# Lanthanide Pyrophosphates as Substrates for the Pyrophosphate-Dependent Phosphofructokinases from *Propionibacterium freudenreichii* and *Phaseolus aureus*: Evidence for a Second Metal Ion Required for Reaction†

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ABSTRACT: In the absence of $Mg^{2+}$, both the dimeric bacterial and tetrameric plant fructose 2,6-bisphosphate-activated pyrophosphate-dependent phosphofructokinases ($PP_i$-PFKs) are inactive at pH 8 and 25 °C. In the presence of a low concentration of $Mg^{2+}$ (5 $\mu$M), both enzymes will utilize a variety of metal–pyrophosphate complexes as reactant in the direction of fructose 6-phosphate (F6P) phosphorylation. The $V_{max}$ values are about 100-fold lower and the $K_m$ values about 10-fold greater than those measured with $MgPP_i$ when lanthanide–$PP_i$ complexes are used as a substrate. In the presence of added $Mg^{2+}$, the $K_m$ values of the above remain essentially unchanged, while $V_{max}$ values increase 10-fold for lanthanide–$PP_i$ complexes. These data, along with the 12–16 order of magnitude increased affinity of the lanthanides for $PP_i$ compared to $Mg^{2+}$, indicate that the $PP_i$-PFKs require two metal ions for catalysis, one to form a chelate with $PP_i$ and a second as an essential activator. With $CePP_i$, an activation constant of about 25 $\mu$M is measured for $Mg^{2+}$. In addition, a number of other divalent (but no tripositive) metal ions serve as activators including $Mn^{2+}$, $Co^{2+}$, $Mo^{2+}$, $Cr^{2+}$, $Fe^{2+}$, and $Ni^{2+}$; activation constants are in the range 20–150 $\mu$M. The exchange-inert $Cr^{III}(PP_i)(H_2O)_4$ complex is not a substrate, but is an inhibitor competitive against $MgPP_i$ with a $K_i$ of 27 $\mu$M. Results are discussed in terms of the possible role of the divalent metal ion activators.

Pyrophosphate-dependent phosphofructokinase ($PP_i$-PFK[1]) catalyzes phosphorylation of the 1-hydroxyl of F6P to give FBP using $MgPP_i$ as the phosphoryl donor. The enzyme from the bacterium *Propionibacterium freudenreichii* is the most thoroughly studied and has been shown to have a rapid equilibrium random kinetic mechanism with dead-end E–$MgPP_i$–$P_i$, E–F6P–$P_i$, and E–FBP–$MgPP_i$ complexes based on initial velocity (Bertagnolli & Cook, 1984), isotope exchange at equilibrium, exchange against forward reaction flux, and positional isotope exchange (Cho et al., 1989) studies. The enzymes from the amoebal parasite *Entamoeba histolytica* (Bertagnolli & Cook, 1984) and the mung bean *Phaseolus aureus* (Bertagnolli et al., 1986a; in the absence and presence of the activator F2,6P) also have a qualitatively similar kinetic mechanism. Bacterial and plant enzymes have a stringent requirement for a $\beta$-D-furanose with the stereochemistry of fructose at C-3 and C-4 for optimum binding (Bertagnolli et al., 1986b).

The pH dependence of the inactivation of the enzyme from *Propionibacterium* by PLP has provided information on the ionization state of reactant phosphate groups and lysine residues involved in substrate binding (Cho & Cook, 1988). These authors suggested that the phosphate of F6P is ionized and hydrogen-bonded to a lysine, while $MgPP_i$ is also fully ionized and likewise hydrogen-bonded to a lysine. The lysine that is hydrogen-bonded to the 6-phosphate of F6P has been identified by chemical modification as Lys-315, while protection of modification Lys-80 and Lys-85 was afforded by $MgPP_i$ (Green et al., 1992). More recent studies making use of site-directed mutagenesis, however, have ruled out a role for the latter two lysine residues in $MgPP_i$ binding and catalysis (Green et al., 1993).

