Yeast Inorganic Pyrophosphatase

I. BINDING OF PYROPHOSPHATE, METAL ION, AND METAL ION-PYROPHOSPHATE COMPLEXES*

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SUMMARY

Because of the requirement for divalent metal ion activation, inorganic pyrophosphatase activity is measured in a complex equilibrium mixture containing free metal ion, free PPi, and metal ion-PPi complexes. Using crystalline yeast inorganic pyrophosphatase the binding to the enzyme of each of these individual components of the equilibrium has been examined. The dissociation constants of the binary Mn2+, Zn2+, Mg2+, Ca2+, respectively. In the absence of free metal ions neither PPi nor CaPPi bind to the enzyme; in the presence of excess Ca2+, CaPPi is bound quite strongly (KD apparent = 7 x 10^-3 M). The enzyme has two binding sites for CaPPi per mole of enzyme dimer. The results of these static binding studies are consistent with the kinetic analyses presented in the following papers.

Catalytic mechanisms and their relationship to details of macromolecular structure have been most intensively investigated with enzymes which catalyze simple hydrolytic reactions (e.g. ribonuclease, chymotrypsin, lysozyme); information gained from study of these hydrolytic enzymes is of significant aid in understanding more complex enzymatic reactions. Regrettably, the small group of enzymes for which the chemical mechanism is known in greatest detail does not include those which catalyze reactions involving polyphosphates, a class of compounds of immense intrinsic importance. The inorganic pyrophosphatase reaction (pyrophosphate phosphohydrolase (EC 3.6.1.1)) provides a system for studying biological catalysis of polyphosphate hydrolysis which is quite simple in the sense that the substrate contains only the minimal structural requirement for reactions of this type (i.e. the phosphoric anhydride bond). The added complexity of the effect of phosphate substituents such as adenosine is absent in this case. We have carried out extensive investigations of this reaction as catalyzed by crystalline inorganic pyrophosphatase obtained from bakers' yeast; previous work on this enzyme has recently been reviewed (1).

Like many other reactions involving polyphosphate compounds (2), the pyrophosphatase reaction as catalyzed by the yeast enzyme requires a divalent metal ion activator (3, 4). Cohn (5) has reported that this enzyme binds activating metal ions in the absence of PPi, making it a type II enzyme according to her classification. Coherent interpretation of the effects of divalent metal ions upon the reaction rate requires information about the strength of interactions between metal ion, substrate, and enzyme. For this simple system, extensive information is presently available concerning the strong complexes formed between PPi and divalent metal ions (6). This apparently simple equilibrium is complicated by the formation with certain metal ions of both mono- and dimetal complexes, and by the ionization of PPi and its metal complexes (6). In this paper we describe our investigations of the binding by the enzyme of the individual components of this equilibrium: uncomplexed metal ions, uncomplexed PPi, and a calcium ion-pyrophosphate (CaPPi) complex. In the following papers we report detailed kinetic studies of the activation of the reaction by Mg2+ and inhibition by Ca2+ (7, 8).

MATERIALS AND METHODS

Reagents—Tris buffer, “Ultra Pure” grade, was obtained from Mann Research Laboratories, diphenylthiocarbazone (dithizone) from Matheson, Coleman, and Bell, and Chelex-100 from BioRad Laboratories. All other reagents, except as noted, were analytical grade from Baker Chemical Company or Mallinckrodt Chemical Works and were used without further purification except where traces of metal ions were extracted from components of buffer solutions (see below).

Isotopes—Tetrasodium 32PPi (specific activity > 1000 Ci per mole) was obtained from New England Nuclear. Paper chromatography (9) showed less than 1% radioactive impurities. *CaCl2 (1.7 x 10^-4 M per mole) was from Amer sham-Searle.

