

# Catalysis of the Hydrolysis of Phosphorylated Pyridines by $\text{Mg}(\text{OH})^+$ : A Possible Model for Enzymatic Phosphoryl Transfer<sup>†</sup>

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**ABSTRACT:** The second-order rate constants for reaction of the  $\text{Mg}^{2+}$  complexes of phosphorylated pyridine monoanions with  $\text{Mg}(\text{OH})^+$  are  $10^4$ – $10^6$ -fold larger than the second-order rate constants for their reaction with water (25 °C, ionic strength 1.5). Of the  $10^6$ -fold rate enhancement with the phosphorylated 4-morpholinopyridine/ $\text{Mg}^{2+}$  complex,  $\sim 10^4$ -fold is attributed to the greater nucleophilicity of  $\text{Mg}(\text{OH})^+$  compared with water. The remaining catalysis of  $\sim 10^2$ -fold is attributed to induced intramolecularity from positioning of the hydroxide ion and phosphoryl group by the  $\text{Mg}^{2+}$  ions. This reaction may provide a model for the role of a metal ion in increasing the concentration of the anions of enolpyruvate and serine and holding the nucleophile in the correct position for phosphoryl transfer in the reactions catalyzed by pyruvate kinase and alkaline phosphatase, for example. Some mechanisms that can provide catalysis of phosphoryl transfer through a metaphosphate-like transition state are reviewed briefly.

The mechanisms for catalysis of phosphoryl transfer reactions by enzymes and the role of  $\text{Mg}^{2+}$  in this catalysis are not well understood. Two mechanisms that can contribute to this catalysis are (1) lowering the  $\text{p}K_a$  of water, which allows deprotonation and formation of the stronger nucleophile,  $\text{Mg}(\text{OH})^+$ , at physiological pH values and (2) providing a template to hold the reactants in place in the transition state (Lowenstein, 1958; Jencks, 1969; Spiro, 1973; Cooperman, 1976; Benkovic & Schray, 1978; Knowles, 1981). In this paper we show that  $\text{Mg}^{2+}$  catalyzes phosphoryl transfer from pyridines (Chart I) to water by these mechanisms. Although the catalysis is not large, the reaction may serve as a model for the role of a metal ion in catalysis by enzymes such as pyruvate kinase and alkaline phosphatase. It is suggested that this is one of several mechanisms that can provide significant catalysis of phosphoryl transfer, even through a transition state that is metaphosphate like.

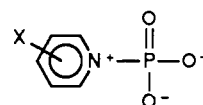
Several examples of rapid phosphoryl transfer to metal hydroxides have been reported [Lipkin et al., 1959; Bamann & Trappmann, 1959; Bruice & Benkovic, 1966; Osterheld, 1972; Cooperman, 1976; Milburn et al. (1985) and references cited therein]. However, many of these are two-phase systems that have been difficult to characterize quantitatively [e.g., Butcher and Westheimer (1955) and Jenkins (1988)]. Ambiguity of analysis because of uncertainty in the geometry for chelation of the bound metal ion has been circumvented in some reactions by the use of Co(III) and other exchange-inert metal ions; several phosphoryl-transfer reactions to the hydroxide ion ligand of a Co(III)/substrate complex have been shown to give large rate enhancements, compared with hydrolysis of the free substrate (Farrell et al., 1969; Jones et al., 1983, 1984; Meyer & Cornelius, 1984; Haight et al., 1985; Milburn et al., 1985, and references cited therein).

In this paper we describe an analysis of the factors that contribute to catalysis by  $\text{Mg}(\text{OH})^+$  in homogeneous solution.

## MATERIALS AND METHODS

**Materials.**  $\gamma$ -Picoline and pyridine were purified by distillation. Aqueous solutions of phosphorylated 4-

Chart I



phosphorylated pyridine (PyrP), X = H

phosphorylated  $\gamma$ -picoline (PicP), X = 4- $\text{CH}_3$

phosphorylated 4-morpholinopyridine (MPP), X = 4-N $\begin{array}{c} \diagup \diagdown \\ \diagdown \diagup \end{array}$

morpholinopyridine, phosphorylated  $\gamma$ -picoline, and phosphorylated pyridine were prepared as described previously (Skoog & Jencks, 1984; Herschlag & Jencks, 1987). 4-Morpholinopyridine was a gift from Dr. Mark Skoog. Solutions of  $\text{MgCl}_2$  were passed through a Millipore filter (0.45  $\mu\text{m}$ ).

**Reactions of Phosphorylated Pyridines.** Reactions of  $1 \times 10^{-4}$  M phosphorylated 4-morpholinopyridine,  $2 \times 10^{-4}$  M phosphorylated  $\gamma$ -picoline, and  $5 \times 10^{-4}$  M phosphorylated pyridine at  $25.1 \pm 0.1$  °C were followed spectrophotometrically at 303, 256–258, and 262 nm, respectively. These reactions were first order for  $>3t_{1/2}$ ; end points were determined after  $\geq 10t_{1/2}$ . Reaction mixtures in the range pH 7.8–8.5 (uncorrected, see below) were used to determine rate constants for reactions with hydroxide ion in the presence of  $\text{MgCl}_2$ ; higher pH values gave precipitation in the presence of  $\text{MgCl}_2$ , presumably of magnesium phosphate. No precipitates were observed in the reaction mixtures. A 5-fold decrease in the initial concentration of phosphorylated  $\gamma$ -picoline in reactions monitored with 4- and 5-cm path lengths gave no significant change ( $<10\%$ ) in the observed second-order rate constant for reaction with hydroxide ion in the presence of  $\text{Mg}^{2+}$ . The ionic strength was maintained at 1.5 or 1.0 with potassium chloride, and the pH was determined at the end of each reaction.

**Estimation of Hydroxide Ion Concentration.** The concentration of hydroxide ion in reaction mixtures was estimated from the observed pH at ionic strength 1.5 (KCl) in the absence of  $\text{MgCl}_2$  and the equation  $[\text{HO}^-] = 10^{\text{pH}-14}$ . Replacement of 1.0 M KCl by 0.33 M  $\text{MgCl}_2$  in order to maintain constant ionic strength gives a decrease in the pH reading of 0.2, and replacement of smaller amounts of KCl gives smaller changes in pH. However, this change in pH is

