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FLUORIDE INHIBITION OF INORGANIC PYROPHOSPHATASE

IV. EVIDENCE FOR METAL PARTICIPATION IN THE ACTIVE CENTER AND A FOUR-SITE MODEL OF METAL EFFECT ON CATALYSIS

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Summary

Atomic spectroscopy of native yeast inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) after gel filtration showed that it only binds activating Mg^{2+} in an easily dissociable manner. Formation of a covalent intermediate between the enzyme and an entire substrate molecule in the presence of fluoride, however, dramatically strengthened the binding of two Mg^{2+} per subunit and eliminated at neutral pH the effect of added metals on protein fluorescence but not on the absorption spectrum, suggesting that different metal binding sites influence the two spectra. This conclusion was confirmed by spectral studies on native enzyme. A third, low-affinity site for Mg^{2+} was found on the enzyme at pH >8. A model of enzyme-substrate-metal interactions was proposed, according to which the fluorescence-controlling site belongs to the active center and substrate can only be bound to it as a 1 : 1 complex with metals.

Introduction

It is becoming more and more apparent that metal ions play a key role in catalysis by metal-activated enzymes and this role is often not confined to substrate activation. Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) furnishes a good example of this. Kinetic studies of this typical metal-activated enzyme have revealed that at least three metal ions per active center participate in catalysis [1—3]. The results obtained by several

equilibrium methods are consistent with the idea of the multiplicity of metal-binding sites on the enzyme [3—10]. The location of these sites and the mechanism of their effect on catalysis, however, remain undefined. The same can be said about the majority of other protein catalysts depending on the presence of cations. This is largely explained by the fact that the enzyme-metal interactions occur in a rapid reversible manner and the effects of different metal sites on activity cannot be studied separately.

In this work, use was made of the unique ability of fluoride to stabilize the binding of Mg^{2+} at some of the available sties on pyrophosphatase for elucidating the role of metals in this enzyme. The results show that pyrophosphatase has three catalytically important metal sites per subunit, of which one belongs to the active center. This paper continues a series of works on inorganic pyrophosphatase inhibition by fluoride [11—13].

Materials and Methods

Materials

The sources of the enzyme and other reagents used in this work were described previously [11,12]. Buffer solutions employed in metal binding studies were prepared on bidistilled water and rendered metal-free by passing through a column with Chelex-100 resin (Bio Rad Laboratories).

Preparation of the covalent enzyme-substrate compound

This was done following, with slight modifications, the procedure described in [12]. A 0.45 ml portion of a solution containing 1 mM PP_i and 0.1 M NaF was added at room temperature to an equal volume of 0.01—1.5 mg/ml pyrophosphatase solution in 0.1 M imidazole-Cl buffer (pH 6.2) which contained 2 mM $MgCl_2$. The extent of substrate incorporation into the enzyme was estimated from its residual activity since the resulting enzyme-substrate compound is inactive [12]. For the highest protein concentrations used, it was found necessary to further add to the inactivation mixture several 50 μl aliquots of a 10 mM PP_i/20 mM $MgCl_2$ solution (pH $\approx$6) to achieve deep inactivation. The mixture was then applied to a 0.9 $\times$ 23 cm Sephadex G-50 (medium) column which had been preequilibrated, unless otherwise noted, with 0.1 M Tris-Cl buffer whose pH was 7.2 if measured at 25° C. The column was developed with the same buffer at 4° C at a flow rate of 0.6 ml/min. The void volume was discarded, and 1.5-ml portion of the eluate containing 90% applied protein was collected and used in further experiments.

Enzyme assay

An aliquot (50 μl, approximately 0.1 μg protein) of enzyme solution was added to 2 ml of reaction mixture which consisted of 0.1 M imidazole-Cl (pH 7.2), 1 mM PP_i and 2 mM $MgCl_2$ at 25° C. The reaction was stopped in 2 min by 1 ml of 0.15 M H_2SO_4. The amount of P_i liberated was measured by an automated procedure [1,3,11].