Enzyme—Only highly purified, crystalline yeast inorganic pyrophosphatase was employed in these studies. A detailed description of the purification and crystallization of the enzyme is being reported elsewhere.1 The purification included release of the enzyme from bakers’ yeast cells either by autolysis in mildly alkaline aqueous suspension (10), or by toluene plasmonalysis (4), followed by ammonium sulfate fractionation, acid precipitat-

1 J. W. Ridlington, J. Sperow, Y. Yang, and L. Butler, Arch. Biochem. Biophys., in press.
tion, DEAE-cellulose column chromatography, and crystallization from ammonium sulfate solution. The specific activity of the crystalline preparations (600 to 650 μmoles of PPi hydrolyzed per min per mg of protein) compares favorably with other purified preparations (4, 10). Examination by disc gel electrophoresis or analytical ultracentrifugation revealed a high degree of homogeneity in the preparation. The enzyme was stored at 3-5°C as a crystalline suspension in ammonium sulfate solution. Concentrated stock enzyme solutions were reasonably stable for several months under refrigeration. Enzyme concentration was determined by ultraviolet absorption using εm,λ = 1.45 (4) and molecular weight 71,000 (1).

Solutions—Doubly deionized distilled water was used in all reagents employed in binding studies. Glass vessels in contact with the enzyme were previously soaked in dichromic acid, soaked in soap solutions, and boiled in deionized water, followed by prolonged soaking in several changes of deionized water. For studies of metal ion binding, the enzyme was dialyzed overnight against 0.1 M Tris, pH 7.65, containing 0.1 mM EDTA. EDTA was removed by dialysis against 0.1 M Tris, pH 7.65, which had been rendered metal-free by dithizone extraction in CCl4 (11, 12).

Fluorometry—Fluorescence measurements were carried out in an Aminco SPF-125 fluorometer equipped with a Xenon lamp and attached Sargent recorder. Full scale deflection on the recorder was adjusted to correspond to 20% change in the intrinsic fluorescence of the unperturbed enzyme in order to enhance the sensitivity of the measurement. Temperature (measured in a temperature-equilibrated fluorescence cell in the optical compartment) was maintained by a Haake constant temperature bath and pump and a KR-30 refrigeration unit from Polyscience Corporation.

To 1.0 ml of metal-free buffer fully temperature-equilibrated in the 1-cm fluorescence cell were added 10 μl of metal-free enzyme to give a final enzyme concentration of approximately 0.3 μM. The exciting wave length was 280 nm; emission was measured at 345 nm. After a steady fluorescence base-line was attained, 5-μl aliquots of metal ion were added every 3 min until no further fluorescence quenching was observed. Only data which had been reproduced at least three times were utilized; experiments in which fluctuations in lamp intensity were not negligible were discarded. Data were corrected for dilution but not for the inner filter effect because the optical density at the exciting wave length (280 nm) was never greater than 0.03 (15).

To obtain values for dissociation constants of metal-ion-enzyme complexes, fluorescence titration data were plotted according to a linear equation first derived by Benesi and Hildebrand (14).

As used here, the equation is

\[ \frac{1}{[M^{2+}]} = A \left( \frac{1}{[E]} \right) - \frac{1}{K_D} \]

where [M2+] represents the total concentration of divalent metal ion (the derivation assumes that the proportion of M2+ bound to the enzyme is insignificant), ΔF is the observed fraction of maximal quenching at a given [M2+], Kp is the dissociation constant, and A is a composite constant which accounts for the protein concentration and the difference in fluorescence of the free enzyme and its complex with M2+. Plots of 1/[M2+] versus 1/ΔF are linear, with ordinate intercept of -1/Kp.

Dilution Rate Measurement of Ligand Binding—In general, our previously described modifications (9) of the dialysis rate technique (15) were employed in this work. In order to minimize adhesion of ligand to the dialysis cell which was made of acrylicite (American Cyanamid, Pearl River, N. Y.), all cell surfaces in contact with ligand were treated with Teflon spray (Rulon, from the Dixon Corporation, Bristol, Rhode Island) when 32P binding was being measured, or with trimethylphenyl ammonium hydroxide (Eastman Organic Chemicals) when 45Ca binding was measured. Buffer was pumped through the lower chamber at a rate of 3.5 ml per min and collected on a fraction collector directly into scintillation vials or planchets for counting. Temperature was maintained at 5-8°C by an ice water bath. Only the first ligand addition contained radioactivity; this ensures maximum sensitivity. In all cases a control experiment without protein was run under exactly the same conditions in order to calibrate the cell (9). Calibration and corrections for volume changes, loss of ligand by dialysis, and altered specific radioactivity were made as previously described (9).

