J. Enzyme Inhibition, 1986, Vol. 1, pp. 113–125
8755–5093/86/0102–0113 $25.00/0

Printed in Great Britain.

INHIBITION AND INACTIVATION OF PYRUVATE PHOSPHATE DIKINASE WITH Cr(III) COMPLEXES OF ADENOSINE 5′-TRIPHOSPHATE AND INORGANIC PYROPHOSPHATE*

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(Received December 2, 1985)

The exchange inert complexes β,γ-bidentate $Cr(H_2O)_4ATP$ and P^1, P^2-bidentate $Cr(H_2O)_4PP$ were found to bind to the *Bacteriodes symbiosus* pyruvate phosphate dikinase ATP and PP binding sites, respectively. The inactivation of the enzyme that was observed with these complexes was shown to involve covalent attachment of the entire complex to the enzyme *via* insertion of enzyme amino acid side chains into the coordination sphere of the Cr(III). Incubation of $Cr(H_2O)_4ATP$ with other proteins also resulted in covalent attachment.

KEY WORDS: Pyruvate phosphate dikinase inhibition, Cr(III) complexes, ATP complexes.

ABBREVIATIONS: Abbreviations used include: PPDK, pyruvate phosphate dikinase; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; MES, 2-(N-Morpholino)ethanesulfonic acid; AMPPNP, adenosine 5′-[β,γ-imido]triphosphate; P_i, othophosphate; PP, inorganic pyrophosphate; ATP, adenosine 5′-triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; NADH, dihydronicotinamide adenine dinucleotide; PEP, phosphoenolpyruvate; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; Pyr, pyruvate.

INTRODUCTION

Pyruvate, phosphate dikinase (E.C. 2.7.9.1) catalyzes the reversible phosphorylation of pyruvate to phosphoenolpyruvate by ATP:

$$\text{pyruvate} + \text{ATP} + P_i \rightleftarrows \text{PEP} + \text{AMP} + \text{PP}.$$

In *B. symbiosus* and *E. hystolytica* pyruvate kinase is absent and pyruvate phosphate dikinase appears to fulfill its role in glycolysis.[3,4] The reaction mechanism proposed by Wood and his collaborators first for the *P. shermanii* enzyme[5] and then later for the *B. symbiosus* enzyme[6] involves three partial reactions and three stable enzyme forms (*vide infra*).

$$\text{ATP} + \text{enzyme} \rightleftarrows \text{AMP} + \text{enzyme-PP}, \qquad (1)$$

*This work was supported by NIH grants GM-28688 and GM-36260 to DD-M. DD-M is a NIH Research Career Development Awardee (ES-00111). Preliminary reports on this work have been presented.[1,2]

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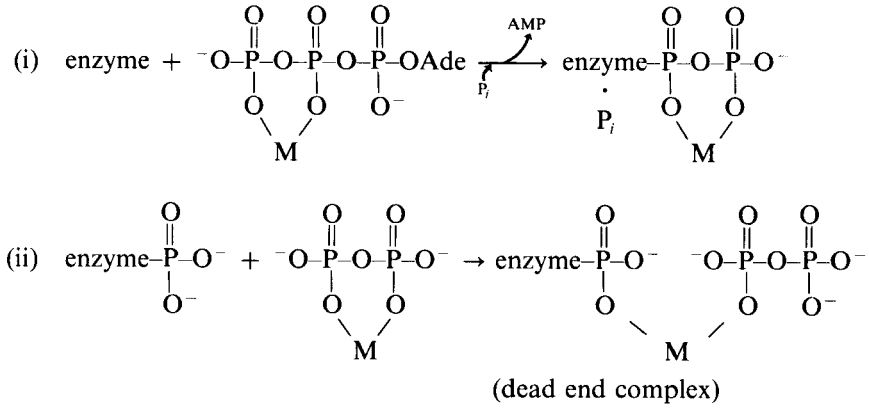
$$P_i + \text{enzyme-PP} \rightleftarrows PP + \text{enzyme-P}, \quad (2)$$

$$\text{pyruvate} + \text{enzyme-P} \rightleftarrows PEP + \text{enzyme}. \quad (3)$$

The kinetic mechanisms of the *P. shermanii* and *B. symbiosus* enzymes have been reported to be nonclassical tri uni uni ping pong, i.e., the three partial reactions take place at three separate sites on the enzyme. The three sites are thought to be connected *via* a histidine residue which swings from one site to the next carrying the covalently attached pyrophosphoryl or phosphoryl group.

Pyruvate phosphate dikinase requires both monovalent and divalent cations for activity.[6] *In vivo*, the requirement for the divalent cation is fulfilled by Mg^{2+}. By analogy to other kinases we expected that the role of Mg^{2+} cofactor is to complex with ATP (or similarly, pyrophosphate) to form the active substrate in the reaction.[7] The purpose of the present study was to determine whether MgATP (likewise, MgPP) is the actual substrate for pyruvate phosphate dikinase by testing the binding affinity of the enzyme towards exchange inert metal–ATP (and metal–PP) complexes as well as the ability of the enzyme to recognize these complexes as substrates. In view of the mechanism of the reaction catalyzed by this enzyme the substrate activity of the exchange inert metal–ATP or metal–PP complexes can not be tested in the classical sense. Specifically, the reaction of bidentate M(III)ATP or M(III)PP complexes with the enzyme is expected to result in enzyme inactivation owing to the formation of an exchange inert "enzyme intermediate" which would be stable to further catalytic processing (see Scheme 1).



SCHEME 1

The studies reported in this paper were largely focussed on examining the ability of pyruvate phosphate dikinase from *B. symbiosus* to recognize the exchange inert complexes of ATP and PP as substrates by testing these complexes as reversible and irreversible inhibitors of the enzyme. Although some of the complexes did prove to be irreversible inhibitors the results obtained from the studies of the mode of reaction of M(III) complexes of ATP and PP with pyruvate phosphate dikinase support an inactivation pathway which involves insertion of the enzyme into the coordination sphere of the metal ion rather than formation of the dead end complex shown in Scheme 1.

