Supplementary Material Available

Figure illustrating the electrophoretic behavior of the isolated PMN-PLA_2 used in these experiments (1 page). Ordering information is available on any current masthead page.

References

Bangham, A. D. (1972) *Annu. Rev. Biochem. 41,* 753-776.
Bangham, A. D., & Dawson, R. M. C. (1958) *Nature (London) 182,* 1292-1293.
Bangham, A. D., & Dawson, R. M. C. (1962) *Biochim. Biophys. Acta 59,* 103-115.
Bangham, A. D., & Horne, R. W. (1964) *J. Mol. Biol. 8,* 660-668.
Brockerhoff, H., & Jenson, R. G. (1974) in *Lipolytic Enzymes,* Academic Press, New York, N.Y.
Davies, J. T., & Rideal, E. K. (1963) in *Interfacial Phenomena,* Academic Press, New York, N.Y.
Dawson, R. M. C. (1964) in Metab. Sigu. Lipids Proceedings Advanced Study Course (Dawson, R. M. C., & Rhodes, D. N., Eds.) p 179, Wiley, London.
Dawson, R. M. C., Hemington, N. L., Miller, N. G. A., & Bangham, A. D. (1976) *J. Membr. Biol. 29,* 179-184.
de Haas, G. H., Slotboom, H. J., Verheij, H. M., Jansen, E. H. J. M., de Araujo, P. S., & Vidal, J. C. (1978) in *Advances in Prostaglandin and Thromboxane Research* (Galli, C., Galli, G., & Porcellati, G., Eds.) Raven Press, New York, N.Y.
Delori, P. J. (1973) *Biochimie 55,* 1031-1045.
Franson, R., Waite, M., & LaVia, M. (1971) *Biochemistry 10,* 1942-1946.
Franson, R., Patriarca, P., & Elsbach, P. (1974) *J. Lipid Res. 15,* 380-388.
Franson, R., Dobrow, R., Weiss, J., Elsbach, P., & Weglicki, W. B. (1978) *J. Lipid Res. 19,* 18-23.
Goldhammer, A. P., Jain, M. K., & Cordes, E. H. (1975) *J. Membr. Biol. 23,* 293-304.
Kamo, N., Miyake, M. K., Kurihara, K., & Kobatake, Y. (1974) *Biochim. Biophys. Acta 367,* 11-23.
Kawauchi, S., Iwanaga, S., Samejima, Y., & Suzuki T. (1971) *Biochim. Biophys. Acta 236,* 142-160.
Patriarca, P., Beckerdite, S., Pettis, P., & Elsbach, P. (1972) *280,* 45-56.
Pieterson, W. A., Volwerk, J. J., & de Haas, G. H. (1974) *Biochemistry 13,* 1439-1445.
Waite, M., & van Deenen, L. L. M. (1967) *Biochim. Biophys. Acta 137,* 498-517.
Weiss, J., Franson, R., Beckerdite, S., Schmeidler, K., & Elsbach, P. (1975) *J. Clin. Invest. 55,* 33-42.
Wells, M. A. (1972) *Biochemistry 11,* 1030-1041.

Nuclear Magnetic Resonance Studies of Inorganic Phosphate Binding to Yeast Inorganic Pyrophosphatase†

Donald J. Hamm and Barry S. Cooperman*

ABSTRACT: Yeast inorganic pyrophosphatase is a dimer of identical subunits. Previous work (Rapoport, T. A., et al. (1973) *Eur. J. Biochem. 33,* 341) indicated the presence of two different Mn^{2+} binding sites per subunit. In the present work, the binding of inorganic phosphate to the Mn^{2+}-inorganic pyrophosphatase complex has been studied by ^1H and ^{31}P nuclear magnetic resonance. Two distinct phosphate sites have been found, having dissociation constants of 0.24 mM and 18 mM. The Mn^{2+}-^{31}P distance from tightly bound Mn^{2+} to phosphate bound in the low affinity site (6.2 Å) is consistent with outer sphere binding. Binding to both phosphate sites can be simultaneously inhibited by the pyrophosphate analogue, hydroxymethanebisphosphonate, providing evidence for the physical proximity of these two sites. The weaker Mn^{2+} site is apparently far from both phosphate sites. From the magnitudes of the dissociation constants found for both phosphate and analogue binding and the recent work of P. D. Boyer and his co-workers (private communication) on enzyme-catalyzed phosphate-water exchange, it appears unlikely that the hydrolysis of enzyme-bound pyrophosphate is the rate-determining step in the overall enzymatic catalysis of pyrophosphate hydrolysis, at least when Mn^{2+} is the required divalent metal ion cofactor.

Phosphoryl transfer is among the most widespread reactions of biochemical importance. The enzymes catalyzing reactions of this type have in common a general requirement for divalent metal ions for activity. Yeast inorganic pyrophosphatase (EC 3.6.1.1, pyrophosphate phosphohydrolase) shares in this requirement and displays several properties which make it attractive as a model for phosphoryl transfer enzymes (Cooperman et al., 1973). The early work on this enzyme was reviewed by Butler (1971), at which time comparatively little was known. Since then our understanding of the enzyme has increased considerably. The enzyme is a dimer made up of identical monomers of molecular weight 33 000-35 000 (Ridlington et al., 1972; Heinrikson et al., 1973). The primary sequence has been determined (Cohen et al., 1978) and an X-ray crystallographic determination of its structure is well underway (Bunick et al., 1978). In addition, important information has been obtained on the binding of divalent metal ions and of pyrophosphate and pyrophosphate analogues (Ridlington & Butler, 1972; Cooperman & Chiu, 1973a; Rapo-

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port et al., 1973; Baykov & Avaeva, 1974), on the identity of essential amino acids at the active site (Heitmann et al., 1972; Cooperman & Chiu, 1973b; Rapoport et al., 1973; Heitmann & Uhlig, 1974), and on the kinetic (Moe & Butler, 1972a,b; Rapoport et al., 1972) and chemical (Cooperman et al., 1973b; Sperow et al., 1973; Konsowitz & Cooperman, 1976; Avaeva et al., 1977) mechanisms of action.

In the present work, we have used ^1H and ^{31}P NMR to study the binding of inorganic phosphate to the enzyme-Mn^{2+} complex, making use of the powerful probe properties of paramagnetic Mn^{2+} (Cohn & Hughes, 1962; Eisinger et al., 1965; Cohn, 1970; Mildvan & Engle, 1972). These studies clearly show the presence of two distinct, though probably adjacent, phosphate sites on the enzyme. One of the enzyme-bound phosphates appears to bind to Mn^{2+} via an outer sphere complex, similar to what has been seen with several other phosphoryl transfer enzymes (Mildvan & Grisham, 1974) while the position of the other phosphate with respect to Mn^{2+} is unclear. Our results are in accord with recent studies on the phosphate–water oxygen exchange reaction also catalyzed by the enzyme, and lead to the interesting conclusion that hydrolysis of enzyme-bound pyrophosphate is unlikely to be the rate-determining step in overall enzyme-catalyzed pyrophosphate hydrolysis, at least when Mn^{2+} is the required divalent metal ion cofactor.