Radioactivity Measurements—32P was counted on a Nuclear-Chicago automatic gas flow planchet counter; 45Ca was counted on a Beckman CPM-100 scintillation counter.

RESULTS

Divalent Metal Ion Binding—On addition of certain divalent metal ions to the highly purified enzyme previously dialyzed against EDTA and then against metal-free buffer there is a significant and reproducible quenching of the intrinsic protein fluorescence. The shape of the emission spectra (maxima at 345 nm) is not appreciably altered by the quenching, nor is the shape of the ultraviolet absorption spectra or the fluorescence excitation spectra (maxima at 280 nm) significantly perturbed by addition of metal ions.

The quenching of fluorescence by divalent metal ion exhibits saturation effects expected of an equilibrium between metal ion and enzyme. Control experiments demonstrated that the fluorescence quenching is rapidly and completely reversed by addition of chelators. The degree of quenching at saturation for Mg2+, Zn2+, and Mn2+, activators of the reaction, and Ca2+, a strong inhibitor, is essentially identical (67 ± 0.5%). Metal ions such as Ba2+ and Sr2+ which neither activate nor inhibit the enzyme had no effect on the fluorescence even at high concentrations (0.01 M). In contrast, Eu3+, a rare earth which is similarly inert catalytically, produced a far greater degree of quenching at saturation than did the normal activating metal ions. The interaction of rare earth metal ions with the enzyme will be described in a future publication.

Data from a series of three replicate fluorescence titrations of the enzyme with Mg2+ is presented in Fig. 1 in the form of the linear reciprocal plot devised by Benesi and Hildebrand (14). Values for the dissociation constant of the metal ion-enzyme complex obtained from this treatment of fluorescence quenching data for several metal ions are shown in Table I.

These data were obtained at ionic strength = 0.2, somewhat lower than the ionic strength = 1.0 utilized in the kinetic measurements (7). Fluorescence titration under the standard conditions of the kinetic assays (0.45 M Tris, pH 7.4; 0.00 M KCl) yielded a dissociation constant for Mg2+ of 80 μM, 5-fold higher than that observed at the lower ionic strength.

Binding of PPi—Attempts to measure binding of free 32P to the enzyme in the absence of added divalent metal ion were complicated by rapid enzymatic hydrolysis of the 32P. Efforts to eliminate rigorously contaminating traces of free metal ions by dialysis of the enzyme against chelators and treatment of all buffer and 32P solutions with Chelex-100 were ineffective in preventing hydrolysis of the 32P, during the exposure to the high concentrations of enzyme necessary for binding experiments.
I/AF

FIG. 1. Linear plot of fluorescence titration with Mg$^{2+}$. Conditions were 0.24 M Tris, pH 7.4, at 30°. Three separate titrations are represented (see symbols). The plot is described under "Materials and Methods"; the ordinate intercept is $-1/K_D$.

Table I

**Dissociation constants for metal ion-enzyme complex**

Conditions for fluorescence measurements were 0.24 M Tris, pH 7.4, approximately 0.5 μM crystalline yeast inorganic pyrophosphatase, 30°. Excitation at 280 nm, emission at 345 nm. These are the average values of several replicate experiments. NMR (nuclear magnetic resonance) measurements are unpublished data supplied by Dr. Barry Cooperman. Conditions were 0.1 M Tris, pH 7.2, 0.1 M KCl, 30°. The kinetic measurement was taken from the following paper (7); conditions were 0.45 M Tris, pH 7.4, 0.6 M KCl, 30°.

| Metal ion | $K_D$ |
|-----------|-------|
| Fluorescence | NMR | Kinetic |
| Mn$^{2+}$ | 2 | 9 |aub |
| Zn$^{2+}$ | 11 | 57 | |
| Mg$^{2+}$ | 16 | 72 | |
| Ca$^{2+}$ | 800 | 160 | |