An acid–base chemical mechanism has been proposed for $PP_i$-PFK based on the pH dependence of kinetic parameters and dissociation constants for dead-end analogs of the reactants (Cho & Cook, 1989). A single base proton shuttle mechanism is proposed in which the base accepts a proton from the 1-hydroxyl concomitant with phosphoryl transfer and then donates it to the inorganic phosphate formed as the second product. The general base is likely Asp-151 as shown by a $4 \times 10^5$-fold decrease in $k_{cat}$ in the D151S mutant compared to wild type (Green et al., 1993). Finally, a dissociative-like transition state for phosphoryl transfer is proposed for $PP_i$-PFK based on the observation that $PP_i$ in which the bridge oxygen is replaced by sulfur is a good substrate (Halkides et al., 1991).

In all of the above studies, it was assumed that a single divalent metal ion was required for reaction. However, it is becoming more apparent that enzymes involved in phosphoryl transfer reactions in some cases require more than a single divalent metal ion. For example, choline kinase (Reinhardt et al., 1984) and the catalytic subunit of the cyclic AMP-dependent protein kinase (Armstrong et al., 1979; Cook et al., 1982) require two $Mg^{2+}$ ions, while pyrophosphatase (Knight et al., 1984) requires three. As a result, it became important to determine whether the $PP_i$-PFK required more than one divalent metal ion. In the present study lanthanide–$PP_i$

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[1] Abbreviations: F6P, $\beta$-D-fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; F2,6P, fructose 2,6-bisphosphate; EDTA, ethylenediaminetetraacetic acid; $PP_i$, inorganic pyrophosphate; PLP, pyridoxal 5′-phosphate; $PP_i$-PFK, inorganic pyrophosphate dependent phosphofructokinase; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

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complexes are used as substrates for the $PP_i$-PFK in the absence and presence of $Mg^{2+}$ ion. The data suggest that there is a requirement for a $Mg^{2+}$ ion as activator in addition to that required for formation of the Mg–$PP_i$ chelate complex. Results are discussed in terms of the mechanism of the $PP_i$-PFK-catalyzed phosphoryl transfer reaction.

## MATERIALS AND METHODS

*Chemicals.* Fructose 6-phosphate, FBP,[1] F2,6P, Taps, EDTA, and NADH were from Sigma. Sodium pyrophosphate was obtained from Mallinkrodt, while all metal ions were obtained as the chloride salts from Aldrich. The $Cr(H_2O)_4$-$PP_i$ was synthesized according to the method of Knight et al. (1981). The lanthanides were purchased as the chloride salts from Aldrich and were gold-label, that is 99.999% pure.

*Enzymes.* Fructose 1,6-bisphosphate aldolase, triose isomerase, α-glycerophosphate dehydrogenase, and crude preparations of bacterial and plant $PP_i$-PFKs were from Sigma. The enzyme from *Propionibacterium freudenreichii* was purified by the method of O'Brien et al. (1975) as modified by Bertagnolli and Cook (1984). The final enzyme preparation had a specific activity of about 325 units/mg, where a unit is defined as the amount of enzyme required to produce 1 μmol of FBP in 1 min at pH 8 and 25 °C. Purified enzyme was stored in 100 mM Pipes, pH 7, containing 20% glycerol at −20 °C. The enzyme from *Phaseolus aureus* was purified by the method of Sabularse and Anderson (1981) as modified by Bertagnolli et al. (1986b). The final enzyme preparation had a specific activity of about 80 units/mg, where a unit is as defined above, but with the inclusion of 1 μM F2,6P. Purified enzyme was stored as above.

*Enzyme Assays.* All assays were carried out in 10 or 100 mM Taps, pH 8, using a Gilford 250 spectrophotometer equipped with a strip chart recorder and a circulating water bath to maintain the temperature of the cell compartment at 25 °C. Reactions were carried out in 1-mL cuvettes with a 1-cm pathlength. In all cases, 1 μM F2,6P was included in assays for the plant enzyme. With the exception that other metal ions were substituted for $Mg^{2+}$ in forming the metal–$PP_i$ chelate complex, and that assays were in some cases carried out as a function of uncomplexed $Mg^{2+}$, assays were carried out as described previously (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986a).

