Investigations of Substrate Specificity and Reaction Mechanism of Several Kinases Using Chromium(III) Adenosine 5′-Triphosphate and Chromium(III) Adenosine 5′-Diphosphate†

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ABSTRACT: β,γ-Bidentate and α,β,γ-tridentate CrATP were tested as inhibitors vs. MgATP of several kinases. Bidentate CrATP served as a much stronger competitive inhibitor of hexokinase and glycerokinase than tridentate CrATP, while tridentate CrATP was a slightly stronger competitive inhibitor of creatine, fructose-6-P, 3-P-glycerate, and acetate kinases. Bidentate and tridentate CrATP were equally effective inhibitors of myokinase and arginine kinase. The substrate activities of bidentate and tridentate CrATP with these kinases were tested with a CD assay sensitive to multiple turnovers and a radioisotopic assay sensitive to fractional turnovers. In each case bidentate and not tridentate CrATP served as a substrate. The relative V_{max} values for $Co(NH_3)_4ATP$, $Cr(H_2O)_4ATP$, and $Cr(NH_3)_4ATP$ with hexokinase at pH 5.9 were 176:9:1. Hexokinase, glycerokinase, creatine kinase, and arginine kinase were specific for the left-hand screw sense (Λ) bidentate isomer, while pyruvate kinase, myokinase, and fructose-6-P kinase were specific for the right-hand screw sense (Δ) isomer. Both ring conformers of the correct screw sense were substrates. Neither hexokinase nor creatine kinase isomerized the ring puckering isomers in the absence of a turnover. The product of the hexokinase reaction was the cis monodentate complex $Cr(H_2O)_4(ADP)$(glucose-6-P), which after release from the enzyme was slowly converted to the trans isomer, which showed no substrate activity in the reverse reaction but was a strong competitive inhibitor vs. MgATP. The rate-limiting step of the kinase–CrATP reaction was determined to be release of the $Cr(H_2O)_4(ADP)$(substrate-P) complex from the closed catalytic form of the enzyme. All of the kinases tested gave a rapid single turnover, except for pyruvate kinase with pyruvate as a substrate, which showed 1.5% turnover (but a full turnover with glycolate), and creatine and arginine kinases, which showed 2–3% turnover. Hexokinase was inhibited 1000-fold more strongly by monodentate CrADP than by bidentate CrADP, while creatine and pyruvate kinases were equally inhibited by both. We conclude that hexo- and glycerokinases release monodentate MgADP after breaking of the coordination bond from phosphorylated substrate to Mg but that all other kinases tested catalyze migration of Mg to give bidentate MgADP as the product. Only one screw sense isomer of MgADP is probably produced, as indicated in the case of creatine kinase by the 20-fold difference in inhibition constants between the two separated isomers of bidentate CrADP.

ATP (as well as ADP, ROPP, and PP_i) exists under physiological conditions primarily as the Mg^{2+} chelate, and it is this form of the nucleotide which is the substrate for most ATP-utilizing enzymes. In solution MgATP exists as a rapidly equilibrating mixture of isomers, only one of which presumably possesses the proper structure and stereochemistry for reaction with a specific enzyme. Through knowledge of the structure and stereochemistry of the Mg^{2+} substrate and product complexes, one should be able to trace the movement of the metal during the reaction and thus gain a great deal of information about the mechanism of and the catalytic role of the metal in the enzyme-catalyzed reaction. To this end a number of exchange-inert cobalt(III) and chromium(III) nucleotide complexes have been prepared in this laboratory [DePamphilis & Cleland, 1973; Janson & Cleland, 1974; Danenberg & Cleland, 1975; Cornelius et al., 1977; Cleland & Mildvan, 1979; see also Dunaway-Mariano & Cleland (1980)]. The stereo- and structural specificity of yeast hexokinase for MgATP has recently been shown to be (Λ) β,γ-bidentate[1] by demonstrating the substrate activity of this isomeric form of $Co(NH_3)_4ATP$ (Cornelius & Cleland, 1978), and the specificity of rabbit muscle pyruvate kinase has been shown to be (Δ) β,γ-bidentate by demonstrating the substrate activity of this isomeric form of CrATP (Dunaway-Mariano et al., 1979). Li et al. (1978) have also used $Co(NH_3)_4ATP$ isomers to show that the pyrophosphate transferring enzyme P-ribose-PP synthetase is specific for (Δ) β,γ-bidentate MgATP.

The present studies describe the specificities of a series of kinases. Evidence is presented which supports a common reaction mechanism and catalytic role of Mg^{2+} in the mechanism for all kinases, except for the form of MgADP which is the substrate for the reverse reaction.

Materials and Methods[2]

$Co(NH_3)_4ATP$ and $Co(NH_3)_4ADP$ were prepared according to Cornelius et al. (1977) and purified on CHpA columns prior to use. Chromium(III) complexes of ATP and ADP were prepared as described in Dunaway-Mariano & Cleland (1980). All enzymes were from Sigma Chemical Co.: glycerokinase (*Candida mycoderma*), pyruvate kinase (rabbit muscle type III), hexokinase (baker's yeast, F-300), creatine kinase (rabbit

† From the Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706. *Received July 31, 1979.* This work was supported by a National Institutes of Health Postdoctoral Fellowship (GM-06598-01) to D.D.M. and prior to Dec 31, 1978, by grants from the National Institutes of Health (GM-18938) and the National Science Foundation (BMS-16134) and after Jan 1, 1979, by a grant from the National Science Foundation (PCM-23345) to W.W.C. A preliminary report on this work has been presented (Dunaway-Mariano, 1978).

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[1] The screw sense nomenclature is that of Cornelius & Cleland (1978). In this system, the reference axis is a line through the metal perpendicular to the chelate ring, and the bond from the chelate ring to the rest of the molecule is the skew line defining either a left-hand (Λ) or a right-hand (Δ) helix.

[2] Abbreviations used: BHEP, Cl salt of *N*,*N*′-bis(hydroxyethyl)-piperazine; Pipes, K salt of piperazine-*N*,*N*′-bis(2-ethanesulfonate); Mes, K salt of 2-(*N*-morpholino)ethanesulfonate; Hepes, K salt of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate; CHpA, cycloheptaamylose cross-linked with epichlorohydrin.

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muscle type I), acetate kinase (*Escherichia coli*), arginine kinase (lobster tail muscle), fructose-6-P kinase (rabbit muscle type III), myokinase (rabbit muscle), and 3-P-glycerate kinase (yeast type IV). CHpA gel was prepared by the method of Cornelius & Cleland (1978).

Monodentate CrADP. This compound was prepared by the method of Danenberg & Cleland (1975), with the Dowex-50-X2-H^+ column chromatography carried out at 4 °C using 0.1 M sodium acetate, pH 4, over a 5-h period. CrADP eluted from the column as a broad peak, the first third of which was monodentate and rest of which was bidentate. The fractions comprising the front edge of the peak had a pH of 2.5–3.5 and were used in the inhibition studies. The bidentate CrADP used for comparison was from the column fractions collected just behind those containing the monodentate CrADP. The inhibition studies were carried out immediately after elution of the CrADP from the column using the assays described below.

Dowex-50 Assay for Substrate Activity of CrATP. Reaction mixtures (0.25 mL) containing ^{14}C- or ^{3}H-labeled substrates and enzyme were incubated for a specified time, and the reaction was terminated by addition of 20 μL of $HClO_4$ and 2 drops of CCl_4 to the vortexing mixture. The solution was then diluted to 1 mL with cold water, filtered through a glass wool plug, and adsorbed onto a 25 × 0.5 cm column of Dowex-50-X2-H^+ (100–200 mesh) at 4 °C. The column was washed with 75 mL of 10 mM HCl, followed by either 75 mL of 1 M HCl or 100 mL of water. The 5-mL fractions were assayed for ^{14}C, and the relative amounts of unreacted substrate and product were determined.

CD Assay for Substrate Activity of Chromium and Cobalt ATP Complexes. Molar ellipticities are expressed in deg $cm^2/dmol$. The ellipticities of the reaction mixtures containing 1 mM bidentate CrATP and appropriate substrate and enzyme (5.3 mL in 10-cm microcylindrical cells at room temperature) were monitored at 575 nm and at pH 5.9 by using a sensitivity setting of 0.5–2 mdeg/cm and a chart speed of 0.1–0.2 cm/min. The reaction velocities were calculated assuming the total change in molar ellipticity to be 775 [an average for the two bidentate ring conformers; see Dunaway-Mariano & Cleland (1980)]. Reactions of 1.3 mM $Co(NH_3)_4ATP$ at pH 7 or 8 were monitored at 560 nm, and the total change in molar ellipticity during reaction was assumed to be 800 (Cornelius & Cleland, 1978). Reaction of 1.1 mM $Cr(NH_3)_4ATP$ at pH 7 was monitored at 520 nm, and the total change in molar ellipticity was assumed to be 800 on the basis of the similarities between $Co(NH_3)_4ATP$ and $Cr(NH_3)_4ATP$.

