individual $Rh(H_2O)_nATP$ isomers bind to the kinases, the isomers were used as dead end inhibitors of the kinase-catalyzed reaction. The inhibition constant (K_i) for each isomer was determined from the initial velocity data measured as a function of MgATP concentration (fixed concentration of cosubstrate) in the presence and absence of the $Rh(H_2O)_nATP$ isomer as described under Materials and Methods. In each instance competitive inhibition was observed, except for the case of α,β,γ-tridentate $Rh(H_2O)_3ATP$ inhibition of *Candida mycoderma* glycerokinase, where uncompetitive inhibition was observed. The K_i values obtained are reported in Table II along with those reported earlier for β,γ-bidentate $Cr(H_2O)_4ATP$ and what has been presumed to be α,β,γ-tridentate $Cr(H_2O)_3ATP$.

DISCUSSION

In the present study the enzymes hexokinase, glycerokinase, adenylate kinase, pyruvate kinase, and creatine kinase were used to evaluate the substrate activities of the stereoisomers of β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_3ATP$ (Chart I). The MgATP substrate structure for each of these enzymes had been previously tested using β,γ-bidentate $Co(NH_3)_4ATP$, β,γ-bidentate $Cr(H_2O)_4ATP$, and (what we presume to be) α,β,γ-tridentate $Cr(H_2O)_3ATP$. Furthermore, each enzyme (with the exception of glyceroki-

Table II: Inhibition Constants[a] Measured for $Rh(H_2O)_nATP$ and $Cr(H_2O)_nATP$[b] Complexes

kinase	active substr	K_m, MgATP (μM)	K_i (μM) b-RhATP[c]	t-RhATP	b-CrATP	t-CrATP
hexokinase	Λ-Bid[d]	35 ± 2	1.7 ± 0.1 Λ, 0.8 ± 0.1 Δ, 30 ± 2	5.6 ± 0.2	0.069 ± 0.007 Λ, 0.029 Δ, 0.18	120 ± 10
glycerokinase	Λ-Bid	65 ± 6	20 ± 1 Λ, 15 ± 3 Δ, 19 ± 4	100 ± 12	0.14 ± 0.02	240 ± 20
acetate kinase	Λ-Bid[e]	125 ± 18	33 ± 5 Λ, 24 ± 5 Δ, 34 ± 6	94 ± 9	900 ± 100	180 ± 20
F-6-P kinase	Δ-Bid[b]	35 ± 4	17 ± 2 Λ, 18 ± 2 Δ, 15 ± 2	ND[g]	140 ± 20	58 ± 9
adenylate kinase	Δ-Bid	200 ± 20	6.0 ± 1 Λ, 30 ± 2 Δ, 2 ± 0.1	ND	11.7 ± 0.7 (Λ, 20)[h] (Δ, 11)[h]	11 ± 1
creatine kinase	Λ, exo-Trid[d]	300 ± 30	20 ± 1	56 ± 2	68 ± 6	18.3 ± 0.6
arginine kinase	Λ, exo-Trid[f]	3500 ± 150	85 ± 12	170 ± 30	89 ± 7	68 ± 9

[a] All K_i values were calculated for competitive inhibition except for t-RhATP inhibition of glycerokinase, where the K_i was calculated from the intercept effect observed for the uncompetitive inhibition. [b] Result from Dunaway-Mariano and Cleland (1980b). [c] b-RhATP, a mixture of the Δ and Λ isomers of β,γ-bidentate $Rh(H_2O)_4ATP$; t-RhATP, a mixture of the four stereoisomers of α,β,γ-tridentate $Rh(H_2O)_3ATP$; b-CrATP, a mixture of the Δ and Λ isomers of β,γ-bidentate $Cr(H_2O)_4ATP$; t-CrATP, a mixture of the four isomers of α,β,γ-tridentate $Cr(H_2O)_3ATP$. [d] Λ-Bid, Λ β,γ-bidentate; Δ-Bid, Δ β,γ-bidentate; Λ, exo-Trid, Λ β-P, exo α-P α,β,γ-tridentate. [e] Result from Romaniuk and Eckstein (1981). [f] Result from Cohn et al. (1982) and Jarori et al. (1989). [g] ND, not determined. [h] Result from Sanders et al. (1989).

nase) had been studied using the ^{17}O–Mn EPR technique (Reed & Leyh, 1980; Reed & Markham, 1984) and/or the Mg(II)/Cd(II) ATPS technique (Cohn, 1982; Eckstein, 1985).[4] Thus, a comparison could be made between the MgATP substrate structures predicted by the substrate activities of the $Rh(H_2O)_nATP$, $Cr(H_2O)_nATP$, and $Co(NH_3)_nATP$ complexes (Table I) and the MgATP substrate structures predicted on the basis of the ^{17}O–Mn EPR and Mg(II)/Cd(II) ATPS studies.