MATERIALS AND METHODS

General

Pyruvate phosphate dikinase was purified from *B. symbiosus* (American Type Culture Center) according to the method of Goss *et al.*[8] The enzyme prepared in this manner was free of myokinase, migrated as a single protein band in SDS PAGE gels (7.5% acrylamide) and had an activity at pH 6.8 and 25°C of 15 units/mg in the direction of ATP synthesis. The activity of the enzyme was measured spectrophotometrically using the standard lactate dehydrogenase/NADH coupled assay. β,γ-Bidentate $Cr(H_2O)_4ATP$ was prepared according to published procedure[9] and purified just prior to use, using high pressure liquid chromatographic techniques (Beckman Model 332 HPLC; Whatman C-18 reverse phase preparative column (50 cm); 0.1 M K^+ methane sulfonate (pH 2.2) isocratic eluant; 4 ml/min flow rate).[10] β,γ-Bidentate $Co(NH_3)_4ATP$,[11] β,γ-bidentate $Cr(H_2O)_4AMPPNP$,[9] P^1,P^2-bidentate $Co(NH_3)_4PP$[11], P^1,P^2-bidentate $Cr(NH_3)_4PP$[12] and P^1,P^2-bidentate $Cr(H_2O)_4PP$[13] were prepared according to known methods. The PP complexes were recrystallized prior to use.

[γ-^{32}P]-ATP was purchased from New England Nuclear, while [^{51}Cr]-$Cr(H_2O)_6^{3+}$ (in HCl), [2,8-^{3}H]-ATP, [8-^{14}C]-AMP and [β-^{32}P]-ATP were purchased from Amersham. All nucleotides, buffers and enzymes were purchased from Sigma Chemical Co.

Enzyme Inhibition Experiments

β,γ-Bidentate $Cr(H_2O)_4ATP$ (1.8 mM) was tested as an inhibitor of pyruvate phosphate dikinase *vs.* AMP (5–50 μM) in the presence of 80 mM NH_4Cl, 2.5 mM $MgCl_2$, 0.5 mM PEP, 1 mM PP, 10 units/ml lactate dehydrogenase, 0.2 mM NADH, and 50 mM K^+MES (pH 6.0). P^1,P^2-Bidentate $Cr(H_2O)_4PP$ was tested as inhibitor *vs.* PP (150–500 μM) in the presence of 5 mM PEP, 1 mM AMP, 10 mM $MgCl_2$, 40 mM NH_4Cl, 0.2 mM NADH, 10 units/ml lactate dehydrogenase and 100 mM K^+MES (pH 6.0). K_i values were calculated from Lineweaver Burke plots of the initial velocity data.

Enzyme Inactivation Experiments

These experiments were carried out by incubating pyruvate phosphate dikinase in 75 mM K^+MES (pH 6.0), 10 mM $MgCl_2$ and 80 mM NH_4Cl at 25°C in the presence and absence of the inactivator. At specific times 10–50 μl aliquots were removed from the incubation mixtures and added to a 1 ml quartz cells at 25°C contaiing AMP (0.5 mM), $MgCl_2$ (5 mM), NH_4Cl (40 mM), PEP (0.5 mM), PP (1 mM), lactate dehydrogenase (10 units/ml), and NADH (0.3 mM). The enzyme activity was monitored by measuring the rate of change in the assay solution absorbance at 340 nm (according to standard procedure).

Enzyme Labelling Experiments

These experiments were carried out by incubating (50–200 μM) pyruvate phosphate dikinase in 150 mM K^+MES (pH 6), 10 mM $MgCl_2$, 40 mM NH_4Cl, and 0.5–15 mM labelled Cr(III) or Co(III) complex at 25°C. After a specified incubation period the 0.5 ml reaction mixtures were chromatographed on a 1.0 × 55 cm Sephadex-G-50

column at 4°C using 10 mM K^+MES (pH 6.0) as eluant. Two and one-half ml fractions were collected and assayed for enzyme by taking A^{280} readings, and for radioisotope by using liquid scintillation techniques. The enzyme-containing fractions were combined, made 6 M in guanidine·HCl and rechromatographed on the Sephadex-G-50 column.

The CPM values measured for the enzyme containing fractions were corrected for quenching. The labelling stoichiometries were calculated by dividing the concentration of enzyme in the fraction (based on fraction absorbance at 280 nm, a factor of 1.0 absorbance unit per 1.3.mg/ml protein and a subunit molecular weight of 94,000 daltons[6]) into the concentration of the label in that fraction (based upon fraction CPM/ml and the known specific activity of the label in CPM/mmole). The concentrations of bovine serum albumin and yeast hexokinase were based on a factor of 1.0 absorbance unit per 1.0 mg/ml and subunit molecular weights of 49,500 and 66,000 daltons. Hexokinase activity was measured by using a standard glucose 6-phosphate dehydrogenase/NADP coupled assay.

β,γ-Bidentate $Cr(H_2O)_4ATP \rightleftarrows [^{14}C]$-AMP Exchange

A 0.5 ml reaction solution 0.5 mM in β,γ-bidentate $Cr(H_2O)_4ATP$, 0.5 mM in [8-^{14}C] AMP (s.a. = 5 mCi/μmole), 10 mM in P_i, 0.5 mM in PP, 14 mM in $MgCl_2$, 20 mM in NH_4Cl, 100 mM in imidazole·HCl (pH 6.0) and 0.27 units/ml in pyruvate phosphate dikinase was incubated at 15°C. Fifty μl aliquots were removed over a 2 h time period and promptly loaded on Dowex-50-X2 (H^+) columns prepared from disposable pipets (resin volume *ca.* 2 ml). The columns were washed with 50 ml of dionized water in order to elute the AMP and then with 15 ml of 6 M HCl in order to elute the $Cr(H_2O)_4ATP$. The radioactivity of the water and acid washes was measured by using liquid scintillation techniques. Control reactions not containing enzyme were run concurrently. A second assay technique was used in which the $Cr(H_2O)_4ATP$ and AMP were separated by using high pressure liquid chromatographic techniques (Altex C-18 reverse phase column, 10 mM K^+ methane sulfonic acid (pH 2.2), flow rate 1 ml/min). The ATP ⇄ [^{14}C]AMP exchange rate was measured under conditions identical to those used to measure the CrATP ⇄ [^{14}C]AMP exchange rate. The exchange was monitored by separating the ATP and AMP using silica gel thin layer chromatogrpahy (2-propanol, H_2O, 14.5 M NH_4OH; 7:2:1) and then assaying them for radioactivity by using liquid scintillation techniques. The exchange rate was calculated according to the method of Yagil and Hoberman.[14] The ATP/AMP exchange reaction was allowed to reach equilibrium (ATP:AMP = 1:1) prior to the addition of [^{14}C]-labelled AMP.