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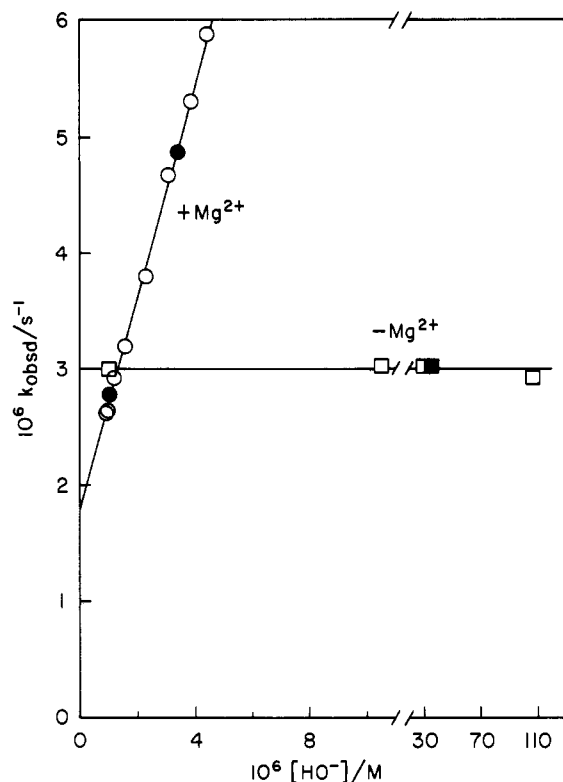


FIGURE 1: Dependence on the concentration of hydroxide ion of the rate constant for disappearance of  $10^{-4}$  M phosphorylated 4-morpholinopyridine in the presence and absence of 0.33 M  $\text{MgCl}_2$  with 0.05 or 0.10 M CHES buffer (open and closed symbols, respectively), at 25 °C and ionic strength 1.5 (KCl).

not accompanied by a change in the acid:base ratio for *p*-nitrophenol or for 4-morpholinopyridine as determined by absorbance spectra in 0.05 M Tris and 0.05 M CHES<sup>1</sup> buffers at pH 7.6 and 9.0, respectively. The following had no effect on this pH change: the use of different buffers (CHES, MOPS, and Tris), an increase in buffer concentration over the range 0.05–0.10 M, and replacement of  $\text{MgCl}_2$  by  $\text{CaCl}_2$ .

## RESULTS

Figure 1 shows that the rate of hydrolysis of phosphorylated 4-morpholinopyridine (MPP)<sup>1</sup> increases linearly with  $(1-5) \times 10^{-6}$  M hydroxide ion in the presence of 0.33 M  $\text{Mg}^{2+}$  but does not increase significantly in the absence of  $\text{Mg}^{2+}$ . These data and similar data for reactions of PicP and PyrP gave the apparent second-order rate constants,  $k_{\text{HO}}^{\text{app}}$ , in Table I for reactions with hydroxide ion in the presence of 0.33 M  $\text{Mg}^{2+}$ . A 2-fold increase in the concentration of CHES buffer does not significantly affect the rate constants for hydrolysis of MPP and PicP with and without  $\text{Mg}^{2+}$  (Figure 1 and data not shown). The hydrolysis of MPP in the presence of 0.33 M  $\text{Ca}^{2+}$  and  $\sim 3 \times 10^{-4}$  M hydroxide ion (0.05 M CAPS buffer, pH 10.4) was found to be  $\sim 50$ -fold faster than in the absence of  $\text{Ca}^{2+}$  (not shown; 25 °C,  $I = 1.0$ , 5 cm path length with  $2 \times 10^{-5}$  M MPP).

Figure 2 shows that the rate constant for hydrolysis increases with increasing  $\text{Mg}^{2+}$  concentration at pH 9.1 but decreases at the lower pH of 8.0. Thus,  $\text{Mg}^{2+}$  catalyzes the reaction with

Table I: Rate Constants for Reactions of  $\text{Mg}^{2+}(\text{OH}^-)$ , Water, and Hydroxide Ion with Phosphorylated Pyridine Monoanions<sup>a</sup>

	substrate or conditions <sup>b</sup>	phosphorylated		
		pyridine	$\gamma$ -picoline	4-morpholino-pyridine
$\text{pK}_{\text{lg}}^c$		5.52	6.33	9.01
$k_{\text{HO}}^{\text{app}d}$	0.33 M $\text{Mg}^{2+}$	$3 \times 10^2$	$1.1 \times 10^2$	0.9
$k_{\text{HO}}^{\text{app}e}$	XPyP·Mg	$5 \times 10^2$	$2 \times 10^2$	1.5
$k_{\text{HO}}^{\text{app}f}$	XPyP·Mg	$2 \times 10^3$	$6 \times 10^2$	5
$k_{\text{Mg-OH}}^g$	XPyP·Mg	5	2	$1.4 \times 10^{-2}$
$k_{\text{HOH}}^{\text{Mg}}$	XPyP·Mg	$1.15 \times 10^{-4}$	$1.48 \times 10^{-5}$	$1.86 \times 10^{-8}$
$k_{\text{Mg-OH}}/k_{\text{HOH}}^{\text{Mg}}$		$4 \times 10^4$	$1 \times 10^5$	$7 \times 10^5$
$k_{\text{HOH}}^h$	XPyRP	$1.93 \times 10^{-4}$	$2.86 \times 10^{-5}$	$5.41 \times 10^{-8}$
$k_{\text{HO}}^i$	XPyRP	$1.5 \times 10^{-2}$	$4.5 \times 10^{-3}$	$3.1 \times 10^{-5}$

<sup>a</sup> Reactions at 25 °C and ionic strength 1.5 (KCl). Rate constants have units of  $\text{M}^{-1} \text{s}^{-1}$  unless noted otherwise;  $[\text{HOH}] = 55.5 \text{ M}$  is used.

<sup>b</sup> XPyRP represents the uncomplexed phosphorylated pyridine monoanion substrates, and XPyP·Mg represents the complexes with  $\text{Mg}^{2+}$ .

<sup>c</sup> At 25 °C and ionic strength 1.0 (from Skoog and Jencks (1984) and references therein).

<sup>d</sup> Each apparent second-order rate constant for the reaction with hydroxide ion was determined from 10 observed rate constants with the pH varied from  $\sim 7$  to 9 in the presence of 0.05 M CHES buffer and 0.33 M  $\text{MgCl}_2$ .

The concentration of hydroxide ion was determined as described under Materials and Methods.

<sup>e</sup> Apparent second-order rate constants for reaction of the  $\text{Mg}^{2+}$ /substrate complex with hydroxide ion in the presence of 0.33 M  $\text{Mg}^{2+}$  determined by division of  $k_{\text{HO}}^{\text{app}}$  by 0.62, which is the fraction of phosphorylated pyridine complexed with  $\text{Mg}^{2+}$  in the presence of 0.33 M  $\text{MgCl}_2$ .

This fraction was determined from  $K_{\text{a}} = 5 \text{ M}^{-1}$  for association of the complex (Herschlag & Jencks, 1989b) with use of eq 2.

