Enhancement of the Rate of Pyrophosphate Hydrolysis by Nonenzymatic Catalysts and by Inorganic Pyrophosphatase*  

To estimate the proficiency of inorganic pyrophosphatase as a catalyst, 31P NMR was used to determine rate constants and thermodynamics of activation for the spontaneous hydrolysis of inorganic pyrophosphate (PPi) in the presence and absence of Mg2+ at elevated temperatures. These values were compared with rate constants and activation parameters determined for the reaction catalyzed by Escherichia coli inorganic pyrophosphatase using isothermal titration calorimetry. At 25 °C and pH 8.5, the hydrolysis of MgPPi2− proceeds with a rate constant of 2.8 × 10−10 s−1, whereas E. coli pyrophosphatase was found to have a turnover number of 570 s−1 under the same conditions. The resulting rate enhancement (2.8 × 1012-fold) is achieved entirely by reducing the enthalpy of activation (ΔH‡ = −16.6 kcal/mol). The presence of Mg2+ ions or the transfer of the substrate from bulk water to dimethyl sulfoxide was found to increase the rate of pyrophosphate hydrolysis by as much as ~106-fold. Transfer to dimethyl sulfoxide accelerated PPi hydrolysis by reducing the enthalpy of activation. Mg2+ increased the rate of PPi hydrolysis by both increasing the entropy of activation and reducing the enthalpy of activation.

Enzymes may have evolved from simpler catalysts that lowered the enthalpy of activation (ΔH‡) of biologically important reactions in the primitive earth. Thus, it is of interest to compare the thermodynamic basis of the rate enhancements produced by enzymes with the thermodynamic basis of the rate enhancements produced by small inorganic catalysts (1).

It is reasonable to suppose that inorganic pyrophosphatase (PPi)2− served as a phosphoryl group donor in primitive biological systems (2). Even today, PPi-dependent proton pumps, sodium pumps, and kinases are found in some bacteria (3–5). PPi hydrolysis plays an important role in the metabolism of modern organisms by removing the PPi that is generated by biosynthetic reactions such as the synthesis of aminoacyl-tRNA. The enzyme (inorganic pyrophosphatase (PPase)) that catalyzes this reaction has an unusual active site that has been termed “mini-mineral,” in that metal ions and structural water molecules appear to be largely responsible for catalysis (6). This enzyme furnishes an opportunity to compare the proficiency of an enzyme that catalyzes one of the simpler reactions in biology with the efficiencies of primitive catalysts that model some of the key chemical features of the modern enzyme’s active site.

Two classes of PPases have been identified, and the tertiary structures of Type I PPases (found in eukaryotes and Escherichia coli) differ significantly from those of Type II PPases (found in some bacteria), suggesting that these two classes evolved independently (7, 8), yet these enzymes have remarkably similar active sites (9, 10). Type I PPases require three Mg2+ ions for activity (10), whereas Type II PPases require four Mn2+ ions (9). Metal ions and water molecules, organized and positioned by the protein, constitute the immediate environment of the bound substrate and have been proposed to assist catalysis in two ways (10). First, the extremely polar active site neutralizes the charged PPi substrate so that the nucleophile can approach. A crystal structure of the Saccharomyces cerevisiae Type I enzyme complex with two bound phosphate ions shows that the oxygen atoms of the two phosphate groups are coordinated by six hydrogen bonds to water, six bonds to the metal ions, and six hydrogen bonds to protein side chains (10). In the Bacillus subtilis Type II enzyme with the substrate analog imidodiphosphate, all three nonbridging oxygen atoms of the phosphoryl group that undergoes attack by water are bound to a Mn2+ ion (9). Second, metal ions coordinate the substrate water molecule, decreasing its pKa value and rendering it more effective as a nucleophile at ordinary pH values. In Type I PPases, the substrate water molecule bridges two metal ions (10), whereas in Type II PPases, the substrate water molecule resides in the center of a plane formed by three metal ions (9).

In the active sites of PPases, metal ions seem to stabilize the transition state to a greater extent than do protein side chains. Of the 14 conserved residues shared by Type I PPases, only two aspartic acid residues decreased kcat by more than ~100-fold when mutated to alanine, and both of those mutations eliminated binding of the two structural Mg2+ ions (11). A water molecule appears to act as a general acid by protonating the leaving phosphoryl group (9, 10). Thus, PPase may constitute an extreme case in which metal ions and water molecules play a direct role in catalysis, whereas the protein moiety serves mainly as a scaffold for organizing the system.

In principle, it does not seem obvious that a protein-based active site could not accelerate PPi hydrolysis. Kinases and phosphatases both require metal ions (generally one and two, respectively), yet these enzymes (which are structurally unrelated to PPases) employ protein side chains as catalytic bases, nucleophiles, or electrostatic catalysts (12). The apparently convergent evolution of Type I and II PPases suggests that, in...
this special case, a primitive mini-mineral catalytic site might suffice to catalyze PP$_i$ hydrolysis.