Experimental Section

Disodium hydroxymethanebisphosphonate (PCHOHP)[1] was a gift from Dr. D. A. Nicholson of Proctor and Gamble. Carrier-free $^{54}Mn^{2+}$ was purchased from New England Nuclear. D_2O (99.8% D) was purchased from Stohler Isotope Chemicals. All other chemicals were reagent grade and used without further purification unless otherwise stated.

Water for stock solutions was glass distilled and passed through a Barnstead standard mixed bed deionizing column. T_1 measurements on the HOD peak of the D_2O showed contamination by paramagnetic metal ions to be extremely small. Stock solutions of Tris (TRIZMA, Sigma) and KCl used for making up buffers were passed over Chelex 100 (Bio-Rad) to reduce trace heavy metal contamination. Solutions of Pipes (ULTROL, Calbiochem) were used without further purification. Stock solutions of inorganic phosphate (P_i) used in the magnetic resonance experiments were prepared by weight, brought to the desired pH, passed over Chelex 100 and standardized by means of a molybdovanadate assay (Simonsen et al., 1946). Solutions of PCHOHP were prepared by weight, shown to be free of organic contaminants and paramagnetic metal ions by ^1H and ^{31}P NMR, and used without further purification. Stock solutions of Mn^{2+} (nonradioactive) were prepared by weight from analytical grade $MnCl_2 \cdot 4H_2O$ in deionized water and standardized either by a direct EDTA titration at pH 10 using Eriochrome Black T as the indicator (West, 1969) or by ESR (Cohn & Townsend, 1954). Radioactivity measurements on solutions containing $^{54}Mn^{2+}$ were made by counting the 0.84 MeV γ emitted by $^{54}Mn^{2+}$ in a well counter coupled to a Northern NS 600 multichannel analyzer (counting efficiency = 10%).

Yeast inorganic pyrophosphatase (35–45 Kunitz units/mg; Kunitz, 1952) was prepared using an improved version[2] of the published procedure (Cooperman et al., 1973) and stored as ammonium sulfate pads. Prior to use in magnetic resonance experiments, the ammonium sulfate was removed by dialysis against several changes of a suitable buffer or deionized water. The small amounts of denatured enzyme present at the end of the dialysis were removed by centrifugation (10 K, 20 min). Enzyme contaminated with Mn^{2+} from previous experiments was routinely regenerated metal free by reprecipitation in 95% ammonium sulfate solutions followed by dialysis of the pad against the desired buffer as above. Enzyme concentration was measured spectrophotometrically using an extinction coefficient of 1.45 at 280 nm (Kunitz, 1952) for a 0.1% solution of inorganic pyrophosphatase and a subunit weight of 35 000 (Cooperman & Chiu, 1973a). Enzyme activity was measured at 30 °C as previously described (Cooperman et al., 1973).

Nuclear Relaxation Rates. The longitudinal ($1/T_1$) proton relaxation rate of water was measured at 24.3 MHz by a pulsed nuclear magnetic resonance (NMR) technique described previously (Cohn & Leigh, 1962). Values of the observed enhancements (Eisinger et al., 1962), ϵ_{obsd}, were calculated from

$$\epsilon_{obsd} = \frac{1/T_1^* - 1/T_{1,0}^*}{1/T_1 - 1/T_{1,0}} \quad (1)$$

where $1/T_1$ and $1/T_{1,0}$ are the observed relaxation rates in the presence and absence of Mn^{2+}, respectively. The terms with asterisks represent the same rates measured in the presence of inorganic pyrophosphatase. In the absence of enzyme, added P_i has essentially no effect on either $1/T_1$ or $1/T_{1,0}$. Sample volumes varied from 50 to 100 μL.

The longitudinal ($1/T_1^P$) and transverse ($1/T_2^P$) phosphorus nuclear magnetic relaxation rates of P_i were measured at 24.3, 40.3, 108.3 and 145.7 MHz by Fourier transform NMR. The measurements at 24.3 MHz were recorded on a Varian NV 14 spectrometer modified for FT operation: at 40.3 MHz on a JEOL PS100; at 109.3 MHz on a Bruker HX 270; and at 145.7 MHz on a Bruker WH 360. The 180°-τ-90° method was used to measure $1/T_1^P$ on all the instruments except the Bruker 270 which used the 90°-τ-90° method with homogeneity spoil (McDonald & Leigh, 1973). The transverse rates were obtained from measurement of the line widths. Sample volumes of 1.5 mL in 8-mm tubes were used at 24.3 MHz and 0.8–2.0 mL in 10-mm tubes at the higher frequencies. The solvent water contained 10–15% D_2O for the purpose of providing a field frequency lock on the deuterium resonance. Sample temperatures of all magnetic resonance experiments were maintained at 25 ± 2 °C (unless stated otherwise) by a thermostated stream of dry air or nitrogen gas. Final temperatures were checked with a calibrated copper-constantin thermocouple (TRI-R Instruments) positioned in the center of the sample area of the probe. Loss of enzyme activity during the NMR experiments which lasted up to 24 h at 25 °C did not exceed 10%. At 40.3 MHz, T_1^P for a sample solution of P_i in standard buffer was 7.0 s.

In early ^{31}P NMR experiments, it was observed that ternary mixtures of enzyme-Mn^{2+}-P_i, freshly prepared from Mn^{2+}-free enzyme which had been stored at 4 °C, showed a marked time dependence for both $1/T_1^P$ and $1/T_2^P$. Similar time-dependent changes, which occur on the binding of metal ion to inorganic pyrophosphatase, have been reported previously (Höhne & Rapoport, 1973; Bruckmann, 1974). Subsequently, therefore, enzyme and Mn^{2+} were preincubated at room temperature overnight. Using this procedure, stable values for the ^{31}P NMR relaxation rates were obtained when the enzyme-Mn^{2+} solutions were titrated with a third component. This precaution was observed for the water relaxation experiments as well.

Unless otherwise indicated, all phosphorus magnetic reso-

[1] Abbreviations used: PCHOHP, hydroxymethanebisphosphonate; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

[2] M. Bond, N. Y. Chiu, & B. S. Cooperman, manuscript in preparation.

TABLE I: Parameter Definitions.

Equilibrium Constants

K_M = [E][Mn]/[EMn]
K_1 = [EMn][P]/[EMnP]
K_2 = [EMnP][P]/[EMnP$_2$]
K_D = [Mn][P]/[MnP]
K_I = [PCHOHP][EMnP]/[PCHOHP-EMnP]
K_I' = [PCHOHP·EMn][P]^2/[EMnP$_2$][PCHOHP]
K_I^* = [PCHOHP][EMn]/[PCHOHP-EMn]

Conservation

$[E]_T$, $[Mn]_T$, $[P]_T$ total concentration in all their forms of enzyme, manganous ion, and inorganic phosphate, respectively

Water Proton Relaxation Rates (PRR)

ϵ_b = enhancement of the binary EMn complex
ϵ_t = enhancement of the ternary EMnP complex
ϵ_q = enhancement of the quaternary EMnP$_2$ complex

^{31}P Relaxation Rates

$T_{1,0}^P$ = relaxation time in the absence of manganous ion
T_{1p}^P = paramagnetic contribution to the observed T_1^P
$1/T_{1p}^P = 1/T_{1,obsd}^P - 1/T_{1,0}^P$
$T_{1b}^P = T_1$ in the binary MnP complex
$T_{1t}^P = T_1$ in the ternary EMnP complex
$T_{1q}^P = T_1$ in the quaternary EMnP$_2$ complex

Dialysis Experiments

$1/T_{1c}^P = 1/T_{1,in}^P - 1/T_{1,out}^P$
$1/T_{2c}^P = 1/T_{2,in}^P - 1/T_{2,out}^P$

nance experiments were carried out at 40.3 MHz in 0.1 M Tris-HCl (pH 7.2) which was 0.1 M in KCl (standard buffer).