Even the dialysis rate technique, which yields a complete binding curve within a few minutes (19), was inadequate. Hydrolysis of $^{32}$PP$_i$ was almost completely prevented by inclusion of a low concentration of chelating agent in the buffer. In experiments carried out in the presence of 0.1 mM EDTA or 0.4 mM 8-hydroxy-5-quinoline sulfonic acid in 0.1 M Tris, pH 7.5, $^{32}$PP$_i$ hydrolysis was negligible in the short time required by the dialysis rate technique employed. However, no $^{32}$PP$_i$ binding was detected. Under these conditions (absence of metal ions) free PP$_i$ is bound to the enzyme very weakly or not at all (lower limit of apparent dissociation constant for enzyme-PP$_i$ complex was calculated to be 0.1 mM).

**Binding of Metal Ion-PP$_i$ Complexes**—In the presence of appreciable concentrations of Mg$^{2+}$, PP$_i$ is rapidly hydrolyzed so that binding of the MgPP$_i$ complexes cannot be measured by conventional equilibrium techniques. However, the enzymatic hydrolysis rate in the presence of Ca$^{2+}$ and absence of Mg$^{2+}$ is sufficiently slow to permit determination of the binding of the CaPP$_i$ complex to the enzyme using the dialysis rate technique.

A typical dialysis rate experiment for measuring binding of $^{32}$PP$_i$ in the presence of a large excess of Ca$^{2+}$ (6 mM) is shown in Fig. 2. Under these conditions virtually all (>99%) of the PP$_i$ is present as the monocalcium complex, and the enzyme is ~90% saturated with free Ca$^{2+}$ ($K_D = 0.8$ mM, from Table I). The plot indicates that CaPP$_i$ is strongly bound to the enzyme, for almost all of the first PP$_i$ addition (final concentration 2.7 μM) was bound by the 2.5 μM enzyme, so that little $^{32}$PP$_i$ was free to dialyze. Subsequent PP$_i$ additions increased the concentration of unbound PP$_i$ sufficiently to give a dialysis rate near that of the control. The binding data, after correction as previously described (9), is presented in the form of a Scatchard plot in Fig. 3. The value for the apparent dissociation constant of the CaPP$_i$-enzyme complex is $7 \times 10^{-2}$ M as determined from this plot. However, CaPP$_i$ is bound so tightly that measurements are experimentally possible (because of the limiting specific radioactivity of $^{32}$PP$_i$) only in the range of CaPP$_i$ concentration near saturation of the enzyme. Estimation of the value of the dissociation constant thus necessitated a rather long extrapolation of the data (Fig. 3). In other experiments values as high as $7 \times 10^{-8}$ M were found for the apparent $K_D$ because of the experimental limitations this should be considered as an upper limit.

Although the tightness of the binding results in uncertainty in the precise value of the dissociation constant, it facilitates determination of the number of binding sites. In several experiments the number of binding sites was observed to be 2.0 ± 0.1 per mole (Fig. 3).

The apparent lack of binding of free PP$_i$ makes feasible a complementary experiment, binding of the $^{40}$CaPP$_i$ complex in


Fig. 3. Scatchard plot of CaPP\textsubscript{1} binding. After correction and calculation of free ligand concentration as previously described (9), the data of Fig. 3 are presented in the form of a Scatchard plot (16).

the presence of a large excess of PP\textsubscript{i} so that effectively all of the \textsuperscript{45}Ca\textsuperscript{2+} is complexed. In a dialysis rate experiment using 14 \textmu M enzyme in 0.1 M Tris buffer, pH 7.05, containing 10 mM PP\textsubscript{i}, titration with \textsuperscript{45}Ca\textsuperscript{2+} resulted in no detectable binding of the CaPP\textsubscript{i} complex. Thus under these conditions (no free Ca\textsuperscript{2+}, all Ca\textsuperscript{2+} present as CaPP\textsubscript{i}) the enzyme apparently binds the CaPP\textsubscript{i} complex very weakly if at all (apparent \( K_D > 10^{-4} \)).

