Yeast Inorganic Pyrophosphatase. II. Magnetic Resonance and Steady-State Kinetic Studies of Metal Ion and Pyrophosphate Analog Binding†

Barry S. Cooperman* and Ning Yu Chiu

ABSTRACT: Magnetic resonance techniques, introduced by Cohn and her coworkers, are employed to measure metal ion binding and pyrophosphate analog binding to inorganic pyrophosphatase. T_1 measurements show that Mn^{2+} binds directly to enzyme, with an enhancement (ϵ_b) of 12.2, and a stoichiometry of two sites per enzyme molecule. Electron spin resonance studies give the same stoichiometry and K_D values. Gd^{3+} is very tightly bound, with an enhancement of 9.3. By a competitive procedure, Mg^{2+}, Ca^{2+}, and Zn^{2+} binding are also measured. Enhancement measurement is also used to monitor Mn^{2+}–pyrophosphate analog binding. The ϵ_t values are smaller than ϵ_b. One of the analogs studied, hydroxymethanebisphosphonate, is shown to be an inhibitor of enzymatic activity, and the relationship between kinetic and magnetic resonance studies is discussed.

Our investigations on the mechanism of action of inorganic pyrophosphatase have, as their immediate goal, the elucidation of the identity and role of amino acid residues at the active site. At this stage of our work, we wish to distinguish between three possible roles for an active-site residue: metal ion binding, pyrophosphate binding and direct involvement in a bond-making or -breaking step, recognizing that a given residue may have more than one role, and further that binding and catalytic roles need not be as independent of one another as this classification scheme would suggest.

In the first paper of this series (Cooperman *et al.*, 1973) we described a new assay method for measurement of inorganic pyrophosphatase activity. In this paper we describe measurement of both metal ion and pyrophosphate analog binding, using magnetic resonance techniques introduced by Cohn and her coworkers (Mildvan and Cohn, 1970; Cohn and Townsend, 1954; Reed and Cohn, 1972), and steady-state kinetics.

Cohn (1963) has previously cited experiments showing Mn^{2+} binding to inorganic pyrophosphatase and more recent studies have shown that other divalent metal ions bind as well (Ridlington and Butler, 1972; Rapaport *et al.*, 1972), in agreement with the results presented here. In addition our results provide strong evidence for two, noninteracting Mn^{2+} binding sites per enzyme molecule and show a clear parallelism between kinetic and magnetic resonance studies of analog binding.

Experimental Section

Materials

N-Ethylmorpholine (Aldrich) was distilled before use. Disodium hydroxymethanebisphosphonate (PCHOP)[1] and methanebisphosphonic acid (PCH_2P) were gifts of Dr. D. A. Nichols of Proctor and Gamble. Tetrasodium imidobisphosphonate decahydrate (PNHP) was a gift of Dr. Ralph Yount. All other chemicals were reagent grade and were used without further purification.

Water for stock solutions was glass distilled and passed through a Barnstead standard mixed-bed deionizing column. PP_i solutions were made up by weight, and standardized by measuring total P_i release on full hydrolysis. Solutions of Tris and KCl used for making up buffers for T_1 and electron spin resonance (esr) measurements were passed over Chelex-100 (Bio-Rad).

Yeast inorganic pyrophosphatase (35–45 Kunitz units/mg) (Kunitz, 1952) was prepared as described in an accompanying article (Cooperman *et al.*, 1973) and stored in the form of ammonium sulfate pads. Prior to use in magnetic resonance or kinetic experiments ammonium sulfate was removed by passage over a G-25 (fine) column equilibrated either with a suitable buffer or with water. In the latter case, the enzyme solution could be lyophilized with no loss in specific activity and made up to any desired concentration in a suitable buffer. Small amounts of denatured enzyme were removed by centrifugation.

Methods

T_1 Measurements. The proton relaxation rate of water was measured at 24.3 MHz at 30° by a pulsed nuclear magnetic resonance (nmr) technique described previously (Cohn and Leigh, 1962). Values of the observed enhancement (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 T_1 and $T_{1,0}$ are the observed relaxation times in the presence and absence of Mn^{2+}, respectively. The terms with asterisks represent the same parameters in the presence of added complexing agent.

Binary Complexes. In solutions of inorganic pyrophosphatase and Mn^{2+}, eq 2–4 are important, where ϵ_b is the en-

$$\epsilon_{obsd} = \frac{[M]}{[M]_T} + \frac{[EM]}{[M]_T}\epsilon_b = \epsilon_b - (\epsilon_b - 1)\frac{[M]}{[M]_T} \quad (2)$$

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 28, 1972.* This work was supported by Research Grant AM-13202 from the National Institutes of Health.