Equilibrium Dialysis. Solutions (1.0 mL) of enzyme and all components except metal ion were dialyzed against 2.5 mL of the same solutions minus enzyme, in 4.0-mL Biovials (Beckman Instruments). A small aliquot of a concentrated Mn^{2+} stock containing a known amount of $^{54}Mn^{2+}$ was then added to the external solution and the vials were capped. The vials were allowed to stand vertically for a few minutes in order that the internal pressure equalized, thus preventing leakage, and then the caps were taped securely in place. The vials were then mounted on the rack of a Psychotherm controlled environment incubator shaker (New Brunswick Scientific) set at 25 ± 2 °C and agitated at 100 rpm for approximately 8 h to establish equilibrium. After equilibration, the entire contents "inside" the sac and an equivalent amount of the "outside" solutions were transferred to separate, preweighed, 10-mm NMR tubes. These samples were counted for $^{54}Mn^{2+}$ content, yielding values for $[Mn^{2+}]_{T,in}$ and $[Mn^{2+}]_{T,out}$. The same samples were then used directly in ^{31}P NMR experiments, yielding values for $T_{1,in}^P$, $T_{1,out}^P$, $T_{2,in}^P$, and $T_{2,out}^P$.

Calculations of Correlation Times and Distance. For protein-Mn^{2+}-phosphorus ligand complexes, the Solomon-Bloembergen equation for the longitudinal relaxation time of the ^{31}P nucleus bound near the paramagnetic site may be simplified to eq 2

$$\frac{1}{T_{1M}} = \frac{B}{r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2}\right] \quad (2)$$

where r is the Mn-^{31}P internuclear distance, ω_I is the nuclear precessional frequency, B is a product of physical constants, and τ_c is the dipolar correlation time (Mildvan & Engle, 1972). The measured T_1 value for a complex is given by eq 3, where τ_m is the mean residence time of the ligand in the Mn^{2+} coordination sphere.

$$T_{1,complex} = T_{1M} + \tau_m \quad (3)$$

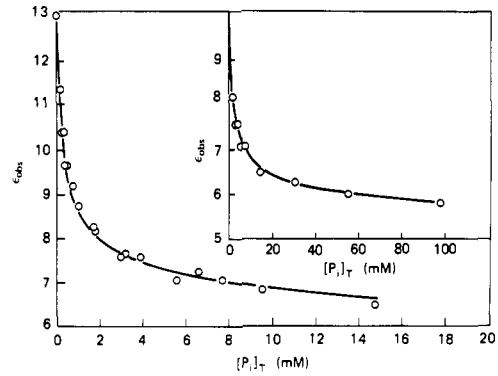


FIGURE 1: Plot of ϵ_{obsd} vs. inorganic phosphate concentration. Aliquots of stock solutions containing 100 μN enzyme, 40 μM Mn^{2+}, and 5, 20, or 200 mM P_i were added successively to a 50-μL sample of 100 μN PPase and 40 μM Mn^{2+}. T_1 measurements were obtained after each addition. The results for the titration at low phosphate concentration are shown in the main figure. In the inset the data are extended to high $[P_i]$. The curves are least-squares fits to eq 6. (See text.) The points are experimental.

Thus, the value for $T_{1,complex}$ may be used to calculate r if τ_c can be determined and τ_m is small compared with T_{1M}. If the former condition is met, then from eq 2 and 3

$$T_{1,complex} = \frac{r^6}{3B\tau_c}[1 + \omega_I^2\tau_c^2] \quad (4)$$

Further, if τ_c is frequency independent, then a plot of $T_{1,complex}$ vs. ω_I^2 yields a straight line, from which τ_c may be calculated as the square root of the slope/intercept ratio (Peacocke et al., 1969).

Results

Both water proton relaxation rates (PRR) and inorganic phosphate ^{31}P relaxation rates were measured for solutions containing either Mn^{2+} and phosphate or enzyme, Mn^{2+}, and phosphate. These results will be presented separately. Parameters used in equations in the Results section are defined either in Table I or in the text.

Water Proton Relaxation Rates. Values of ϵ_{obsd} were measured in solutions containing enzyme, Mn^{2+}, and varying amounts of inorganic phosphate, as shown in Figure 1. The curve obtained is clearly biphasic and provides evidence for the existence of two classes of phosphate binding sites. Under the conditions used in Figure 1, ϵ_{obsd} is given by eq 5, which, to a very good approximation, can be transformed into eq 6.

$$\epsilon_{obsd} = \frac{[Mn]}{[Mn]_T} + \frac{[EMn]}{[Mn]_T}\epsilon_b + \frac{[EMnP]}{[Mn]_T}\epsilon_t + \frac{[EMnP_2]}{[Mn]_T}\epsilon_q \quad (5)$$

$$\epsilon_{obsd} = \frac{K_1K_2\epsilon_b + K_2[P]\epsilon_t + [P]^2\epsilon_q}{\frac{K_1K_2K_M}{[E]_T - [M]_t} + K_1K_2 + K_2[P] + [P]^2} \quad (6)$$

Here the assumptions made are that the concentration of binary MnP complex is small compared with $[Mn]_T$, that the contributions of both Mn and MnP to ϵ_{obsd} are negligible, and that [E], the concentration of unbound enzyme, can be set equal to $[E]_T - [Mn]_T$. These assumptions are valid over the entire concentration range studied in Figure 1. Values of K_1, K_2, ϵ_b, ϵ_t, and ϵ_q were obtained by fitting the data in Figure 1 to eq 6, using a computerized nonlinear least-squares fitting procedure (Lietzke, 1963) and taking K_M equal to 9 μM as previously determined (Cooperman & Chiu, 1973a): K_1, 0.24 ± 0.05 mM; K_2, 14 ± 5 mM; ϵ_b, 14.5 ± 0.5; ϵ_t, 7.7 ± 0.2; ϵ_q, 5.5 ± 0.4. The value of ϵ_b determined here is in reasonable accord