Inhibition Studies. The kinase inhibition studies reported in Table I were carried out at 25 °C and pH 5.8 (50 mM Pipes). The pyruvate kinase–lactate dehydrogenase couple (0.7 mM phosphoenolpyruvate, 0.2 mM NADH, 67 μg/mL pyruvate kinase, and 33 μg/mL lactate dehydrogenase) was used in the glycerokinase, creatine kinase, acetate kinase, myokinase, and arginine kinase catalyzed reactions. The aldolase–3-phosphoglyceraldehyde dehydrogenase couple (both 3 units/mL, plus 1 mM NAD and 13 mM arsenate) was used in the fructose-6-P kinase catalyzed reaction. For 3-phosphoglycerate kinase, 0.2 mM NADH and 67 μg/mL 3-phosphoglyceraldehyde dehydrogenase were used as a couple.

For the studies in Table II, all reactions contained 50 mM Pipes, pH 5.8. The hexokinase–glucose-6-P dehydrogenase couple (100 units/mL hexokinase, 20 units/mL glucose-6-P dehydrogenase, 5–10 mM glucose, and 1 mM NADP) was used in the acetate kinase, creatine kinase, and myokinase catalyzed reactions. Glucose-6-P dehydrogenase (20 units/mL) and 1 mM NADP were used to follow the hexokinase reaction, and lactate dehydrogenase was used to follow pyruvate kinase.

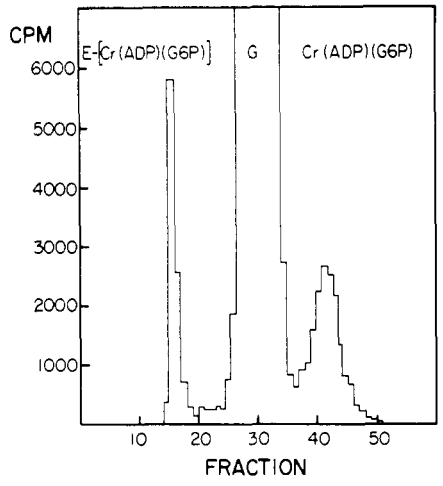

FIGURE 1: Chromatography of a 0.3-mL reaction mixture containing 10 μM hexokinase, 1 mM [^{14}C]glucose, 1 mM bidentate CrATP, and 60 mM Pipes, pH 7, on a 55 × 1.3 cm column of Sephadex G-25 at 4 °C, using 50 mM Pipes, pH 7, as the eluant (1.6-mL fractions). The reaction mixture was incubated for 10 min at 25 °C before being cooled and placed on the column.

Results

Substrate Activity of CrATP with Hexokinase. The substrate activity of bidentate CrATP was first demonstrated by incubating 1 mM CrATP and [^{14}C]glucose with 10 μM hexokinase at pH 7 and 25 °C for 10 min and then chromatographing the reaction mixture on a Sephadex column (Figure 1). The first peak eluted from the column is partly a hexokinase–CrATP–glucose complex but largely a hexokinase–Cr(ADP)(glucose-6-P) complex, as reported by Danenberg & Cleland (1975); the second peak is unconsumed glucose, and the third peak is Cr(ADP)(glucose-6-P). The original reaction mixture was calculated to be 17 μM in free Cr(ADP)(glucose-6-P) and 10 μM in the enzyme–substrate plus enzyme–product complex.

In a more convenient assay, the enzyme was first precipitated from the reaction mixture with acid, thus releasing any bound substrate or product back into the solution, which was then filtered and chromatographed on a small Dowex-50-H^+ column. Unreacted [^{14}C]glucose was eluted with 10 mM HCl, and the product, Cr(ADP)([^{14}C]glucose-6-P), was eluted with 1 M HCl. At pH 6 and 25 °C, bidentate CrATP gave a single turnover on the enzyme (15 μM product) in 1 min. Tridentate CrATP under the same conditions showed no conversion to product in 45 min.

Next, the four individual bidentate CrATP isomers described in Dunaway-Mariano & Cleland (1980) were tested as substrates for hexokinase at pH 6 and 25 °C, and the time courses for the reactions are shown in Figure 2. It was necessary to use pH 6 for these studies, since bidentate CrATP isomers epimerize rapidly at higher pH values [see Dunaway-Mariano & Cleland (1980)]. All four time courses were biphasic, with the amplitude of the burst corresponding to a single turnover on the enzyme. For isomers 1 and 4 the single turnover was complete in less than 30 s, while for isomers 2 and 3 the first-order rate constant for a single turnover was 0.3 min^{-1}. The first-order rate constants for the slow reactions following the single turnover of each of the four isomers ranged from 0.0025 to 0.005 min^{-1}.

The time courses for a single turnover of the four bidentate isomers on hexokinase were examined at pH 5.5 and 0 °C

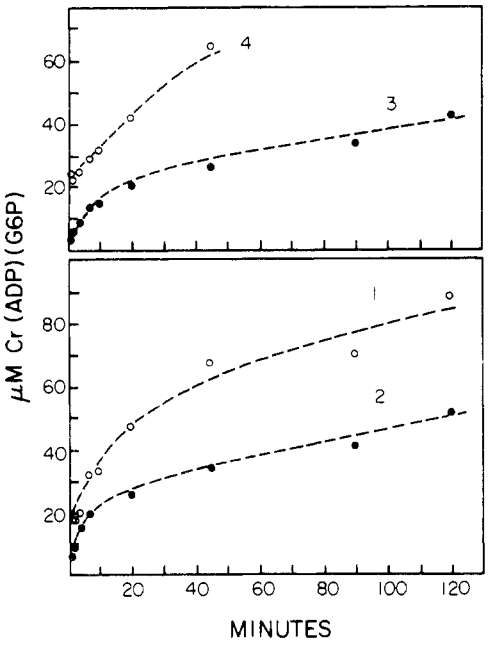

FIGURE 2: Time course for reaction of the four bidentate CrATP isomers (110 μM) with 1.34 mM [^{14}C]glucose and 18 μM hexokinase at 25 °C and pH 6 (20 mM Pipes).

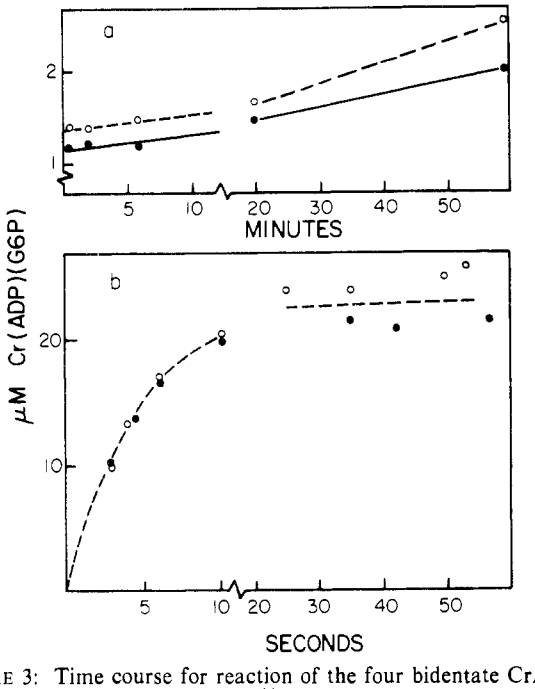

FIGURE 3: Time course for reaction of the four bidentate CrATP isomers (220 μM) with 1.65 mM [^{14}C]glucose and 22 μM hexokinase at 0 °C and pH 5.5 (60 mM Mes). (a) Isomers 2 (●) and 3 (○); (b) isomers 1 (○) and 4 (●).

(Figure 3). Isomers 2 and 3 showed small bursts of product formation which amounted to 5% of a single turnover and corresponded to 1% contamination by isomers 1 and 4. The first-order rate constant for a single turnover of isomer 1 was 0.20 s^{-1} and of isomer 4 was 0.23 s^{-1}.