*Metal Chelate Complex Correction.* Correction for metal–F6P and metal–Taps chelate complexes formed was carried out when necessary according to the method of Bertagnolli and Cook (1984). The dissociation constants considered for metal–$PP_i$ complexes other than $MgPP_i$ are given in Table 5.

*Atomic Absorption Analysis.* In the absence of added $Mg^{2+}$, low levels of activity were observed for both bacterial and plant $PP_i$-PFKs. As a result, it was important to determine whether a background level of $Mg^{2+}$ existed in the reaction components. Atomic absorption analysis made use of a Perkin-Elmer Model 3030 spectrometer with a $Mg^{2+}$ lamp and carbon furnace detection for increased sensitivity. Each sample was run in triplicates against a standard curve obtained using a standardized $Mg^{2+}$ solution. No metal ion was detected in the distilled water, but the following concentrations were detected in the reaction components at the levels normally used in an assay: 100 mM Taps, pH 8, 4.4 μM; 2 mM F6P, 0.45 μM; 0.2 mM NADH, 0.26 μM; FBP aldolase, 0.05 μM; triose isomerase and α-glycerophosphate dehydrogenase, none; $PP_i$-PFK, 0.03 μM. A total endogenous $Mg^{2+}$ concentration of about 5.2 μM was thus present in each reaction mixture, and this was confirmed by analyzing samples of a complete reaction mixture.

Table 1: Kinetic Parameters in the Direction of F6P Phosphorylation for Lanthanide–Pyrophosphate Complexes in the Presence of 5 μM Endogenous $Mg^{2+}$ [a]

| bacterial $PP_i$-PFK | | | | plant $PP_i$-PFK[b] | | |
|---|---|---|---|---|---|---|
| $V_{max}$, mM/min | % $V$ | $K_m$, μM | metal | $V_{max}$, mM/min | % $V$ | $K_m$, μM |
| 0.01220 | | 5 | $Mg^{2+}$ | 0.015 | | 6 |
| | | | Lanthanides | | | |
| 0.00040 | 3.3 | 56 | $Ce^{3+}$ | 0.00012 | 0.8 | 44 |
| 0.00026 | 2.1 | 50 | $Dy^{3+}$ | 0.00017 | 1.1 | 46 |
| 0.00033 | 2.7 | 45 | $Er^{3+}$ | 0.00020 | 1.3 | 42 |
| 0.00039 | 3.2 | 104 | $Eu^{3+}$ | 0.00014 | 0.9 | 56 |
| 0.00035 | 2.9 | 46 | $Gd^{3+}$ | 0.00020 | 1.3 | 53 |
| 0.00026 | 2.1 | 39 | $Ho^{3+}$ | 0.00015 | 1.0 | 54 |
| 0.00028 | 2.3 | 58 | $Nd^{3+}$ | 0.00013 | 0.9 | 44 |
| 0.00025 | 2.0 | 46 | $Sm^{3+}$ | 0.00018 | 1.2 | 58 |
| 0.00029 | 2.4 | 53 | $Tb^{3+}$ | 0.00017 | 1.1 | 47 |
| 0.00036 | 3.0 | 57 | $Tm^{3+}$ | 0.00020 | 1.3 | 49 |
| 0.00027 | 2.2 | 51 | $Yb^{3+}$ | 0.00013 | 0.9 | 40 |

[a] Assays were carried out at pH 8 in 100 mM Taps at 25 °C with 2 mM F6P and 0.06 units of $PP_i$-PFK. Reaction was initiated with the 1:1 metal–pyrophosphate complex (10–100 μM). $K_m$ is for the metal–pyrophosphate complex. [b] Plus 1 μM F2,6P.

*Data Processing.* Reciprocal initial velocities were plotted vs reciprocal reactant concentration and all plots were linear. Data were fitted using appropriate rate equations and FORTRAN programs developed by Cleland (1979). Individual saturation curves obtained by varying either divalent metal or metal–$PP_i$ complex were fitted using eq 1, while initial velocity patterns obtained by varying uncomplexed divalent metal at different fixed levels of metal–$PP_i$ complex were fitted using eq 2, and the inhibition pattern for $Cr(H_2O)_4$-$PP_i$ was fitted using eq 3. In eqs 1–3, $v$ is the initial velocity,

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

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

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

$V$ is the maximum velocity, $A$ and $B$ are reactant (or metal) concentrations, $K_a$ and $K_b$ are Michaelis constants for $A$ and $B$, while $K_{ia}$ and $K_{is}$ are the inhibition constant for $A$ and slope, respectively.