[1] Abbreviations used are: PCHOHP, hydroxymethanebisphosphonate; PCH_2P, methanebisphosphonate; PNHP, imidobisphosphonate.

$$[M] = \frac{-B \pm \sqrt{B^2 + K_D[M]_T}}{2} \quad (3)$$

$$B = K_D + [E] - [M]_T$$

$$K_D = \frac{[E][M]}{[EM]} \quad (4)$$

hancement of Mn^{2+} bound to enzyme, K_D is the dissociation constant of the Mn^{2+}–enzyme complex, and $[M]_T$ and $[E]_T$ are the total concentrations of Mn^{2+} and enzyme, expressed as total Mn^{2+} binding sites, respectively. Although the amount of protein is a known quantity, the value of $[E]_T$ is somewhat uncertain since there are disputes in the literature both as to the molecular weight and subunit structure of inorganic pyrophosphatase (Butler, 1971). ϵ_{obsd} is thus a function of three unknowns, ϵ_b, K_D, and $[E]_T$. Initial estimates of ϵ_b and K_D were determined by graphical methods. Best-fit values of ϵ_b, K_D, and $[E]_T$ were obtained using the Lietzke computerized, non-linear least-squares-fitting procedure described previously (Cooperman and Buc, 1972).

Dissociation constants for other, diamagnetic, metal ions were obtained by titration of a Mn^{2+}–enzyme solution with a second metal ion. Under these conditions free Mn^{2+} is given by eq 5, where K_D' is the dissociation constant of the second

$$[Mn^{2+}] = \frac{-C \pm \sqrt{C^2 + 4D}}{2} \quad (5)$$

$$C = B + \frac{K_D}{K_D'}[N]_T$$

$$D = K_D[M]_T\left(\frac{[N]_T}{K_D'} + 1\right)$$

metal ion–enzyme complex and $[N]_T$ is the total concentration of second metal ion. Best-fit values of K_D' were obtained using the Lietzke procedure.

In solutions where Mn^{2+} binding to buffer is not negligible, ϵ_{obsd} is given by

$$\epsilon_{obsd} = \frac{1}{[M]_T}[(EM)\epsilon_B + (M) + (MB)\epsilon_B] \quad (6)$$

where ϵ_B is the enhancement of Mn^{2+} when bound to buffer. If, as is true in these studies, ϵ_B is very close to 1, eq 6 simplifies to eq 7, and [M] is given by eq 8.

$$\epsilon_{obsd} = \epsilon_B - a(\epsilon_B - 1)\frac{[M]}{[M]_T} \quad (7)$$

$$[M] = \frac{-B' \pm \sqrt{(B')^2 + \frac{4K_D[M]_T}{a}}}{2} \quad (8)$$

where $a = ([M] + [MB])/[M]$ and $B' = K_D + ([E]_T - [M]_T)/a$.

The value of a was determined by esr measurement of Mn^{2+} concentration in solutions of Mn^{2+} and buffer (Cohn and Townsend, 1954).

Ternary Complexes. In solutions of enzyme, Mn^{2+}, and pyrophosphate analog I, the relevant equilibria are, in addition to K_D (eq 4)

$$K_1 = \frac{[M][I]}{[MI]} \quad (9)$$

$$K_2 = \frac{[E][MI]}{[EMI]} \quad (10)$$

$$K_I = \frac{[E][I]}{[EI]} \quad (11)$$

A formally identical set of equilibria have been used to analyze binding data for pyruvate kinase (Mildvan and Cohn, 1966). ϵ_{obsd} is given by eq 12, where ϵ_a and ϵ_t are the enhancements of the MI and EMI complexes, respectively.

$$\epsilon_{obsd} = \frac{[M]}{[M]_T} + \frac{[MI]}{[M]_T}\epsilon_a + \frac{[EM]}{[M]_T}\epsilon_b + \frac{[EMI]}{[M]_T}\epsilon_t \quad (12)$$

For both PCHOHP and PCH_2P it was assumed that the values of ϵ_a and K_1 did not differ significantly from those determined previously for PP_i (see Results). K_D, ϵ_b and $[E]_T$ were determined in the binary studies. ϵ_{obsd} is thus a function of three unknowns, ϵ_t, K_2, and K_I. Reed *et al.* (1970) have recently described a computerized, numerical method for analysis of a system of this type and this method was applied directly to evaluate the three unknown parameters.

Electron Spin Resonance Measurements. In a relatively dilute (<5 mg/ml) solutions of inorganic pyrophosphatase, free Mn^{2+} concentration was measured by the intensity of its esr spectrum with a Varian Model V-4502 spectrometer at 9.5 GHz (X band) at 30°. Values of K_D and $[E]_T$ were determined from eq 3 using the Lietzke procedure.

Electron spin resonance spectra of bound Mn^{2+} in concentrated enzyme solutions were recorded with a Varian Model E3 spectrometer at 9.5 GHz.

Inorganic Pyrophosphatase Assay. Assays were run at 30° as previously described (Cooperman and Mark, 1971). Some assays were done using only partially purified (15 Kunitz units/mg) enzyme.

Standard Buffer. Unless otherwise indicated, all kinetics and magnetic resonance experiments were carried out in 0.1 M Tris-HCl buffer (pH 7.2), which was 0.1 M in KCl.

Results and Discussion

Binary Complex of Mn^{2+} and Pyrophosphatase. T_1 measurements of Mn^{2+} binding to pyrophosphatase were made at fixed enzyme concentration and varying Mn^{2+} and at fixed Mn^{2+} and varying enzyme. Sample data are shown in Figure 1. Electron spin resonance measurements were made at fixed enzyme and varying Mn^{2+}. Values of K_D and ϵ_b in the pH range 6–8 are summarized in Table I. Electron spin resonance measurements on the buffers used at pH's 6.0, 7.0, and 7.2 showed no significant binding of Mn^{2+} by buffer. The pH 8.0 buffer showed weak Mn^{2+} binding (as evidenced by a 20% reduction in expected Mn^{2+} signal intensity) and in this case eq 7 and 8 were used in place of eq 2 and 3 in evaluating K_D, ϵ_b, and $[E]_T$.