The catalytic equilibrium constant [E–cis-Cr(ADP)(glucose-6-P)]/[E–CrATP–glucose] for hexokinase at pH 5.5 and 0 °C was determined to be 16 by incubating [^{14}C]glucose (65 μM) for 30 min with sufficient excess hexokinase (200 μM) and isomer 4 of bidentate CrATP (0.8 mM) so that all of the glucose was adsorbed on the enzyme. The thermodynamic equilibrium constant for the reaction, [*cis*-Cr(ADP)(glucose-6-P)]/([CrATP][glucose]), could not be determined

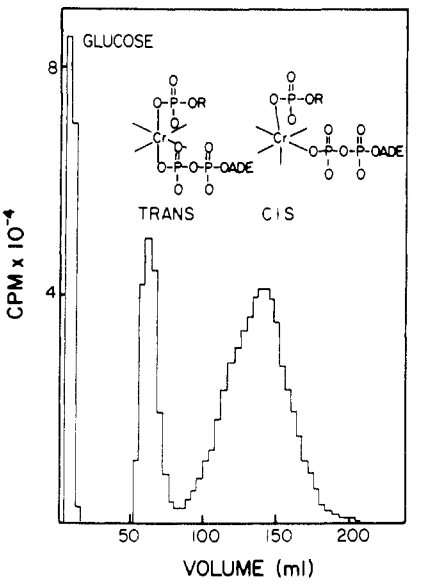

FIGURE 4: Chromatography of a 0.27-mL reaction mixture containing 210 μM hexokinase, 0.8 mM [^{14}C]glucose, and 0.9 mM bidentate CrATP isomer 4 on a 30 × 0.5 cm column of Dowex-50-H^+ at 4 °C. The reaction mixture was incubated for 30 min at 25 °C in 55 mM Pipes, pH 6, and terminated by adding 40 μL of $HClO_4$ and 2 drops of CCl_4, vortexing, diluting to 2 mL with water, and centrifuging. The column was eluted with 45 mL of 10 mM HCl, followed by water.

because *cis*-Cr(ADP)(glucose-6-P) isomerized into the trans isomer when released into solution, as described below.

Substrate Activity of Cr(ADP)(glucose-6-P) with Hexokinase. Cr(ADP)(glucose-6-P) was prepared by incubating bidentate CrATP isomer 4 and glucose with hexokinase at 25 °C for 30 min. The reaction mixture was chromatographed on a Dowex-50-H^+ column in the usual manner except that Cr(ADP)(glucose-6-P) was eluted as two peaks from the column by using water (Figure 4). The first product eluted from the column (which has the lower γ-phosphate pK_a) was *trans*-Cr(ADP)(glucose-6-P), while the second product eluted was the corresponding cis isomer. When the incubation time was only several minutes, the product was largely cis, but when the incubation was for several hours at 25 °C so that most of the product present had dissociated from the enzyme, the product was almost entirely trans. It appears that the isomerization occurs only in solution and not on the enzyme and that the equilibrium strongly favors the trans form but is reversible as shown by the following data.

Freshly prepared *cis*- and *trans*-Cr(ADP)(glucose-6-P) isomers (6.8 μM) when incubated in 50 mM Mes, pH 5.9, with 8 μM hexokinase at 4 °C gave the following yields of glucose after removal of protein and chromatography on Dowex-50-H^+ in the usual way: trans, 0.5% in 30 min and 11% in 18 h; cis, 12% in 30 min and 43% in 18 h. Under the same conditions, but in the absence of hexokinase, these complexes decomposed with liberation of glucose-6-P (not adsorbed on Dowex-50-H^+ but adsorbed on Dowex-1-Cl^- at pH 7) to the extent of 0.2–0.3% after 30 min and 6.5% after 18 h. After a 6-h period at pH 6.5 and 4 °C, a solution of *cis*-Cr(ADP)(glucose-6-P) contained 1% glucose-6-P, 36% *trans*-Cr(ADP)(glucose-6-P), and 63% of the cis isomer.

The *cis*- and *trans*-Cr(ADP)(glucose-6-P) isomers (prepared as in Figure 4, except that 80 μM hexokinase and 200 μM CrATP isomers 1 and 4 were used; excess CrATP will bleed from the column and contaminate the products) were tested as inhibitors vs. MgATP of hexokinase at pH 5.8 (50 mM Pipes; glucose-6-P dehydrogenase was used to follow the reaction). At saturating levels of glucose (20 mM), the trans

isomer gave competitive inhibition, K_{is} = 0.57 ± 0.04 μM, while at 13 μM glucose (about $K_m/8$), it gave noncompetitive inhibition, K_{is} = 5 ± 1 μM and K_{ii} = 2.8 ± 0.4 μM. The cis isomer in the presence of 13 μM glucose also gave noncompetitive inhibition, K_{is} = 0.6 ± 0.1 μM and K_{ii} = 0.9 ± 0.2 μM. It appears that in the absence of glucose, both cis and trans complexes can be present on the enzyme at the same time as MgATP and presumably occupy the sugar binding site. As previously observed for CrATP by Danenberg & Cleland (1975), the inhibitory power of both the cis and trans isomers in the hexokinase–MgATP–glucose reaction mixtures increased as the reaction proceeded.

Substrate Activity of $Co(NH_3)_4ATP$, $Cr(NH_3)_4ATP$, and $Cr(H_2O)_4ATP$ with Hexokinase Determined by CD Measurements. The relative reactivities of $Co(NH_3)_4ATP$, $Cr(NH_3)_4ATP$, and $Cr(H_2O)_4ATP$ with hexokinase and glucose were determined by monitoring changes in ellipticities at 560, 520, and 575 nm, respectively. At pH 7 the turnover number for $Cr(NH_3)_4ATP$ was 0.033 min^{-1} and for $Co(NH_3)_4ATP$ was 5.8 min^{-1}. At pH 5.9 the turnover number was 2.9 min^{-1} for $Co(NH_3)_4ATP$ and 0.14 min^{-1} for $Cr(H_2O)_4ATP$. As the $Cr(NH_3)_4ATP$ and $Cr(H_2O)_4ATP$ reactions proceeded, the ellipticity increased in a positive direction, while with $Co(NH_3)_4ATP$ it increased in a negative direction.

The ATPase activity of 28 μM hexokinase at pH 5.7 (10 mM Pipes) was examined by using 0.8 mM bidentate isomer 4 as the substrate in the presence and absence of 16 mM lyxose by following the disappearance of ellipticity at 575 nm. The turnover number at 15 °C for CrATP in the presence of lyxose was 0.023 min^{-1} and in its absence was 0.020 min^{-1}. Bidentate CrATP isomer 1 (0.18 mM) was then reacted with a stoichiometric amount of hexokinase at 25 °C, and the change in ellipticity at 575 nm was measured. The half-life of this reaction was about 10 min, but when lyxose was added after the reaction had reached 50% conversion, reaction of the CrATP isomer was complete within 1 min. The reaction product, $Cr(H_2O)_4(ADP)(P_i)$, was isolated from the reaction mixture by CHpA chromatography at pH 5.6, 4 °C. CrATP, like MgATP, is thus hydrolyzed in the absence of a sugar acceptor, and the process is stimulated greatly by lyxose. The rates with MgATP [~1 min^{-1} at optimum pH (Colowick, 1973)] and CrATP [~0.3 min^{-1}, correcting for the drop in activity at pH 5.7 (Viola & Cleland, 1978)] are in fact similar, except that with CrATP, subsequent release of $Cr(ADP)(P_i)$ is rate limiting for multiple turnovers.

Inhibition of Hexokinase with CrADP. β-Monodentate CrADP and α,β-bidentate CrADP were tested at pH 5.6 as inhibitors vs. MgATP of hexokinase. Both complexes gave competitive inhibition (monodentate CrADP, K_{is} = 3.2 ± 0.2 μM; bidentate CrADP, K_{is} = 3.3 ± 0.3 mM). The inhibitory power of monodentate CrADP increased during the reaction, while that of bidentate CrATP remained constant. Bidentate CrADP isomers I and II, separated on a cycloheptaamylose column as described in Dunaway-Mariano & Cleland (1980), both gave competitive inhibition, and the K_{is} values are listed in Table II.