Spectral studies

Protein fluorescence was measured with an Aminco-Bowman SPF spectro-

photofluorimeter which was equipped with a deuterium lamp to enhance stability. Excitation and emission wavelengths were 255 and 355 nm, respectively.

Ultraviolet difference absorption spectra were recorded with a Spekord (Karl Zeiss, Jena) spectrophotometer using a 80—100% transmittance scale.

Results

Mg^{2+} content of native and F^--inactivated pyrophosphatase

It has been reported previously [11] that Mg^{2+}-supported PP_i hydrolysis by pyrophosphatase in the presence of fluoride is accompanied by gradual inactivation of the enzyme. The removal of the inhibitor and substrate by gel filtration did not result in immediate reactivation since 2 mol/mol PP_i and fluoride remained firmly bound to the enzyme [12].

The results presented in Fig. 1 shows that the F^--treated pyrophosphatase contains, in addition to PP_i and fluoride, appreciable amounts of gel filtration-resistant magnesium. When corrected for the background Mg^{2+} in the elution buffer, the mean content of the bound metal in the combined protein fraction was 3.7 ± 0.1 g-atoms per mol of molecular weight 65 000 [14—17]. This corresponded to 3.9 atoms per inactive enzyme molecule or 1.95 atoms per subunit. It should be pointed out that the contamination of the eluting buffer with Mg^{2+} was rather high although it had been treated with a chelating resin.

In a control experiment, Mg^{2+} content of native pyrophosphatase was determined. The enzyme was incubated with $MgCl_2$ and PP_i essentially under the same conditions as for Fig. 1, but in the absence of NaF, and subjected to gel filtration. The enzyme was fully active after such treatment. The amount of the protein bound Mg was less than 0.1 g-atom/mol in this case. Since this was at variance with the results of Butler and coworkers [15] who reported that pyrophosphatase contains extremely tightly bound 'structural magnesium', the analysis of native pyrophosphatase for Mg^{2+} was repeated many times but always

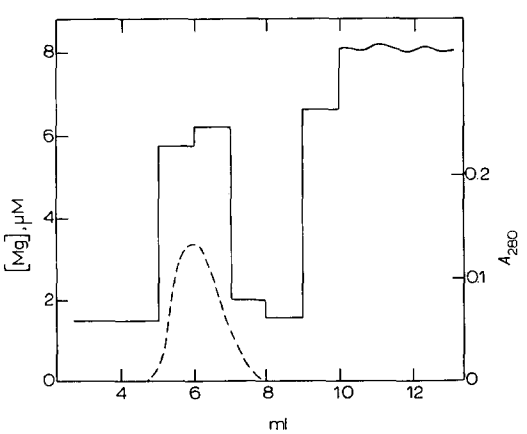

Fig. 1. Gel filtration of F^--inactivated pyrophosphatase. 0.16 mg inactive enzyme was prepared. Magnesium concentration in 1 ml fractions was determined by atomic absorption spectroscopy with a Hitachi 207 apparatus. Enzyme activity after gel filtration was 5% of the original value. Absorbance at 280 nm (- - - - -) was monitored using an LKB model II Uvicord.

with the same results. Higher specific activity of our enzyme preparation (1000 compared to 650 IU/mg [15]) may indicate higher degree of purity, and thus provide a reasonable explanation of the discrepancy.

Since pyrophosphatase inactivation by fluoride is accompanied by substrate incorporation into enzyme active site [12], it is natural to assume that the tightening of protein-metal bonds in F^--treated enzyme was due to direct shielding effect of the bound PP_i molecule. This suggestion was confirmed by the observation that enzyme-substrate compound decomposition rendered the bound metal readily dissociable. Thus, incubation of F^--inactivated pyrophosphatase for 4 h at 20° C after removal of the substrate, inhibitor and metal excess led to almost complete dissappearance of the bound substrate and metal, as determined by gel filtration analysis.