<sup>f</sup> Apparent third-order rate constants ( $\text{M}^{-2} \text{s}^{-1}$ ) for reaction of the  $\text{Mg}^{2+}$ /substrate complex that is first order in hydroxide ion and in  $\text{Mg}^{2+}$ , determined by division of  $k_{\text{HO}}^{\text{app}}$  by  $[\text{Mg}^{2+}] = 0.33 \text{ M}$ .

<sup>g</sup> The second-order rate constant for reaction of  $\text{Mg}(\text{OH})^+$  with the  $\text{Mg}^{2+}$  complex of phosphorylated pyridine (Scheme I and eq 1), determined from  $k_{\text{HO}}^{\text{app}}$  and eq 3.

<sup>h</sup> Rate constants from Herschlag and Jencks (1987).

<sup>i</sup> Rate constants from Herschlag and Jencks (1989a).

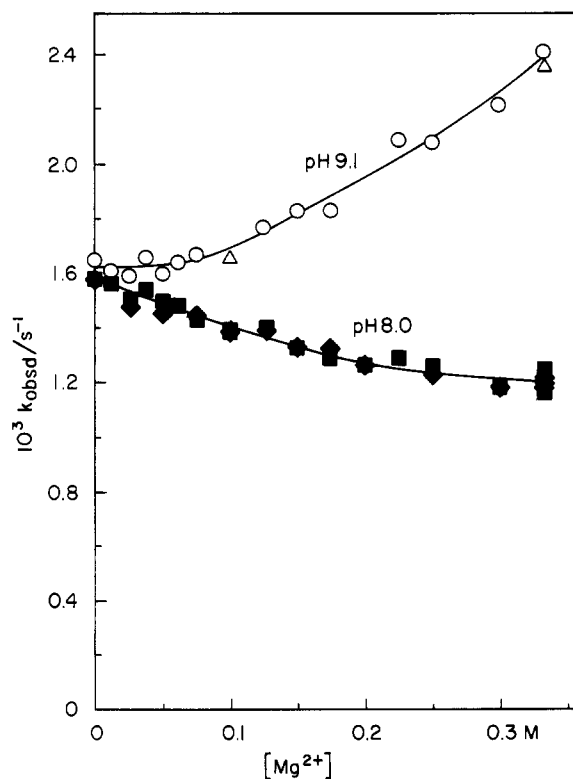


FIGURE 2: Dependence on the concentration of  $\text{Mg}^{2+}$  of the observed rate constant for disappearance of  $2 \times 10^{-4}$  M phosphorylated  $\gamma$ -picoline in 0.05 M CHES buffer, pH 9.1 (O,  $\Delta$ ; separate experiments) and pH 8.0 ( $\blacksquare$ ,  $\blacklozenge$ ; separate experiments) at 25 °C. The ionic strength was maintained with KCl at 1.0 (O,  $\Delta$ ,  $\blacksquare$ ) or 1.5 ( $\blacklozenge$ ).

<sup>1</sup> Abbreviations: MPP, phosphorylated 4-morpholinopyridine monoanion; PicP, phosphorylated  $\gamma$ -picoline monoanion; PyrP, phosphorylated pyridine monoanion; XPyP·Mg, the complex of a substituted phosphorylated pyridine monoanion with  $\text{Mg}^{2+}$ ; CHES, 2-(cyclohexylamino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; en, ethylenediamine.

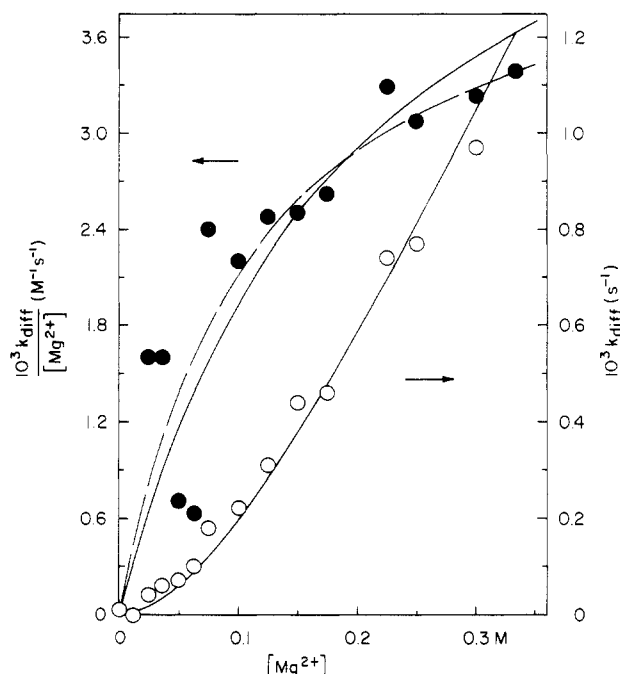
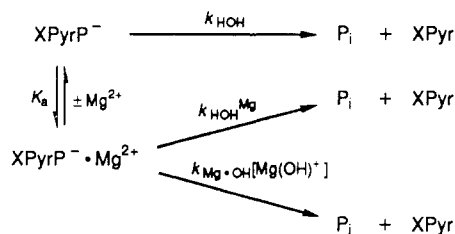


FIGURE 3: Dependence on  $\text{Mg}^{2+}$  concentration of the rate constants for disappearance of  $2 \times 10^{-4}$  M phosphorylated  $\gamma$ -picoline (PicP) catalyzed by hydroxide ion. The open symbols are  $k_{\text{diff}}/[\text{Mg}^{2+}]$ , the difference in the values of  $k_{\text{obsd}}$  at pH 9.1 and 8.0 from Figure 2 (right scale), and the closed symbols are these rate constants divided by  $[\text{Mg}^{2+}]$  (left scale). The upper solid line is the nonlinear least-squares fit to  $k_{\text{diff}}/[\text{Mg}^{2+}]$  with  $K_a = 5 \text{ M}^{-1}$  for the association of phosphorylated  $\gamma$ -picoline and  $\text{Mg}^{2+}$ . The fit without fixing the value of  $K_a$  gives  $K_a = 9 \text{ M}^{-1}$ , shown by the dashed line. The values of  $k_{\text{diff}} = k_{\text{obsd}}(\text{pH } 9.1) - k_{\text{obsd}}(\text{pH } 8.0) - (0.05 \times 10^{-3} \text{ s}^{-1})$  include the small correction for the buffer-dependent reaction (see text).

Scheme I



hydroxide ion (Figure 1) but inhibits the reaction with water (Herschlag & Jencks, 1987).