Hexokinase. As indicated in Table I, hexokinase is specific for the Λ β,γ-bidentate $Rh(H_2O)_4ATP$ complex. Consistent with this finding is the observation that Λ β,γ-bidentate $Co(NH_3)_4ATP$ is a substrate for hexokinase while Δ β,γ-bidentate $Co(NH_3)_4ATP$ is not (Cornelius & Cleland, 1978), nor are the γ-monodentate $Co(NH_3)_5ATP$ (Knight, 1984) and α,β,γ-tridentate $Co(NH_3)_3ATP$ (this study) complexes. Likewise, studies with the $Cr(H_2O)_nATP$ complexes showed the Λ β,γ-bidentate $Cr(H_2O)_4ATP$ isomer to be a much faster reacting substrate than the Δ β,γ-bidentate $Cr(H_2O)_4ATP$ isomer and the α,β,γ-tridentate $Cr(H_2O)_3ATP$ isomers not to be substrates (Dunaway-Mariano & Cleland, 1980b). The results from the substrate specificity tests with the exchange-inert metal–ATP complexes uniformly point to a Λ β,γ-bidentate $Mg(H_2O)_4ATP$ structure, which is consistent with the prediction made on the basis of the Mg(II)- and Cd(II)-controlled hexokinase stereospecificity toward ATPβS and ATPαS diastereomers (Jaffe & Cohn, 1979).

Glycerokinase. Like hexokinase, glycerokinase was found to be specific for the Λ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer (Table I), but within the $Co(NH_3)_nATP$ series, neither the β,γ-bidentate $Co(NH_3)_4ATP$ (Dunaway-Mariano & Cleland, 1980b) nor α,β,γ-tridentate $Co(NH_3)_3ATP$ (this study) complexes displayed substrate activity with glycerokinase. Previous studies with the $Cr(H_2O)_nATP$ isomers, however, showed that the Λ β,γ-bidentate $Cr(H_2O)_4ATP$ isomer reacts much faster with glycerokinase than does the Δ β,γ-bidentate $Cr(H_2O)_4ATP$ isomer and that the α,β,γ-tridentate $Cr(H_2O)_3ATP$ isomers do not react at all (Dunaway-Mariano & Cleland, 1980b). From these results a consistent picture of Λ β,γ-bidentate $Mg(H_2O)_4ATP$ as the glycerokinase substrate emerges.

Adenylate Kinase. As indicated in Table I, adenylate kinase is specific for the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer. In agreement with this observation is the earlier report that the Δ isomer is the preferred isomer of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex (the β,γ-bidentate $Co(NH_3)_4ATP$ Δ and Λ isomers are substrate inactive) and that the α,β,γ-tridentate $Cr(H_2O)_3ATP$ is not a substrate for adenylate kinase (Dunaway-Mariano & Cleland, 1980b).

The structure of MgATP bound in the active site of myokinase has also been examined using several techniques other than the exchange-inert metal–ATP technique. Specifically, on the basis of ^{31}P spin-relaxation measurements, Ray et al. (1988) concluded that the structure of MgATP bound in the myokinase active site is β,γ-bidentate, and the results obtained from Mg(II)/Cd(II) ATPβS/ATPαS studies (Tomasselli & Noda, 1981, 1983; Tomasselli et al., 1984) and Mn–^{17}O–ATP EPR studies (Kalbitzer et al., 1983) indicate that the configuration at the β-P is Δ. Thus, the results from each of these studies provide evidence for Δ β,γ-bidentate $Mg(H_2O)_4ATP$ as the myokinase substrate.

Pyruvate Kinase. Unlike hexokinase, glycerokinase, and myokinase, for which the probes of the MgATP substrate structure produced straightforward, consistent results, pyruvate kinase proved to be complex. Previous studies carried out using the Co^{III}- and $Cr^{III}ATP$ complexes showed that neither the β,γ-bidentate $Co(NH_3)_4ATP$ (Dunaway-Mariano & Cleland, 1980b) nor the α,β,γ-tridentate $Co(NH_3)_3ATP$ (this study) complexes serve as substrate for pyruvate kinase. Substrate activity tests carried out with the β,γ-bidentate $Cr(H_2O)_4ATP$ and α,β,γ-tridentate $Cr(H_2O)_3ATP$ complexes (Dunaway-Mariano et al., 1979; Dunaway-Mariano & Cleland, 1980b) revealed the Δ β,γ-bidentate $Cr(H_2O)_4ATP$ complex as the only active isomer (Table I). On the other