In this work, we sought to determine the magnitude and thermodynamic origin of the rate enhancements produced by \textit{E. coli} PPase and by simple inorganic catalyst models of the enzyme’s inorganic core. We compared the intrinsic reactivities of PP$_i$$^\text{4-}$, PP$_i$$^\text{3-}$, PP$_i$$^\text{2-}$, and MgPP$_i$$^\text{2-}$ (the true substrate for PPase) (13). We also determined the rate constants and activation parameters for the hydrolysis of PP$_i$ by \textit{E. coli} PPase. In addition, we examined the rate of PP$_i$ hydrolysis in dimethyl sulfoxide (DMSO), a dipolar aprotic solvent chosen to explore the effect of transferring this substrate from solvent water to a somewhat polar nonaqueous environment.

**EXPERIMENTAL PROCEDURES**

\textit{PP$_i$, Hydrolysis—}Chemicals were purchased from Sigma. To measure the rate constants for the hydrolysis of PP$_i$, hydrolysis between pH 5 and 9.5, sodium pyrophosphate (0.2 M) and various anionic buffers (1 M, pH 5–9.5; see below) were sealed in quartz tubes under vacuum and incubated in convection ovens (Model 47900, Barnstead Thermolyne Corp.) at temperatures ranging from 70 to 150 °C ($\pm$1.5 °C as indicated by an ASTM thermometer) for varying periods of time. Solutions were buffered with potassium acetate (pH 5.0–6.3), sodium arsenate (pH 6.5–7), or ethyl phosphonate (pH 8.3–8.9). In experiments carried out at high pH values, PP$_i$ (0.2 M) and potassium hydroxide (KOH; 1–2 M) were sealed in Teflon-lined stainless steel bombs for incubation.

After reaction, samples (0.3 ml) were prepared for analysis by the addition of D$_2$O (0.3 ml) containing hexamethylphosphoramide (1.8 $\times$ 10$^{-5}$ M) as an internal integration standard. The integrated intensities of the $^3$P signals arising from PP$_i$ and inorganic phosphate (PO$_4$) were measured using a Varian 600-MHz spectrometer. Data were acquired for a minimum of four transients and processed using SpinWorks (14).

Experiments to determine the rate constants for MgPP$_i$$^\text{2-}$ hydrolysis were performed under three sets of experimental conditions and analytic methods. In the first method, the reaction mixture contained 1 $\times$ 10$^{-7}$ M Mg$^{2+}$, 2 $\times$ 10$^{-7}$ M PP$_i$, and 1 $\times$ 10$^{-7}$ M ethyl phosphonate (pH 7.5). The increase in PO$_4$ was monitored using $^3$P NMR as described above. The $^3$P signal arising from PP$_i$ was broadened in the presence of Mg$^{2+}$ and was not used to determine rate constants. In the second method, the reaction mixture contained 1 $\times$ 10$^{-2}$ M Mg$^{2+}$, 2 $\times$ 10$^{-3}$ M PP$_i$, and 2 $\times$ 10$^{-3}$ M ethyl phosphonate (pH 8.0). The amount of PO$_4$ that was liberated by the hydrolysis of PP$_i$ was measured using a colorimetric acid-molybdate assay (15). To prepare samples, 5 $\times$ 10$^{-3}$ ml of reaction mixture was mixed with 0.5 ml of water and added to a solution (0.5 ml) containing 1.2 M hydrosulfuric acid, 5% ascorbic acid, and 0.4 M ammonium molybdate. Samples were incubated at 60 °C for 30 min. The characteristic blue color that develops in the presence of inorganic phosphate was monitored spectrophotometrically at 820 nm using a Hewlett-Packard 8452A diode array spectrophotometer. Separate control experiments showed that PP$_i$ and ethyl phosphonate did not contribute to the absorbance at 820 nm and that PP$_i$ was not hydrolyzed during the acid incubation.

In the third method for measuring MgPP$_i$$^\text{2-}$ hydrolysis, the reaction mixture contained 2 $\times$ 10$^{-4}$ M Mg$^{2+}$, 2 $\times$ 10$^{-5}$ M PP$_i$, and 2 $\times$ 10$^{-3}$ M sodium borate (pH 8.7). After incubation, the concentration of PO$_4$ was measured using the acid-molybdate assay, in which 0.5 ml of reaction mixture was incubated with 0.5 ml of acid-molybdate reagent.

Titrations of the reaction mixtures showed that MgPP$_i$$^\text{2-}$ is the major ionic species as the pH is varied between 7.5 and 8.7. Under all three experimental conditions, PP$_i$ is expected to be fully complexed with Mg$^{2+}$ at all temperatures ($K_D$ = ~5 $\times$ 10$^{-3}$ M at 25 °C, $\Delta H$ = 3.7 kcal/mol) (16). No precipitate was visible in the starting material. After incubation, a small amount of white precipitate was present in a fraction of the samples. The precipitate dissolved when the acid-molybdate reagent was added.

\textit{Pyrophosphatase Reaction—}Soluble PPase from \textit{E. coli} was purchased from Sigma. Enzyme assays were performed using isothermal titration calorimetry (VP-ITC system, MicroCal, Northampton, MA) as described by Todd and Gomez (17). To determine $k_{cat}$, the enzyme (6.6 $\times$ 10$^{-10}$ to 6.6 $\times$ 10$^{-9}$ M) was prepared in HEPES buffer (3 $\times$ 10$^{-3}$ M, pH 8.4) with magnesium chloride (3 $\times$ 10$^{-3}$ M) and degassed for 10 min to prevent bubble formation during the experiment. After thermal equilibration and a 200-s time lag to establish a baseline, PP$_i$ (5 $\times$ 10$^{-3}$ M) was injected into the reaction mixture (1.45 ml) to initiate the reaction. The final concentration of PP$_i$ (4.2 $\times$ 10$^{-5}$ M) was ~10-fold greater than its $K_m$ value for PPase (2.5 $\times$ 10$^{-4}$ M) (18).