The data in Figure 3 show that the hydrolysis of PicP at pH 7–9 follows the rate law of eq 1. This rate law can be

$$v = [k_{\text{HOH}}\text{Mg} + k_{\text{Mg} \cdot \text{OH}}[\text{Mg}(\text{OH})^+]][\text{PicP} \cdot \text{Mg}] + k_{\text{HOH}}[\text{PicP}] \quad (1)$$

accounted for by the reactions shown in Scheme I: the complex of PicP with  $\text{Mg}^{2+}$  (eq 2) reacts with  $\text{Mg}(\text{OH})^+$  and with

$$K_a = [\text{PicP} \cdot \text{Mg}] / [\text{PicP}][\text{Mg}^{2+}] \quad (2)$$

water. The open symbols in Figure 3 give  $k_{\text{diff}}$ , the difference between the observed rate constants at pH 9.1 and 8.0 (Figure 2); only reactions that involve hydroxide ion contribute to the rate constant  $k_{\text{diff}}$ . The rate constants at pH 9.1 were corrected for a small rate increase of 3% ( $0.05 \times 10^{-3} \text{ s}^{-1}$ ) at pH 9.1 compared to pH 8.0 in the absence of  $\text{Mg}^{2+}$  (Figure 2, intercept) that results from a small effect of CHES base on the rate (unpublished experiments); this small correction does not affect the conclusions from these data. The open symbols in Figure 3 show that the dependence of the rate constants,  $k_{\text{diff}}$ ,

on the concentration of  $\text{Mg}^{2+}$  is greater than first order.

Division of  $k_{\text{diff}}$  by the concentration of  $\text{Mg}^{2+}$  gives the solid symbols in Figure 3. There is significant scatter in these rate constants, especially at low concentrations of  $\text{Mg}^{2+}$ , because they represent a small difference between two larger rate constants that is divided by the small concentration of  $\text{Mg}^{2+}$ . Nevertheless, the rate constants are clearly inconsistent with a straight line through the origin and show that the data follow saturation behavior. The upper solid line in Figure 3 is a nonlinear least-squares fit to the data with an association constant of  $K_a = 5 \text{ M}^{-1}$  (eq 2) that was determined independently (Herschlag & Jencks, 1987, 1990). The dashed line shows the best fit to the data without the value of  $K_a$  fixed; this fit gives a value of  $K_a = 9 \text{ M}^{-1}$  but is not significantly better than the fit with  $K_a = 5 \text{ M}^{-1}$ .

The saturation behavior of  $k_{\text{diff}}/[\text{Mg}^{2+}]$  with increasing  $\text{Mg}^{2+}$  concentration is consistent with a reaction of hydroxide ion that is dependent on two  $\text{Mg}^{2+}$  ions: one that saturates to form a complex with the substrate and one that gives a linear rate increase with increased concentration. Thus, the transition state contains two  $\text{Mg}^{2+}$  ions, hydroxide ion, and the substrate so that the observed reaction can be accounted for by the reaction of  $\text{Mg}(\text{OH})^+$  with the phosphorylated pyridine/ $\text{Mg}^{2+}$  complex, as shown in Scheme I. Although the data can be fully accounted for by this reaction, a small contribution to the rate from a hydroxide ion dependent reaction that involves a single  $\text{Mg}^{2+}$  ion cannot be excluded.

The rate constants  $k_{\text{Mg} \cdot \text{OH}}$  for reaction of the  $\text{Mg}^{2+}$  complexes of MPP, PicP, and PyrP (Scheme I; eq 1) in Table I were determined from the data as follows. The values of  $k_{\text{HO}}^{\text{app}}$  obtained with 0.33 M  $\text{Mg}^{2+}$  (Table I) were first divided by 0.62, the fraction of substrate present as the  $\text{Mg}^{2+}$  complex in the presence of 0.33 M  $\text{Mg}^{2+}$ , to give the rate constants  $k_{\text{HO}}^{\text{app}'}$ , which are the apparent second-order rate constants for reaction of the phosphorylated pyridine/ $\text{Mg}^{2+}$  complex with hydroxide ion in the presence of 0.33 M  $\text{Mg}^{2+}$  (Table I). The value of 0.62 was calculated from  $K_a = 5 \text{ M}^{-1}$  for formation of the complex (eq 2) (Herschlag & Jencks, 1990). This apparent second-order rate constant,  $k_{\text{HO}}^{\text{app}'}$ , was then divided by the concentration of  $\text{Mg}^{2+}$ , 0.33 M, to give the third-order rate constant,  $k_{\text{HO}}^{\text{app}''}$ , which is first order in  $\text{Mg}^{2+}$ , hydroxide ion, and the  $\text{Mg}^{2+}$ /substrate complex (Table I). Finally, the rate constant,  $k_{\text{HO}}^{\text{app}''}$ , was converted to the second-order rate constant,  $k_{\text{Mg} \cdot \text{OH}}$ , for reaction of  $\text{Mg}(\text{OH})^+$  with the  $\text{Mg}^{2+}$ /substrate complex with use of eq 3 and  $\text{p}K_a = 11.5$  for  $\text{Mg}(\text{H}_2\text{O})_6^{2+}$  (Baes & Mesmer, 1976; Childs, 1970).

$$k_{\text{HO}}^{\text{app}''}[\text{HO}^-][\text{Mg}^{2+}] = k_{\text{Mg} \cdot \text{OH}}[\text{Mg}(\text{OH})^+] \quad (3)$$

## DISCUSSION

**Catalysis by Increased Nucleophilicity.** The second-order rate constants for reaction of the  $\text{Mg}^{2+}$  complexes of phosphorylated pyridines with  $\text{Mg}(\text{OH})^+$  are  $\sim 10^4$ – $10^6$ -fold larger than the second-order rate constants for reaction with water (Table I). The solid line in Figure 4 shows the dependence of  $\log k_2$  on the  $\text{p}K_a$  of the nucleophile for the reactions of anionic oxygen nucleophiles with  $\text{MPP} \cdot \text{Mg}$  (Herschlag & Jencks, 1989b). The slope of  $\beta_{\text{nuc}} = 0.29$  and the increase in  $\text{p}K_a$  for  $\text{Mg}(\text{OH})^+$  compared with water of  $12.6 - (-1.3) = 13.9$  give a rate enhancement from the increased nucleophilicity of  $\text{Mg}(\text{OH})^+$  of  $10 \exp[0.29 \times 13.9] = 10^4$  (dashed line, Figure 4). The  $\text{p}K_a$  for  $\text{Mg}(\text{H}_2\text{O})_6^{2+}$  of 12.6 was obtained by statistical correction for the 12 ionizable protons; if the six water molecules are not equivalent, then the catalysis calculated for increased nucleophilicity would be  $\sim 2$ -fold smaller and that for induced intramolecularity correspondingly larger