From the known $A_{280\,nm}$ values of the solutions used, the fact that a 0.1% solution of inorganic pyrophosphatase has an $A_{280\,nm}$ of 1.45 (Kunitz, 1952), the best-fit values of $[E]_T$ allow a calculation of the combining weight for Mn^{2+}. The data in Figure 1 give a value of $3.5 \pm 0.3 \times 10^4$ molecular weight units, and the esr data give $3.6 \pm 0.4 \times 10^4$ molecular weight units. Thus two independent experiments give essentially

TABLE I: Metal Ion Binding to Inorganic Pyrophosphatase.

Metal Ion	pH	K_D (μM)	K_D, rel	ϵ_b	Method	No. of Determinations
Mn^{2+}	6.0[a]	31 ± 11			Esr	13
Mn^{2+}	7.0[b]	12 ± 4			Esr	11
Mn^{2+}	7.2[c]	9.0 ± 1.3	1.0 (1.0)[e]	12.2 ± 0.4	T_1	31
Mn^{2+}	8.0[d]	6.1 ± 3.0		15.5 ± 2.1	T_1	9
Mg^{2+}	7.2[c]	83 ± 16	9.2 (8.0)[e]		T_1	29
Ca^{2+}	7.2[c]	166 ± 22	18.4 (400)[e]		T_1	9
Zn^{2+}	7.2[c]	57 ± 2	6.3 (5.5)[e]		T_1	6
Gd^{3+}	7.2[c]	0.079 ± 0.010	0.0087	9.3	T_1	14

[a] 0.16 M 2-(*N*-morpholinoethanesulfonate)–NaOH. [b] 0.18 M *N*-Ethylmorpholine·HCl. [c] Standard buffer. [d] 0.2 M *N*-Ethylmorpholine·HCl. [e] Figures in parentheses were determined by Ridlington and Butler (1972).

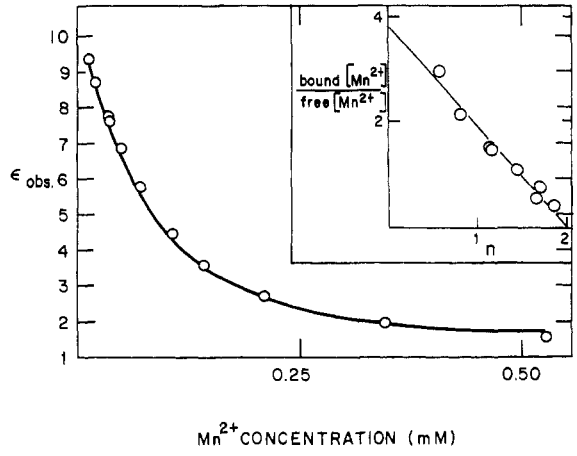

FIGURE 1: Titration of inorganic pyrophosphatase with Mn^{2+}; standard buffer. Small volumes of stock solutions of $MnCl_2$ were added successively to 100-μl samples of an inorganic pyrophosphatase solution ($A_{280\ nm}$ equal to 1.78) and T_1* was measured after each addition. Total volume of additions to any one sample never exceed 6 μl. T_1 values were determined by adding the same volumes of $MnCl_2$ solutions to buffer. $[E]_T$ values used in fitting data to eq 2 and 3 include small dilution correctons. Curve is theoretical, using best-fit values summarized in Table I. Points are experimental. Best fit value of $[E]_T$ in $A_{280\ nm}$ = 1.78 solution: 3.54 ± 0.35 × 10^{-5} N. Inset: Scatchard plot; n is defined as the number of moles of Mn^{2+} bound per 70,000 g of enzyme.

the same values for combining weight and for K_D (Table I). Since the published estimates for the molecular weight of inorganic pyrophosphatase vary between 63,000 (Schachman, 1952; Ridlington *et al.*, 1972) and 71,000, these results indicate a stoichiometry of two apparently identical Mn^{2+} binding sites per molecule.[2]

The Scatchard (1948) plot in Figure 1 illustrates the lack of cooperativity in Mn^{2+} binding. Other evidence that the enzyme is at least functionally a dimer comes from work demonstrating a stoichiometry of two for $CaPP_i$ binding (Ridlington and Butler, 1972), from carboxypeptidase digestion results (Avaeva and Akhmedov, 1970), and from the finding of a single subunit of mol wt 3.5 × 10^4 on gel electrophoresis in the presence of sodium dodecyl sulfate (Ridlington *et al.*, 1972). By two other criterion, however, the stoichiometry of reactive SH groups (Negi *et al.*, 1971) and tightly bound Mg^{2+} (Ridlington *et al.*, 1972), the enzyme appears to be monomeric (Butler, 1971). This may be because the enzyme is made up of two subunits which are similar but not identical. However, there are now several enzymes known which seem to have identical subunits and yet display functional asymetry, for example, the lactic acid and malate dehydrogenases (Tsernoglu *et al.*, 1971) and acetoacetate decarboxylase (O'Leary and Westheimer, 1968), and inorganic pyrophosphatase may provide another example of this as yet poorly understood phenomenon.