## RESULTS

*Lanthanide–Pyrophosphates as Substrates for the $PP_i$-PFK.* In the absence of any added metal ions in the direction of FBP formation, there is a rate that is 16% of that obtained with a total added $Mg^{2+}$ concentration slightly greater than the pyrophosphate concentration. Atomic absorption analysis of all solutions used and of the complete reaction mixture indicated a contamination with 5.2 μM $Mg^{2+}$. Of the 5.2 μM $Mg^{2+}$, 4.4 μM $Mg^{2+}$ (85%) was found as a contamination of the Taps buffer. The rate could be essentially eliminated when the buffer concentration was decreased from 100 to 10 mM.

The $PP_i$-PFK reaction is reported to be activated by several divalent metal ions at a concentration of 10 mM in the order $Mn^{2+} > Mg^{2+} > Co^{2+}$ (Reeves et al., 1976). At 10 mM buffer concentration, none of the preformed trivalent lanthanide–pyrophosphate complexes shown in Table 1 served as a substrate (phosphoryl donor). At 100 mM buffer, however, the results shown in Table 1 for bacterial and plant

Table 2: Kinetic Parameters in the Direction of F6P Phosphorylation for Lanthanide–Pyrophosphate Complexes in the Presence of 2mM added $Mg^{2+}$ [a]

| bacterial $PP_i$-PFK | | | | plant $PP_i$-PFK[b] | | |
|---|---|---|---|---|---|---|
| $V_{max}$, mM/min | % $V$ | $K_m$, $\mu$M | metal | $V_{max}$, mM/min | % $V$ | $K_m$, $\mu$M |
| 0.0750 | | 4.6 | $Mg^{2+}$ | 0.0700 | | 7 |
| | | | Lanthanides | | | |
| 0.0063 | 8.4 | 58 | $Ce^{3+}$ | 0.0015 | 2.1 | 64 |
| 0.0021 | 2.8 | 47 | $Dy^{3+}$ | 0.0011 | 1.6 | 42 |
| 0.0037 | 4.9 | 53 | $Er^{3+}$ | 0.0013 | 1.9 | 45 |
| 0.0037 | 4.9 | 76 | $Eu^{3+}$ | 0.0010 | 1.4 | 54 |
| 0.0026 | 3.5 | 44 | $Gd^{3+}$ | 0.0009 | 1.3 | 40 |
| 0.0027 | 3.6 | 41 | $Ho^{3+}$ | 0.0013 | 1.9 | 55 |
| 0.0034 | 4.5 | 56 | $Nd^{3+}$ | 0.0012 | 1.7 | 40 |
| 0.0019 | 2.5 | 48 | $Sm^{3+}$ | 0.0011 | 1.6 | 62 |
| 0.0032 | 4.3 | 53 | $Tb^{3+}$ | 0.0014 | 2.0 | 40 |
| 0.0041 | 5.5 | 56 | $Tm^{3+}$ | 0.0011 | 1.6 | 39 |
| 0.0076 | 10.0 | 60 | $Yb^{3+}$ | 0.0011 | 1.6 | 44 |

[a] Assays were carried out using conditions identical to those given in Table 1 with the exception that 2 mM $Mg^{2+}$ was added. $K_m$ is for the metal–pyrophosphate complex. [b] Plus 1 $\mu$M F2,6P.

enzymes are obtained. The $V_{max}$ value reported for $Mg^{2+}$ is for the rate obtained with pyrophosphate in the absence of added metal (that is, 5 $\mu$M $Mg^{2+}$) and the $K_m$ value is that reported previously with $Mg^{2+}$ in a slight excess of $PP_i$ (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986a). Kinetic parameters for all of the lanthanide–pyrophosphates are remarkably similar with maximum rates for the bacterial enzyme that are 30–50 times lower and those for the plant enzyme that are 75–125 times lower than that obtained with endogenous $Mg^{2+}$ alone.