Glycerokinase. The substrate activities of CrATP and $Co(NH_3)_4ATP$ with glycerokinase were tested by using both the Dowex-50-H^+ and CD assays. Tridentate CrATP and $Co(NH_3)_4ATP$ showed no activity. On the other hand, the turnover number for bidentate CrATP at pH 5.9 and 25 °C in the CD assay was 0.38 min^{-1}. The single turnover time courses for reaction of the individual bidentate CrATP isomers at pH 5.5 and 0 °C were measured with the Dowex column assay (Figure 5). Isomers 2 and 3 showed a small burst of

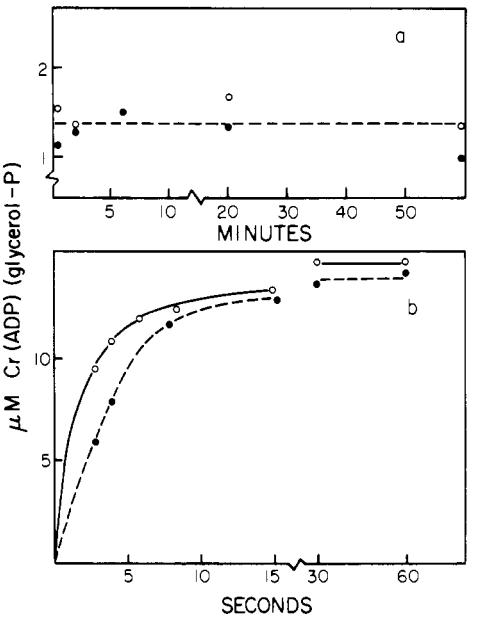

FIGURE 5: Time course for reaction of the four bidentate CrATP isomers (220 μM) with 1.8 mM [^{14}C]glycerol and 12 μM glycerokinase at pH 5.5 (60 mM Mes) and 0 °C. (a) Isomers 2 (○) and 3 (●); (b) isomers 1 (○) and 4 (●).

Table I: Inhibition Constants of CrATP Isomers as Competitive Inhibitors vs. MgATP of Several Kinases

kinase	K_{is} (μM) bidentate CrATP[c]	K_{is} (μM) tridentate CrATP[d]
hexokinase	0.069 ± 0.007[a]	120 ± 10
glycerokinase	0.14 ± 0.02	240 ± 20
pyruvate kinase[b]	135	600
creatine kinase	68 ± 6	18.3 ± 0.6
acetate kinase	900 ± 100	180 ± 20
fructose-6-P kinase	140 ± 20	58 ± 9
3-phosphoglycerate kinase	63 ± 7	28 ± 3
arginine kinase	89 ± 7	68 ± 9
myokinase	11.7 ± 0.7	11 ± 1

[a] Value from Dunaway-Mariano & Cleland (1980). [b] These values from Dunaway-Mariano et al. (1979) represent K_m values for the enolization of pyruvate. [c] A mixture of all four bidentate isomers was used. [d] A mixture of tridentate isomers was used.

product formation which amounted to 10% of a single turnover on the enzyme and corresponded to 0.6% contamination by isomers 1 and 4. The first-order rate constants for a single turnover of isomers 1 and 4 were 0.34 and 0.28 s^{-1}, respectively.

Bidentate CrATP and tridentate CrATP were tested as inhibitors vs. MgATP of glycerokinase at pH 5.9. Both complexes showed competitive inhibition, and the inhibition constants are listed in Table I.

Pyruvate Kinase. Dunaway-Mariano et al. (1979) reported that pyruvate kinase used bidentate CrATP and not tridentate CrATP or bidentate $Co(NH_3)_4ATP$ as a substrate in the reaction with glycolate. On the basis of single turnover experiments, it was concluded that the substrate activity resulted from reaction of the Δ screw sense isomers (2 and 3) and not the Λ ones (1 and 4). In the present study, the CD assay was used to confirm this result and to measure the rate of release of the product, Cr(ADP)(P-glycolate). At pH 5.9 and 25 °C the ellipticity at 575 nm increased in the predicted negative direction, and the turnover number was 0.028 min^{-1}. As expected, $Co(NH_3)_4ATP$ at pH 7 showed no activity.

The relative reactivities of bidentate CrATP isomers 2 and 3 with pyruvate kinase and glycolate were determined by

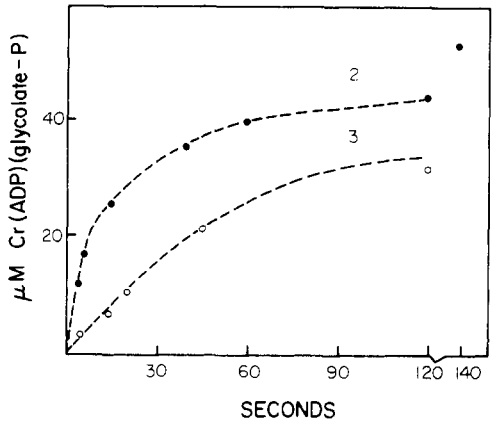

FIGURE 6: Time course for reaction of 0.46 mM bidentate CrATP isomer 2 or 3 with 10.6 mM [^{14}C]glycolate and 50 μM pyruvate kinase at pH 6 and 25 °C. The reactions contained 100 mM KCl, 4 mM $MnCl_2$, and 60 mM Pipes.

measuring the time courses for a single turnover at pH 6 and 25 °C with the Dowex column assay (Figure 6). A control containing no pyruvate kinase showed no product formation. The first-order rate constant for a single turnover of isomer 2 was 0.10 s^{-1} and of isomer 3 was 0.02 s^{-1}. Bidentate CrATP isomers 1 and 4, incubated for 10 min under the same conditions, showed no significant reaction.

The substrate activity of 1 mM Δ bidentate CrATP (mixture of isomers 2 and 3) with 0.68 mM pyruvate kinase was also tested with 0.53 mM [^{14}C]pyruvate at pH 5.8 (100 mM Pipes, 4 mM $MnCl_2$, and 75 mM KCl). After 15 min at 25 °C the enzyme was precipitated with $HClO_4$, and the filtrate was chromatographed at 4 °C on a 30 × 0.6 cm column of Dowex-1-X2-Cl^- equilibrated with 10 mM BHEP, pH 5, by using as the eluant 350 mL of a linear gradient (0–0.3 M) of KCl in the same buffer, followed by 1 M HCl. Unreacted [^{14}C]pyruvate eluted at 40 mM KCl, and traces of pyruvate dimer eluted at 100 mM KCl. The product, $Cr(H_2O)_4$(ADP)([^{14}C]P-enolpyruvate), was eluted with 1 M HCl. The ratio of apparent product to the total pyruvate used was 0.02 for the complete reaction and 0.008 for a control lacking CrATP. The net amount of product was 10 μM, corresponding to 1.5% of a turnover on the enzyme.

Monodentate and bidentate CrADP were tested at pH 5.9 as inhibitors of pyruvate kinase. Both showed competitive inhibition vs. MgADP (monodentate CrADP, K_{is} = 3.0 ± 0.3 mM; bidentate CrADP, K_{is} = 3.4 ± 0.3 mM). The two bidentate CrADP epimers were tested individually as inhibitors of pyruvate kinase, and the K_{is} values are listed in Table II.

Fructose-6-P Kinase. Three assay methods were used to demonstrate the substrate activity. First, in a 0.17-mL volume, 0.44 mM bidentate Cr[^{14}C]ATP was incubated at pH 5.9 (75 mM Pipes) with 20 mM fructose-6-P and 1000 units/mL kinase for 1 h at 25 °C and chromatographed, after removal of protein with $HClO_4$, at 4 °C on a 20 × 0.5 cm column of Dowex-50-H^+. The product, Cr([^{14}C]ADP)(fructose-di-P) was eluted with 60 mL of water, and the unconsumed Cr-[^{14}C]ATP was eluted with 1 M HCl. The radioactivity in the water wash was 45% more than that in a control containing no enzyme, corresponding to 24 μM product.

Second, 0.75 mM CrATP (bidentate or tridentate) was incubated with 0.44 mM [^{14}C]fructose-6-P and 7000 units/mL kinase in a 0.28-mL volume at pH 6 (80 mM Pipes) and 25 °C for 15 min and chromatographed, after removal of protein with $HClO_4$, on a Dowex-1 column as described above for pyruvate kinase. The enzyme used for this assay was exposed to 3-P-glycerate kinate and 3-P-glyceraldehyde dehydrogenase, 3-P-glycerate, NADH, arsenate, and $MgCl_2$ to remove traces of ATP before use. Unreacted fructose-6-P was eluted at 30 mM KCl, and the product was eluted at 80 mM. The ratio of radioactive product to total radioactivity was 0.18 for a control lacking CrATP, 0.35 for the complete reaction mixture with bidentate CrATP, and 0.15 with tridentate CrATP. After correction for the control, the bidentate reaction contained 75 μM product.