\textit{PP$_i$, Hydrolysis in Wet DMSO—}A saturated solution of sodium pyrophosphate (~2 $\times$ 10$^{-4}$ M) was prepared in dry DMSO, and water (~1%, v/v) was added. The precise concentration of water in the reaction mixture was measured by comparison with pyrazine, which was added as an internal integration standard, using $^1$H NMR. Hydrolysis reactions were conducted in sealed quartz tubes at elevated temperatures (70–120 °C) as described above. After incubation, the reaction mixture was diluted 1:50 with water and analyzed using the acid-molybdate assay. To prepare samples, 0.5 ml of the aqueous solution was added to the acid-molybdate reagent (0.5 ml). The concentration of PO$_4$ was determined by comparison with a standard curve. (Standard samples also contained 2% DMSO.) Separate control experiments showed that the added DMSO did not affect the results of the acid-molybdate assay.

**RESULTS**

\textit{Spontaneous Hydrolysis of PP$_i$$^\text{+}$, PP$_i$$^\text{3-}$, and PP$_i$$^\text{2-}$—}In buffered solution from pH 5 to 14, the hydrolysis of PP$_i$ was monitored using $^3$P NMR and followed simple first-order kinetics to completion under all conditions. Rate constants for PP$_i$ hydrolysis were determined at several temperatures by measuring the relative intensities of the peaks arising from PP$_i$ and PO$_4$ as a function of time. Linear regression of Arrhenius plots of reaction rates plotted as a logarithmic function of the reciprocal of absolute temperature (Kelvin), constructed from data obtained at nine pH values between 5.0 and 14.3 (representative plots are shown in Fig. 2), yielded $R^2$ values of $>$0.98. Fig. 3 shows values of $\Delta G^\circ$ (extrapolated to 25 °C) and $\Delta H^\circ$, estimated from each Arrhenius plot as a function of pH (Fig. 2). These pH profiles were used to estimate the individual activation parameters for

Catalysis of Pyrophosphate Hydrolysis
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TABLE 1

<table>
<thead>
<tr>
<th>Catalyst</th>
<th>ΔG°</th>
<th>ΔH°</th>
<th>ΔS°</th>
<th>k25</th>
<th>Rate enhancement*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPi</td>
<td>35.0 ± 0.2</td>
<td>33.9 ± 0.4</td>
<td>-1.1 ± 0.6</td>
<td>1.2 × 10⁻¹³</td>
<td>560</td>
</tr>
<tr>
<td>PPi⁻</td>
<td>31.2 ± 0.4</td>
<td>30.8 ± 1.6</td>
<td>-0.4 ± 2.0</td>
<td>6.7 × 10⁻¹⁰</td>
<td>5.9 × 10⁴</td>
</tr>
<tr>
<td>PPi²⁻</td>
<td>28.5 ± 0.1</td>
<td>29.1 ± 0.4</td>
<td>0.6 ± 0.5</td>
<td>7.1 × 10⁻⁹</td>
<td>2.3 × 10⁴</td>
</tr>
<tr>
<td>MgPPi²⁻</td>
<td>30.4 ± 0.4</td>
<td>29.3 ± 1.0</td>
<td>-1.1 ± 1.4</td>
<td>2.8 × 10⁻¹⁰</td>
<td>2.0 × 10⁻² (6.5 × 10¹¹)</td>
</tr>
<tr>
<td>E. coli PPase</td>
<td>13.7 ± 0.1</td>
<td>12.7 ± 0.2</td>
<td>-1.0 ± 0.3</td>
<td>570</td>
<td></td>
</tr>
<tr>
<td>PPi⁴⁻ in DMSO</td>
<td>29.0 ± 0.2</td>
<td>18.4 ± 0.4</td>
<td>-10.6 ± 0.6</td>
<td>3.0 × 10⁻¹⁰</td>
<td>1.4 × 10⁴</td>
</tr>
</tbody>
</table>

* Rate enhancements were obtained by comparison with the rate constant for PPi⁴⁻ hydrolysis, except in the case of E. coli PPase, for which the rate enhancement was obtained by comparison with the rate constant for MgPPi²⁻ hydrolysis. The rate enhancement provided by E. coli PPase at 37 °C is shown in parentheses.

A

B

FIGURE 1. A, rate constants for the hydrolysis of PPi⁴⁻, PPi³⁻, PPi²⁻, and MgPPi²⁻ were determined in this work. The Kd or pKd values are shown for each species. The conformation of MgPPi²⁻, that is shown is the most stable in solution, although not necessarily the reactive species, B, reaction diagram for the proposed enzyme reaction. In the PPase active site, the nucleophilic water molecule is thought to be coordinated by two Mg²⁺ ions, which lower the pKα of the water molecule, facilitating proton transfer to a base within the active site. The hydrolysis reaction is hypothesized to proceed through an associative mechanism, in which the attack by the nucleophile occurs prior to the phosphate-phosphate bond breaking. In this work, the thermodynamic effect of the two Mg²⁺ ions on water deprotonation was examined.