*Stimulation of the Rate Obtained with Lanthanide–Pyrophosphates by Addition of 2 mM $Mg^{2+}$.* Repetition of the above experiments using the same amount of enzyme in the presence of 2 mM $Mg^{2+}$ resulted in the data shown in Table 2. A significant increase in the $V_{max}$ is observed with stimulation of 7–15-fold and 5–10-fold for the bacterial and plant enzymes, respectively. The ratio of the maximum rates obtained for the lanthanide–pyrophosphates with 2 mM $Mg^{2+}$ with respect to that obtained with 2 mM $Mg^{2+}$ and pyrophosphate did not significantly change compared with the ratio obtained with the lanthanide–pyrophosphate and 5 $\mu$M endogenous $Mg^{2+}$ with respect to that obtained with 5 $\mu$M endogenous $Mg^{2+}$ and pyrophosphate, nor did the $K_m$ values for the lanthanide–pyrophosphates.

The exchange-inert $Cr(H_2O)_4PP_i$ complex was prepared to determine whether it could serve as a phosphoryl donor. No significant activity was observed when the exchange-inert complex was used in the absence or presence of 2 mM added $Mg^{2+}$. If the complex is a substrate, it likely only works under single turnover conditions. When tested as an inhibitor, the $CrPP_i$ was found to be competitive vs $MgPP_i$, with a $K_i$ of 27 $\mu$M (data not shown).

The initial rate was obtained as a function of added $Mg^{2+}$ concentration (5 $\mu$M to 1 mM), at different fixed concentrations of $CePP_i$ as the phosphoryl donor and a fixed concentration of F6P, under conditions where the endogenous $Mg^{2+}$ concentration was fixed by using 10 mM Taps at a very low level (0.8 $\mu$M) such that no background rate was observed. Under these conditions, maximum velocities essentially identical to those reported in Table 2 were obtained. Double-reciprocal plots of $1/v$ versus $1/Mg^{2+}$ at different phosphoryl donor concentrations intersect to the left of the ordinate on the abscissa (Figure 1), indicative of a sequential kinetic mechanism, and consistent with the rapid equilibrium random

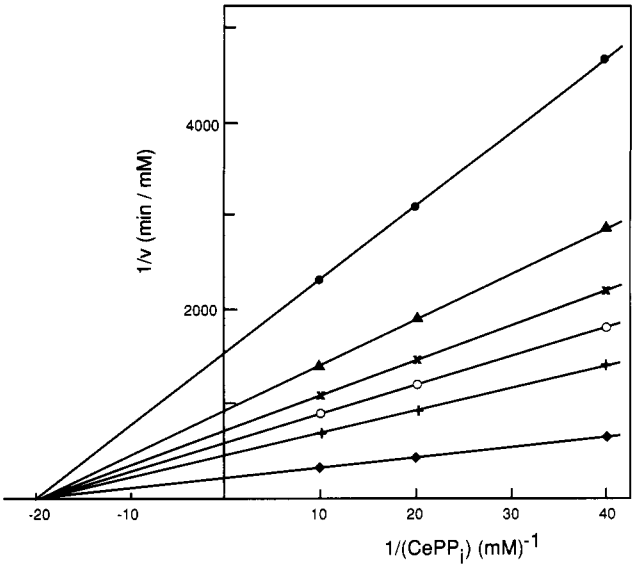


FIGURE 1: Initial velocity pattern obtained by varying $CePP_i$ at different levels of $Mg^{2+}$. Data were obtained at pH 8 and 25 °C. The $CePP_i$ concentrations are as indicated, while the $Mg^{2+}$ concentrations are as follows: ●, 10 $\mu$M; ▲, 20 $\mu$M; ×, 50 $\mu$M; ○, 100 $\mu$M; +, 250 $\mu$M; ♦, 1 mM.