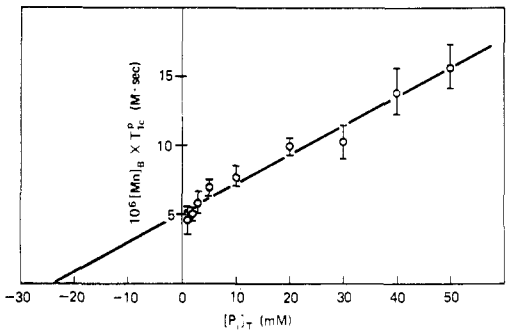


FIGURE 2: Plot of $[Mn]_B T_{1c}$ vs. inorganic phosphate concentration. Small aliquots of a concentrated P_i solution were added to a 1-mL solution containing 100 μN enzyme, 15 μM Mn^{2+}, 10% D_2O, and standard buffer. $T_{1,obsd}{}^P$ values were measured after each addition. Values for $T_{1c}{}^P$ and $[Mn]_B$ were obtained by small corrections applied to $T_{1p}{}^P$ and $[Mn]_T$ (see text). The solid line is a least-squares fit to the data. Points are experimental.

with the value (12.2 ± 0.4) previously reported (Cooperman & Chiu, 1973a).[3] The value of K_1 is similar to the value of 1.2 mM for phosphate binding, previously determined from flow dialysis studies under comparable conditions (Rapoport et al., 1973). Curves similar to those in Figure 1 were also obtained at other pH values and other enzyme concentrations (data not shown). The results at pH 8.0 indicated slightly tighter binding of phosphate compared with those at pH 7.2, while pH 6.0 binding appears considerably weaker. These results are in accord with the earlier studies of Rapoport et al. (1973).

^{31}P Relaxation Rates: Binary Solution. In solutions of manganous ion and inorganic phosphate, $T_{1p}{}^P$ is given by eq 7 (Navon et al., 1970)

$$T_{1p}{}^P = \frac{T_{1b}{}^P}{[Mn]_T}[[P] + K_D] \quad (7)$$

A plot of $T_{1p}{}^P$ vs. [P] yielded a good straight line allowing calculation of $T_{1b}{}^P$ (1.0×10^{-4} s) and K_D (20 ± 2 mM). At 100 mM inorganic phosphate, plots of $1/T_{1p}{}^P$ and $1/T_{2p}{}^P$ were linear functions of manganous ion concentration and yielded a T_{1p}/T_{2p} ratio of 190 (data not shown). These values are similar to those obtained by Ray & Mildvan (1973) for methyl phosphonate binding to manganous ion under analogous conditions; $T_{1b}{}^P$, 1.25×10^{-4} s, K_D, 15 mM, T_{1p}/T_{2p}, 328.

^{31}P Relaxation Rates: Ternary Solution. Phosphate Titration. The PRR work described above shows the presence of two binding sites on the enzyme for inorganic phosphate. Therefore, in solutions of enzyme, manganous ion, and inorganic phosphate eq 8 applies, which can be transformed into eq 9.

$$\frac{[P]}{T_{1,obsd}{}^P} = \frac{1}{T_{1,0}{}^P}[P] + \frac{1}{T_{1b}{}^P}[MnP] + \frac{1}{T_{1t}{}^P}[EMnP] + \frac{1}{T_{1q}{}^P}[EMnP_2] \quad (8)$$

$$[Mn]_B T_{1c}{}^P = \frac{T_{1q}{}^P\left[\left(\frac{K_1}{[P]} + 1\right)K_2 + [P]\right]}{\frac{T_{1q}{}^P}{T_{1t}{}^P}\frac{K_2}{[P]} + 1} \quad (9)$$

Utilization of the dialysis method permits us to evaluate $1/T_{1c}{}^P$ (Table I) by subtracting out the contributions to $1/T_{1,obsd}{}^P$ of inorganic phosphate and the MnP complex, and to evaluate

[3] Different preparations of enzyme showed small differences in measured ϵ_b values.

TABLE II: Phosphate Titration of Enzyme-Mn^{2+}.[a]

$[P]_T$ (mM)	$[Mn]_{in}$ (μM)	$[Mn]_{out}$ (μM)	$[Mn]_B$ (μM)	$1/T_{1,in}$ (s^{-1})	$1/T_{1,out}$ (s^{-1})	$1/T_{1c}$ (s^{-1})
10	55.8	3.00	52.8	11.36	1.33	10.03
20	55.1	3.30	51.8	7.14	1.20	5.94
35	54.0	3.70	50.3	5.40	1.06	4.34
50	52.9	4.10	48.8	3.85	0.91	2.94

[a] $[E]_T$ = 100 μN.

$[Mn]_B$, defined as $[Mn^{2+}]_{T,in}$ minus $[Mn^{2+}]_{T,out}$. In the concentration ranges discussed in this section, $[Mn]_B$ is equal to the sum of [EMn], [EMnP], and [$EMnP_2$]. A plot of $[Mn]_B T_{1c}{}^P$ vs. [P] (Table II) for [P] in the range 10-50 mM yielded a good straight line. In this range, $K_1/[P]$ may be ignored (see above), and the fact that a good straight line is obtained is evidence that $1 \gg (T_{1q}{}^P K_2)/(T_{1t}{}^P[P])$ and therefore that $T_{1q}{}^P/T_{1t}{}^P \ll 1$. Thus, in this concentration range, eq 9 simplifies to eq 10

$$[Mn]_B T_{1c}{}^P = T_{1q}{}^P[K_2 + [P]] \quad (10)$$

permitting evaluation of $1/T_{1q}{}^P$ and K_2 from the straight line plot. From four experiments of this kind we obtained an average $1/T_{1q}{}^P$ value of $4.4 \pm 0.8 \times 10^3$ s^{-1} and an average K_2 value of 18.4 ± 4.4 mM. We consider this value of K_2 inherently more reliable than the value obtained by PRR measurement since it was obtained directly rather than via a curve-fitting procedure. Nevertheless, it is comforting that both determinations agree within experimental error.

We next extended the measurement of $T_1{}^P$ to more dilute solutions of inorganic phosphate, in an attempt to evaluate $T_{1q}{}^P/T_{1t}{}^P$. These results are presented in Figure 2. In this experiment the dialysis technique was not used. Rather, inorganic phosphate was added serially to a stock solution of enzyme and Mn^{2+}, and correction factors were applied to the measured $[Mn]_T$ and $1/T_{1p}{}^P$ values to obtain estimates of $[Mn]_B$ and $1/T_{1c}{}^P$. This procedure has the advantage of being much more economical with enzyme, and its use is justified in the present instance.[4]

Within the accuracy of our measurements there is no clear deviation from a straight line for $[Mn]_B T_{1c}{}^P$ vs. [P] down to 1 mM phosphate. It is true, however, that the measured points at 1 mM and 2 mM phosphate do fall below the least-squares fitted line. Furthermore, the deviation seen must be an underestimate of that due to the denominator in eq 9, since at the low phosphate concentrations $K_1/[P]$, though not large, is no longer negligible compared with one. We conclude that $T_{1q}{}^P/T_{1t}{}^P$ must be quite small ($\leq$0.04), although our data do not permit an accurate estimate of this ratio to be made.