The esr spectrum of enzyme bound Mn^{2+} is shown in Figure 2. Proof that this spectrum is not due to free Mn^{2+} comes from the observed temperature independence of the spectrum, as well its overall shape, both properties being

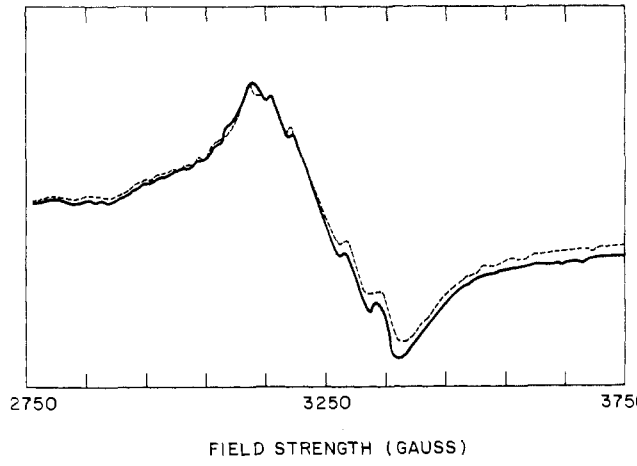

FIGURE 2: Temperature dependence of esr spectrum of Mn^{2+} bound to inorganic pyrophosphatase. (- - -) 1 and 15°. (——) 25 and 30°. Spectra were taken on a 46-μl sample, 1.37 mM in enzyme, 0.65 mM in $MnCl_2$, in a 0.05 M hydroxyethylpiperazineethanesulfonic acid buffer (pH 8.0).

[2] Ridlington *et al.* (1972) have recently presented evidence for a single tightly bound, slowly dialyzing, Mg^{2+} per molecule (mol wt 71,000) of enzyme, which may be involved in maintaining enzyme structure, as distinct from more weakly bound Mg^{2+} which plays a more direct role in catalysis. The binding we observe is almost certainly to the latter sites, as evidenced by the magnitude of the K_D values (Table I) and the rapidity with which equilibrium is established (within 1 min at 30°). We have not as yet tested our enzyme preparation for Mg^{2+} content, but it is noteworthy that a purification step used by Ridlington *et al.* and not used in this work, is the incubation of crude enzyme with 50 mM Mg^{2+} at pH 4.8.

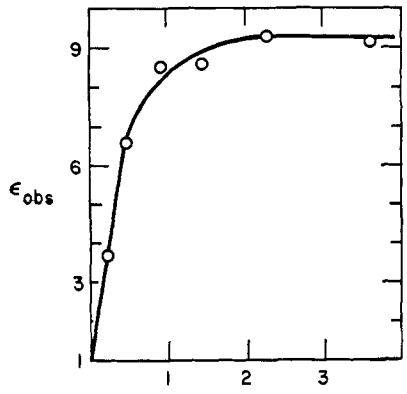

FIGURE 3. Titration of Gd^{3+} with inorganic pyrophosphatase; standard buffer. 1 μl of 1 mM $GdCl_3$ was added to 100 μl of varying concentrations of inorganic pyrophosphatase and T_1* was measured. Line is not theoretical.

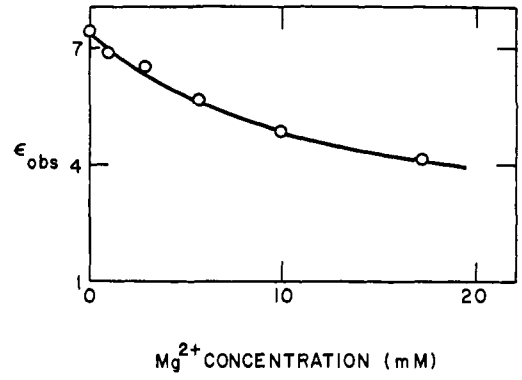

FIGURE 4: Titration of Gd^{3+}–inorganic pyrophosphatase solution with Mg^{2+}; standard buffer. Small volumes of stock solutions of $MgCl_2$ were added successively to 101 μl of stock solution of 0.99 mM $GdCl_3$ and 1.4×10^{-5} N inorganic pyrophosphatase (combining mol wt 3.5×10^4)and T_1* was measured after each addition. Solid line was calculated using eq 2 and 5 ($K_D' = 8.3 \times 10^{-5}$ M, $\epsilon_b = 9.3$), using the best-fit value for the Gd^{3+} dissociation constant (see text). Points are experimental. Values of $[E]_T$ and $[M]_T$ used in fitting procedure include small dilution corrections.

quite different for free Mn^{2+} (Reed and Ray, 1971). The spectrum shown is for a solution having a ratio of Mn^{2+} to binding sites of 0.5. At higher ratios the spectrum is unchanged, except that above 0.75 peak characteristic of free Mn^{2+} begin to appear. If the two Mn^{2+} ions bound to enzyme were within 15 Å of each other, one would have expected to see some change in the spectrum from a spin–spin interaction (Leigh, 1970). The failure to observe a change is evidence that the binding sites are quite far from one another and this is in accord with the observed noncooperativity of Mn^{2+} binding.