The hydrolyses of PPi²⁻, PPi³⁻, and PPi⁴⁻ (Table 1) as described below.

In the pH range between 5 and 14.3 over which experiments were conducted, PPi exhibits two pKα values of 6.6 and 9.4 at 25 °C (Fig. 1) (19). Values for the free energies of activation of hydrolysis (25 °C) approached a constant value at the lower end of this range, consistent with hydrolysis of a single species, PPi²⁻, and in accord with a previous description of PPi hydrolysis measured at pH values between 0 and 7 (20). The activation parameters for the hydrolysis of PPi²⁻ reported in Table 1 were measured directly at pH 5.0.

Rate constants for PPi⁴⁻ hydrolysis were also measured directly at high pH values. As in the case of other phosphate esters and phosphate anhydrides (21, 22), each additional negative charge decreased the rate constant for PPi₁ hydrolysis. Even at pH values as high as 13, ~4 pH units above the pKα value of PPi³⁻, the observed rate constants reflect the hydrolysis of a small population of PPi³⁻ that reacts several orders of magnitude more rapidly than does the species predominating at pH 13, PPi⁴⁻. To evaluate the rate of PPi⁴⁻ hydrolysis, rate constants for PPi hydrolysis were determined under conditions of increasing pH until the observed rate constant did not change, at a pH value of 14.0 (1 M KOH). Reaction rates in 1 and 2 M KOH (but not 0.1 M KOH) did not differ significantly (Figs. 2 and 3). Data obtained at pH 14 and 14.3 were combined into a single Arrhenius plot to determine the activation parameters for PPi⁴⁻ hydrolysis (Fig. 2).

Experiments were conducted at a constant ionic strength of 1.0 except for those carried out in 2 M KOH. In the pH range from 5 to 13, changing ionic strength showed only a small effect on the rate of PPi hydrolysis (~1.5-fold increase in rate as the ionic strength was adjusted from 0.1 to 1.0 with KCl). In solutions containing 1 M KOH and 1 M potassium chloride, the

The observed rate constant might also represent the kinetically indistinguishable reaction between PPi⁴⁻ and OH⁻.
observed rate constants for PP$_3^-$ hydrolysis were identical within experimental error to those measured in 1 and 2 M KOH.

Rate constants for PP$_3^{2-}$ and PP$_3^{-}$ hydrolysis were measured directly at pH 5.0 and 14.0, respectively, but Fig. 3 shows that there is no pH value at which PP$_3^{2-}$ is the only species that contributes to the observed rate constant. To determine $\Delta G^\ddagger$ for the hydrolysis of PP$_3^{2-}$, the data shown in Fig. 3 were fit to a theoretical curve described by Equation 1,

$$\Delta G^\ddagger = (35.0 \text{ kcal/mol})[\text{PP}_3^{4-}] + x \times [\text{PP}_3^{2-}]$$

$$+ (28.5 \text{ kcal/mol})[\text{PP}_3^{2-}] \quad \text{(Eq. 1)}$$

using the application CurTiPot (23), with $pK_a$ values of 6.6 and 9.4 for PP$_3^{2-}$ and PP$_3^{4-}$, respectively. A similar analysis was performed to estimate $\Delta H^\ddagger$ for PP$_3^{2-}$ hydrolysis, and the value of $T\Delta S^\ddagger$ for PP$_3^{2-}$ hydrolysis was calculated using Equation 2.

$$\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger \quad \text{(Eq. 2)}$$

The estimated activation parameters for PP$_3^{2-}$ hydrolysis are shown in Table 1. Experimental errors in $\Delta G^\ddagger$ and $\Delta H^\ddagger$, estimated using the sum of squared errors, are 0.4 and 1.6 kcal/mol, respectively. The approximate relationship between temperature and rate constant for PP$_3^{2-}$ hydrolysis is shown as a dashed line in Fig. 2, alongside Arrhenius plots for PP$_3^{2-}$ and PP$_3^{4-}$ hydrolysis.