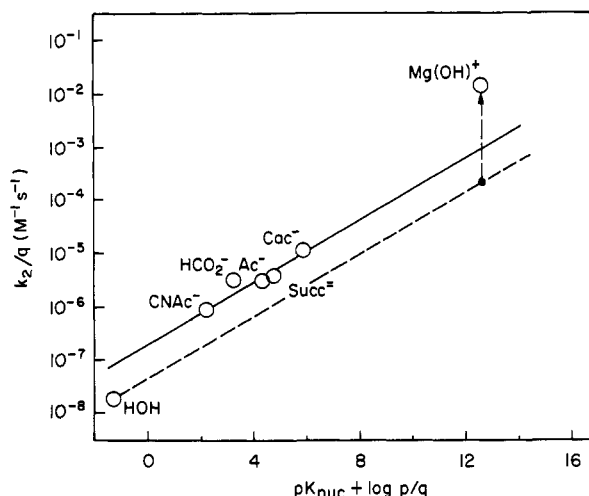


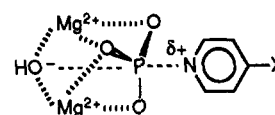
FIGURE 4: Dependence of  $\log k_2$  for the disappearance of the phosphorylated 4-morpholinopyridine/ $\text{Mg}^{2+}$  complex ( $\text{MPP}\cdot\text{Mg}^+$ ),  $10^{-4}$  M, on the  $\text{pK}_a$  of the oxygen nucleophile. The upper solid line of slope ( $\beta_{\text{nuc}}$ ) 0.29 is the best fit to the rate constants for reaction with substituted acetate ions (Herschlag & Jencks, 1989b), and the dashed line is drawn with the same slope through the rate constant for reaction with water. Abbreviations:  $\text{CNAc}^-$ , cyanoacetate;  $\text{Cac}^-$ , cacodylate. The rate constants and  $\text{pK}_a$  values are statistically corrected [Table I and Herschlag and Jencks (1989b)].

(see below). The increase in nucleophilicity of  $10^4$ -fold corresponds to an increase in rate of  $10^4/55 \approx 200$  for 1 M nucleophile relative to liquid water, which is 55 M.

Lowering the  $\text{pK}_a$  of a basic nucleophile provides an advantage for catalysis by an enzyme near neutral pH. Hydroxide ion is, of course, a stronger nucleophile than water, but its ability to provide catalysis at pH 7 is small because of its low concentration.  $\text{Mg}^{2+}$  decreases the  $\text{pK}_a$  of water and gives a large concentration of  $\text{Mg}(\text{OH})^+$ , compared with hydroxide ion, at moderate pH values. This gives a rate advantage when the dependence of the rate on the basicity of the nucleophile is not very large ( $\beta_{\text{nuc}} < 1.0$ ) and the pH is close to the  $\text{pK}_a$  of  $\text{Mg}(\text{OH})^+$ . For example, a decrease in the  $\text{pK}_a$  of a nucleophile by 1 unit, with a Brønsted slope of  $\beta_{\text{nuc}} = 0.3$  for nucleophiles of increasing basicity, gives a 2-fold decrease in the rate constant at a pH below its  $\text{pK}_a$ . However, the concentration of hydroxide ion or another basic nucleophile is decreased by 10-fold, so that there is a 5-fold advantage for the nucleophile of lower  $\text{pK}_a$ . With a fixed pH and  $\beta_{\text{nuc}} < 1.0$ , the fastest reaction will occur with a nucleophile that has a  $\text{pK}_a$  value close to the pH of the experiment. A nucleophile that is a stronger base will be present at a low concentration so that the reaction will be slow, while a weaker base will be present at a high concentration but will have a smaller second-order rate constant.

One of the  $\text{Zn}^{2+}$  ions of alkaline phosphatase may decrease the  $\text{pK}_a$  of the serine hydroxyl group to provide a stronger nucleophile than free serine;  $\text{SerO}^-\cdot\text{Zn}^{2+}$ , unlike free  $\text{SerO}^-$ , can be present at a high concentration at neutral pH (Jones et al., 1983; Sowadski et al., 1985). Similarly, a  $\text{Mg}^{2+}$  or  $\text{Mn}^{2+}$  ion bound to pyruvate kinase could decrease the  $\text{pK}_a$  of enolpyruvate to give the more nucleophilic enolate ion (Lodato & Reed, 1987). The same role has been proposed for the  $\text{Zn}^{2+}$  ion of carbonic anhydrase [for reviews, see Davis (1961), Buckingham (1977), Lipscomb (1983), and Fersht (1985)] and for  $\text{Zn}^{2+}$  and other metal ions in a number of model reactions for addition to carbonyl substrates [Sigman & Jorgensen, 1974; Wooley, 1975; Buckingham, 1977; Hipp & Busch, 1978; Satchell and Satchell (1978) and references cited therein].

Chart II



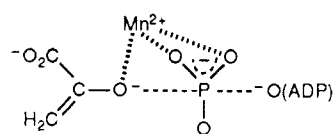
**Catalysis by Induced Intramolecularity.** The solid line in Figure 4 shows that  $\text{Mg}^{2+}$  provides modest catalysis of the reactions of MPP with carboxylate anions, compared to water (Herschlag & Jencks, 1990). The second-order rate constant for the reaction of  $\text{Mg}(\text{OH})^+$  with  $\text{MPP}\cdot\text{Mg}$  falls  $10^{1.9}$ -fold above the dashed line of slope  $\beta_{\text{nuc}} = 0.29$  in Figure 4, so that  $\sim 10^2$ -fold of the total rate enhancement of  $10^{5.9}$ -fold (Table I) may be attributed to induced intramolecularity from interaction of at least one of the  $\text{Mg}^{2+}$  ions with both the hydroxyl and phosphoryl oxygen atoms in the transition state (Chart II). If there were no interactions of the  $\text{Mg}^{2+}$  ions in the transition state that do not also occur in the ground state the rate constant would be expected to fall on the dashed line in Figure 4. The comparison was made with water, rather than acetate ion, because the reaction with acetate anions is itself subject to catalysis by induced intramolecularity; see below and Herschlag and Jencks (1990).

The inhibition by  $\text{Mg}^{2+}$  of phosphoryl transfer between uncharged bases suggests that there is no electrophilic catalysis by an interaction of  $\text{Mg}^{2+}$  with the phosphoryl oxygen atoms and is consistent with a metaphosphate-like transition state in which there is donation of charge from the phosphoryl oxygen atoms to the phosphorus atom [Herschlag and Jencks (1987) and references cited therein]. This suggests that the positive deviation of the rate with  $\text{Mg}(\text{OH})^+$  cannot be accounted for by electrophilic catalysis. In contrast, metal ions can provide electrophilic catalysis of addition to carbonyl compounds by polarization of the  $\text{C}=\text{O}$  bond, as well as by holding a nucleophile in position to add to the carbonyl group of the substrate [e.g., Breslow and Chipman (1965), Breslow et al. (1975), Buckingham et al. (1970), Buckingham (1977), Hay and Clark (1977), Hipp and Busch (1978), Boreham et al. (1979), Suh et al. (1982), and Fife and Przystas (1986)]. These mechanisms cannot always be distinguished [e.g., Pocker and Meany (1967), Satchell and Satchell (1978), and Fife and Przystas (1985)]. Both mechanisms may contribute to the fast reaction of  $\text{Ca}(\text{OH})^+$  with *p*-nitrophenyl methylphosphonate, which presumably has a more associative transition state with more charge on the phosphoryl oxygen atoms compared with the phosphoryl oxygen atoms in the reactions of phosphorylated pyridines (Behrman, 1970a,b).