Binary Complexes of Inorganic Pyrophosphatase and Other Metal Ions. Titration of Mn^{2+}–pyrophosphatase solutions with added metal ion led to decreases in ϵ_{obsd}. Values of K_D, calculated using eq 5, are summarized in Table I. Implicit in the calculation is the assumption that the stoichiometry of binding sites is the same for metal ion as it is for Mn^{2+}. The value of K_D for Mg^{2+} (83 μM) agrees within a factor of two with one determined under similar conditions from a steady-state kinetic analysis (Rapoport *et al.*, 1972) which is evidence that the metal ion binding monitored by T_1 measurements is kinetically significant. The relative affinities of Mg^{2+} and Zn^{2+} with respect to Mn^{2+} are similar to those measured by fluorescence quenching under somewhat different conditions (Ridlington and Butler, 1972). The relative Ca^{2+} affinities differ considerably and the reasons for this are unclear, although based on relative affinities for simple ligands (Sillen and Martell, 1964), the twofold stronger binding of Mg^{2+} found by magnetic resonance is more reasonable than the 50-fold stronger binding found by fluorescence quenching.

Studies on metal ion binding proteins consistently show that Zn^{2+} is bound more tightly than Mn^{2+}, which is in turn bound more tightly than Mg^{2+} (Mildvan and Cohn, 1965; Cottam and Ward, 1969; Bright, 1967; Malmstrom, 1953; Ray, 1969; Peck and Ray, 1969), and this order is what would be expected on the basis of results with simpler ligands (Sillen and Martell, 1964). It is thus highly unusual that inorganic pyrophosphatase binds Zn^{2+} only about as well as it does Mg^{2+} and more poorly than it does Mn^{2+}. This cannot be due to ionic radius considerations (Bright, 1967), since Zn^{2+} (0.74 Å) falls in between Mn^{2+} (0.80 Å) and Mg^{2+} (0.65 Å). It may be that the release of Mn^{2+} on addition of Zn^{2+} is really due to a more complicated process than that of simple competition described by eq 5. Ridlington *et al.* (1972) have found that incubation of enzyme with Zn^{2+} leads to a loss of activity although this is not seen with Mg^{2+} or Mn^{2+}. Schlesinger and Coon (1960) demonstrated some time ago that in the presence of Zn^{2+}, ATP is a substrate (albeit a poor one) whereas in the presence of Mg^{2+} it is not. It remains for future work to determine whether these observations are linked to the anomalously weak Zn^{2+} binding.

The potential usefulness of the fluorescent and magnetic properties of the lanthanide metal ions as structural probes (Reuben, 1973) prompted T_1 measurements of Gd^{3+} binding to pyrophosphatase (Figure 3). The definition of ϵ_{obsd} is the same as in eq 1, with Gd^{3+} replacing Mn^{2+}. Although the binding is too tight to estimate a dissociation constant directly from this data, a value of ϵ_b of about 9.3, similar to the 12.2 found for Mn^{2+}, is indicated. Titration of a Gd^{3+}–pyrophosphatase solution with Mg^{2+} (Figure 4) shows that Mg^{2+} competes for the Gd^{3+} site. Taking the dissociation constant for Mg^{2+} to be equal to 8.3×10^{-5} M (Table I), and assuming the stoichiometry of Gd^{3+} and Mg^{2+} sites to be the same, yields an estimate of 8×10^{-8} M for the Gd^{3+} dissociation constant.

Ternary Complexes of Enzyme–Mn^{2+}–Pyrophosphate Analogs. Rapid PP_i hydrolysis prevents direct observation, *via* the T_1 measurement technique, of the ternary complex, enzyme–Mn^{2+}–PP_i PCH_2P, PCHOHP, and PNHP, though structurally similar to PP_i (Larsen *et al.*, 1969), are not substrates for the enzyme and we have used these compounds to demonstrate formation of the corresponding ternary complexes, enzyme–Mg^{2+}–PP_i analog.

Before discussing the ternary complexes it will be necessary to discuss the chemistry of binary solutions consisting of Mn^{2+} and PP_i (or PP_i analogs), which is complex. We have previously shown (Cooperman and Mark, 1971) that in dilute (0.1–0.5 mM) solutions of Mn^{2+} and PP_i (or PP_i analogs) large aggregates are formed, having an Mn^{2+}:PP_i ratio of approximately 1.7. In the concentration range cited, which is a relevant one to the inorganic pyrophosphatase studies, aggregation is rather slow (half-lives of minutes are common) and disaggregation is extremely slow, taking many hours. Thus, although aggregation is reversible, equilibration is not