**PP$_3^-$ Hydrolysis in the Presence of Mg$^{2+}$**—Most evidence indicates that MgPP$_2^{2-}$ is the true substrate of soluble PPases (13). At its estimated concentration in vivo $(0.5–1.3 \times 10^{-3} \text{ M PP}}$_3$ in \text{E. coli})$ (24), most PP$_3$ is expected to be associated with Mg$^{2+}$ ($\sim 1 \times 10^{-3} \text{ M free}$) (25) because $K_{d} \sim 5 \times 10^{-3} \text{ M at 25 °C}$ (16). There is strong kinetic evidence that one of the three active site Mg$^{2+}$ ions is bound along with PP$_3$ (26). We wished to estimate the rate of MgPP$_2^{2-}$ hydrolysis at ambient temperatures to serve as a benchmark for comparison with the rate of the enzyme-catalyzed reaction. Because the reduced solubility of the MgPP$_2$ complex at elevated temperatures limited the accuracy of individual rate determinations, experiments were conducted using both $^{31}$P NMR and the acid-molybdate assay. Rates were determined at Mg$^{2+}$ concentrations ranging from $2 \times 10^{-4}$ to $1 \times 10^{-2} \text{ M Mg}^{2+}$ and at pH values between 7.5 and 8.7. Sixty-one data points were obtained and used to construct an Arrhenius plot (Fig. 4). There was considerable scatter in the data set ($R^2 = 0.88$), but the prediction intervals associated with the extrapolation were $\pm 0.4 \text{ kcal/mol for } \Delta G^\ddagger$ at 25 °C and $\pm 1.0 \text{ kcal/mol for } \Delta H^\ddagger$, with 95% confidence. Data collected under different conditions were distributed randomly around the best fit line. In the presence of Mg$^{2+}$, the rate of PP$_3$ hydrolysis increased by a factor of $\sim 0.1$ with each pH unit increase (data not shown). In contrast, the rate of PP$_3$ hydrolysis in the absence of Mg$^{2+}$ decreased rapidly with increasing pH. This behavior is consistent with the view that MgPP$_2^{2-}$, rather than PP$_3^{2-}$, is the major species that contributes to rate constants observed between pH 7.5 and 8.7.
Catalysis of Pyrophosphate Hydrolysis

**PP$_i$ Hydrolysis by Pyrophosphatase**—As in earlier work on several kinase reactions (22), isothermal titration calorimetry furnished a sensitive continuous assay in experiments with *E. coli* PPase at pH 8.4. At this pH value, PP$_i$ hydrolysis is the slowest step, rather than substrate binding or product release (18). In kinetic experiments using isothermal titration calorimetry, the enzyme reaction releases an amount of heat directly proportional to the amount of substrate turned over, and the instrument output reflects the energy required to balance the heat generated by the reaction (17). Fig. 5A shows the calorimeter output upon substrate injection in the presence (black trace) and absence (blue trace) of PPase. In this trace, the initial positive spike represents the heat of injection. After substrate mixing, the trace returned to the baseline when no enzyme was present (blue trace). When PPase was present in the reaction mixture, the resulting exothermic reaction resulted in a negative displacement from the baseline. As substrate was consumed, the rate at which heat was generated subsided, and the trace returned to the baseline.

The total heat released by the reaction (the area in the shaded region in Fig. 5A) is given by Equation 3,

$$\int_0^t \frac{dQ}{dt} \, dt$$

and the velocity ($dP/dt$) is given by Equation 4,

$$\frac{dP}{dt} = \frac{[PP_i]}{[PP_i]_0} \cdot \frac{dQ}{dt}$$

where $dQ/dt$ is the difference between the baseline and the heat released at any time $t$ (Fig. 5A), $[PP_i]_0$ is the initial substrate concentration, and the integral represents the area in the shaded region of Fig. 5A, the total heat released by the hydrolysis of 4.2 × 10$^{-5}$ M PP$_i$ (17).

The concentration of PP$_i$ at any time $t$ was obtained by multiplying $[PP_i]_0$ by the integrated area between substrate injection and time $t$, divided by the total area in the well (Equation 5).

$$[PP_i] = [PP_i]_0 \cdot \frac{1 - \int_0^t \frac{dQ}{dt} \, dt}{\int_0^\infty \frac{dQ}{dt} \, dt}$$

Continuous plots of the velocity at every time $t$ versus $[PP_i]_0$ were generated at temperatures between 10 and 50 °C. To obtain values for $K_m$ and $V_{max}$, these data were fit to the Michaelis-Menten equation using SigmaPlot (Fig. 5B), and values of $k_{cat}$ were determined by dividing $V_{max}$ by the concentration of enzyme used in each experiment. These $k_{cat}$ values were used to construct an Arrhenius plot (Fig. 6) that yielded the thermodynamics of activation shown in Table 1. The $k_{cat}$ value of 570 s$^{-1}$ measured for PPase at 25 °C in this work is slightly larger than a literature value of 290 s$^{-1}$ (18).

**PP$_i$ Hydrolysis in DMSO**—Because transfer of the substrate from bulk water to nonpolar solvents greatly accelerates the second-order rate constant for hydrolysis of phosphate monoesters and diesters (27, 28), we sought to determine whether desolvation might also increase the rate of PP$_i$ hydrolysis. PPase has an unusually polar active site (10), so we chose to model PP$_i$ desolvation in the active site of PPase by transferring PP$_i$ from bulk water to a polar aprotic solvent, DMSO.

In water, PP$_i$ hydrolysis proceeds essentially to completion (29), but when the concentration of H$_2$O was reduced from 55.5

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**Figure 5. Measurement of $k_{cat}$ using isothermal titration calorimetry.** A, the reaction was initiated by injecting 5 × 10$^{-3}$ ml of PP$_i$ into 1.4S ml of reaction mixture (positive spike at 100 s). The upper trace (blue) shows the instrument output in the absence of PPase. When PPase is present in the reaction mixture (lower trace, black), heat is generated in proportion to substrate turnover. The velocity ($dP/dt$) at any time was determined using Equation 4. Data were fit to the Michaelis-Menten equation using SigmaPlot, from which $k_{cat}$ and $K_m$ values were extracted.
to \( \sim 0.56 \) for the DMSO experiments, we found that a temperature-dependent equilibrium between PP\(_i\) and PO\(_4\) was established. To determine values for the equilibrium constants, reactions were run until equilibrium was reached (as determined from the liberation of PO\(_4\)) at temperatures ranging from 110 to 160 \( ^\circ \text{C} \) (see Equation 6). We used these data to construct a van’t Hoff plot (data not shown) that furnished an estimated \( K_{\text{eq}} \) value of \( 1.1 \times 10^{9} \) favoring PP\(_i\) hydrolysis in DMSO at 25 \( ^\circ \text{C} \), compared with a value of \( 7.6 \times 10^{6} \) in water (29).