Table 3: Comparison of the Percent Stimulation with Other Divalent Metal Ions as Activators Relative to $Mg^{2+}$ Using $CePP_i$ as the Phosphoryl Donor[a]

| bacterial $PP_i$-PFK | metal | plant $PP_i$-PFK[b] | bacterial $PP_i$-PFK | metal | plant $PP_i$-PFK[b] |
|---|---|---|---|---|---|
| 6 | Ba | 7 | 26 | Mo | 38 |
| 6 | Ca | 7 | 3 | Ni | 20 |
| 0 | Cd | 0 | 0 | Pb | 0 |
| 23 | Co | 27 | 3 | Pd | 0 |
| 37 | Cr | 15 | 0 | Sn | 0 |
| 5 | Cu | 4 | 0 | Sr | 0 |
| 9 | Fe | 9 | 0 | Zn | 0 |
| 230 | Mn | 201 | | | |

[a] Assays were carried out at pH 8 in 10 mM Taps at 25 °C with 2 mM F6P, 50 $\mu$M $CePP_i$, 50 $\mu$M $M^{2+}$, and 0.06 units of $PP_i$-PFK. The rates with other divalent metals are relative to $Mg^{2+}$, which was arbitrarily set at 100%. Reaction was initiated with the 1:1 metal pyrophosphate complex (10–100 $\mu$M). None of the trivalent metal ions were activators at 50 $\mu$M. Percentages below 9% were considered very weak activators. [b] Plus 1 $\mu$M F2,6P.

kinetic mechanism proposed for the $PP_i$-PFKs (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986a; Cho et al., 1988). The apparent $K_m$ value for $Mg^{2+}$ is its activation constant ($K_{act}$) and is the dissociation constant for $Mg^{2+}$ from the E–F6P–$CePP_i$–Mg complex. Using $CePP_i$ as the phosphoryl donor, the $K_{act}$ value for $Mg^{2+}$ is 26 ± 3 $\mu$M, measured for the bacterial enzyme, while a value of 53 ± 20 $\mu$M is measured for the plant enzyme. In the case of a rapid equilibrium random kinetic mechanism, the $K_{i_{Mg}}$ value is the dissociation constant for $Mg^{2+}$ from the E–F6P–Mg complex, and these values are equal to the $K_{act}$ values in all of the above.

*Specificity of the Activator Site.* The $CePP_i$ complex was used to screen other divalent metal ions for their ability to activate the $PP_i$-PFK. Significant stimulation was observed with 50 $\mu$M metal ion (Table 3) according to the following order: $Mn^{2+} > Mo^{2+}$, $Co^{2+}$, $Cr^{2+} > Fe^{2+}$, $Ni^{2+}$. Little or no stimulation was observed with the following metal ions: $Ba^{2+}$, $Ca^{2+}$, $Cd^{2+}$, $Cu^{2+}$, $Pb^{2+}$, $Pd^{2+}$, $Sn^{2+}$, $Sr^{2+}$, or $Zn^{2+}$. Activation constants for all of the above divalent metal ion activators are in the 50–100 $\mu$M range for the bacterial and plant enzymes using $CePP_i$ (Table 4). The exception is $Ni^{2+}$, which has

Table 4: Activation Constants for Divalent Metal Activators from Table 3[a]

| bacterial $PP_i$-PFK | metal | plant $PP_i$-PFK[b] | bacterial $PP_i$-PFK | metal | plant $PP_i$-PFK[b] |
|---|---|---|---|---|---|
| 22 | Co | 105 | 43 | Mn | 88 |
| 65 | Cr | 59 | 92 | Mo | 101 |
| 19 | Fe | 24 | 129 | Ni | 252 |

[a] Assays were carried out at pH 8 in 10 mM Taps at 25 °C with 2 mM F6P, 50 μM $CePP_i$, and 0.06 units of $PP_i$-PFK. The divalent metals were varied over the concentration range 20–200 μM. $K_{act}$ values are in μM. NR, no rate was detected. [b] Plus 1 μM F2,6P.

activation constants of 130 and 250 μM for bacterial and plant enzymes, respectively, when $CePP_i$ is the phosphoryl donor.[2]