$Co(NH_3)_4ATP$ Substrate Activity

[^{32}P-γ-P]-β,γ-Bidentate $Co(NH_3)_4ATP$ (0.069 mM) was incubated with 7.8 mM $MgCl_2$, 26 mM NH_4Cl, 216 μM pyruvate phosphate dikinase and 160 mM imidazole·HCl (pH 6.9) at 25°C for 3 h. The reaction mixture (320 μl) was then chromatographed on a 1 × 55 cm Sephadex-G-50 column (10 mM K^+ MES, pH 6 as eluant). The Co(III) containing fractions were combined, concentrated *in vacuo* to 3 ml and divided into two portions. Fifty μmoles of EDTA (pH 5) were added to the first portion, which was then heated at 100°C for 1 min, cooled, made 21 mM in ammonium molybdate, acidified with HCl and extracted with an equal volume of

isobutanol/benzene (1:1). The organic and aqueous layers were assayed for ^{32}P using liquid scintillation techniques. The second portion was treated in the same manner except that yeast inorganic pyrophosphatase (50 μM) and $MgCl_2$ (10 mM) were added and the mixture incubated at 25°C for 2 h prior to the addition of the EDTA.

RESULTS

Inactivation Studies

$Cr(H_2O)_4ATP$ was found to be a competitive inhibitor *vs.* AMP and to have a K_i value of 0.7 mM. When tested as an inactivator of pyruvate phosphate dikinase the data shown in Figure 1 were obtained. These data indicate that the inactivation process does not proceed *via* a single pathway, i.e., more than one rate process is evident from the semi-log plot. Secondly, the inactivation reaction does not appear to follow saturation kinetics which suggests that the formation of a Michaelis complex between the enzyme and the $Cr(H_2O)_4ATP$ is not required for inactivation.

The inactivation reaction was carried out in the presence of several different agents (EDTA, DTT, ATP, AMP, Mn^{2+}, PEP) in order to probe the possible inactivation sites on the enzyme. The inactivation rate data obtained are presented in Figures 2 and 3.

Next, the properties of P^1,P^2-bidentate $Cr(H_2O)_4PP$ as an inactivator were

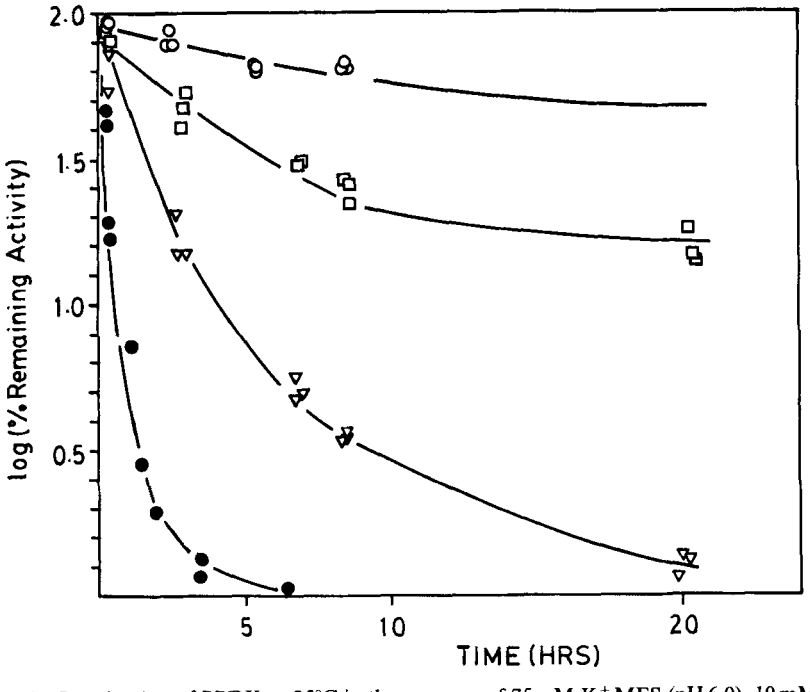


FIGURE 1 Inactivation of PPDK at 25°C in the presence of 75 mM K^+ MES (pH 6.0), 10 mM $MgCl_2$, 80 mM NH_4Cl and (○) 0.2 mM $Cr(H_2O)_4ATP$, (□) 0.8 mM $Cr(H_2O)_4ATP$, (△) 5 mM $Cr(H_2O)_4ATP$, (●) 16 mM $Cr(H_2O)_4ATP$.