Rate constants were determined for PP\(_i\) hydrolysis in DMSO at temperatures between 70 and 120 \( ^\circ \text{C} \). At each temperature, we used the observed rate constant (\( k_{\text{obs}} \)) and the equilibrium constant for PP\(_i\) hydrolysis estimated from the van’t Hoff plot (\( K_{\text{eq}} \)) to calculate a value for the rate constant for PP\(_i\) hydrolysis (\( k_1 \)) according to the relationships in Equations 6–8.

\[
K_{\text{eq}} = \frac{[\text{PP}][\text{H}_2\text{O}]}{[\text{PO}_4][\text{PP}]} = \frac{k_{-1}}{k_1} \quad \text{(Eq. 6)}
\]

\[
k_{\text{obs}} = k_1 + k_{-1} \quad \text{(Eq. 7)}
\]

\[
k_1 = \frac{k_{\text{obs}}}{K_{\text{eq}} + 1} \quad \text{(Eq. 8)}
\]

These results were used to construct an Arrhenius plot that yielded the rate constant and activation parameters for hydrolysis of PP\(_i\) in DMSO at 25 \( ^\circ \text{C} \) (Fig. 7 and Table 1).

Because H\(_2\)O does not ionize significantly in DMSO (pK\(_a\) of 32) (30), no source of protons is available to generate PP\(_i\)\(^{-3}\). Thus, the observed rate constant is expected to reflect PP\(_i\)\(^{-4}\) hydrolysis. As an additional control, hydrolysis in DMSO was also monitored in the presence of 0.1 or 1 M KOH in the reaction mixture. As expected, the rate constant for PP\(_i\) hydrolysis in DMSO, corrected for the relative concentrations of H\(_2\)O, did not change significantly.

**Thermodynamic Basis of Water Activation by Mg\(^{2+}\)** —Crystal structures of PPses from different organisms show that the nucleophilic water molecule is coordinated by two Mg\(^{2+}\) ions in the active site (10). This arrangement presumably activates the water for nucleophilic attack by decreasing its pK\(_a\) value; this water molecule has a pK\(_a\) value that has been estimated as \( \sim 8 \) in the yeast enzyme (31) and \( \sim 6.5 \) in the *E. coli* enzyme (32).

To determine the extent to which two Mg\(^{2+}\) ions would be expected to increase the pK\(_a\) of a water molecule in solution and the thermodynamic basis for water molecule activation, we adopted the following approach.\(^4\) In the case of a single Mg\(^{2+}\) ion, titration of 0.1 M MgCl\(_2\) with KOH showed that the apparent pK\(_a\) value of MgH\(_2\)O is \( \sim 10 \), i.e. that Equation 9 applies.

\[
K_1 = \frac{[\text{H}^+][\text{MgOH}^+]}{[\text{MgH}_2\text{O}^{2+}]} = 1 \times 10^{-10} \quad \text{(Eq. 9)}
\]

With this information, in addition to the ionization constant of water (Equation 10) and the published dissociation constant of MgOH\(^+\) (33) (Equation 11), the dissociation constant of ligand water (Equation 12) was calculated using the relationship described by Equations 13 and 14, yielding a value for \( K_3 \) (Equation 15).

\[
K_w = \frac{[\text{H}^+][\text{OH}^-]}{[\text{H}_2\text{O}]} = 1 \times 10^{-14} \quad \text{(Eq. 10)}
\]

\[
K_2 = \frac{[\text{OH}^-][\text{Mg}^{2+}]}{[\text{MgOH}^+]} = 400 \quad \text{(Eq. 11)}
\]

\(^4\) In solution, Mg\(^{2+}\) is present as hexaaquamagnesium (Mg(H\(_2\)O\(_{6}\))\(^{2+}\)). For simplicity, only water molecules explicitly involved in the equilibria are included in these equations. In expressions that use [H\(_2\)O], mole fraction is used to express [H\(_2\)O] because the other components are comparatively dilute, and \( X_{\text{H}_2\text{O}} \sim 1 \).
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\[ K_3 = \frac{[\text{Mg}^{2+}][\text{H}_2\text{O}]}{[\text{MgH}_2\text{O}^{2+}]} \]  
(Eq. 12)

\[
\frac{[\text{H}^+][\text{MgOH}^+]}{[\text{MgH}_2\text{O}^{2+}]} = \frac{[\text{MgOH}^+][\text{H}^+][\text{OH}^-][\text{Mg}^{2+}][\text{H}_2\text{O}]}{[\text{H}_2\text{O}][\text{MgH}_2\text{O}^{2+}]} \]  
(Eq. 13)

\[
K_1 = \frac{K_aK_3}{K_2} \]  
(Eq. 14)

\[
K_5 = \frac{[\text{Mg}^{2+}][\text{H}_2\text{O}]}{[\text{MgH}_2\text{O}^{2+}]} = 2.5 \times 10^{-7} \]  
(Eq. 15)