The enhanced reactivity of magnesium hydroxide from induced intramolecularity can be described in two equivalent ways: (1)  $\text{Mg}^{2+}$  provides a template for the transition state that holds the hydroxyl and phosphoryl groups in place in the transition state through electrostatic interactions. (2) The hydroxyl and phosphoryl oxygen atoms chelate  $\text{Mg}^{2+}$  in the transition state. There is a smaller loss of entropy for binding of  $\text{Mg}^{2+}$  to the phosphoryl and hydroxyl groups in the transition state than in the ground state because the chelating phosphoryl and hydroxyl groups are already held together in a favorable geometry for chelation in the transition state. Thus, even though the loss in entropy to form the transition state should be the same, to a first approximation, for the reaction of MPP with hydroxide ion and the reaction of  $\text{MPP}\cdot\text{Mg}$  with  $\text{Mg}(\text{OH})^+$ , the catalysis by  $\text{Mg}^{2+}$  represents an entropic advantage.

The catalysis by  $\text{Mg}^{2+}$  of phosphoryl transfer from a pyridine to acetate ion provides another example of a rate advantage from induced intramolecularity, with an effective

Chart III



molarity of 10 M (Herschlag & Jencks, 1990). The effective molarity is the rate advantage, compared with a standard state of 1 M, from positioning of the reactants by  $\text{Mg}^{2+}$  in order to decrease the entropic barrier of the reaction (Page, 1973, 1977; Jencks, 1975; Kirby, 1980).

EPR studies have shown that the  $\text{Mn}^{2+}$  of pyruvate kinase is chelated by oxygen atoms of the  $\gamma$ -phosphoryl group of ATP and oxalate ion, an analogue of enolpyruvate anion (Lodato & Reed, 1987). This suggests that the  $\text{Mn}^{2+}$  ion may contribute to catalysis by induced intramolecularity, as shown in Chart III, as well as by decreasing the  $\text{pK}_a$  of the enol. Analogous catalysis is possible with alkaline phosphatase by chelation of the nucleophilic oxygen atom of serine and the oxygen atoms of the phosphoryl group by  $\text{Zn}^{2+}$  at the active site (Jones et al., 1983, 1984; Sowadski et al., 1985). If  $\text{Mg}^{2+}$  coordinates the  $\gamma$ - and  $\beta$ -phosphoryl groups of ATP in the transition state of a kinase reaction,  $\text{Mg}^{2+}$  would help to hold the reactants in place in the reverse reaction between ADP and the phosphorylated substrate. In the kinase direction  $\text{Mg}^{2+}$  can stabilize the development of negative charge on the leaving  $\beta$ -phosphoryl group of ADP (Bruice & Benkovic, 1966; Jencks, 1969; Cooperman, 1976).

**Reactions of PicP and PyrP.** The rate enhancement of  $10^{3.5}$  from increased nucleophilicity in the reactions of  $\text{PicP}\cdot\text{Mg}$  and  $\text{PyrP}\cdot\text{Mg}$  with  $\text{Mg}(\text{OH})^+$ , compared with water (Table I,  $k_{\text{MgOH}}^{\text{Mg}}$  and  $k_{\text{HOH}}^{\text{Mg}}$ ), is 3-fold less than that for MPP. This reflects the smaller value of  $\beta_{\text{nuc}} = 0.25$  for reactions of oxygen nucleophiles with PicP and PyrP compared with  $\beta_{\text{nuc}} = 0.29$  for reactions with MPP (Herschlag & Jencks, 1989b). Correction of the total rate advantage with  $\text{Mg}(\text{OH})^+$  ( $k_{\text{MgOH}}^{\text{Mg}}/k_{\text{HOH}}^{\text{Mg}}$ , Table I) for the rate advantage from increased nucleophilicity gives factors of  $10^{1.5}$  and  $10^{1.1}$  for catalysis by induced intramolecularity with PicP and PyrP, respectively, which are 2- and 6-fold smaller than that with MPP. The smaller amount of catalysis compared with MPP may result from an unfavorable electrostatic interaction between the  $\text{Mg}^{2+}$  of  $\text{Mg}(\text{OH})^+$  and the positive charge on the pyridine; electron donation from the 4-morpholino substituent decreases this positive charge in MPP.

The dependence of  $\log k_{\text{MgOH}}^{\text{Mg}}$  on the  $\text{pK}_a$  of the pyridine leaving group gives a slope of  $\beta_{\text{lg}} = -0.74$  (not shown). This value is similar to  $\beta_{\text{lg}} = -0.82$  and  $-0.79$  for phosphoryl transfer to trifluoroethoxide and hydroxide ions in the absence of  $\text{Mg}^{2+}$ , which have  $\text{pK}_a$  values similar to that of  $\text{Mg}(\text{OH})^+$  (Herschlag & Jencks, 1989a,b).

**Mechanisms for Stabilization of a Metaphosphate-like Transition State by Enzymes.** Although it might seem that phosphoryl transfer through a metaphosphate-like transition state would be difficult to catalyze (Hassett et al., 1982), a number of possible mechanisms for such catalysis have been described (Cooperman, 1982; Herschlag & Jencks, 1987, 1989b, 1990) and some have been observed in model reactions, such as that described above.

(1) Enzymes that catalyze phosphoryl-transfer reactions appear to stabilize the development of negative charge on leaving groups by general acid catalysis or by metal ion catalysis. It is of interest to consider the possible advantages and disadvantages of these mechanisms in different enzymatic reactions.

There is evidence that several kinases that catalyze phosphoryl transfer to ROH substrates, such as phosphofructokinase and hexokinase, have a base near the nucleophilic oxygen atom (Anderson et al., 1978; Evans & Hudson, 1979; Evans et al., 1981; Redina & Cleland, 1984; Hellinga & Evans, 1987). The advantage from general base catalysis of proton removal may not be immediately apparent because there is little bond formation to ROH and, presumably, little change in proton affinity in a dissociative transition state. However, proton removal by a general base is required in order to avoid the formation of the unstable product  $\text{RO}(\text{H}^+)\text{PO}_3^{2-}$  and may help to bind the nucleophilic hydroxyl group in the correct position for reaction. The advantage from this catalysis is more readily apparent in the reverse reaction, which requires general acid catalysis to protonate the leaving atom [Cooperman, 1982; Herschlag and Jencks (1990) and references cited therein].