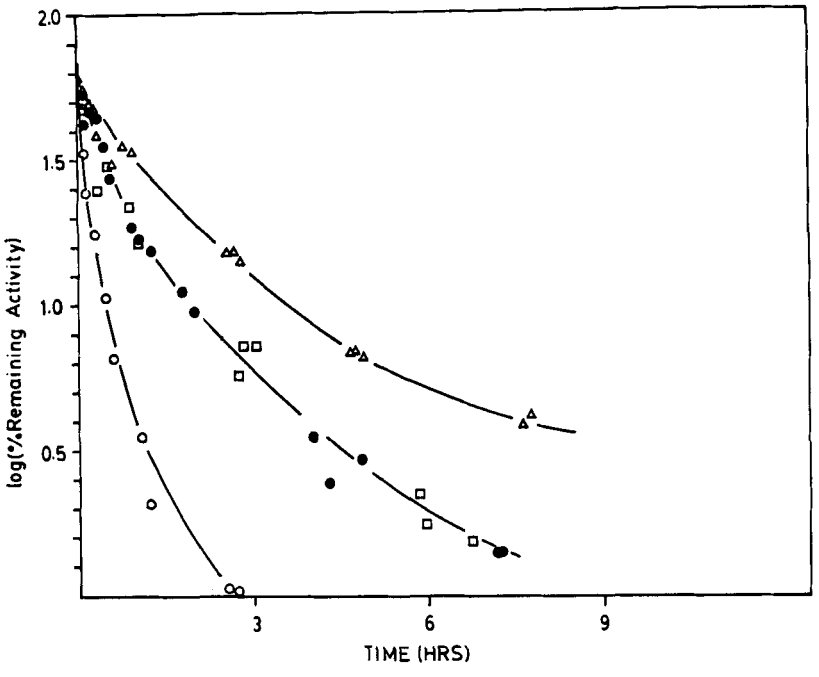


FIGURE 2 Inactivation of PPDK at 25°C in the presence of 75 mM K^+ MES (pH 6.0), 10 mM $MgCl_2$, 80 mM NH_4Cl and 10 mM $Cr(H_2O)_4ATP$ (●), 10 mM $Cr(H_2O)_4ATP$ and 11 mM PEP (○), 10 mM $Cr(H_2O)_4ATP$ and 11 mM ATP (□), 10 mM $Cr(H_2O)_4ATP$ and 14 mM AMP (△).

examined. Although $Cr(H_2O)_4PP$ was found to be a competitive inhibitor of pyruvate phosphate dikinase *vs.* MgPP (K_i = 0.70 mM) the inactivation observed with $Cr(H_2O)_4PP$ is not first order nor does the rate of inactivation follow saturation kinetics (Figure 4). In contrast to $Cr(H_2O)_4PP$, $Cr(NH_3)_4ATP$ (Figure 5) did not inactivate the enzyme. $Cr(H_2O)_4AMPPNP$, on the other hand, was found to be a moderately good inactivator.

Labelling Studies

Incubation of pyruvate phosphate dikinase with [^{51}Cr,^{32}P]-$Cr(H_2O)_4PP$ followed by Sephadex G-50 column chromatography revealed that *ca.* 5Crl$(H_2O)_4PP$ molecules were bound per subunit of enzyme (Table I). When the enzyme was denatured and passed back through the column 2–3$Cr(H_2O)_4PP$ molecules per subunit remained associated (Table I). On a separate occasion the labelling stoichiometry was measured using [^{32}P]-$Cr(H_2O)_4PP$ and shown to accompany loss of enzyme activity. After 3.5 h of incubation, 2.3Crl$(H_2O)_4PP$ molecules were found to be bound per enzyme molecule and this degree of modification corresponded to an 83% loss of enzyme activity. Further incubation (24 h) more than doubled the number of $Cr(H_2O)_4PP$ molecules attached to the enzyme but did not further reduce its activity.

Incubation of $Cr(H_2O)_4ATP$ labelled at the adenine moiety with ^3H and at the γ-phosphoryl moiety with ^{32}P with pyruvate phosphate dikinase resulted in the

TABLE I
Covalent labelling of pyruvate phosphate dikinase, bovine serum albumin and hexokinase with radio-labelled, exchange inert M(III)ATP and M(III)PP complexes

Complex	Conc	[E]	Time	Chromatography[d]	Label
[γ-^{32}P]–$Co(NH_3)_4ATP$	1 mM	69 μM[a]	4 h	1st	None
[^{32}P]–$Co(NH_3)_4PP$	1 mM	52 μM[a]	4 h	1st	None
[^{51}Cr; ^{32}P]–$Cr(H_2O)_4PP$	1 mM	68 μM[a]	1.5 h	1st 2nd	5.5 (^{51}Cr); 4.5 (^{32}P) 2.8 (^{51}Cr); 1.8 (^{32}P)
[^{32}P]–$Cr(H_2O)_4PP$	5 mM	45 μM[a]	3.5 h 24 h	1st 1st 2nd	2.3 (^{32}P) 7.2 (^{32}P) 4.8 (^{32}P)
[^{3}H,γ-^{32}P]–$Cr(H_2O)_4ATP$	11 mM	90 μM[a]	1 h 22 h	2nd 1st 2nd	5.6 (^{3}H); 6.5 (^{32}P) 28 (^{3}H); 34 (^{32}P) 18 (^{3}H); 18 (^{32}P)
[^{51}Cr,β-^{32}P]–$Cr(H_2O)_4ATP$	1 mM	100 μM[a]	1 h 22 h	1st 2nd	1.2 (^{51}Cr); 1.2 (^{32}P) 0.8 (^{51}Cr); 0 8 (^{32}P)
[^{3}H,γ-^{32}P] $Cr(H_2O)_4ATP$	8 mM	150 μM[b]	6 h	1st 2nd	2.3 (^{3}H); 2.6 (^{32}P) 2.0 (^{3}H); 1.8 (^{32}P)
[^{3}H,γ-^{32}P] $Cr(H_2O)_4ATP$	8 mM	150 μM[c]	6 h	1st 2nd	0.7 (^{3}H); 0.7 (^{32}P) 0.5 (^{3}H); 0.5 (^{32}P)

[a]PPDK. [b]BSA. [c]Hexokinase. [d]1st refers to the first Sephadex G-50 chromatography of the incubation mixture while 2nd refers to the Sephadex G-50 chromatography of the denatured enzyme.

incorporation of the two radio-labels in a 1:1 ratio. The stoichiometry of radiolabel to enzyme was 5:1 (1 h incubation) and the resulting loss of subunit enzyme activity, 44%. After 22 h of incubation the stoichiometry of label to enzyme had increased to 28–34 prior to denaturization, 18 of which remained intact following denaturization. Under reaction conditions where the complex:enzyme ratio was smaller (Table I) the ^{51}Cr, ^{32}P incorporation into pyruvate phosphate dikinase from [^{51}Cr,β-^{32}P]-$Cr(H_2O)_4ATP$ was measured. As indicated in Table I the stoichiometry of labelling observed was *ca.* 1 and the ^{51}Cr : ^{32}P incorporation was 1:1. The ammonia substituted complexes, [γ-^{32}P]-$Co(NH_3)_4ATP$ and [^{32}P]-$Co(NH_3)_4PP$ were also examined as modifiers. Neither complex became covalently attached to the enzyme.