**DISCUSSION**

It was known that the enzyme binds divalent metal ions even in the absence of PP\textsubscript{i}, for Cohn (5) observed enhancement of the rate of relaxation of water protons by Mn\textsuperscript{2+} in the presence of the enzyme but absence of PP\textsubscript{i} and we\textsuperscript{1} have noted enhanced stability of the enzyme on addition of small amounts of Mg\textsuperscript{2+}. It was originally intended to measure binding of \textsuperscript{45}Ca\textsuperscript{2+} directly, and binding of other divalent metal ions by competition with \textsuperscript{45}Ca\textsuperscript{2+} (17). However, binding experiments utilizing equilibrium dialysis and dialysis rate techniques indicated that Ca\textsuperscript{2+} is rather weakly bound to the enzyme (\( K_D > 0.1 \text{ M} \)) compared to other divalent metal ions. Thus routine measurements of binding of \textsuperscript{45}Ca\textsuperscript{2+} in competition with other divalent metal ions would require large amounts of enzyme and lack the desired precision.

Although only a moderate degree of protein fluorescence quenching is observed on binding divalent metal ions, the effect is reproducible and adequate for determination of dissociation constants by fluorescence titration. The fluorescence spectrum is typical of tryptophan-containing proteins (13); presumably the quenching is due to alteration of the environment of 1 or more tryptophan residues on binding of divalent metal ions.

The binding of free metal ions to the enzyme has also been determined by Cooperman,\textsuperscript{2} measuring the relaxation rate of the nuclear magnetic resonance of water protons in the presence of paramagnetic ion Mn\textsuperscript{2+}, and with binding of other metal ions determined by competition with Mn\textsuperscript{2+}. Cooperman's results, presented in Table I, agree exactly with the values obtained by fluorescence titration with respect to the relative strength of the binding of Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, and Zn\textsuperscript{2+}. The \( K_D \) values obtained fluorometrically for these metals have values approximately 5-fold smaller than those obtained by the magnetic resonance technique; this is likely due to differences in the conditions of the binding measurements. In particular, it appears from limited measurements made at high ionic strength that K\textsuperscript{+} from the KCl used to adjust the ionic strength competes very weakly with the divalent cations for the binding site; however, K\textsuperscript{+} does not significantly affect the enzyme's fluorescence. For Ca\textsuperscript{2+}, the \( K_D \) as measured fluorometrically is 5-fold greater, rather than 5-fold less, than that observed with the magnetic resonance technique. The reason for this difference is unknown, but it may be related to the fact that Mg\textsuperscript{2+}, Mn\textsuperscript{2+}, and Zn\textsuperscript{2+} are activators, and Ca\textsuperscript{2+} is an inhibitor. It is conceivable that the mode of binding Ca\textsuperscript{2+} is somewhat different from that of the other metals, causing fluorescence quenching only when a second, less tight binding site is filled. It was also noted that at relatively high concentrations (approximately 1 mM) Zn\textsuperscript{2+} causes an enhancement of protein fluorescence, reversing the quenching observed at much lower Zn\textsuperscript{2+} concentrations. This enhancement was observed only with Zn\textsuperscript{2+}. In general agreement the between the two types of binding measurements is good, considering the independence of the techniques and the difference in conditions of measurement. The apparent strength of Mg\textsuperscript{2+} binding determined statically is much greater than that determined kinetically. This is partially due to the much higher ionic strength in which the kinetic measurements were made; other possible reasons for the apparent difference are discussed in the following paper (7).

Comparison of the relative strength of binding divalent cations by the enzyme with the relative rates of the enzymatic reaction as activated by the same divalent cations reveals an inverse relationship. The most strongly bound of the activating ions, Mn\textsuperscript{2+}, is the least effective activator, and Mg\textsuperscript{2+}, which produces the highest rates, is the most weakly bound. Estimation of the dissociation constant for Mg\textsuperscript{2+} from kinetic measurements indicates even weaker binding than shown here (7). This study demonstrates that, at least, tight binding of divalent metal ions to the enzyme is not an adequate description of the role of the metal ion. Moreover, the relatively weak binding of free Ca\textsuperscript{2+} does not account for its strong inhibition of the reaction (8), suggesting that inhibition is due to a CaPP\textsubscript{i} complex rather than to free Ca\textsuperscript{2+}. This suggestion is consistent with the observed tight binding of CaPP\textsubscript{i} in the presence of excess Ca\textsuperscript{2+}, and is fully confirmed by the kinetic studies of CaPP\textsubscript{i} inhibition (8).