Alkaline phosphatase has  $\text{Zn}^{2+}$  ions in a position where they may stabilize the oxygen atoms of the nucleophile and leaving group, instead of general acid-base catalysis (Sowadski et al., 1985). The  $\text{Zn}^{2+}$  ions presumably provide catalysis by the same mechanisms that are described here for  $\text{Mg}^{2+}$ , especially by stabilizing the anionic oxygen atom of the attacking and leaving groups and by providing electrostatic interactions that hold the substrate firmly in place in the transition state; an arginine residue probably also contributes to this stabilization (Butler-Ransohoff et al., 1988). This enzyme is nonspecific and has an open active site in which the leaving group appears to protrude into the solvent (Sowadski et al., 1985), so that these interactions are important in order to hold the reacting atoms in place. Specific kinases generally surround their specific substrates and can hold them in position for catalysis through multiple interactions in the active site (Anderson et al., 1979; Herschlag, 1988).

$\text{Mg}^{2+}$  and other dications can stabilize the developing negative charge on leaving groups of moderate basicity, such as ADP in kinase reactions. However, general acid catalysis is not important for weakly basic leaving groups like ADP because there is no thermodynamic advantage for proton transfer from an acid of  $\text{pK}_a \sim 7$  to a leaving group with a similar or lower  $\text{pK}_a$  (Jencks, 1972).

(2) Catalysis of phosphoryl transfer by induced intramolecularity is significant for the reactions described here, in spite of the dissociative, metaphosphate-like transition state for these reactions. An effective molarity of 10 M has been estimated for catalysis by  $\text{Mg}^{2+}$  of phosphoryl transfer from a pyridine to acetate ion by acting as a template to assemble the reactants in the transition state [Herschlag & Jencks, 1990; see also Jencks (1962), Benkovic & Schray (1978), Herschlag & Jencks (1986, 1989b)]. The effective molarity is the rate advantage, compared with a standard state of 1 M, from positioning of the reactants by  $\text{Mg}^{2+}$  in order to overcome the entropic barrier of the reaction (Page, 1973). It is not known how large a rate increase can be obtained from induced intramolecularity for phosphoryl transfer at the active site of an enzyme or in an intramolecular reaction with optimal geometry, but this increase is almost certainly much larger than 10 M.

(3) The enzyme could stabilize the trigonal bipyramidal transition state of the phosphoryl-transfer reaction by specific binding interactions; this would correspond to strain or destabilization of the tetrahedral ground state, in which these interactions do not occur. We are not aware of strong evidence for this geometric stabilization for simple phosphoryl transfer, but site-directed mutagenesis of tyrosyl tRNA synthetase

suggests that binding interactions of the leaving pyrophosphoryl group with the active site are developed only in the bipyrimidyl transition state (Leatherbarrow et al., 1986).

*Is the Transition State for Enzyme-Catalyzed Phosphoryl Transfer Dissociative or Associative?*<sup>2</sup> Although there is strong evidence that the transition state for nonenzymatic phosphoryl transfer is metaphosphate-like [see Herschlag and Jencks (1986, 1989a) and references cited therein], Knowles (1980) has concluded that the data for enzyme-catalyzed phosphoryl-transfer reactions do not distinguish between mechanisms with associative or dissociative transition states. However, it has been suggested more recently that the enzyme-catalyzed reactions are associative (Hassett et al., 1982; Hall & Williams, 1986; Mildvan & Fry, 1987). The following summary of the available data reviews and extends Knowles' original arguments. We conclude that there is no basis at this time for the suggestion that enzymes change the metaphosphate-like transition state of the chemical reaction to a more associative transition state. It should be noted that a change to a more associative mechanism on the enzyme would require even greater transition-state stabilization at the active site than is calculated from the ratio of the rate constants of the enzymatic and uncatalyzed reactions because a more associative nonenzymatic reaction is not observed; i.e., it has a higher barrier than the observed metaphosphate-like reaction.

(1) It has been suggested that an enzyme or bound metal ion could promote a change to a more associative transition state, with more bonding to the nucleophile and leaving group (Kirby & Jencks, 1965; Benkovic & Dunikoski, 1971; Williams & Naylor, 1971; Benkovic & Schray, 1973; Mildvan & Grisham, 1974; Mildvan, 1979; Mildvan & Fry, 1987). However, an observed transition state represents the lowest energy pathway to products and it is difficult to change the structure of the reaction surface to produce a different transition state of still lower energy. Ligation of  $Mg^{2+}$  or  $Ca^{2+}$  does not cause a significant change in the amount of bonding in the nonenzymatic transition state, and a change in the strength of the nucleophile by 18 pK units, from water to hydroxide ion, gives an increase in the bond order to a pyridine leaving group in the transition state of only 0.2, as estimated by  $\beta_{lg}$  (Herschlag & Jencks, 1987; Herschlag & Jencks, 1989a,b).

(2) The small values of  $\beta_{lg} = -0.2$  and  $-0.35$  for reactions of phosphate esters with alkaline phosphatase and calcineurin are consistent with an associative transition state but could also rise from an electrostatic interaction between a metal ion and the leaving group at the active site, proton donation to the oxygen atom of the leaving group (Williams et al., 1973; Martin et al., 1985; Hall & Williams, 1986), or a partially rate-limiting conformational change or diffusional encounter with the enzyme.

(3) The decrease in the rate for catalysis by alkaline phosphatase upon substitution of a nonbridge oxygen atom by sulfur in the substrate might arise from a more associative transition state for the enzymatic reaction than for the non-enzymatic reaction, because this substitution facilitates the dissociative nonenzymatic hydrolysis of phosphate monoesters but slows the more associative hydrolysis of phosphate triesters

(Breslow & Katz, 1968). However, differences in the length of P-S and P-O bonds and in the hydrogen-bonding ability, metal ion affinity, Van der Waals radii, and polarizability of sulfur and oxygen also could account for the slow reaction of the sulfur-substituted substrate (Knowles, 1980; Frey & Sammons, 1985). The differential reactivity of sulfur-substituted diastereomers of oligonucleotides and of ATP with several endonucleases (Eckstein, 1985) and kinases [e.g., Cohen (1982)], respectively, shows that factors other than chemical reactivity of the substrate can affect the rates of enzyme-catalyzed reactions.

(4) The slightly inverse  $^{18}O$  isotope effect for the hydrolysis of glucose-6-phosphate catalyzed by alkaline phosphatase is consistent with a dissociative transition state (Weiss & Cleland, 1989).