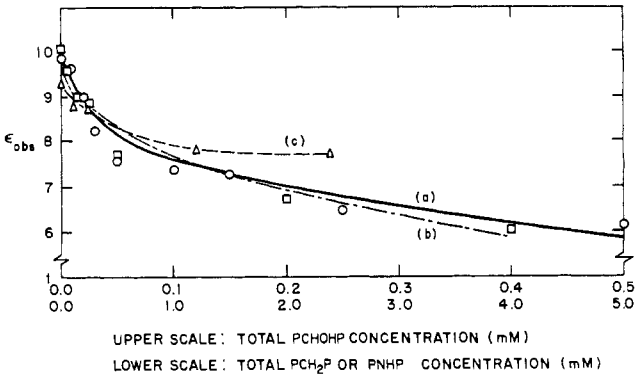

FIGURE 5: Titration of Mn^{2+}–inorganic pyrophosphatase solution with added PP_i analogs. Standard buffer: (a) to 50-μl samples of a stock solution 3.0×10^{-5} M in $MnCl_2$ and 5.8×10^{-5} N in inorganic pyrophosphatase (combining mol wt 3.5×10^4) was added 10 μl of PCHOHP solutions of varying concentration and T_1* was measured. Curve is theoretical (eq 12), using parameter values in text. Points are experimental (○). (b) To 25-μl samples of stock solution 3.1×10^{-5} M in $MnCl_2$ and 6.2×10^{-5} N in inorganic pyrophosphatase was added 5 μl of PCH_2P solutions of varying concentration and T_1* was measured. Curve is theoretical (eq 12) using parameter values in text. Points are experimental (□). (c) Small volumes of PNHP solutions were added successively to a stock solution 2.5×10^{-5} M in $MnCl_2$ and 4.4×10^{-5} N in inorganic pyrophosphatase and T_1* was measured after each addition. Total dilution never exceeded 5%, except for last point (10%). Points are experimental. Curve is not theoretical (△).

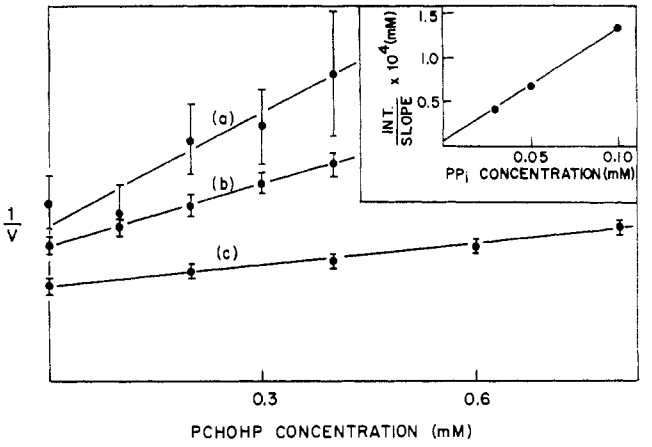

FIGURE 6: Inhibition of inorganic pyrophosphatase by PCHOHP. Enzyme velocity expressed in arbitrary units, buffer 0.24 M Tris-HCl–0.8 M KCl (pH 7.4). (a) [PP_i], 0.03 mM; [Mg^{2+}], 1.5 mM plus [PCHOHP]. (b) [PP_i], 0.05 mM; [Mg^{2+}], 1.5 mM plus [PCHOHP]. (c) [PP_i], 0.10 mM; [Mg^{2+}], 1.1 mM. Inset: secondary plot of intercept/slope against [PP_i].

readily achieved. Although a formal rate law describing aggregation was not obtained, it was clear that the rate of aggregation was dependent on the concentrations of both the 1:1 Mn^{2+}:PP_i complex, and free Mn^{2+}.

These considerations led us to design the ternary complex experiments in the following manner. Binary Mn^{2+}–enzyme solutions were made up first. Enzyme was present in excess over Mn^{2+}, and at a total concentration which was five- to tenfold higher than K_D, so that little free Mn^{2+} was present. A titration curve was constructed by adding varying amounts of PP_i analog to this binary solution. For each sample the value of T_1 was quite stable within at least the first 10 min after addition of PP_i analog, so that aggregation was of little importance in this time period. Thus the ϵ_{obsd} values obtained could be analyzed in terms of the rapidly established equilibria (eq 4 and 9–11). The dependence of ϵ_{obsd} as a function of PP_i analog concentration is shown in Figure 5. For PCHOHP and PCH_2P these results were treated as described earlier yielding the following values for K_2, K_I, and ϵ_t: PCHOHP, 0.8 μM, 20 μM, 8.30 ± 0.6; PCH_2P, 63 μM, 630 μM, 10.3 ± 0.5.[3]

For the closely coupled multiple equilibrium system present in solutions of Mn^{2+}, inorganic pyrophosphatase, and analog it is inherently extremely difficult to obtain unique values for K_2, K_I, and ϵ_t (Reed *et al.*, 1970), so that it would be unreasonable to attach great significance to the values presented above. Nevertheless, certain qualitative aspects of these studies are clear. (a) The affinity of inorganic pyrophosphatase for Mn^{2+}–PCHOHP is considerably greater than it is for Mn^{2+}–PCH_2P. (b) The values of ϵ_t for the analogs are smaller than the value of ϵ_b. Although this would be consistent with coordination of PP_i analog to Mn^{2+} in the ternary complex, the differences are small and could arise from a variety of other effects (Mildvan and Cohn, 1970). Studies on the ^{31}P and ^1H resonances of these analogs should clarify this point. (c) A theoretical model utilizing the four equilibria (eq 4 and 9–11) is consistent with the observed data. If eq 11 were not invoked, ϵ_{obsd} would be predicted to reach a limiting value at high analog concentration instead of falling gradually as it is observed to do.