Table II: Inhibition Constants of Mono- and Bidentate CrADP Isomers as Competitive Inhibitors vs. MgADP of Several Kinases

enzyme	monodentate CrADP (mM)	bidentate CrADP (mM) isomer I[a]	bidentate CrADP (mM) isomer II[a]
pyruvate kinase	3.0 ± 0.3	3.8 ± 0.4	1.16 ± 0.06
acetate kinase		0.068 ± 0.008	0.061 ± 0.006
myokinase		1.3 ± 0.1	0.92 ± 0.07
hexokinase[b]	0.0032 ± 0.0002	1.8 ± 0.08	2.1 ± 0.1
creatine kinase	0.027 ± 0.001	0.0073 ± 0.0003	0.15 ± 0.01

enzyme	bidentate $Cr(NH_3)_4$ADP (mM) isomer I[a]	bidentate $Cr(NH_3)_4$ADP (mM) isomer II[a]
creatine kinase	0.199 ± 0.006	1.0 ± 0.3

enzyme	bidentate $Co(NH_3)_4$ADP (mM) isomer I[a]	bidentate $Co(NH_3)_4$ADP (mM) isomer II[a]
creatine kinase	6.7 ± 0.6	>10

[a] The isomers are labeled in order of elution from CHpA columns. [b] Vs. MgATP.

Third, the substrate activities of bidentate CrATP and $Co(NH_3)_4$ATP were tested by using the CD assay. At pH 7, $Co(NH_3)_4$ATP showed no activity. On the other hand, with 1 mM bidentate CrATP, 25 μM enzyme, and 60 mM fructose-6-P at pH 5.9, the ellipticity at 575 nm increased with time in the negative direction, showing that the active bidentate isomers have the Δ configuration. The turnover number was 0.38 min^{-1}.

Bidentate and tridentate CrATP were competitive inhibitors vs. MgATP of fructose-6-P kinase, and the K_{is} values are listed in Table I.

Myokinase. The substrate activity of CrATP with myokinase was tested by incubating 0.72 mM [^{3}H]AMP, 0.8 mM bi- or tridentate CrATP, and 4 mg/mL enzyme at pH 5.5 (30 mM Mes) and 25 °C for 30 min and chromatographing the mixture, after removal of protein, on a 30 × 0.5 cm column of Dowex-50-H^+ by using 25 mM KCl in 10 mM HCl as the eluant. AMP was eluted as a broad peak with the first 100 mL of eluant, and the product, $Cr(H_2O)_4(ADP)_2$, was eluted just behind this peak. Controls containing no enzyme or no CrATP showed no product. Under conditions where 83 μM product was formed from bidentate CrATP, none was formed from tridentate CrATP.

The stereospecificity of myokinase was determined by using 1.2 mM mixtures of bidentate CrATP isomers 2 and 3 or 1 and 4 as substrates in the above assay at pH 6, after preincubation with 0.21 mM pyruvate kinase, 33 mM glycolate, 120 mM KCl, and 12 mM $MnCl_2$ in a 0.105-mL volume for 5 min at 4 °C to allow time for removal of residual Δ isomers from the isomer 1 and 4 mixture prior to addition of 1 mg of myokinase and further incubation for 3 min. Fifty-five micromolar product was formed from isomers 2 and 3, and only 3 μM product was formed from isomers 1 and 4. In a CD assay, the ellipticity of a solution containing 1.3 mM bidentate CrATP, 10 mM AMP, and 436 units/mL myokinase at pH

5.9 and 25 °C increased in the negative direction [turnover = 0.006 mM/(min·unit·μL of enzyme)], showing Δ specificity. No substrate activity was seen with $Co(NH_3)_4ATP$ at pH 7 by the CD assay.

Bidentate and tridentate CrATP were competitive inhibitors vs. MgATP of myokinase, and the K_{is} values are listed in Table I. Bidentate CrADP isomers I and II both gave competitive inhibition vs. MgADP, and the K_{is} values are listed in Table II.

3-Phosphoglycerate Kinase. In the CD assay no product formation was observed with 1 mM bidentate CrATP, 100 mM 3-P-glycerate, and 800 units/mL kinase at pH 5.9. Bidentate and tridentate CrATP were competitive inhibitors vs. MgATP, and the K_{is} values are listed in Table I.

Arginine Kinase. While no product formation from bidentate CrATP was observed at pH 5.9 in a CD assay, small quantities of the product, $Cr(H_2O)_4(ADP)$(arginine-P), were detected in single turnover experiments. The 1 mM CrATP isomers were reacted with 0.86 mM [^{14}C]arginine (purified by passage over Dowex-1-Cl^-) and 1.9 mM arginine kinase at pH 6 and 25 °C for 20 min. After removal of protein, the mixtures were chromatographed on Dowex-1-Cl^- columns as described for pyruvate kinase by using 50 mL of 10 mM BHEP, pH 6, to elute the arginine and 1 mM HCl to elute the product. The radioactivity in the HCl eluate of controls lacking enzyme or CrATP, or of a mixture containing tridentate CrATP, was only 2% of that in the mixture containing bidentate CrATP, which produced 50 μM product (2.6% turnover).

The 0.5 mM mixtures of bidentate CrATP isomers 2 and 3 or 1 and 4 were incubated with 0.8 mM [^{14}C]arginine and 0.34 mM kinase in the same assay at pH 5.8. In addition, the bidentate isomer 2 and 3 reaction contained 0.6 mM glucose and 30 μM hexokinase, and the isomer 1 and 4 reaction contained 90 μM pyruvate kinase, 14 mM glycolate, 10 mM $MnCl_2$, and 100 mM KCl to remove traces of contaminating isomers of opposite screw sense. Seven micromolar product was seen from isomers 1 and 4 (2% turnover), and 1 μM product was seen from isomers 2 and 3. The 2–3% turnover is a minimum value, since at pH values below 6 arginine kinase denatures rapidly and precipitates from the reaction mixtures. Bidentate CrATP and tridentate CrATP were competitive inhibitors vs. MgATP, and the K_{is} values are reported in Table I.

Acetate Kinase. Neither bidentate CrATP at pH 5.9 nor $Co(NH_3)_4ATP$ at pH 8 showed activity in the CD assay with acetate and acetate kinase. In a radioisotopic assay, 0.76 mM bidentate Cr[^{14}C]ATP was incubated with 22 mg/mL kinase in the absence of acetate at pH 6.5 and 4 °C for 30 min and then chromatographed on a 55 × 1.3 cm column of Sephadex G-25 at 4 °C with 50 mM Pipes, pH 6.5, as the eluant. The elution profile and that of a control without enzyme are shown in Figure 7. Twenty hours after elution, 14% of the adenine in the enzyme-containing fractions was covalently bound to the enzyme, and the rest was noncovalently bound. It thus appears that CrATP, like MgATP (Anthony & Spector, 1971), can phosphorylate the enzyme to give what is presumably Cr(ADP)(E–P). The identity of the second sharp peak in the elution profile is not known. It may result from breakdown of Cr(ADP)(E–P) or possibly from reaction of CrATP with traces of acetate. Further work is needed, but it is clear that acetate kinase does react with bidentate CrATP and that determination of the stereospecificity should be possible.

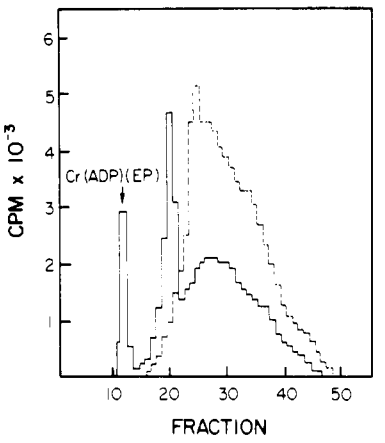

FIGURE 7: Elution profile from a Sephadex G-25 column of a reaction mixture containing ^{14}C-labeled bidentate CrATP and acetate kinase (—) or CrATP alone (---). Elution was with 50 mM Pipes, pH 6.5, at 4 °C.

Bidentate and tridentate CrATP were competitive inhibitors vs. MgATP, and the K_{is} values are shown in Table I.

Creatine Kinase. No substrate activity was seen in a CD assay with bidentate CrATP and creatine. The 1.5 mM bidentate or 2.1 mM tridentate CrATP was incubated with 1.45 mM [^{14}C]creatine (purified by passage over Dowex-1-Cl^-) and 1.8 mM kinase at pH 6 and 25 °C for 1 h, and the reaction mixtures were chromatographed, after removal of protein, on Dowex-1-Cl^- columns as described for pyruvate kinase. Unconsumed creatine was eluted with 50 mL of 10 mM BHEP, pH 5.5, and the product, $Cr(H_2O)_4(ADP)$(creatine-P), was eluted with 1 M HCl. The tridentate CrATP reaction mixture and a control not containing enzyme gave no product, while the bidentate CrATP reaction contained 23 μM product (1.3% turnover). When mixtures of pure screw sense bidentate isomers were used, 21 μM product (1% turnover) was obtained from isomers 1 and 4, but only 1 μM product was obtained from isomers 2 and 3, which had been preincubated with glucose and hexokinase to remove traces of Λ isomers.