The addition of a second Mg\(^{2+}\) ion coordinating the same water molecule would be expected to change the pK\(_a\) value of that water according to the following relationship.

\[
\frac{[\text{H}^+][\text{Mg}_2\text{OH}^{3+}]}{[\text{MgH}_2\text{O}^{2+}]} = \frac{[\text{H}^+][\text{Mg}_2\text{OH}^{3+}][\text{Mg}^{2+}][\text{H}_2\text{O}]}{[\text{MgH}_2\text{O}^{2+}][\text{MgOH}^-][\text{Mg}^{2+}][\text{H}_2\text{O}]} = \frac{K_aK_3}{K_5} \]  
(Eq. 16)

The values of \(K_4\) and \(K_5\) are unknown,\(^5\) but it seems reasonable to suppose that, as a rough approximation, \(K_4 = K_6\) and \(K_3 = K_5\). This approximation would be valid if the bond between the Mg\(^{2+}\) ion and the water molecule is ionic so that it maintains a positive and negative end. The bond length of 0.21 nm in a typical hexaaqua-Mg\(^{2+}\) complex is consistent with the presence of an ionic bond (\(0.23\) nm), as suggested in another discussion of hexaaquamanganese complexes (34). Equation 16, combined with the assumption that \(K_2 = K_4\) and \(K_3 = K_5\), implies that a water molecule that bridges two Mg\(^{2+}\) ions has a pK\(_a\) value of \(\approx 6\), roughly comparable with the pK\(_a\) values of the substrate water molecules in the active sites of yeast and \(E.\) coli PPases.

To determine the thermodynamic basis of substrate water activation, we wished to measure the effect of temperature on the pK\(_a\) value of MgH\(_2\)O\(^{2+}\). A solution of MgCl\(_2\) (0.1 m) was titrated with KOH to pH 10 (the pK\(_a\) value for MgH\(_2\)O\(^{2+}\)), and the temperature of the solution was slowly increased while the pH was monitored. These results were used to construct a van’t Hoff plot (Fig. 8), from which we obtained the thermodynamic parameters in Table 2. If it is assumed that MgH\(_2\)O\(^{2+}\) bonds are ionic, as discussed above, then the thermodynamic effects of complexation of H\(_2\)O by a second Mg\(^{2+}\) ion are expected to be additive and can be estimated by adding \(\Delta \Delta H\) and \(T \Delta \Delta S\) to the \(\Delta H\) and \(T \Delta S\) values determined for the first Mg\(^{2+}\) ion (Table 2).

These results, when adjusted for the thermodynamics of ionization of water (36), indicate that Mg\(^{2+}\) ions can produce a rate enhancement as large as \(\approx 10^6\) and that this rate enhancement is entropic in origin.

DISCUSSION

Spontaneous and Enzyme-catalyzed PP\(_i\) Hydrolysis—At ambient temperature, PP\(_i\)\(^{4-}\) hydrolysis proceeds with a rate constant of \(\approx 1.2 \times 10^{-13}\) s\(^{-1}\), \(\Delta H = 33.9\) kcal/mol, and \(T \Delta S = -1.1\) kcal/mol. Compared with the rate constant for PP\(_i\)\(^{3-}\) hydrolysis, the first protonation accelerates PP\(_i\) hydrolysis by a factor of 560, and the second by an additional factor of \(\approx 100\). Both \(\Delta H\) and \(T \Delta S\) become more favorable upon protonation. These effects of pH resemble those reported for methyl phosphate and ATP (21, 22).

MgPP\(_i\)\(^{2-}\) hydrolysis proceeds with a rate constant of \(2.8 \times 10^{-10}\) s\(^{-1}\) at 25°C, \(\Delta H = 29.3\) kcal/mol, and \(T \Delta S = -1.1\) kcal/mol. This represents a rate enhancement of \(2300\)-fold compared with PP\(_i\)\(^{4-}\) hydrolysis. The rate constants for MgPP\(_i\)\(^{2-}\) hydrolysis are similar to those estimated for PP\(_i\)\(^{3-}\) hydrolysis (Table 1), suggesting that Mg\(^{2+}\) complexation may increase the rate of PP\(_i\) hydrolysis by neutralizing negative charge, as does PP\(_i\) protonation.