## DISCUSSION

*Requirement for Two Metal Ions for Catalysis and Binding.* In the absence of added $Mg^{2+}$, no activity is observed for the $PP_i$-PFKs from bacteria and plant using the lanthanide–$PP_i$ chelate complexes as phosphoryl donors. However, in the presence of even 5 μM $Mg^{2+}$, lanthanide–pyrophosphate complexes give a low level of activity, and the observed activity is increased substantially in the presence of 2 mM added $Mg^{2+}$ such that 2.5–10% and 1.3–2% of the $V_{max}$ obtained with $Mg^{2+}$ alone in slight excess of $PP_i$ is obtained for bacterial and plant enzymes, respectively. The $K_m$ values for the lanthanide–$PP_i$ chelate complexes are on the order of 40–75 μM irrespective of the enzyme studied. In agreement with the lack of quantitative difference between the two enzymes, the $K_m$ values for $MgPP_i$ are about 5 and 6 μM, respectively, for bacterial and plant enzymes (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986a).

It is possible that the above activity observed for the metal–$PP_i$ complexes in the presence of added $Mg^{2+}$ is a result of disproportionation according to the following equilibrium.

$$\text{lanthanide–}PP_i^- + Mg^{2+} \rightleftharpoons MgPP_i^{2-} + \text{lanthanide(3+)} \quad (4)$$

However, in order for a significant amount of $MgPP_i$ to be formed, the stability constants for the $MgPP_i$ and lanthanide–$PP_i$ complexes must be reasonably close to one another. As can be seen in Table 5 there is a difference of 12–16 in the stability constants for the two, indicating that the affinity of $PP_i$ for the trivalent lanthanides is 12–16 orders of magnitude greater than for $Mg^{2+}$; that is, virtually no free $PP_i$ will be present when the trivalent lanthanide and $PP_i$ are at stoichiometric concentrations. As a result, $MgPP_i$ could only be formed if there was a small amount of free $PP_i$ available in the reaction mixture because the lanthanide/$PP_i$ ratio was slightly less than 1. The latter was ruled out by small incremental additions of free lanthanide(3+) to chelate any free $PP_i$ present, with no change in the amount of activity or the values of kinetic parameters observed. In addition, as shown in Tables 1 and 2, the $K_m$ values measured for the lanthanide–$PP_i$ complexes did not change as the concentration of free $Mg^{2+}$ was increased, a result inconsistent with disproportionation.

Thus, the $PP_i$-PFKs from bacteria and plants appear to require two metal ions for binding and activity, one to form the metal–$PP_i$ complex and a second to serve as an essential activator. It is likely that the requirement at both sites is for divalent and not monovalent cations since the high background levels of $K^+$ present in the buffer do not give activity in the presence of lanthanide–$PP_i$s. There is an endogenous activity obtained with the complete assay in the absence of added $Mg^{2+}$, but this activity is eliminated by low (μM) amounts of EDTA, and $Mg^{2+}$ alone will give full maximum activity.

[2] Attempts to measure the activation constant for $Mg^{2+}$ by varying $MgPP_i$ at different fixed levels of free $Mg^{2+}$ gave differences too small to be considered significant. This aspect is currently being pursued.

Table 5: Properties of Metal Ions and Stability Constants for Metal–Pyrophosphate Complexes