The binding of CaPP\textsubscript{i}, in the presence of excess Ca\textsuperscript{2+} can be analyzed according to the following scheme:

\[
E \overset{K_{CaPP_1}}{\rightleftharpoons} E(CaPP_1) \overset{K_{CaPP_1}}{\rightleftharpoons} E(CaPP_i)(CaPP_1)
\]

where \( K_{apparent}^{CaPP_1} = K_{CaPP_1}^{apparent} \left[ 1 + \frac{K_{CaPP_1}}{[Ca^{2+}]} \right] \).

Using the observed value of \( 8 \times 10^{-4} \text{ M} \) for \( K_{CaPP_1} \), the dialysis rate binding study, which was carried out at \( 6 \times 10^{-4} \text{ M} \ Ca^{2+} \), gives a value for the true dissociation constant, \( K_{CaPP_1} \), of \( 6 \times 10^{-9} \text{ M} \). This value does not take into account the various ionic forms of CaPP\textsubscript{i} which are present at pH 7.4, but is a composite of the values for each of the species present.

The fluorometric titration does not permit direct determination of the number of binding sites for metal ion per mole of enzyme. However, Cooperman\textsuperscript{3} found two sites per mole for binding.

\textsuperscript{2} B. Cooperman, personal communication.

\textsuperscript{3} B. Cooperman, personal communication.
divalent metal ions, and we find two binding sites per mole for the CaPP_i complex using flow dialysis (Fig. 3). It has been reported that this enzyme is a dimer (18, 19) and we have observed that it contains 2 apparently identical subunits (1). Thus there is good agreement between the number of binding sites for metal ion and CaPP_i, and the number of subunits.

Although free PP_i has been postulated to be an inhibitor of the reaction (7), our studies suggest that free PP_i is not bound by the enzyme in the absence of divalent metal ions. In order to prevent PP_i hydrolysis while making these measurements it was necessary to carry out these studies of the binding of free 3’PP_i in the presence of chelators, so that the apparent lack of binding could conceivably be due to competition by chelator for the PP_i binding site. Indeed, fluorometric evidence was obtained for binding of EDTA to the enzyme in the absence of added divalent cations (1). In contrast, the other chelator employed in these studies, 8-hydroxy-5-quinoline sulfonic acid, appears not to bind to the enzyme, as judged by several criteria including polarization of fluorescence of the chelator molecule, which should be strongly affected by binding to the enzyme. The chelators were similarly effective in preventing 32PP_i hydrolysis; the large difference in their chemical structures makes it unlikely that both effectively compete with PP_i for a site on the enzyme. Thus it appears likely that their common effect is scavenging of traces of endogenous divalent metal ions which would otherwise allow PP_i hydrolysis to take place, rather than the prevention of PP_i binding by competing for a PP_i binding site.

These results suggest that of the several components of the equilibrium between divalent metal ions and PP_i which are present during enzymic PP_i hydrolysis, only free metal ions are bound by the enzyme in a simple straightforward manner. Binding of other components of the equilibrium, although quite strong, must be preceded by binding of divalent metal ion. This apparent lack of binding free PP_i or CaPP_i in the absence of free divalent metal ion is consistent with the kinetic model proposed by Moe and Butler (7) in which free metal ion must bind to the enzyme before substrates or inhibitors can be bound. Binding and kinetic measurements do not exclude the binding of free PP_i; what is shown by these studies is that PP_i can bind to the enzyme only after a metal ion has bound. In the following papers kinetic evidence for binding of free PP_i, competitive with binding of metal ion-PP_i complexes, will be presented.

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