[4] The ^{17}O–Mn EPR method locates Mn(II) coordination sites on ^{17}O-labeled phosphoryl groups in enzyme–substrate or enzyme–inhibitor complexes by testing for superhyperfine ^{17}O–Mn spin–spin coupling. The Mg(II)/Cd(II) ATPβS/ADPαS method tests for Mg(II) and Cd(II) coordination to the β-P and α-P oxygen and sulfur atoms, respectively, by testing the enzyme stereospecificity toward the ATPβS and ATPαS diastereoisomers. Reversal of stereospecificity in the Mg(II) vs Cd(II)-activated enzyme indicates coordination.

hand, the substrate activity tests carried out on the β,γ-bidentate $Rh(H_2O)_4ATP$ and α,β,γ-tridentate $Rh(H_2O)_3ATP$ complexes produced unexpected results. Both the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ and the Δ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomers proved to be substrates (Table I). As shown in Scheme I, the products generated from these reactions suggest that α-P ligand exchange does not occur prior to or following turnover on the enzyme. Specifically, pyruvate kinase catalyzes phosphoryl transfer to the glycolate from the bidentate $Rh(H_2O)_4ATP$ complex to generate the $Rh(H_2O)_4(ADP)$(glycolate-P) product complex in which the ADP is coordinated via the β-P and from the tridentate complex to generate the $Rh(H_2O)_3(ADP)$(glycolate-P) product complex in which the ADP is coordinated via the β-P and α-P.

The results from tests of $Mg(H_2O)_nATP$ pyruvate kinase substrate structure using a variety of other techniques point to an α,β,γ-tridentate structure. Following early ^{31}P NMR experiments which were suggestive of Mg(II) coordination to the α-, β-, and γ-phosphoryl groups of pyruvate kinase bound ATP (Sloan & Mildvan, 1976; Gupta & Mildvan, 1977), recent EPR studies (Lodato & Reed, 1987; Buchbinder & Reed, 1990) of pyruvate kinase complexes with oxalate, ATP, and divalent metal ions have provided convincing evidence that MgATP binds to the enzyme as the α,β,γ-tridentate complex. Furthermore, the reactivity pattern observed for Mg(II) and Cd(II) complexes of ADPβS (Jaffe & Cohn, 1978) have indicated that the active isomer of the α,β,γ-tridentate $Mg(H_2O)_3ATP$ complex has the Δ β-P configuration in agreement with the results obtained with the tridentate $Rh(H_2O)_3ATP$ isomers.

Assuming then that pyruvate kinase recognizes the Δ β-P α,β,γ-tridentate $Mg(H_2O)_3ATP$ isomer as its natural substrate, why does this enzyme catalyze the reaction of both the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer and the Δ β-P, exo α-P α,β,γ-tridentate $Rh(H_2O)_3ATP$ isomer? One possible explanation is that the enzyme binds the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ isomer in the tridentate conformation with the α-P close to, but not directly coordinated to, the metal ion and that this arrangement is adequate for turnover. The fact that glycolate can substitute for pyruvate in the phosphorylation reaction would suggest that pyruvate kinase has a rather relaxed substrate specificity.

A final issue raised by comparing the pyruvate kinase substrate activities of the $Cr(H_2O)_nATP$ and $Rh(H_2O)_nATP$ complexes is why the α,β,γ-tridentate $Cr(H_2O)_3ATP$ fails to undergo catalytic turnover. This failure may be an indication that the structure of this material is something other than α,β,γ-tridentate, a possibility discussed earlier, or that its tridentate chelate structure differs significantly from that of the α,β,γ-tridentate $Rh(H_2O)_3ATP$ complex. At present we cannot distinguish between these two possibilities.

Creatine Kinase. Tests of the $Mg(H_2O)_nATP$ substrate structure of creatine kinase using the exchange-inert metal–ATP complexes are difficult because of the unfavorable equilibrium both on and off the enzyme and because of the rather large K_m for creatine. Nevertheless, small amounts of product formed by the creatine kinase catalyzed reaction of the exchange-inert metal–ATP complexes and creatine can be observed. As indicated in Table I, both the Δ and Λ β,γ-bidentate $Rh(H_2O)_4ATP$ isomers were found to be substrate inactive and only the Λ β-P, exo α-P isomer of the α,β,γ-tridentate complex was found to be active. This result suggests that the natural substrate for creatine kinase is the Λ β-P, exo α-P α,β,γ-tridentate $Mg(H_2O)_3ATP$ complex, in agreement with the results obtained using ^{31}P NMR relaxation (Jarori et al., 1985), ^{17}O–Mn EPR (Reed & Leyh, 1982; Leyh et al., 1985; Smithers et al; 1989; Frey et al., 1987) and the Mg(II)/Cd(II) ATPβS/ATPαS (Burgers & Eckstein, 1980) techniques.