K_{is} values for bidentate and tridentate CrATP as competitive inhibitors vs. MgATP of creatine kinase are in Table I. Monodentate CrADP and bidentate CrADP both gave competitive inhibition vs. MgADP (monodentate, K_{is} = 27 ± 1 μM; bidentate, K_{is} = 25 ± 1 μM). The pure α-phosphate epimers of $Co(NH_3)_4ADP$, $Cr(NH_3)_4ADP$, and $Cr(H_2O)_4ADP$ were also tested vs. MgADP, and the K_{is} values are listed in Table II.

An atttempt was made to examine the conformations of the chelate rings of bidentate CrATP isomers 1 and 4 while bound to the active site of creatine kinase by measuring the CD spectra of solutions containing 0.28 mM creatine kinase, 27 mM creatine, and 0.25 mM pure CrATP isomer at pH 5.9, 25 °C. Unfortunately, because of the limited solubility of creatine and the unfavorable dissociation constant for the E–CrATP–creatine complex (K_i = 14 mM for creatine at pH 7; Schimerlik & Cleland, 1973), both the binary E–CrATP and ternary E–CrATP–creatine complexes were present. The CD spectra of the isomer 1 and 4 solutions were quite similar in that both showed maxima at 600 nm (–) and 440 nm (+) which are quite shifted from those of the free CrATP isomer [575 nm (–) and 450 nm (+)]. However, the amplitudes of these maxima differed for the two enzyme-bound isomers (isomer 1, −1500 and +600; isomer 4, −625 and +350), showing that the two isomers are not bound to the enzyme in the same conformation. Interestingly, the positive ellipticities at 675 and 640 nm of isomers 1 and 4 disappear upon binding

Table III: Stereospecificity of Several Kinases toward Bidentate CrATP Isomers[a]

kinase	CD assay[b]	^{14}C assay[c]	apparent % turnover[d]
hexokinase	Λ	Λ	100
glycerokinase	Λ	Λ	100
fructose-6-P kinase	Δ	[e]	100
myokinase	Δ	Δ	100
pyruvate kinase	Δ	Δ	100 or 1.5[f]
creatine kinase	–	Λ	2
arginine kinase	–	Λ	3

[a] None of the kinases shown gave substrate activity with tridentate CrATP. [b] Where Λ or Δ is given, the specificity was determined from the change in ellipticity at 575 nm. (–) means no activity in the CD assay. [c] Where Λ or Δ is given, bidentate CrATP isomers 1 and 4 or 2 and 3 were tested separately for substrate activity. The myokinase assay used [^{3}H]AMP. [d] Product produced in ^{14}C assay relative to enzyme concentration. The values of 100 are approximate (for example, with hexokinase where the catalytic equilibrium constant on the enzyme is 16, the exact value would be 94), and the observed product concentration increased somewhat with incubation time in those cases where a positive CD assay result shows that product is released at a finite rate from the enzyme. [e] Activity seen with mixed bidentate isomers, but no separate test with isomers 1 and 4 or 2 and 3. [f] Glycolate, 100; pyruvate, 1.5.

of the isomers to the enzyme.

Discussion

Metal and Ligand Specificity. In the present study we found that a number of kinases will accept bidentate Cr$(H_2O)_4$ATP as a substrate, while only hexokinase will accept Co$(NH_3)_4$ATP. The inertness of Co$(NH_3)_4$ATP toward most kinases is likely a result of poor binding interactions between the ammonia ligands (which can not serve as hydrogen-bond acceptors) and kinase active-site residues. Similar results were reported by Janson & Cleland (1974), who found that the K_{is} value of Cr$(H_2O)_n(NH_3)_m$ATP varied in direct proportion to m/n. With hexokinase, we found V_{max} for the cobalt(III) and chromium(III) complexes to decrease in the order Co$(NH_3)_4$ATP, Cr$(H_2O)_4$ATP, and Cr$(NH_3)_4$ATP. As discussed in more detail below, the low V_{max} of Cr$(H_2O)_4$ATP results from a very low product release step, rather than a slow catalytic step, but we do not know what the slow step is for the tetraammine complexes.

Stereospecificity and Structural Specificity. As indicated in Table III, each of the kinases tested used β,γ-bidentate CrATP as a substrate, but none uses tridentate CrATP. Since tridentate CrATP binds as well or better than bidentate CrATP to each of the kinases except hexokinase and glycerokinase, the inertness of the tridentate complex may derive from inability of the β,γ-chelate ring, due to steric restraints placed on it by the fused α,β-chelate ring, to adopt the geometry of the transition state for the reaction.

The stereospecificities of the kinases were examined by using purified bidentate CrATP isomers or with the CD assay. As indicated in Table III, hexokinase, glycerokinase, creatine kinase, and arginine kinase are specific for the Λ screw sense (isomers 1 and 4), while pyruvate kinase, myokinase, and fructose-6-P kinase require the Δ screw sense (isomers 2 and 3).

It is interesting to compare these assignments with the results obtained by the use of ATPβS. Since Mg^{2+} strongly prefers to form coordination bonds to oxygen, as opposed to sulfur, the A diastereomer (*S*) of ATPβS will exist almost entirely as the Δ Mg complex, and the B diastereomer (*R*) will exist as the Λ Mg complex (Jaffe & Cohn, 1978). By this criterion hexokinase has Λ specificity (Jaffe & Cohn, 1978), and pyruvate kinase and myosin are Δ and acetate kinase Λ (Eckstein & Goody, 1976). In addition, P. M. J. Burgers and F. Eckstein (personal communication) have recently shown Λ specificity for creatine kinase using ATPβS. This result, as well as the assignment for hexokinase by Jaffe & Cohn (1978), has been confirmed by showing reversal of specificity (that is, a change from isomer B to isomer A) when Cd^{2+} is the metal. Since Cd^{2+} prefers to coordinate sulfur rather than oxygen, this apparent reversal (which is only apparent, since both CdATPβS, isomer A, and MgATPβS, isomer B, exist almost entirely as Λ chelates) provides confirmation that the isomer specificity results from metal specificity and not from enzyme preference for an oxygen in the noncoordinated β position when a hydrogen bond must form to this position. Armstrong et al. (1979) have in fact found that RNA polymerase uses both isomers of ATPβS with Mg^{2+}, but only the B isomer with Cd^{2+}, and have ascribed this result to formation of a hydrogen bond from the enzyme to the free β-oxygen (that is, the preference of Mg^{2+} for oxygen over sulfur is balanced by the preference of the enzyme for oxygen over sulfur in the hydrogen-bonding position). A similar situation may be responsible for the use of both isomers of MgATPβS by myokinase (Eckstein & Goody, 1976), which has Δ specificity. The results from the use of ATPβS, when confirmed by Mg^{2+}–Cd^{2+} reversal, thus appear to determine accurately the screw sense specificity of the enzyme for MgATP, but caution must be exercised if Cd^{2+} is not used in addition to Mg^{2+}, since it is only with the CdATPβS complexes, where the uncoordinated position on the β-phosphate is still an oxygen, that unambiguous results can be expected.

A second interesting observation which emerged from the stereospecificity studies is that while the half-lives for a single turnover are dramatically different (1000-fold at a minimum), hexokinase does react with both Λ bidentate CrATP and Δ bidentate CrATP (see Figure 2). At pH 6 and 25 °C, the first-order rate constant for a single turnover of Δ bidentate CrATP is 0.3 min^{-1}. In contrast, Δ bidentate Co$(NH_3)_4$ATP showed no reactivity toward hexokinase (Cornelius & Cleland, 1978). Thus, the enzyme catalyzes epimerization to the Λ isomer in the case of Δ bidentate CrATP but not in the case of Δ bidentate Co$(NH_3)_4$ATP. In Dunaway-Mariano & Cleland (1980) we reported that nonenzymatic epimerization of Cr$(H_2O)_4$ATP is subject to base catalysis. While three or more hydroxide ions appeared to participate in the overall process, the simplest mechanism can still be considered to involve deprotonation of a water ligand, expulsion of the β-phosphate, rotation, reinsertion, and finally protonation of the hydroxide ligand. At pH 6 this process is quite slow, but at pH 6.7 it would occur at 0.3 min^{-1}, the observed enzymatic rate. Hexokinase may catalyze this process by having some group in a position capable of deprotonation of a water ligand in Δ Cr$(H_2O)_4$ATP. In contrast, due to the high ammonia ligand pK_a value, Co$(NH_3)_4$ATP is stable to epimerization up to pH 8 at least and thus stable to epimerization in the hexokinase active site. Since hexokinase can catalyze epimerization of CrATP, it is possible that it does so even faster with MgATP. This would allow both Λ and Δ MgATP to act as substrates and eliminate the halving of V_{max} that would result from nonproductive binding of Δ MgATP, assuming that the two isomers are bound equally tightly. Note that glycerokinase does not seem to catalyze epimerization (compare Figures 3a and 5a).