The extent of diamagnetic contribution to $1/T_{1q}{}^P$ can be estimated by determining the corresponding value for the Mg^{2+} complex since Mg^{2+} is known to bind competitively with

[4] The results shown in Table II were quite reproducible. Thus, for solutions containing $\geq$10 mM inorganic phosphate, $[Mn]_T$ and $1/T_{1p}{}^P$ data could be corrected directly. At 10 mM inorganic phosphate, the correction in $[Mn]_T$ to get $[Mn]_B$ is 5%, and in $1/T_{1p}{}^P$ to get $1/T_{1c}{}^P$ is 10.6%. From the trends in Table II, both of these corrections will decrease as $[P_i]$ is decreased. Furthermore, as $[P_i]$ is lowered, the experimental error in determining $1/T_{1p}{}^P$ increases because of signal-to-noise problems. For example, we estimate this error to be ±12% at 2 mM P_i and ±11% at 1 mM P_i. We estimated corrections to $[Mn]_T$ and $1/T_{1p}{}^P$ by extrapolating results such as those shown in Table II to lower $[P_i]$, and using the $T_{1b}{}^P$ and K_D values determined as described. Even large relative errors in estimating these correction factors should lead to only 1-2% errors in estimating $[Mn]_B$ and $1/T_{1c}{}^P$, or much less than the experimental error in determining $1/T_{1p}{}^P$.

TABLE III: Mn^{2+} Titration of Enzyme-Phosphate.[a]

$[Mn]_{out}$ (μM)	$[Mn]_B$ (μM)	$[E]_T$ (μM)	$1/T_{1c}^P$ (s^{-1})
14.8	120	114	8.26
23.4	168	106	8.26
36.0	187	110	8.62

[a] $[E]_T$ = 100 μN; $[P]_T$ = 50 mM.

Mn^{2+} (Cooperman & Chiu, 1973a). T_1 measurements (not shown) on solutions containing 100 mM inorganic phosphate, 100 μN enzyme, and varying Mg^{2+} (50–500 μM) lead to an estimated value of 0.09×10^3 s^{-1}, which is within the error for $1/T_{1q}^P$ and so can be considered negligible.

Mn^{2+} Titration. In the absence of added ligand, inorganic pyrophosphatase has been shown to have two Mn^{2+} binding sites per subunit, the affinities of which differ by approximately one order of magnitude (Rapoport et al., 1973). The results presented so far have been for conditions where $[E]_T > [Mn]_T$ and thus reflect primarily the tight Mn^{2+} site. In order to test the effect of the weak Mn^{2+} site on the measured T_1^P, we prepared a series of samples by equilibrium dialysis in which the concentration of Mn^{2+} bound to enzyme exceeded the enzyme subunit concentration, and measured the T_1^P of these samples. The data are summarized in Table III from which it is clear that there is little or no contribution to $1/T_{1c}^P$ from Mn^{2+} bound in the weak site. Making the reasonable assumption that the tighter site is saturated when much of the second site is filled allows calculation of $1/T_{1q}^P$ (equal to $1/T_{1c}^P[P]_T/[E]_T$) of 3.8×10^3 s^{-1}. This value agrees very well with the value derived above from the phosphate saturation results and strengthens the conclusion that $1/T_{1q}^P$ is determined only by Mn^{2+} bound in the tight site. It should be noted that the data in Table III allows estimation of a dissociation constant for Mn^{2+} binding to $EMnP_2$ of approximately 15 μM. This may be compared with the dissociation constant of Mn^{2+} binding to EMn of 78 μM (Rapoport et al., 1973).

Hydroxymethanebisphosphonate (PCHOHP) Titration. The effect of PCHOHP on T_{1p}^P in a solution of enzyme, Mn^{2+}, and different amounts of inorganic phosphate is shown in Figure 3. We have previously shown that PCHOHP is a competitive inhibitor of the enzyme and binds to the EMn complex (Cooperman & Chiu, 1973a,b). The results in Figure 3 demonstrate that PCHOHP binding displaces the weakly bound phosphate, which, as we have seen, is entirely responsible for the observed effect of EMn on T_1^P. The slope of the line for 10 mM phosphate is 2.6 times as large as that for 50 mM phosphate. A simple model which accounts for this result is that PCHOHP binding can result in displacement of only the weak site phosphate, to form the quaternary complex PCHOHP·EMnP, or of both phosphates, to form the ternary complex PCHOHP·EMn. From this model we can derive the expression for T_{1p}^P shown below.

$$T_{1p}^P = \frac{T_{1q}^P}{[Mn]_T} \times \left[[P] + K_2 + \left[\frac{K_2}{K_1} + \frac{K_1'}{[P]} \right] [PCHOHP] \right] \quad (11)$$

Here it is assumed that both [Mn] and [MnP] make little contribution to $[Mn]_T$ and that relaxation via MnP is small compared with relaxation via $EMnP_2$, both assumptions being justified under the conditions described in Figure 3. In eq 11 the PCHOHP·EMnP complex gives rise to the term K_2/K_1, and the PCHOHP·EMn complex gives rise to the term $K_1'/$

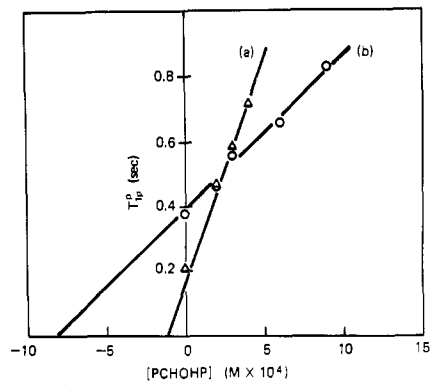


FIGURE 3: Plot of T_{1p}^P vs. PCHOHP concentration. Small volumes of a stock solution of PCHOHP (pH 7.2) were added successively to 1.0-mL samples of a ternary mixture of PPase, Mn^{2+}, and P_i. Dilution did not exceed 1%. The ternary mixture was composed of: 100 μN PPase, 40 μM Mn^{2+}, 10% D_2O, standard buffer; (a) 10 mM P_i or (b) 50 mM P_i. T_1^P values were obtained after each addition. Points are experimental. Lines are least-squares fits to the data according to eq 11. (See text.)

[P]. Thus, the dependence of the slope in Figure 3 on phosphate concentration provides evidence for the ternary complex, and the fact that the slope is not proportional to $1/[P]$ provides evidence for the quaternary complex. Setting K_2 equal to 18 mM (see above), initial estimates of K_1 and K_1' were obtained from the abscissa intercepts and slope ratio in Figure 3. Best fit values for these parameters were obtained by a least-squares fitting procedure (Lietzke, 1963) yielding a value for K_1 of 4.3 $\pm$ 0.6 $\times 10^{-4}$ M, and for K_1' of 2.07 $\pm$ 0.12 M. The direct dissociation constant for PCHOHP binding to EMn, K_1^* (Table I), is given by the ratio K_1K_2/K_1' and can now be calculated as equal to 2.1 μM [$(18 \times 10^{-3})(0.24 \times 10^{-3})/2.07$]. This agrees very well with the value of 2 μM which can be calculated from our previous results measuring directly PCHOHP binding to EMn (Cooperman & Chiu, 1973a),[5] providing strong additional support for our model. That PCHOHP can displace two phosphates to form the ternary complex is, of course, totally reasonable. The formation of the quaternary PCHOHP·EMnP complex is also not unexpected, given the work of Höhne & Heitmann (1974) showing that, in the presence of Mn^{2+}, tripolyphosphate ion is a substrate for inorganic pyrophosphatase.

pH and Temperature Dependence. It is clear from Table II that under conditions where $[P]_t \gg [E]_t > [Mn]_t$ and $[E]_t > K_M$, $1/T_{1c}^P$ is well approximated by $1/T_{1p}^P$. The variation in $1/T_{1p}^P$ and in T_{1p}^P/T_{2p}^P as a function of pH is shown in Figure 4. Most of our $1/T_{1p}^P$ measurements are taken at pH 7.2, which as can be seen falls in a region where $1/T_{1p}^P$ is essentially independent of pH. The decrease in $1/T_{1p}^P$ with decreasing pH can plausibly be ascribed to weaker phosphate binding to EMn. The corresponding rise in T_{1p}^P/T_{2p}^P reflects an increased concentration of the binary MnP complex in solution as pH is lowered.