The finding that the tridentate but not the bidentate $Rh(H_2O)_nATP$ complex is the substrate for creatine kinase deviates from the results obtained using the $Cr(H_2O)_nATP$ complexes as probes (Table I). Specifically, the α,β,γ-tridentate $Cr(H_2O)_3ATP$ complex was shown to be substrate inactive while the Λ stereoisomer of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex was found to be substrate active (Dunaway-Mariano & Cleland, 1980b). In the present study we retested the activity of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex by using $Cr(H_2O)_4ATP$ that had been pretreated with alkaline phosphatase to remove contaminating ATP which, through its conversion to creatine phosphate, could lead to a false product reading with the assay system used.[5] Substrate activity of the $Cr(H_2O)_4ATP$ complex was observed, thus confirming the previous result (Dunaway-Mariano & Cleland, 1980b) and raising the question of why this kinase turns over tridentate $Rh(H_2O)_3ATP$ but not tridentate $Cr(H_2O)_3ATP$ (the same unanswered question which arose with pyruvate kinase) and the question of why it catalyzes the reaction of bidentate $Cr(H_2O)_4ATP$ but not bidentate $Rh(H_2O)_4ATP$.

Previous studies of the $Mg(H_2O)_nADP$ substrate structure for creatine kinase [operating in the $Mg(H_2O)_3ATP$-forming direction] suggest that it is Δ α,β-bidentate (Dunaway-Mariano & Cleland, 1980b; Shorter et al., 1987; Reed & Leyh, 1980; Leyh et al., 1982). If we assume that creatine kinase can (like pyruvate kinase) catalyze phosphoryl transfer from either the α,β,γ-tridentate or β,γ-bidentate metal–ATP complex and that the metal–ADP complex ultimately formed has the α,β-bidentate chelate structure, then the apparent reactivity of the β,γ-bidentate $Cr(H_2O)_4ATP$ complex vs the unreactivity of the β,γ-bidentate $Rh(H_2O)_4ATP$ complex may reflect a reaction step which follows phosphoryl transfer to creatine and which involves insertion of the α-P into the coordination sphere of the metal to form a thermodynamically more stable α,β-bidentate $metal^{III}ADP$ complex. The greater ligand exchange rate at the Cr(III) vs Rh(III) center (Eigen & Wilkins, 1965) may allow α-P insertion to occur in the enzyme-bound Cr(III) complex where it cannot in the Rh(III) complex.[6]

Kinase Binding Affinities. While kinases generally recognize one $Mg(H_2O)_nATP$ isomer as substrate, $Mg(H_2O)_nATP$ in solution exists as a rapidly equilibrating mixture of structural and stereochemical isomers [$k \sim 7 \times 10^3$ s^{-1}

[5] The method used to separate the [^{14}C]creatine from the ^{14}C-labeled $Rh(H_2O)_n$(ADP)(creatine-P) complex does not also separate [^{14}C]-creatine-P. Thus, ^{14}C-labeled material in the product fraction can arise from reaction of contaminating MgATP as well as from the $Rh(H_2O)_nATP$ complex that is being tested.

[6] Evidence of enzyme-catalyzed ligand exchange at the Cr(III) center of the $Cr(H_2O)_4ATP$ complex has been observed in several instances. For example, the ability of hexokinase to catalyze the phosphorylation of glucose with the Δ β,γ-bidentate $Cr(H_2O)_4ATP$ stereoisomer when it cannot catalyze the phosphorylation with the Δ β,γ-bidentate $Rh(H_2O)_4ATP$ or Δ β,γ-bidentate $Co(NH_3)_4ATP$ stereoisomers can be rationalized by more facile ligand exchange at the Cr(III) vs Co(III) or Rh(III) center (Dunaway-Mariano & Cleland, 1980b). In addition, the $Cr(H_2O)_4ATP$ complex inactivates certain kinases, e.g., pyruvate phosphate dikinase (Ciskanik & Dunaway-Mariano, 1986) and glutamine synthetase (Ransom et al., 1985), by a process involving insertion of an enzyme amino acid side chain into the coordination sphere of the bound Cr(III) complex.