Bovine serum albumin and yeast hexokinase were also incubated with [^{3}H,γ-^{32}P]-$Cr(H_2O)_4ATP$ and like pyruvate phosphate dikinase, they became covalently labelled (see Table I). Unlike the labelled pyruvate phosphate dikinase the labelled hexokinase retained 100% of its activity.

Exchange Studies

$Cr(H_2O)_4ATP \rightleftarrows$ [^{14}C]AMP exchange was examined at pH 6 is the presence of Mg^{3+}, NH_4^+, P_i, PP and pyruvate phosphate dikinase. The exchange reaction was monitored by chromatographically separating the $Cr(H_2O)_4ATP$ and AMP and assaying each for radioactivity. After a 4 h period, 2% of the original radioactivity present in AMP was found to be associated with $Cr(H_2O)_4ATP$ compared to 1.4% observed in the

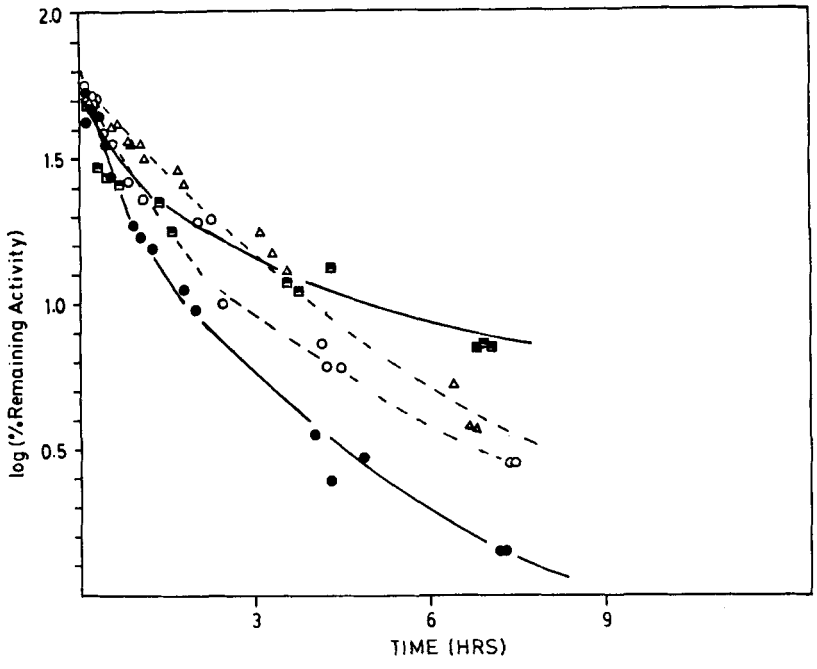


FIGURE 3 Inactivation of PPDK at 24°C in the presence of 75 mM K^+ MES (pH 6.0), 10 mM $MgCl_2$, 80 mM NH_4Cl and 10 mM $Cr(H_2O)_4ATP$ (●), 10 mM $Cr(H_2O)_4ATP$ and 0.5 mM EDTA (○), 10 mM $Cr(H_2O)_4ATP$ and 0.2 mM DTT (△), 10 mM $Cr(H_2O)_4ATP$ and 1 mM $MnCl_2$ (□).

control exchange reaction (lacking enzyme). Under the same reaction conditions but in the presence of 10 fold less enzyme the ATP ⇄ [^{14}C]-AMP exchange reaction (monitored by using silica gel thin layer chromatographic techniques and HPLC techniques) took place at a rate of 1.1 μM AMP converted into ATP per min.

$Co(NH_3)_4ATP$ Substrate Activity

MgATP reportedly pyrophosphorylates pyruvate phosphate dikinase. The labelling studies described above showed that the β,γ-pyrophosphoryl unit of [γ-^{32}P]-$Co(NH_3)_4ATP$ does not become covalently attached to the enzyme. In order to determine whether our inability to isolate the $Co(NH_3)_4PP$ labelled enzyme may have been due to the extreme lability of the enzyme-$Co(NH_3)_4PP$ linkage we incubated the $Co(NH_3)_4ATP$ complex with the enzyme, removed the enzyme from the reaction mixture using Sephadex-G-50 column chromatography and analyzed the mixture for $Co(NH_3)_4PP$ and for $Co(NH_3)_4(ADP)(P_i)$. The presence of the $Co(NH_3)_4(ADP)(P_i)$ complex (resulting from P_γ–O–P_β bond cleavage) could be detected by treating the complex with EDTA in order to release the $^{32}P_i$ and then assaying for $^{32}P_i$ using a standard phosphomolybdate extration procedure. The presence of the [^{32}P]–$Co(NH_3)_4PP$ complex (resulting from cleavage at the β-P) could be detected by treating the complex with yeast inorganic pyrophosphatase to first convert it to [^{32}P]–$Co(NH_3)_4(P_i)_2$ and then with EDTA to convert it to $^{32}P_i$.[15] We found using

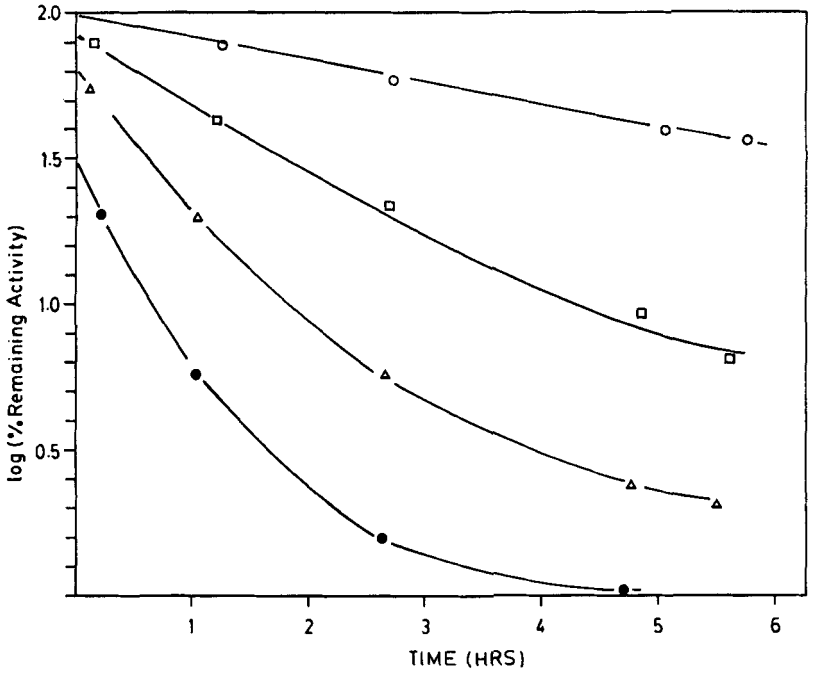


FIGURE 4 Inactivation of PPDK at 25°C in the presence of 75 mM K^+ MES (pH 6.0), 10 mM $MgCl_2$, 80 mM NH_4Cl and (○) 1 mM $Cr(H_2O)_4PP$, (□) 3 mM $Cr(H_2O)_4PP$, (△) 10 mM $Cr(H_2O)_4PP$ and (●) 20 mM $Cr(H_2O)_4PP$.