(5) It has been suggested that the close proximity of the phosphorus and nucleophilic atoms in the active sites of enzymes with bound substrates or substrate analogues, as determined from X-ray crystallography and NMR, provides evidence against a metaphosphate intermediate (Mildvan, 1979; Mildvan & Fry, 1987). However, these techniques do not provide distances in the transition state and it is doubtful that distances and force constants can be estimated with sufficient accuracy to distinguish between the small differences that correspond to the different bond orders of transition states with more or less associative character. Furthermore, the fact that the volumes of activation for the hydrolysis of acetyl phosphate and 2,4-dinitrophenyl phosphate are near zero or negative (Di Sabato et al., 1962; Ramirez et al., 1986) suggests that the active site need not be significantly larger than the substrate in order to accommodate a dissociative transition state. It is important that the entering group be held firmly in place for nucleophilic attack even with a dissociative bipyrimidyl transition state, as described above, so that a larger active site would not be advantageous for catalysis. It should be noted that there is evidence against the formation of a metaphosphate intermediate for reactions in aqueous solution, despite the metaphosphate-like character of the transition state (Buchwald et al., 1984; Bourne & Williams, 1984; Skoog & Jencks, 1984; Herschlag & Jencks, 1989a).

(6) The absence of racemization of the transferred phosphoryl group in several kinase reactions and the lack of positional isotope exchange of the  $\beta$ -oxygen atoms of ATP in the absence of the second substrate give no indication of a fully dissociative reaction mechanism with a metaphosphate intermediate (Cullis, 1987; Mildvan & Fry, 1987).

(7) It has been suggested that the presence of a general base in position to abstract a proton from the substrate of a kinase reaction is evidence for an associative mechanism, because general base catalysis would not be expected in a metaphosphate mechanism (Mildvan & Fry, 1987). However, a base is probably required to accept a proton from the product, whatever the nature of the transition state (see above and Herschlag & Jencks, 1989b).

(8) It is conceivable that an enzyme could promote a change to a somewhat more associative transition state by holding the reactants in place in order to overcome the larger entropic barrier of the more associative reaction (Haake & Allen, 1980). Evidence for a pentavalent intermediate has been reported in the intramolecular cleavage of a diester bond in RNA, in which the reacting groups are held in place (Breslow & Labelle, 1986; Anslyn & Breslow, 1989), while the intramolecular reactions of phosphate diesters appear to be concerted (Kirby & Younas, 1970; Ba-Saif et al., 1989). The incorporation of oxygen from solvent in the intramolecular

<sup>2</sup> In this paper we use "dissociative" to refer to a reaction that has a metaphosphate-like transition state and "associative" to refer to a transition state in which the total bonding to incoming and departing groups is increased in the transition state relative to the reactants. Although dissociative is sometimes used to denote an  $S_N1$  reaction and associative to denote an  $S_N2$  or addition-elimination reaction, here they do not denote the molecularity of the reaction.

reaction of *cis*-Co<sup>III</sup>(en)<sub>2</sub>(OH)(*p*-nitrophenyl phosphate) to form Co<sup>III</sup>(en)<sub>2</sub>PO<sub>4</sub> suggests that a pentavalent phosphorane intermediate is formed through an associative transition state with bound Co(III) (Jones et al., 1983). However, there may be significant covalent character of the strong Co(III)–O(O) bond that makes the substrate resemble a phosphate diester, rather than a monoester.

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## Conformations of the Coenzymes and the Allosteric Activator, ADP, Bound to NAD<sup>+</sup>-Dependent Isocitrate Dehydrogenase from Pig Heart†

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**ABSTRACT:** NAD<sup>+</sup>-dependent isocitrate dehydrogenase from pig heart is an allosteric enzyme that is activated by ADP and is inhibited by NADPH in the presence of NADH. Transferred nuclear Overhauser effect measurements, made at a range of times to ensure that observed effects are due to direct dipole-dipole transfer and not to spin diffusion, were used to determine the conformations of pyridine nucleotide coenzymes and of the allosteric effector ADP. For NAD<sup>+</sup>, significant effects were observed on the N2 proton (on the nicotinamide ring) when the N1' proton (on the nicotinamide ribose) was saturated and on the N6 proton when the N2' proton was saturated, indicating that the conformation of the nicotinamide-ribose moiety is anti. The anti conformation is expected because of the stereospecificity of NAD<sup>+</sup>-dependent isocitrate dehydrogenase and is the same as for NADP<sup>+</sup>-dependent isocitrate dehydrogenase. For the adenosine moiety of NAD<sup>+</sup>, the predominant nuclear Overhauser effect on the A8 proton is found when the A2' proton is saturated. This result implies that the adenine-ribose bond is anti with respect to the ribose. Previous kinetic and binding studies of ADP activation have shown an influence of divalent metal ions. The conformation of bound ADP, in the presence of Mg<sup>2+</sup> and/or Ca<sup>2+</sup>, is found to be anti about the adenine-ribose bond. The 3'H-8H distance increases when Ca<sup>2+</sup> is added to the Mg-ADP-enzyme complex. Changes in the 4'H-1'H distance upon addition of isocitrate are indicative of interactions between the ADP activator site and the isocitrate site. <sup>31</sup>P NMR of ADP in the presence of enzyme and Mg<sup>2+</sup> demonstrates a resonance attributable to the bound  $\beta$ -phosphate. This resonance, observed only in the presence of Mg<sup>2+</sup>, titrates with a lower pK than free ADP, but no changes are seen with different combinations of divalent metals. The conformational analysis and <sup>31</sup>P NMR results suggest that the allosteric effects of ADP in the presence of divalent metals are propagated through changes in the enzyme conformation which are manifested at the isocitrate site.

**M**ammalian heart tissue contains two forms of the enzyme isocitrate dehydrogenase. Both NAD<sup>+</sup>-dependent isocitrate dehydrogenase [isocitrate:NAD<sup>+</sup> oxidoreductase (decarboxylating), EC 1.1.1.41] and NADP<sup>+</sup>-dependent isocitrate dehydrogenase (EC 1.1.1.42) are located in the mitochondria,

and, in contrast to other tissues, a cytoplasmic form is absent or present at low concentrations in heart (Plaut & Gabriel, 1983). An understanding of how substrates bind to these enzymes is sought as part of the elucidation of the roles of both enzymes in heart metabolism. The stereochemistry of the isocitrate dehydrogenase reaction is the same for both enzymes (Nakamoto & Vennesland, 1960; Colman, 1983) with hydride transfer to the *pro-R* position of reduced nicotinamide. The configuration of the coenzyme bound to the NADP<sup>+</sup>-de-

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