(Pecoraro et al., 1984)]. How the "correct" isomer is selected from among this mixture is not known. The selection process may occur at the binding step or at the catalytic step. In the latter case, unless the enzyme can catalyze (or at least not hinder) the isomerization of an inactive isomer to the active one, the enzyme will have to search for the correct isomer by repeating cycles of isomer binding and release.

In the present study the binding affinities of the Rh-$(H_2O)_n$ATP isomers were measured toward kinases for which the active $Mg(H_2O)_n$ATP isomer had been previously identified for the purpose of determining whether the substrate specificity observed for turnover is also observed for substrate binding. The kinases tested which recognize β,γ-bidentate $Mg(H_2O)_4$ATP as substrate are hexokinase, glycerokinase, acetate kinase, adenylate kinase, and fructose-6-phosphate kinase (Table II). Of these kinases, only hexokinase and adenylate kinase were found to bind the substrate-active bidentate $Rh(H_2O)_4$ATP stereoisomer more tightly (30- and 15-fold, respectively) than the inactive bidentate $Rh(H_2O)_4$-ATP stereoisomer. Tridentate $Rh(H_2O)_3$ATP (as a mixture of stereoisomers) binds to hexokinase, glycerokinase, and acetate kinase 4–6-fold less tightly than the active bidentate stereoisomer. Creatine kinase and arginine kinase, two kinases which recognize the Λ β-P, exo α-P isomer of α,β,γ-tridentate $Mg(H_2O)_3$ATP as substrate, bind the bidentate $Rh(H_2O)_4$-ATP stereoisomers (as a mixture) twice as tightly as the tridentate $Rh(H_2O)_3$ATP stereoisomers (as a mixture). On the other hand, the Δ α,β-bidentate $Rh(H_2O)_4$ADP (Shorter et al., 1987) and Δ α,β-bidentate $Cr(H_2O)_4$ADP isomers (Dunaway-Mariano & Cleland, 1980b) bind to creatine kinase and arginine kinase ~20-fold tighter than do the Λ isomers. Taken together these results suggest that in some but not all cases isomer discrimination takes place at the stage of binding, and for those kinases which do show such discrimination in binding the isomers of $Rh(H_2O)_n$ATP, the level of discrimination falls between a factor of 10 and 30.

Finally, we found that in several instances the binding behavior of the $Rh(H_2O)_n$ATP complexes differed somewhat from those previously reported for the $Cr(H_2O)_n$ATP complexes (Dunaway-Mariano & Cleland, 1980b). First, the bidentate $Rh(H_2O)_4$ATP complex did not display slow tight binding inhibition of hexokinase as had been reported for bidentate $Cr(H_2O)_4$ATP (Danenberg & Cleland, 1975) nor did it bind as tightly (Table II). On the other hand, the tridentate $Rh(H_2O)_3$ATP complex was bound much more tightly than the tridentate $Cr(H_2O)_3$ATP complex. Glycerokinase, like hexokinase, was found to bind the bidentate $Cr(H_2O)_4$ATP complex tighter than the bidentate $Rh(H_2O)_4$-ATP complex. Fructose-6-phosphate kinase, on the other hand, binds the bidentate $Rh(H_2O)_4$ATP complex significantly tighter than it does the bidentate $Cr(H_2O)_4$ATP complex. Finally, adenylate kinase displayed greater stereospecificity in binding the two β,γ-bidentate $Rh(H_2O)_4$ATP isomers than had been noted for the two $Cr(H_2O)_4$ATP stereoisomers (Sanders et al., 1989). These results indicate that some kinases do recognize structural and/or electronic differences between the $Rh(H_2O)_n$ATP and $Cr(H_2O)_n$ATP complexes. This observation is in contrast to the behavior exhibited by these kinases toward the α,β-bidentate $Rh(H_2O)_4$ADP vs α,β-bidentate $Cr(H_2O_4)$ADP complexes, where little to no differences in binding affinities were found (Shorter et al., 1987).

Conclusions. The $Rh(H_2O)_n$ATP complexes have proven to be superior to the $Cr(H_2O)_n$ATP and $Co(NH_3)_n$ATP complexes as probes of kinase MgATP substrate structure. We found greater consistency between the substrate structure predicted using the $Rh(H_2O)_n$ATP isomers as probes and that predicted using the ^{17}O–Mn EPR and Mg(II)/Cd(II) ATPS methods than has been previously observed using the Cr-$(H_2O)_n$ATP or $Co(NH_3)_n$ATP complexes as probes. Moreover, the ability to determine the purity and structure of both the $Rh(H_2O)_n$ATP reactant and product complexes by ^{31}P NMR offers, as exemplified by the pyruvate kinase study, quite an advantage.

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