| metal ion | ionic radius, Å | log $K$[a] | metal ion | ionic radius, Å | log $K$[a] |
|---|---|---|---|---|---|
| | | Alkaline Earth | | | |
| $Mg^{2+}$ | 0.66 | 5.45 | $Sr^{2+}$ | 1.12 | 3.3 |
| $Ca^{2+}$ | 0.99 | 5.4 | $Ba^{2+}$ | 1.34 | NA |
| | | Transition | | | |
| $Fe^{2+}$ | 0.74 | NA | $Mn^{2+}$ | 0.80 | NA |
| $Co^{2+}$ | 0.72 | 6.1 | $Cr^{2+}$ | 0.89 | NA |
| $Ni^{2+}$ | 0.69 | 5.9 | $Al^{3+}$ | 0.51 | NA |
| $Cu^{2+}$ | 0.72 | 7.6 | $Cr^{3+}$ | 0.63 | NA |
| $Zn^{2+}$ | 0.74 | 8.7 | $Fe^{3+}$ | 0.64 | 7.0 |
| $Mo^{2+}$ | 0.78 | NA | $Sc^{3+}$ | 0.732 | NA |
| | | Lanthanide | | | |
| $Ce^{3+}$ | 1.03 | 17.2 | $Nd^{3+}$ | 0.995 | 15.0 |
| $Dy^{3+}$ | 0.908 | 20.6 | $Sm^{3+}$ | 0.964 | 20.2 |
| $Er^{3+}$ | 0.81 | 21.3 | $Tb^{3+}$ | 0.923 | 20.5 |
| $Eu^{3+}$ | 0.95 | 20.3 | $Tm^{3+}$ | 0.87 | NA |
| $Gd^{3+}$ | 0.938 | 20.5 | $Yb^{3+}$ | 0.858 | 17.5 |
| $Ho^{3+}$ | 0.894 | 20.9 | | | |

[a] $K$ = [ML]/[M][L], where M is the metal ion and L is the ligand, in this case $PP_i$. Values are obtained at variable ionic strength. NA, not available. Data from Smith and Martell (1979) and CRC Handbook of Chemistry and Physics, 71st ed.

*Specificity for the Divalent Metal Ion Activation Site.* A number of divalent metal ions will substitute for $Mg^{2+}$ as an activator, while trivalent metal ions apparently cannot fulfill this role. Generally, it is the smaller (0.6–0.7 Å ionic radius), harder divalent metal ions such as $Mg^{2+}$, $Mn^{2+}$, and $Co^{2+}$ that appear to be the best activators, while larger and softer metal ions such as $Cd^{2+}$ either do not activate at all or do so poorly.

*Role of the Divalent Metal Ion Activator.* Initial velocity patterns obtained by varying the concentrations of $CePP_i$ and $Mg^{2+}$ simultaneously are indicative of a rapid equilibrium random kinetic mechanism. These data are in agreement with earlier initial velocity data obtained with $Mg^{2+}$ alone (Bertagnolli & Cook, 1984; Bertagnolli et al., 1986). In addition, the equality of $K_{i_{Mg}}$ and $K_{Mg}$ values indicates that the binding of $Mg^{2+}$ is unaffected by the presence of the metal–$PP_i$ complex. Thus, if the uncomplexed divalent metal ion interacts with $CePP_i$, the interactions cannot be very strong, at least in the ground state.

The activating divalent metal ion could play a role in maintaining the active structure of the $PP_i$-PFK, or be involved in binding and/or catalysis. There is no evidence that the activator plays any role in the initial binding of reactants, but there certainly could be an effect on binding the transition state. In this regard, the mutagenesis studies of Green et al. (1993) are of interest. A low but significant sequence similarity was found to exist between the *Escherichia coli* ATP-PFK and the *Propionibacterium freudenreichii* $PP_i$-PFK (Ladror et al., 1991). The longest common sequence between the two is T-I-D-N-D, significant because it is at the active site of the ATP-PFK based on X-ray crystallography (Shirakihara & Evans, 1988). Indeed, site-directed mutagenesis indicates that the first Asp in the above sequence is the general base that abstracts a proton from the 1-hydroxyl

of F6P in the phosphoryl transfer reaction for both the ATP-PFK (Hellinga & Evans, 1987) and the $PP_i$–PFK (Green et al., 1992). The crystal structure of the *E. coli* enzyme suggests that the second Asp in the above sequence interacts with $Mg^{2+}$, which in turn interacts with the polyphosphate chain of ATP (Hellinga & Evans, 1987). Mutagenesis of the equivalent Asp in the $PP_i$-PFK (Green et al., 1992), gives a mutant protein that behaves in a manner similar to that obtained for the ATP-PFK enzyme, and thus the Asp may have a similar function. The latter certainly could be the site for the activator which interacts with the reactant(s) in the transition state resulting in its stabilization and facilitation of catalysis. However, a purely structural role for the second metal ion cannot at this time be ruled out and more work will be required on its role.

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