conditions sensitive enough to detect a single turnover on the enzyme that pyruvate phosphate dikinase did not convert $Co(NH_3)_4ATP$ to $Co(NH_3)_4(ADP)(P_i)$ nor to $Co(NH_3)_4PP$ and AMP.

DISCUSSION

The original purpose of our studies was to determine whether ATP or the Mg^{2+} complex of ATP is the actual substrate in the pyruvate phosphate dikinase reaction. The method that is routinely used to determine the substrate structure of MgATP in enzymic reactions, namely the Mg^{2+}/Cd^{2+}-ATPβS method[16] could not be used in the present case since ATPβS is not a substrate for pyruvate phosphate dikinase. For this reason we chose to study the substate specificity of pyruvate phosphate dikinase using exchange inert metal–nucleotide complexes (to test MgATP structure) and metal–PP complexes (to test MgPP structure).

The basic assumption that one makes in utilizing the exchange inert M(III)ATP complexes as probes of Mg(II)ATP substrate structure is that the relative binding affinities and enzymic reactivities observed for the exchange inert M(III)ATP complexes are representative of those of the corresponding Mg(II)ATP complex. Because of the multiple phosphoryl transfer steps involved in the pyruvate phosphate dikinase reaction the substrate activities of M(III)ATP and M(III)PP complexes could not be tested in the conventional manner. Instead, the participation of the exchange inert

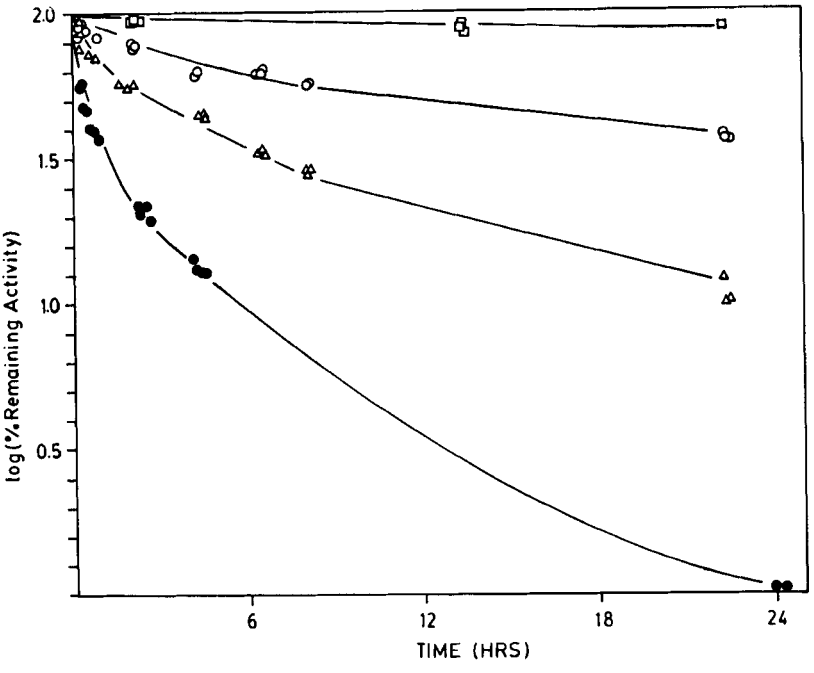
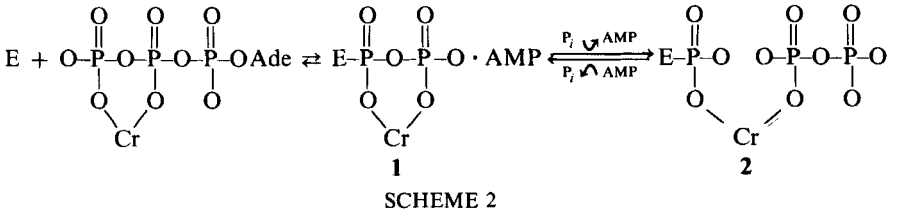


FIGURE 5 Inactivation of PPDK at 25°C in the presence of 75 mM K^+ MES (pH 6.0), 10 mM $MgCl_2$, 80 mM NH_4Cl and (□) 6.4 mM $Cr(NH_3)_4PP$, (○) 20 mM $Co(NH_3)_4ATP$, (□) 8.5 mM $Co(NH_3)_4PP$, (●) 6.1 mM $Cr(H_2O)_4AMPPNP$.

complex in a given partial reaction was tested by testing its ability to undergo isotopic exchange with the product of that partial reaction and/or by testing its ability to form a covalent dead end complex with the enzyme.

Of the kinases studied to date those which act on exchange inert M(III)ATP complexes recognize β,γ-bidentate $Cr(H_2O)_4ATP$ as substrate. Thus, our study of pyruvate phosphate dikinase substrate specificity was initiated using bidentate $Cr(H_2O)_4ATP$ as a probe. $Cr(H_2O)_4ATP$ was found to be a competitive inhibitor *vs* AMP and its K_i value at pH 6 was evaluated as 0.70 mM. This value compares quite closely with the K_i value (0.72 mM) determined for MgATP as a competitive inhibitor *vs* AMP at pH 7.[17] Next, we tested the ability of pyruvate phosphate dikinase to catalyze the isotope exchange of [^{14}C]-AMP with bidentate $Cr(H_2O)_4ATP$ in the presence and absence of P_i. We envisioned that such an exchange process could take place according to Scheme 2. Although [^{14}C]-AMP ⇄ ATP exchange could be demonstrated [^{14}C]-AMP ⇄ $Cr(H_2O)_4ATP$ exchange could not be.