The variation in $1/T_{1p}^P$ as a function of temperature is shown in Table IV, allowing calculation of a $\Delta H_{obsd}^{\ddagger}$ of 4.2 kcal/mol.

Frequency Dependence. As we have seen, our derived value for $1/T_{1q}^P$ reflects, to a good approximation, solely the interaction between the tight Mn site and the low affinity phosphate site in the quaternary $EMnP_2$ complex. We can therefore use measured T_1^P values to calculate the Mn^{2+}–^{31}P distance, r, between these two sites. Values of T_{1c}^P and T_{2c}^P at several

[5] Given the errors involved in both determinations, the closeness of this agreement must be considered to be fortuitous.

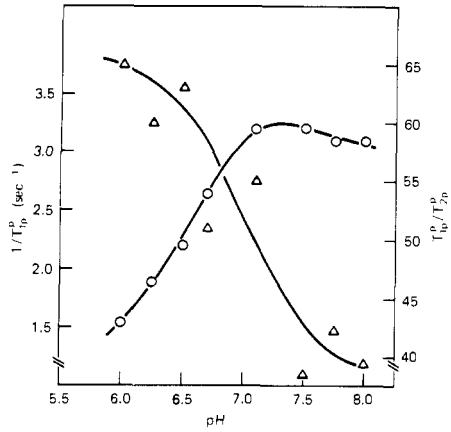


FIGURE 4: Dependence of T_{1p}^{p} and T_{1p}^{p}/T_{2p}^{p} on pH. Two starting solutions, identical in composition except for pH, were preincubated at room temperature overnight and admixed in appropriate portions to obtain different final pH values, and T_{1p}^{p} and T_{2p}^{p} were determined. The two starting solutions contained 100 μN PPase, 33.3 μM Mn^{2+}, 10% D_2O, 90 mM KCl, and 45 mM P_i and were adjusted either to pH 6.0 or pH 8.0. $1/T_{1p}^{p}$ (○); T_{1p}^{p}/T_{2p}^{p} (△).

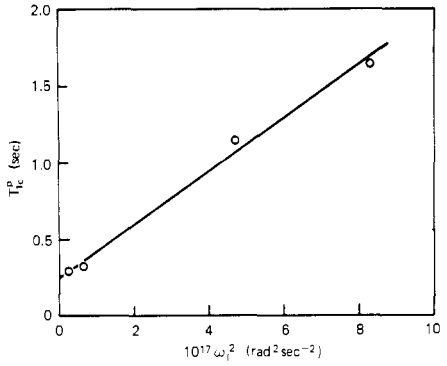


FIGURE 5: Frequency dependence of T_{1c}^{p}. T_{1c}^{p} was measured at 24.3, 40.3, 108.3, and 145.7 MHz on samples prepared by equilibrium dialysis as described in the Experimental Section. The sample inside the dialysis sac initially contained 100 μN PPase, 15% D_2O, 50 mM P_i, and standard buffer. The outside solution was identical except that it contained, in addition, 48.8 nmol of Mn^{2+} (2500 cpm of ^{54}Mn). The same samples were used for all frequencies. The points are experimental and represent the average of two experiments. The line is a least-squares fit.

frequencies are listed in Table V. Since $T_{1c}^{p} > T_{2c}^{p}$, τ_m must be small compared with T_{1M} and $1/T_{1q}^{p}$ may be set equal to $1/T_{1M}$ (eq 3). The $\Delta H^{\ddagger}$ value of 4.2 kcal/mol is also in accord with this conclusion. With this equality and combining eq 4 and 10, we obtain eq 12

$$T_{1c}^{p} = \left[\frac{[P] + K_2}{[Mn]_B}\right]\frac{r^6}{3B_c}[1 + \omega_I^2\tau_c^2] \qquad (12)$$

A plot of T_{1c}^{p} against ω_I^2 (Figure 5), using the data presented in Table V, yields a good straight line. Thus, in this frequency range τ_c is essentially independent of frequency and may be calculated as equal to $2.6 \pm 0.3 \times 10^{-9}$ s. This value, combined with the value of $1/T_{1q}^{p}$ at 40.3 MHz already determined (4.4×10^3 s^{-1}), leads to a calculated value for r of 6.2 Å (eq 2). The error in r is determined by the sixth roots of the errors in T_{1M} and in the dispersion term for τ_c. The error in T_{1q}^{p} should not exceed 30%, corresponding to an error in r of 4.5% or ±0.3 Å. At values of $\omega_I\tau_c$ close to one, as is the case at 40.3 MHz, r is especially insensitive to errors in τ_c. Thus, a reasonable estimate of the error in r is ±0.4 Å.

TABLE IV: Temperature Dependence of $1/T_{1p}^{p}$.

temp (°C)	$1/T_{1p}^{p}$ (s^{-1})
25	2.36
15	1.82
3	1.38

TABLE V: Frequency Dependence of T_{1c}^{p} and T_{2c}^{p}.

ν_I (MHz)	T_{1c} (s)	T_{2c} (s)
24.3	0.27 ± 0.03	
40.3	0.33 ± 0.02	0.044 ± 0.037
108.3	1.15 ± 0.15	0.055 ± 0.010
145.7	1.64 ± 0.12	0.031 ± 0.008