Inhibition of Inorganic Pyrophosphatase by PP_i Analogs. Study of inhibition of inorganic pyrophosphatase by PP_i analogs is complicated by the fact that the true substrate is the 1:1 M^{2+}–PP_i complex (and to a lesser extent the M_2^{2+}–PP_i complex) (Rapaport *et al.*, 1972; Baykov *et al.*, 1972; Moe and Butler, 1972a,b). Thus an observed inhibitory effect could be due either to true competitive inhibition or to metal ion sequestration. Careful determination of all of the relevant dissociation constants in solutions of metal ion and PP_i, or PP_i analog, can in principle allow calculation of the concentration of the various species in solution, but this would be exceedingly difficult given the multiplicity of species in solutions of metal ion, PP_i, and PP_i analog (including the 1:1, 1:2, and 2:1 complexes of metal ion and PP_i (or PP_i analog), the mixed PP_i–metal ion–PP_i analog complex, and free metal ion, PP_i, and PP_i analog).

In the present study we have adopted the simpler approach of seeking a region of metal ion concentration over which enzymatic velocity is essentially independent of metal ion concentration and measuring PP_i analog effects in this region. With Mg^{2+} as metal ion, and PP_i concentrations in the range of 0.5–1.5 $\times 10^{-4}$ M, there is little change in rate for variation in Mg^{2+} concentration between 0.2 and 1.5 $\times 10^{-3}$ M, in agreement with previous workers (Moe and Butler, 1972a). Working in this range, added PCHOHP was found to decrease the enzymatic rate in a manner consistent with competitive inhibition and inconsistent with either simple noncompetitive (K_i (slope) equal to K_i (intercept)) or uncompetitive inhibition (Figure 6). Equation 13 can therefore be applied,

[3] Under the conditions of this experiment (excess enzyme over Mn^{2+}, total concentrations high with respect to K_2 and K_D), determination of these values does not require precise knowledge of the values of ϵ_A and K_I. In view of this, and since determination of these parameters is complicated by the aggregation phenomenon, the K_I and ϵ_A values for the PP_i analogs were assumed to be the same as those previously determined for PP_i (ϵ_A = 1.2, K_I = 2.3×10^{-5} M) (Cooperman and Mark, 1971).

where K_M, V_{max}, K_i, [S], and [I] have their usual meanings (Dixon and Webb, 1964).

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M\left[1 + \frac{[I]}{K_i}\right]}{V_{max}[S]} \quad (13)$$

A secondary plot (Figure 6, insert) of the intercept:slope ratio against [S] (eq 14) yielded a value for K_i/K_M of 12 ± 1, but the

$$\frac{\text{intercept}}{\text{slope}} = \frac{K_i[S]}{K_M} + K_i \quad (14)$$

intercept was too poorly determined to yield a good value for K_i. However, under virtually identical conditions, Moe and Butler (1972a) have determined K_M to be 10 μM, so that K_i may be estimated as 120 μM. In constructing the plots in Figure 6, [I] was assumed equal to $[PCHOP]_T$ and [S] was assumed equal to $[PP_i]_T$. This assumption is reasonable, since under our experimental conditions these concentrations are quite similar to those of the 1:1 complexes with Mg^{2+}, which are most likely the true inhibitor and substrate for the enzyme.

In a comparable recent study,[4] PNHP also gave results consistent with competitive inhibition. By contrast, we have found that PCH_2P and ADP give no observable inhibition at concentrations up to 20 times that of PP_i. Negi *et al.* (1972) have recently reported that at pH 6.0 Mg^{2+}–PCH_2P is a competitive inhibitor of the enzyme, with a K_i about tenfold larger than the K_M. This apparent disagreement with our results might be due to differences in pH, since we have shown that Mg^{2+}–PCHOHP binds more tightly to the enzyme at pH 6.0 than at pH 7.4 (Cooperman and Chiu, 1973). However, it is well to point out that Negi's studies were done at very high PCH_2P concentrations (12.5–22 mM) and at Mg^{2+} to PCH_2P ratios of just slightly over one, where formation of Mg_2^{2+}–PCH_2P complexes would be significant (Kabachnik *et al.*, 1967), so that the observed inhibition could be a metal ion sequestration effect.