SCHEME 2

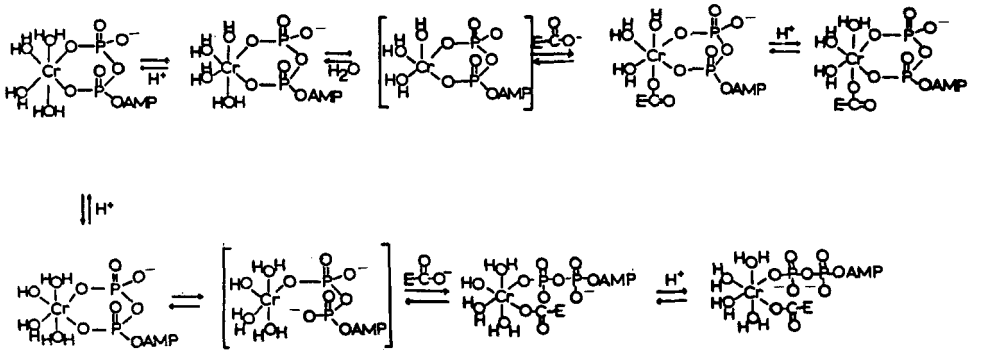
The failure of the $Cr(H_2O)_4ATP$ to undergo exchange could have been due to failure of the reaction to proceed at a measurable rate in the forward direction or the reverse direction.

As an alternate approach to determining whether enzyme forms **1** or **2** shown in Scheme 2 are formed in the presence of bidentate $Cr(H_2O)_4ATP$ the ability of the metal-nucleotide complex to inactivate the enzyme as a catalyst of the AMP + PP + PEP → ATP + P_i + Pyr reaction was tested. As indicated by the data shown in Figure 1 bidentate $Cr(H_2O)_4ATP$ does indeed inactivate the enzyme. If, however, the inactivation was solely resulting from the reaction shown in Scheme 1 the inactivation process should: (i) show first order kinetics, (ii) show saturation kinetics, and (iii) be inhibited by the presence of pyruvate phosphate dikinase substrates. As indicated by the data shown in Figures 1 and 2 none of these criteria are met. In addition, we found that while the ATP analog, AMPPNP neither underwent isotopic exchange with [^{14}C]-AMP (data not shown) or inactivated the enzyme, the β,γ-bidentate $Cr(H_2O)_4AMPPNP$ complex did inactivate enzyme (Figure 5).

The question that we next addressed was: how does bidentate $Cr(H_2O)_4ATP$ inactivate pyruvate dikinase if it is not *via* formation of the dead end complexes **1** and **2** shown in Scheme 2? Initially, we suspected that the $Cr(H_2O)_4ATP$ sample might be contaminated with $Cr(H_2O)_6^{3+}$ or a sulfhydryl oxidizing agent, either of which could lead to inactivation of the enzyme. Thus, protection by DTT, EDTA and Mn^{2+} was tested. As indicated by the data shown in Figure 3 these agents offered only partial protection of the enzymatic activity. Covalent attachment of $Cr(H_2O)_4ATP$ to the enzyme was then tested using $Cr(H_2O)_4ATP$ radiolabelled at the metal, polyphosphate and adenine moieties. The data shown in Table 1 indicate that the entire $Cr(H_2O)_4ATP$ molecule covalently attaches to the enzyme. Furthermore, given ample reaction time more than one $Cr(H_2O)_4ATP$ molecule attached per subunit.

The next question that was considered is: through which atom(s) does the $Cr(H_2O)_4ATP$ covalently attached itself to the enzyme? For the purpose of testing the role of adenosine moiety in covalent modification P^1,P^2-bidentate $Cr(H_2O)_4PP$ was prepared. When tested as a competitive inhibitor *vs.* MgPP at pH 6 a K_i value of 0.70 mM was obtained. This value closely compares to the K_i value of 0.36 mM measured for the competitive inhibitor Mg(imidodiphosphate) at pH 7.[17] Although $Cr(H_2O)_4PP$ binds to enzyme active site it, like $Cr(H_2O)_4ATP$, appears to inactivate the enzyme by reaction at sites other than the active site (see Figure 4). In addition, as with $Cr(H_2O)_4ATP$ the $Cr(H_2O)_4PP$ in its entirety covalently attaches to the enzyme and it does so with a stoichiometry greater than 1:1 (see Table I). On the basis of these results we suspected that the attachment of the $Cr(H_2O)_4ATP$ or $Cr(H_2O)_4PP$ took place at the Cr(III) center and that it occurred at multiple sites on the enzyme. This type of "covalent" attachment would require insertion of an enzyme amino acid residue into the coordination sphere of the Cr(III) with displacement of a H_2O ligand. Previous studies have shown that ligand exchange does occur in $Cr(H_2O)_4$polyphosphate complexes and that this exchange, being base catalyzed, probably involves deprotonation of a water ligand with ensuing expulsion of a second ligand.[9] The pK_a for the water ligands of $Cr(H_2O)_4ATP$ is *ca.* 7. Thus, at pH 6, the pH at which the labelling experiments were carried-out, there will exist a small amount of the $Cr(H_2O)_3(OH)(ATP)$ complex. This complex should readily lose a ligand to form a pentavalent species which in turn could react with a nucleophilic residue according to Scheme 3. In order to explore this possibility we examined the properties of $Cr(NH_3)_4PP$, a complex which is not subject to base catalyzed ligand exchange at

pH 6. The data shown in Figure 5 indicate that this complex does in fact fail to inactivate pyruvate phosphate dikinase.