Discussion

This study presents the first clear evidence for two phosphate binding sites per subunit of inorganic pyrophosphatase. It having been previously shown that there are also two Mn^{2+} sites per subunit, the effect of enzyme bound Mn^{2+} on the relaxation times of bound phosphate potentially involves four different Mn^{2+}-P_i interactions. As we have seen the more weakly bound Mn^{2+} is without effect on T_{1c}^{p}, so that the problem simplifies to a consideration of the interaction of only the tightly bound Mn^{2+} with the phosphate sites. These sites differ substantially (75-fold) in their affinities for phosphate. Relaxation by enzyme-bound Mn^{2+} is effective at the low affinity site, leading to a longitudinal relaxation rate of 4.4×10^3 s^{-1}, and permitting calculation of an Mn^{2+}-P distance of 6.2 Å. This distance falls within the expected range of 5.6–6.6 Å for a Mn phosphate second sphere complex (Mildvan & Grisham, 1974). Relaxation by enzyme-bound Mn^{2+} is much less effective at the high affinity site, which could be due either to slow dissociation of phosphate from the high affinity site, i.e., $\tau_m \gg T_{1M}$ in eq 3 or to a high T_{1M} value if the high affinity phosphate site is relatively far from tightly bound Mn^{2+}. Neither of these possibilities can be excluded at present. From the results in Figure 2, $T_{1t} > 25T_{1q}$, i.e., $T_{1t} > 7 \times 10^{-3}$ s. Given the value of K_1 (0.24 mM), in order for τ_m to dominate T_{1t} the rate constant for phosphate binding to the high affinity site would have to be $<6 \times 10^5$ M^{-1} s^{-1}. This is much slower than would be expected for simple phosphate binding to a protein, supporting the idea that T_{1M} dominates T_{1t}. On the other hand, the PRR enhancement value for the ternary complex, ϵ_t (7.7), is much lower than that for the binary complex, ϵ_b (14.5), and is suggestive of a direct coordination of the high affinity phosphate to Mn^{2+}. Such direct coordination would lead to a T_{1M} value much less than 7×10^{-3} s, so that τ_m would have to dominate T_{1t}. This would be possible if the rate-determining step in phosphate binding to the high affinity site were not the binding itself but rather a conformational change of the enzyme either preceding or following initial enzyme–phosphate complex formation (vide infra).[6]

The finding that PCHOHP can simultaneously (via K_1') displace both phosphates from the enzyme is a strong indication that the two phosphate sites are adjacent to one another. This conclusion is supported by recent work by Boyer and his co-workers[7] on the phosphate–water oxygen exchange reaction

[6] In experiments not presented, we have shown enzyme-Mn^{2+} to have no observable effect on the T_1 of the methylene protons of methylene bisphosphonate, another pyrophosphate analogue, under conditions where this analogue is known to bind (Cooperman & Chiu, 1973a). This presumably reflects slow exchange.

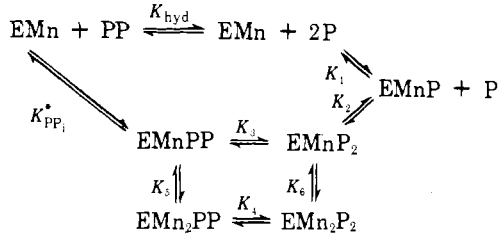
[7] P. D. Boyer, private communication.

catalyzed by the enzyme. They have shown that the dependence of the rate of this exchange on phosphate concentration is consistent with a two-site model for phosphate binding and with dissociation constants of the order of magnitude of those estimated here. They also have evidence that the exchange reaction proceeds via pyrophosphate synthesis. Since Cohn (1958) has shown that the exchange proceeds 500 times faster than the complete reversal of the hydrolysis reaction, exchange via pyrophosphate synthesis is possible only if the rate of release of enzyme-bound pyrophosphate is slow compared with the rate of pyrophosphate synthesis. If we consider the two phosphate sites as subsites for pyrophosphate binding, then, by analogy, there might well be a slow step in the release of phosphate bound in the high affinity subsite, permitting τ_M to dominate T_{1t}.

The large difference in the affinities of the sites for phosphate may provide a clue to their functional roles in pyrophosphate hydrolysis. Phosphoryl transfer from a phosphate monoester, which pyrophosphate may be considered to be, is known to be strongly dependent on the conjugate acidity of the leaving group, the Brønsted β value for monoester dianions have a value of -1.23 (Kirby & Varvoglis, 1967; Miller & Ukena, 1969). Given the structure of pyrophosphate, much of its affinity for the enzyme must be due to favorable electrostatic interactions. For instance, it is known that at least one arginine is essential for pyrophosphate binding (Cooperman & Chiu, 1973b; Bond, Chiu, & Cooperman, manuscript in preparation), and we have recently shown that this arginine is directly involved at the high affinity phosphate subsite.[8] Since the conjugate acidity of an anion binding to a cationic template will increase with the binding affinity, a greater catalytic efficiency is to be expected if the leaving phosphoryl group is bound to the higher affinity subsite. This leaves the lower affinity subsite as providing the locus for nucleophilic water attack. From the proximity of the two phosphate sites, the lack of effect of the more weakly bound Mn^{2+} on $1/T_{1c}^{P}$ is evidence against the direct interaction of the second metal ion with either phosphate. This supports the conclusion reached earlier (Höhne & Rapoport, 1973) that the requirement of a second metal ion for maximal enzymatic activity is due to an allosteric effect.

Inorganic pyrophosphatase-catalyzed hydrolysis of pyrophosphate proceeds with a solvent kinetic isotope effect of two (Konsowitz & Cooperman, 1976). This suggests that the rate-determining step in the reaction is the hydrolysis itself, rather than a step involving either binding or conformational change. However, this suggestion may be in error in the light of our present results if the phosphate–water exchange reaction proceeds via pyrophosphate synthesis. The arguments here are as follows. Enzyme-catalyzed pyrophosphate hydrolysis requires two bound metal ions. In Scheme I, the equilibrium constant for enzyme-bound pyrophosphate hydrolysis, K_4, is equal to K_3K_5/K_6. K_3, in turn, is equal to $K_{hyd}K_{PP_i}{}^*/K_1K_2$, where K_1 and K_2 are as defined in Table I, $K_{PP_i}{}^*$ is equal to $[EMn][PP_i]/[EMnPP_i]$, and K_{hyd} is equal to $[P_i]^2/[PP_i]$. K_1 (0.24 mM) and K_2 (18 mM) have been evaluated in this paper. On the basis of competitive inhibition studies on enzyme-catalyzed PP_i hydrolysis with Mn^{2+} as the required divalent metal ion cofactor, we have previously estimated PCHOHP binding to be five times stronger than PP_i binding (Cooperman & Chiu, 1973a), so that we can estimate a value of 10 μM for $K_{PP_i}{}^*$. Lastly, K_{hyd} may be estimated from the data of Flodgaard & Fleron (1974) as equal to 1.6×10^4 M, leading to a calculated value for K_3 of 3.7×10^4. With Mg^{2+} as the cofactor, the turnover number for pyrophosphate hydrolysis at pH 7.2 and 25 °C is approximately 300 s^{-1}. With Mn^{2+}, the corresponding value is 18 s^{-1}, as estimated from the results of Butler & Sperow (1977). If it is assumed that K_4 is equal to K_3, and that k_4, the rate constant for hydrolysis of enzyme-bound pyrophosphate, is equal to the turnover number, we can calculate a value for k_{-4}, the rate constant for synthesis of enzyme-bound pyrophosphate, of 5×10^{-4} s^{-1}. This is some four orders of magnitude too slow to explain the water–phosphate oxygen exchange rates measured by Cohn (1958) and by Boyer and his coworkers.[7] Thus, either the assumptions made in our calculations are invalid, or hydrolysis of enzyme-bound pyrophosphate proceeds much faster than the rate-determining step despite the solvent kinetic isotope effect, or water–phosphate oxygen exchange does not proceed via pyrophosphate synthesis. Here it should be noted that the solvent kinetic isotope effect was determined using Mg^{2+} as the required divalent metal ion cofactor, and the effect with Mn^{2+} might be different. The least certain value in our calculation of K_3 is that for $K_{PP_i}{}^*$. However, on the basis of other estimates for this parameter, both from kinetic and direct binding studies (Cooperman & Chiu, 1973a), we are very unlikely to be more than a factor of 10 in error (i.e., $K_{PP_i}{}^* > 1$ μM). Our estimates of K_1 and K_2 are subject only to relatively small errors. Flodgaard & Fleron (1974) measured an apparent constant for pyrophosphate hydrolysis under conditions very close to those used in this study, and Boyer and his co-workers have confirmed their measurement.[7] Thus, a factor of three as the estimate of the error in K_{hyd}/K_1K_2 is generous, leading to a minimal value for K_3 of 10^3. In order to reduce K_4 below this number sufficiently to allow both enzyme-bound pyrophosphate hydrolysis to be rate-determining and phosphate–water exchange to proceed via pyrophosphate synthesis, K_6 would have to be several hundred times larger than K_5. This is possible but unlikely from our evidence that the second metal ion is not directly involved at the active site.