Hexokinase, glycerokinase, pyruvate kinase, and creatine kinase were tested for specificity toward the two chelate ring conformers of the active bidentate CrATP substrate. As shown

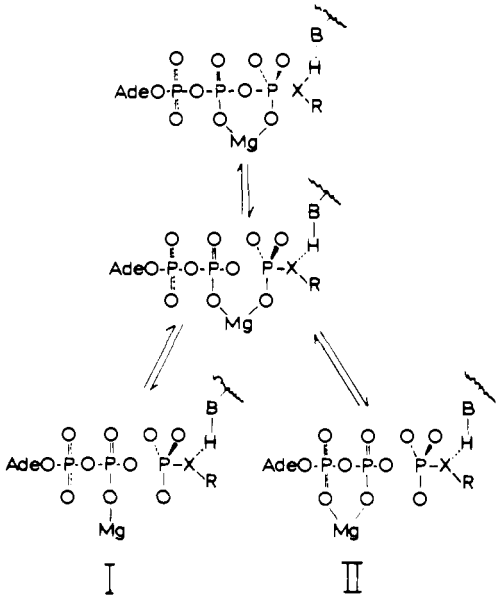

FIGURE 8: Proposed mechanisms of kinases.

in Figure 3, hexokinase reacts with the two Λ isomers at almost the same rate. In addition, as reported in the first paper of this series, the two Δ conformers and the two Λ conformers have nearly equal inhibition constants.[3] Like hexokinase, glycerokinase reacts equally well with both Λ conformers (see Figure 5). Thus, with glycerokinase and hexokinase it is likely that the two conformers are bound in a common catalytic conformation. We tried to demonstrate this point by showing that hexokinase catalyzes the interconversion of the two conformers in the presence or absence of lyxose. Instead we observed ATPase activity by hexokinase, with the rate of the chemical reaction stimulated greatly by lyxose but with the overall rate limited by release of the product, $Cr(H_2O)_4$-(ADP)(P_i).

In contrast, pyruvate kinase in the presence of glycolate showed a fivefold difference in reactivity toward the two Δ conformers (Figure 6). We do not know, however, whether this specificity would still exist with the natural substrate, pyruvate. The CD studies on the binding of the Λ conformers to creatine kinase indicate that the two conformers do not bind to the enzyme in the same manner. However, since both E–CrATP and E–CrATP–creatine complexes were present, it cannot be stated with certainty that the two isomers are bound in different conformations in the ternary complex. The present limited data suggest that conversion of the different ring conformers to a common geometry occurs only during the conformation change of the enzyme to the closed configuration from which the chemical reaction occurs and not in the open configuration of the enzyme from which reactants are free to dissociate.

Kinase Reaction Mechanisms. In Figure 8 are shown two reaction pathways which we propose are open to kinases. Mechanism I is typified by hexokinase, while mechanism II is typified by creatine kinase. The features of these two mechanisms will be described by using these two enzymes as examples.

Mechanism I. β,γ-Bidentate MgATP is bound to the hexokinase–glucose complex in an extended conformation as evidenced by the X-ray work of Steitz et al. (1977) and by the 3 orders of magnitude difference in the inhibition constants of tridentate and bidentate CrATP (Table I). Upon substrate binding hexokinase undergoes isomerization to a closed configuration which forces the chelate ring of the metal nucleotide complex into the catalytically active conformation. We do not know the details of this conformation, except that the coordination bonds between metal and phosphates are not broken. The 6-hydroxyl group of the bound glucose is activated by hydrogen bonding to an active site residue, which is identified by the pH studies of Viola & Cleland (1978) as a carboxyl group and by the X-ray work of Anderson et al. (1978) as an aspartate. The activated glucose then displaces the β-phosphate from the γ-phosphate with inversion of configuration by what is probably an S_N2 mechanism (Orr et al., 1978; Blattler & Knowles, 1979). The transition state for this process is stabilized by delocalization of negative charge onto the magnesium and onto other enzyme groups hydrogen bonded to the other two oxygens of the γ-phosphate. These hydrogen-bonding interactions presumably form only during the conformation change that establishes the catalytic configuration, or otherwise considerable ATPase activity would be expected.

The product of the transphosphorylation step is the *cis*-$Mg(H_2O)_4$(ADP)(glucose-6-P) complex, as demonstrated by isolation of the corresponding chromium complex from the reaction with CrATP. The enzyme at this stage is in a closed conformation, as indicated by the very slow release (Figures 2 and 3) of Cr(ADP)(glucose-6-P). In the next step, the bond between the magnesium and the glucose-6-P breaks, the enzyme reassumes the open conformation, and β-monodentate MgADP is released from the enzyme. Monodentate rather than bidentate MgADP is believed to be the reaction product since (1) the K_{is} of monodentate CrADP is 1000-fold smaller than that for bidentate CrADP, (2) the bismonodentate complex *cis*-$Cr(H_2O)_4$(ADP)(glucose-6-P) shows very tight binding to free hexokinase and the trans isomer shows very tight binding to the hexokinase–glucose complex, and (3) even partial reversal of the stereospecificity of ATPαS formation from ADPαS does not occur when Mg, which chelates the α-phosphate oxygen in bidentate MgADPαS, is replaced with Cd, which chelates the α-phosphate sulfur in the bidentate complex (Cohn, 1979). An interesting feature of this mechanism is the obvious connection between bidentate MgATP being bound in the extended conformation so that the metal is not close to the α-phosphate and the product being the β-monodentate MgADP rather than α,β-bidentate MgADP.

Mechanism II. This mechanism, exemplified by that of creatine kinase, will be described in the ADP to ATP direction. In the first step, the Δ screw sense isomer of bidentate MgADP binds to the enzyme–creatine-P complex, as evidenced by the specificity for MgATPαS, isomer B, and even stronger specificity for CdATPαS, isomer A (P. M. J. Burgers and F. Eckstein, personal communication), the observed binding specificity of the two bidentate CrADP epimers (Table II), and tighter binding of the correct bidentate isomer than of monodentate CrADP.[4] The enzyme assumes a closed con-

[3] The K_{is} values of the Λ isomers are 6–10-fold lower than those of the Δ isomers, since the catalytic reaction rapidly produces 16 times as much E–Cr(ADP)(glucose-6-P) in equilibrium with the initial E–CrATP–glucose complex. The production of E–Cr(ADP)(glucose-6-P) from the Δ isomers is slow enough so that initial velocities are affected only by formation of E–CrATP–glucose.

[4] The lack of binding specificity between the two bidentate CrADP isomers seen with acetate and myokinases in Table II and the small specificity seen with pyruvate kinase do not mean that these enzymes are not specific for one screw sense isomer but only that the local geometry of the active site permits nonproductive binding of the inactive isomer, as is the case with bidentate CrATP isomers and hexokinase (see footnote 3 and the data in Table I of Dunaway-Mariano & Cleland (1980)).

formation in association with displacement of the α-phosphate from the coordination sphere of the metal and insertion of creatine phosphate to give the *cis*-$Mg(H_2O)_4$(creatine-P)-(ADP) intermediate. Since tridentate CrATP is bound even better than bidentate CrATP (Table I), it appears that the three phosphates are not in an extended configuration, but rather are so placed that only a small movement of the metal is needed to change from α,β to β,γ coordination. Phosphate transfer, which presumably is catalyzed in a manner similar to that in the hexokinase reaction, produces β,γ-bidentate MgATP and creatine as final products. The acid–base catalyst in this case is not aspartate, but on the basis of unpublished experiments in this lab by George Kenyon and Paul Cook it appears to be a histidine.