Inhibition studies with Mn^{2+} as metal ion were limited by complexities introduced by the aggregation phenomenon. We have previously shown that the aggregates are not substrates for pyrophosphatase, although the 1:1 Mn^{2+}–PP_i complex is (Cooperman and Mark, 1971). Thus a successful assay depends on avoiding a preincubation of Mn^{2+} and PP_i and using rather high pyrophosphatase concentrations, so that 1:1 complex can be hydrolyzed before aggregation has had time to proceed. Using this procedure, reliable initial velocities can be obtained, at least for a limited range of Mn^{2+} concentration. At an initial PP_i concentration of 1.5×10^{-4} M, initial velocity is virtually independent of Mn^{2+} concentration in the range $1–2 \times 10^{-4}$ M in standard buffer. The specific activity obtained with Mn^{2+} is about 10% of that found with Mg^{2+}. Added PCHOP had an inhibitory effect at very low concentrations so that kinetic inhibition studies could be made in the absence of complications introduced by aggregation effects. For example, at 0.15 mM PP_i and 0.2 mM Mn^{2+}, bringing PCHOHP concentration to 0.02 mM decreased the initial velocity by approximately 40%. Treating the data as above for Mg^{2+} as cofactor give a value for K_i/K_m of 0.2. From the data of Kunitz (1952) and Moe and Butler (1972a,b) the K_m for Mn–PP_i can be approximated as 2 ± 1 μM, so that K_i for Mn–PCHOHP is approximately 0.4 μM. Given the inherent uncertainties in evaluating both quantities, this value of K_i must be considered to be essentially indistinguishable from that obtained for K_2 (0.8 μM), which is evidence that the binding processes monitored by T_1 measurements are kinetically significant. Additional qualitative evidence supporting this view comes from the observation that in the same concentration range, PCH_2P had no inhibitory effects, *i.e.*, both kinetic and T_1 studies show PCHOHP to be more tightly bound than PCH_2P.

One of the more interesting aspects of inorganic pyrophosphatase is the difference in its behavior toward Ca^{2+} and Mg^{2+}, two ions having similar chemical properties. Thus whereas Mg^{2+} is the most efficient divalent metal ion cofactor, the enzyme is totally ineffective with Ca^{2+} (Moe and Butler, 1972b) and this despite the fact that Ca^{2+}–PP_i binds at least 50–100 times more tightly to the enzyme than Mg^{2+}–PP_i (Ridlington and Butler, 1972). In our own studies, we have shown that we can quite drastically change (by a factor of about 60) the relative affinities of PCHOHP and PP_i for enzyme (measured by K_M/K_i) by switching from Mg^{2+} to Mn^{2+}, and further, that for the Mn^{2+}–PP_i analog complexes, the simple substitution of an –OH (PCHOHP) for an –H (PCH_2P), leads to a marked increase in affinity for the enzyme. A possible explanation is suggested by Calvo's (1965, 1968) work on the crystal structure of divalent metal ion complexes of PP_i, which showed that the conformation of the six-membered ring formed in the complex is predominantly staggered boat for smaller cations such as Mg^{2+}, but predominantly eclipsed boat for larger cations such as Ca^{2+} (Mildvan, 1970).

Thus, if one hypothesizes that the staggered form is substrate but that the eclipsed form is bound more tightly, a rationale can be constructed for the observations discussed above. Hypotheses along similar lines might be invoked to explain the unexpected ineffectiveness of the phosphonate analogs of ATP as inhibitors of some enzymes utilizing ATP as substrate (Larsen *et al.*, 1969). Determination of the crystal structures of the metal ion complexes of the phosphonate analogs of PP_i would provide a basis for evaluating the validity of this hypothesis.

Acknowledgment

We thank Dr. George Reed for use of his computer analysis for the ternary solution data and for several helpful discussions; Dr. Mildred Cohn for use of her pulsed nmr and esr equipment; Mr. Robert Bruckmann for taking esr spectra of bound Mn^{2+}; Miss Charlotte Cheston and Miss Diana Mark for performing some enzyme assays; and Dr. Larry Butler for making his results available to us prior to publication.

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Yeast Inorganic Pyrophosphatase. III. Active-Site Mapping by Electrophilic Reagents and Binding Measurements†

Barry S. Cooperman* and Ning Yu Chiu

ABSTRACT: The effects of electrophilic reagents on the enzymatic activity of inorganic pyrophosphatase are studied. Phenylglyoxal incubation results in complete inactivation, while incubation with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride leads to a retention of 7% activity. Trinitrobenzenesulfonate, *O*-methylisourea, and iodoacetic acid are ineffective as inactivating agents. The rates of both inactivation processes are virtually unaffected by added Mg^{2+}, but Mg^{2+}–hydroxymethanebisphosphonate complex, an inhibitor of enzymatic activity, slows the rate considerably. This protective effect is used to measure inhibitor binding and the dissociation constant so obtained is found comparable to a K_i value found previously from steady-state kinetic measurements (Cooperman, B. S., and Chiu, N. Y. (1973), *Biochemistry 12*, 1670). Magnetic resonance measurements show that incubation of enzyme with both inactivating reagents has only a minor effect of Mn^{2+} binding, but that binding of hydroxymethanebisphosphonate to the phenylglyoxal-inactivated enzyme has been abolished. These results and those of related studies are used to construct a plausible enzymatic mechanism.

In the first two papers of this series we presented methods for measuring three functions of yeast inorganic pyrophosphatase: enzymatic activity, divalent metal ion binding, and pyrophosphate analog binding. In this paper we begin using these methods to construct a preliminary structure–function map of the active site. Our strategy has been to (1) determine the sensitivity of the enzyme to a wide range of "group specific" electrophilic reagents, and for those reagents which inactivate the enzyme; (2) test whether inactivation is inhibited by Mg^{2+} or pyrophosphate analog added either separately or together; and (3) determine whether inactivation can be

† From the Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received August 28, 1972.* This work was supported by Research Grant AM-13202 from the National Institutes of Health.