SCHEME I



In conclusion, the results presented here have allowed us to define rather precisely the position of tightly bound Mn^{2+} with respect to the low affinity phosphate site and have provided evidence that the two phosphate sites are close to one another and that weakly bound Mn^{2+} is not close to either site. On the other hand, we do not know the position of tightly bound Mn^{2+} with respect to the high affinity phosphate subsite. In addition, the magnitudes of the dissociation constants measured for the binding of phosphate and the competitive inhibitor, PCHOHP, taken together with the results of Boyer and his co-workers,[7] suggest that the hydrolysis of enzyme-bound pyrophosphate is not the rate-limiting step in the overall enzymatic reaction, at least when Mn^{2+} is the required divalent metal ion cofactor.

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[8] B. Springs & B. S. Cooperman, unpublished results.

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References

Avaeva, S. M., Bakuleva, N. P., Baratova, L. A., Nazarova, T. I., & Fink, N. Yu. (1977) *Biochim. Biophys. Acta 482,* 173.

Baykov, A. A., & Avaeva, S. M. (1974) *Eur. J. Biochem. 47,* 57.

Bruckmann, R. H. (1974) M.S. Thesis, University of Pennsylvania, Philadelphia, Pa.

Bunick, G., McKenna, G. P., Scarbrough, F. E., Uberbacher, E. C., & Voet, D. (1978) *Acta Crystallogr.* (in press).

Butler, L. G. (1971) *Enzymes, 3rd Ed. 4,* 529.

Butler, L. G., & Sperow, J. W. (1977) *Bioinorg. Chem. 7,* 141.

Cohen, S. A., Sterner, R., Keim, P. S., & Heinrikson, R. L. (1978) *J. Biol. Chem. 253,* 889.

Cohn, M. (1958) *J. Biol. Chem. 230,* 369.

Cohn, M. (1970) *Q. Rev. Biophys. 3,* 61.

Cohn, M., & Hughes, T. R. (1962) *J. Biol. Chem. 237,* 176.

Cohn, M., & Leigh, J. S. (1962) *Nature (London) 193,* 1037.

Cohn, M., & Townsend, J. (1954) *Nature (London) 173,* 1090.

Cooperman, B. S., & Chiu, N. Y. (1973a) *Biochemistry 12,* 1670.

Cooperman, B. S., & Chiu, N. Y. (1973b) *Biochemistry 12,* 1676.

Cooperman, B. S., Chiu, N. Y., Bruckmann, R. H., Bunick, G. J., & McKenna, G. P. (1973) *Biochemistry 12,* 1665.

Eisinger, J. Shulman, R. G., & Szymanski, B. M. (1962) *J. Chem. Phys. 36,* 1721.

Eisinger, J., Fawaz-Estrup, F., & Shulman, R. G. (1965) *J. Chem. Phys. 42,* 43.

Flodgaard, H., & Fleron, P. (1974) *J. Biol. Chem. 249,* 3465.

Heinrikson, R. L., Sterner, R., Noyes, C., Cooperman, B. S., & Bruckmann, R. H. (1973) *J. Biol. Chem. 248,* 2521.

Heitmann, P., & Uhlig, H. J. (1974) *Acta Biol. Med. Germ. 32,* 565.

Heitmann, P., Möllerke, C. H., & Uhlig, H. J. (1972) *Acta Biol. Med. Germ. 29,* 551.

Höhne, W. E., & Heitmann, P. (1974) *Acta Biol. Med. Germ. 33,* 1.

Höhne, W. E., & Rapoport, T. A. (1973) *Eur. J. Biochem. 33,* 323.

Kirby, A. J., & Varvoglis, A. G. (1967) *J. Am. Chem. Soc. 89,* 415.

Konsowitz, L. M., & Cooperman, B. S. (1976) *J. Am. Chem. Soc. 98,* 1993.

Kunitz, M. (1952) *J. Gen. Physiol. 35,* 423.

Lietzke, M. H. (1963) *A Generalized Least Squares Program for the IBM 7090 Computer,* ORNL-3259, Office of Technical Service, U.S. Department of Commerce, Washington, D.C.

McDonald, G. G., & Leigh, J. S., Jr. (1973) *J. Magn. Reson. 9,* 358.

Mildvan, A. S., & Engle, J. L. (1972) *Methods Enzymol. 26,* 654.

Mildvan, A. S., & Grisham, C. M. (1974) *Struct. Bond. (Berlin) 20,* 1.

Miller, D. L., & Ukena, T. (1969) *J. Am. Chem. Soc. 91,* 3050.

Moe, O. E., & Butler, L. G. (1972a) *J. Biol. Chem. 247,* 7308.

Moe, O. E., & Butler, L. G. (1972b) *J. Biol. Chem. 247,* 7315.

Navon, G., Shulman, R. G., Wyluda, B. G., & Yamane, T. (1970) *J. Mol. Biol. 51,* 15.

Peacocke, A. R., Richards, R. E., & Sheard, B. (1969) *Mol. Phys. 16,* 177.

Rapoport, T. A., Höhne, W. E., Reich, J. G., Heitmann, P., & Rapoport, S. M. (1972) *Eur. J. Biochem. 26,* 237.

Rapoport, T. A., Höhne, W. E., Heitmann, P., & Rapoport, S. (1973) *Eur. J. Biochem. 33,* 341.

Ray, W. J., Jr., & Mildvan, A. S. (1973) *Biochemistry 12,* 3733.

Ridlington, J. W., & Butler, L. G. (1972) *J. Biol. Chem. 247,* 7303.

Ridlington, J. W., Yang, Y., & Butler, L. (1972) *Arch. Biochem. Biophys. 153,* 714.

Simonsen, D. G., Wertman, M., Westover, L. M., & Weil, J. W. (1946) *J. Biol. Chem. 166,* 747.

Sperow, J. W., Moe, O. A., Ridlington, J. W., & Butler, L. G. (1973) *J. Biol. Chem. 248,* 2062.

Swift, T. J., & Connick, R. E. (1962) *J. Chem. Phys. 37,* 307.

West, T. S. (1969) *Complexometry,* B.D.H. Chemical Ltd., Poole, England.