The basic difference between the two kinase mechanisms is the form of MgADP used. From the stability constants of MgAMP and MgADP [60 and 1620, respectively (Frey & Stuehr, 1972)], the ratio of monodentate MgADP to bidentate MgADP in solution can be estimated to be 1:27. Thus, it is quite reasonable that hexokinase, an enzyme which in vivo is responsible for glucose-6-P production, avoids product inhibition through its specificity for monodentate MgADP. Conversely, creatine kinase must have a tighter afffinity for MgADP than MgATP in order to function properly in the rephosphorylation of MgADP by creatine-P under conditions in the cell where MgATP is always present at levels above 1 mM, and this is accomplished by having the substrate be the major coordination form of MgADP in solution.

On the basis of the specificity for bi- vs. tridentate CrATP, and mono vs. bidentate CrADP, it appears that glycerokinase reacts by the type I mechanism, while all of the other kinases in Table II probably react by the type II mechanism. While the type I mechanism so far is seen only for enzymes catalyzing reactions which are irreversible in vivo, the type II mechanism is seen not only for enzymes catalyzing formation of MgATP, such as pyruvate, acetate, and 3-P-glycerate kinases, but also for ones catalyzing reactions that go both ways in vivo (creatine kinase and myokinase) and ones such as fructose-6-P kinase which are thought to be irreversible. Only further work will show whether the above pattern is general or simply an accident of evolution.

Kinase Catalytic Equilibrium Constant. Recent ^{31}P NMR studies have indicated that the equilibrium constants for the chemical interconversions of enzyme-bound reactants for arginine, creatine, adenylate, pyruvate (with both pyruvate and glycolate), and 3-P-glycerate kinases are all very close to 1 (Nageswara Rao et al., 1979). Thus, despite the diverse nature of these kinases, and despite the drastically different values of the equilibrium constants involved (over 10^4 difference between pyruvate and glycolate with pyruvate kinase, for example), the affinities for the reactants have been so adjusted that equal amounts of substrates and products will be bound at equilibrium. As described by Albery & Knowles (1976), such a situation leads to the highest possible rates for an enzyme-catalyzed reaction, since the size of all the barriers in the free energy profile which must be overcome is reduced to the lowest possible size.

Interestingly, when CrATP is substituted for MgATP as the substrate, the catalytic equilibrium constants vary considerably from 1. For reactions involving phosphorylation of alcohols or phosphates (fructose-6-P kinase, pyruvate kinase with glycolate as the substrate, hexokinase, glycerokinase, and myokinase), the value is high enough (16 with hexokinase and glucose) to see nearly a full turnover on the enzyme. This can be explained by the free energy diagram in Figure 9. We

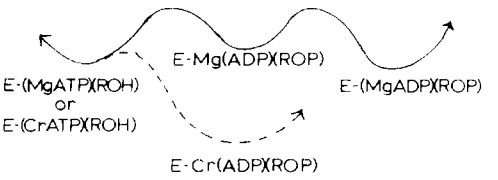

FIGURE 9: Free energy profiles for kinase reactions with either MgATP or CrATP as the substrate.

presume the intermediate E–Mg(ADP)(ROP) complex normally is less stable than either E–(MgADP)(ROP) or E-(MgATP)(ROH), which have equal stability, because the three molecules held together in the complex (Mg, ADP, and ROP) have little affinity for each other when only monodentate coordination to Mg is involved, and the enzyme must supply the energy to keep them from flying apart.[5] With the corresponding E–Cr(ADP)(ROP) complex, however, the inert coordination bonds keep the three molecules together, and the difference in ΔS for dissociation of one molecule rather than three separate ones causes this complex to be much more stable and to lie below E(CrATP)(ROH) in the free energy profile.

Three exceptions were found to this pattern. For pyruvate kinase with pyruvate, only 1.5% turnover was seen. Since CrATP is a very efficient activator for detritiation of pyruvate by pyruvate kinase (Dunaway-Mariano et al., 1979), it seems unlikely that a decreased level of the enolate of pyruvate in the presence of CrATP is the explanation. Possibly the EMg(ADP)(PEP) complex lies so far above E(MgADP)(PEP) and E(MgATP)(pyruvate) on the free energy profile that even after changing Mg to Cr it still lies above these complexes. The other two exceptions are creatine and arginine kinases, where 2–3% turnover was seen. These are the only kinases where the group being phosphorylated has a positive charge, and it is tempting to postulate that during the reaction the charge on the guanidinium group comes closer to the extra positive charge on chromium. There is no other obvious reason for the low percent turnover, since the equilibrium constants for these phosphorylations with MgATP are not far from unity in solution as well as on the enzyme.

Conclusions

It is clear from the studies reported here that bidentate chromium nucleotides are generally able to act as substrates for kinases and that the use of separated screw sense isomers, or in favorable cases the use of the CD assay, allows ready determination of the absolute screw sense specificity of the enzyme for MgATP. By contrast, the screw sense specificity for MgADP in those cases where bidentate MgADP is the substrate can be determined only when, as with creatine kinase, there is not pronounced nonproductive binding of the inactive isomer. A comparison of mono- and bidentate CrADP as inhibitors, however, allows one to tell whether mono- or bidentate MgADP is the normal substrate, and in the latter cases the use of α-S substituted nucleotides with Mg and Cd as metal activators should allow the absolute screw sense of MgADP to be determined. Knowledge of the screw sense specificity of both MgATP and MgADP defines most of the degrees of freedom of the three phosphates on the enzyme and with even a low-resolution X-ray structure should allow determination of the tripolyphosphate geometry during the catalytic reaction.

[5] The NMR studies of Nageswara Rao et al. (1979) presumably determine the sum of EMg(ADP)(ROP) and E(MgADP)(ROP) so that the former could actually make up a sizable proportion of this total in some or all of the cases.

It remains for chromium nucleotides to be tested as substrates for the numerous enzymes other than kinases which use MgATP or other nucleotide triphosphates as substrates. Studies similar to those in the present paper should allow determination of the structural and stereospecificity for these enzymes as well and will probably yield considerable information concerning their reaction mechanisms. In particular, where an enzyme group becomes an inner sphere ligand of Mg during the reaction, chromium nucleotides should fail to act as substrates even for a small percentage of a turnover. The results reported in the present work show that it is practical to detect as little as 1% turnover and, by suitable enzymatic treatment to remove traces of opposite screw sense isomers, to prepare screw sense isomers of bidentate CrATP of sufficient purity to allow determination of the stereoselectivity of the enzyme.

References

Anderson, C. M., Stenkamp, R. E., McDonald, R. C., & Steitz, T. A. (1978) *J. Mol. Biol. 123*, 207.

Anthony, R. S., & Spector, L. B. (1971) *J. Biol. Chem. 246*, 6129.

Armstrong, V. W., Yee, D., & Eckstein, F. (1979) *Biochemistry 18*, 4120.

Blattler, W. A., & Knowles, J. R. (1979) *J. Am. Chem. Soc. 101*, 510.

Cleland, W. W., & Mildvan, A. S. (1979) *Adv. Inorg. Biochem. 1*, 163.

Cohn, M. (1979) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 38*, 786.

Colowick, S. P. (1973) *Enzymes, 3rd Ed. 9*, 1.

Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279.

Cornelius, R. D., Hart, P. A., & Cleland, W. W. (1977) *Inorg. Chem. 16*, 2799.

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

DePamphilis, M. L., & Cleland, W. W. (1973) *Biochemistry 12*, 3714.

Dunaway-Mariano, D. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1420.

Dunaway-Mariano, D., & Cleland, W. W. (1980) *Biochemistry* (preceding paper in the issue).

Dunaway-Mariano, D., Benovic, J. L., Cleland, W. W., Gupta, R. K., & Mildvan, A. S. (1979) *Biochemistry 18*, 4347.

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685.

Frey, C. M., & Stuehr, J. E. (1972) *J. Am. Chem. Soc. 94*, 8898.

Jaffe, E. K., & Cohn, M. (1978) *Biochemistry 17*, 652.

Janson, C. A., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2562, 2567, 2572.

Li, T. M., Mildvan, A. S., & Switzer, R. L. (1978) *J. Biol. Chem. 253*, 3918.

Nageswara Rao, B. D., Kayne, F. J., & Cohn, M. (1979) *J. Biol. Chem. 254*, 2689.

Orr, G. A., Simon, J., Jones, S. R., Chen, G. J., & Knowles, J. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2230.

Schimerlik, M. I., & Cleland, W. W. (1973) *J. Biol. Chem. 248*, 8418.

Steitz, T. A., Anderson, W. F., Fletterick, R. J., & Anderson, C. M. (1977) *J. Biol. Chem. 252*, 4494.

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