SCHEME 3

It thus became clear that the $Cr(H_2O)_4ATP$ normally used to probe kinase substrate recognition could not be used with pyruvate phosphate dikinase. Conceptionally, $Co(NH_3)_4ATP$ might be used in place of $Cr(H_2O)_4ATP$, however in practice it is very difficult to obtain $Co(NH_3)_4ATP$ samples which are not contaminated with $Cr(NH_3)_3(H_2O)ATP$ and $Cr(NH_3)_2(H_2O)_2ATP$. Like $Cr(H_2O)_4ATP$, the $Cr(NH_3)_3(H_2O)ATP$ and $Cr(NH_3)_2(H_2O)_2ATP$ could undergo ligand exchange with the enzyme and for this reason we chose to continue our studies with $Co(NH_3)_4ATP$ which can be obtained in quite pure form. As before we had hoped to detect catalysis of the $Co(NH_3)_4ATP$ complex by detecting formation of the Co(III) derivatives of the dead end complexes **1** and **2** shown in Scheme 2. $Co(NH_3)_4ATP$ was first tested as an inactivator of the enzyme. As indicated by the data shown in Figure 5 a moderate degree of inactivation was observed. However, $Co(NH_3)_4PP$ also inactivated the enzyme to a moderate extent and the inactivation by either complex could be shown to correspond to the appearance of Co(II) in the sample (data not shown). Specifically, sulfhydryls, catalyze the reduction of the Co(III)-ATP and Co(III)-PP complexes and in turn become oxidized.[15] Oxidation of the sulfhydryl groups of pyruvate kinase dikinase leads to loss of activity.[6] Consistent with this mode of inactivation is the observation (reported in Table I) that neither $Co(NH_3)_4ATP$ nor $Co(NH_3)_4PP$ become covalently attached ot the enzyme. This latter observation also suggests that the $Co(NH_3)_4ATP$ complex is unable to form "stable" dead end complexes with the enzyme analogous to those shown in Scheme 2. It was considered possible that once formed the dead end complexes decomposed regenerating free enzyme for further catalysis. For this reason we tested pyruvate phosphate dikinase catalyzed cleavage of the polyphosphate moiety of the $Co(NH_3)_4ATP$ complex under conditions sensitive enough to allow detection of a single turnover on the enzyme. Formation of $Co(NH_3)_4(ADP)(P_i)$ which would result from P_γ–O–P_β cleavage or formation $Co(NH_3)_4(PP)$ or $Co(NH_3)_4(P_i)(PP)$ which would result form P_β–O–P_α cleavage could not be detected. Thus, $Co(NH_3)_4ATP$ is not recognized by pyruvate phosphate dikinase as substrate. Since a number of kinases which have been shown to catalyze the reaction of bidentate $Cr(H_2O)_4ATP$ will not catalyze the reaction of bidentate $Co(NH_3)_4ATP$, these results do not rule out bidentate MgATP as the pyruvate phosphate dikinase substrate.

Although the present studies have not allowed us to define the forms of ATP or PP recognized as substrate for pyruvate phosphate dikinase they do reveal that $Cr(H_2O)_4ATP$, a complex often used as a paramagnetic probe in n.m.r. and e.s.r. based active site distance measurements and as a probe of substrate recognition, will undergo ligand exchange reactions with protein amino acid side chains. As indicated by the data shown in Table I when $Cr(H_2O)_4ATP$ was incubated with yeast hexokinase and bovine serum albumin (arbitrarily selected proteins) covalent labelling did occur. Hexokinase, unlike pyruvate phosphate dikinase, did not lose catalytic activity as a result of the covalent modification. These results should be taken into consideration in planning experiments in which $Cr(H_2O)_4ATP$ or similar Cr(III) complexes are to be incubated with proteins for extended periods of time. Moveover, experiments designed to test for the intermediacy of a phosphoryl enzyme in a phosphoryl transfer reaction either by testing $Cr(H_2O)_4ATP$ as a covalent modifier[7] or as an inactivator[18] should in the absence of demonstrated catalytic turnover of the complex, be viewed with some reservation.

REFERENCES

1. L. Ciskanik and D. Dunaway-Mariano, *Fed. Proc. Fedn. Am. Socs. Exp. Biol.* **41**, 907 (1982).
2. L. Ciskanik and D. Dunaway-Mariano, *Fed. Proc. Fedn. Am. Socs. Exp. Biol.* **42**, 1922 (1983).
3. R. E. Reeves, *J. Biol. Chem.* **243**, 3203 (1960).
4. R. E. Reeves, R. A. Muzies, and D. S. Hsu, *J. Biol. Chem.* **243**, 5465 (1968).
5. Y. Milner and H. G. Wood, *J. Biol, Chem.* **251**, 7929 (1976); Y. Milner, G. Michaels, and H. G. Wood, *J. Biol. Chem.* **253**, 878 (1978).
6. B. R. Moskovitz and H. G. Wood, *J. Biol. Chem.* **253**, 884 (1978); G. Michaels, Y. Milner, B. R. Moskovitz, and H. G. Wood, *J. Biol. Chem.* **253**, 7656 (1978).
7. D. Dunaway-Mariano and W. W. Cleland, *Biochemistry* **19**, 1506 (1980).
8. N. H. Gross, C. T. Evans, and H. G. Wood, *Biochemistry* **19**, 5805 (1980).
9. D. Dunaway-Mariano and W. W. Cleland, *Biochemistry* **19**, 1496 (1980).
10. K. J. Gruys and S. M. Schuster, *Anal. Biochem.* **125**, 66 (1982).
11. R. D. Cornelius, P. A. Hart, and W. W. Cleland, *Inorg. Chem.* **16**, 2779 (1977).
12. W. B. Knight, S. -J. Ting, S. Chuang, D. Dunaway-Mariano, T. Harmony, and M. Sundaralingam, *Arch. of Biochem. Biophys.* **227**, 302 (1983).
13. E. A. Merrit, M. Sundaralingam, and D. Dunaway-Mariano, *J. Am. Chem. Soc.* **103**, 3565 (1981).
14. G. Yagil and H. D. Hoberman, *Biochemistry* **8**, 352 (1969).
15. T. P. Haromy, W. B. Knight, D. Dunaway-Mariano, and M. Sundaralingam, *Biochemistry* **21**, 6950 (1982).
16. For a recent review see F. Eckstein, *Ann. Rev. Biochem.* **54**, 367 (1985).
17. H. C. Wang and D. Dunaway-Mariano, unpublished data.
18. H. Pauls, B. Bredenbrocker, and W. Schorer, *Eur. J. Biochem.* **109**, 323 (1980).