Compared with MgPP\(_i\)\(^{2-}\) hydrolysis as a benchmark reaction, \(E.\) coli PPase accelerates the reaction by a factor of \(2.0 \times 10^{12}\) at 25°C (6.5 × 10\(^13\) at 37°C), somewhat larger than the factor of 10\(^10\) estimated earlier from experiments at a single elevated temperature (90°C) (13). The activation parameters

\(^5\) These values cannot be measured directly because a structure in which a single water molecule bridges two magnesium ions is unlikely to form in solution, i.e. it is energetically unfavorable to bring two charged Mg\(^{2+}\) ions into such close proximity. As a first approximation, treating the Mg\(^{2+}\) ions as point charges and applying Coulomb’s law, placement of two Mg\(^{2+}\) ions 0.42 nm apart (or two MgH\(_2\)O bond lengths) would require overcoming a barrier of \(-4.3\) kcal/mol. This suggests that the active site of PPase is organized in such a way as to stabilize a catalytically favorable, but energetically unfavorable, arrangement of metal ions.
Catalysis of Pyrophosphate Hydrolysis

Our present results are consistent with the view that enzymes excel not only in catalytic power but also in having evolved the ability to minimize unfavorable changes in $\Delta S^\ddagger$ (48, 49). These experiments furnish no direct indication of the physical origin of these thermodynamic effects. However, it seems reasonable to suppose that PPase positions substrate water in an orientation that is optimal for reaction but is energetically disfavored in DMSO.

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indicate that the rate enhancement, like that generated by many hydrolytic enzymes (37), is entirely enthalpic in origin ($\Delta H^\ddagger = 16.6$ kcal/mol). The values of $\Delta S^\ddagger$ are virtually identical for reaction on the enzyme and in solution.

**Nonenzymatic Catalysis of PP$_4$ Hydrolysis**—The similarities between independently evolved PPases raise the possibility that simple inorganic catalysts that mimic some of the features of the active site features might furnish a starting point for the evolution of PPases. Our results show that, after correction of the apparent first-order rate constants for the relative concentrations of H$_2$O, the hydrolysis of PP$_4$ is accelerated by a factor of $1.4 \times 10^6$ when it is transferred from bulk aqueous solution to DMSO. This rate enhancement is due to a decrease in $\Delta H^\ddagger$ of 15.5 kcal/mol, partly compensated by a decrease in $\Delta S^\ddagger$ of 9.5 kcal/mol. This behavior of PP$_4$ is comparable with that of phosphate monoesters, which are hydrolyzed much more rapidly in DMSO than in water due to a decrease in $\Delta H^\ddagger$ (38).

It is also of interest to estimate the energetic effects of coordination of the substrate water molecule by two Mg$^{2+}$ ions. Such an arrangement would be expected to reduce the pK$_a$ of the water molecule by $\sim$6 units as discussed under “Results.” Table 2 shows that that change in $\Delta G$ results from an increase in $\Delta S$. Activation of the nucleophilic water molecule would increase the rate by a factor of $10^6$ only if PP$_4$ hydrolysis proceeded through a fully associative transition state on the enzyme. In contrast to some kinases, which appear to proceed through a dissociative transition state (39), crystal structures of reaction intermediates (40) and deuterium kinetic isotope effects (41) suggest that the transition state in the Type 1 PPase reaction has significant associative character (Fig. 1).

Regardless of these structural details, it is evident that the rate enhancement generated by two Mg$^{2+}$ ions can be substantial. It is also worth noting that two-metal water activation mechanisms have also been implicated in catalysis by other hydrolytic enzymes, including inositol-polynucleotide 1-phosphatase, myo-inositol monophosphatase, phospholipase C, alkyl sulfatase, aminopeptidases, arginase, glutamate carboxypeptidase, urease, and dinitrogen reductase regulatory protein (35, 42–47).

**Nonenzymatic Catalysts and Entropy/Enthalpy Compensation**—In nonenzymatic catalysis, changes in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ frequently oppose each other. In the case of PP$_4$ hydrolysis, transfer from water to DMSO decreases the activation barrier by 6.0 kcal/mol at 25 °C. This change results from a 15.5 kcal/mol decrease in $\Delta H^\ddagger$ compensated by a 9.5 kcal/mol decrease in $\Delta S^\ddagger$. Entropy/enthalpy compensation has also been observed in the catalysis of ATP hydrolysis by Mg$^{2+}$ ions (21), in the catalysis of phosphate ester hydrolysis by cerium ion (1), and in the catalysis of amino acid decarboxylation by pyridoxal phosphate (1). In contrast, only a few enzymes are known to decrease $\Delta S^\ddagger$, and even in those cases, the difference is relatively modest, amounting to only a few kcal/mol (37).

$^{6}$MgPP$^{2−}$ hydrolysis in DMSO could not be measured because the complex is insoluble in DMSO. The $\Delta H^\ddagger$ value for PP$_4$ hydrolysis in DMSO is 10.9 kcal/mol lower than that for MgPP$^{2−}$ hydrolysis in water.

$^{7}$ Of 12 single-substrate and hydrolytic enzymes from the literature for which activation parameters have been reported for both the enzyme-catalyzed and the spontaneous reactions, only two reduce $\Delta S^\ddagger$ by $>1$ kcal/mol. Orotidine monophosphate decarboxylase reduces $\Delta H^\ddagger$ by 26.3 kcal/mol and reduces $\Delta S^\ddagger$ by 6.7 kcal/mol. Alkaline phosphatase reduces $\Delta H^\ddagger$ by 34.3 kcal/mol and reduces $\Delta S^\ddagger$ by 4.6 kcal/mol. For the other enzymes, $\Delta S^\ddagger$ departs only slightly from the value for the uncatalyzed reaction (1, 37).
Catalysis of Pyrophosphate Hydrolysis