Spectral properties of the metal complexes of the enzyme and enzyme-substrate compound

It is known that metal binding changes the absorption [6,9] and fluorescence emission [4] spectra of pyrophosphatase. Interestingly, the dissociation constants of the enzyme-metal complexes derived from these effects differ considerably. Thus, fluorimetry in Butler's group at pH near neutrality gave a value of 16 μM [4], while the absorption spectroscopy in Rapoport's [6] and our laboratories [9] yielded values of 150 and 110 μM, respectively, for Mg^{2+} complexes of pyrophosphatase. An even larger difference was observed for inhibitory cation, Ca^{2+}. The published values for the dissociation constants for it are 800 μM (fluorimetry [4]) and 19 μM (absorption spectroscopy [9]). These discrepancies might mean that different metal-binding sites influence upon the two spectra insofar as experiments using flow dialysis technique showed that pyrophosphatase does have two different sites for metals per subunit [6]. It was not clear, however, if one could compare the binding constants obtained in different laboratories.

To clarify this, the determination of the dissociation constants for several metals was performed by fluorimetric procedures and the results were compared with those obtained by Ridlington and Butler [4] who used the same method. It is seen from Table I that two sets of constants for Mg^{2+}, Ca^{2+} and Mn^{2+} are in fair agreement with each other. Only for Zn^{2+} was the difference beyond experimental error, but could be fully explained by the complexation of this cation by an unprotonated Tris species [18] whose concentration was smaller in the conditions used in this work (lower buffer concentration, pH and temperature). The results of this experiment show, therefore, that the binding properties of different pyrophosphatase preparations are identical and confirm the suggestion that the fluorescence and absorption spectra of this enzyme are controlled by different metal-binding sites. Below, we shall refer to these sites as sites μ_1 and μ_2, respectively.

Studies of the Mg^{2+}-promoted protein spectrum alterations at pH 8.5 revealed the existence of one more metal-binding site with much lower affinity (site μ_3). Addition of 10 mM Mg^{2+} to the enzyme equilibrated with 0.5 mM Mg^{2+} did not alter the protein absorption spectrum at pH 7.2 (Fig. 2a) because site μ_2 was already nearly saturated. At pH 8.5, on the contrary, a marked effect was observed over a broad wavelength range. A dissociation

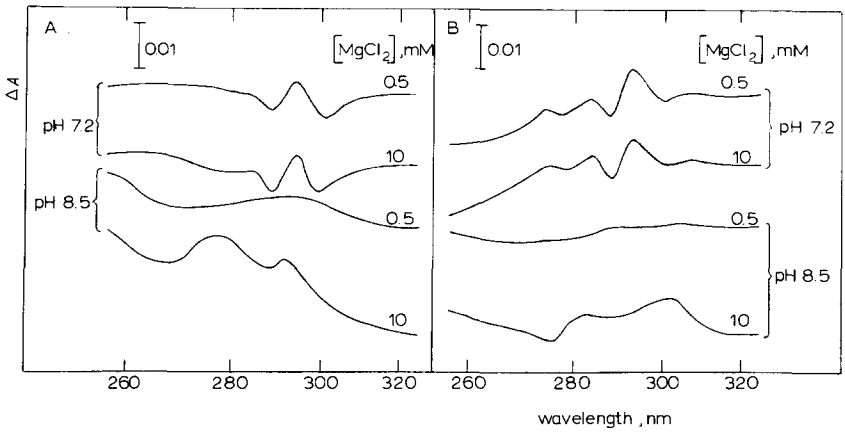

Fig. 2. Difference absorption spectra of native pyrophosphatase (a) and pyrophosphatase-substrate compound (b) generated by Mg^{2+}. Conditions: 25°C, 0.1 M (pH 7.2) or 0.2 M (pH 8.5) Tris-Cl, 0.7 mg/ml enzyme ($A_{280} = 1.0$). The enzyme-substrate compound with 25% residual activity was prepared; 0.2 M Tris-Cl (pH 8.5 when measured at 25°C) was used for gel filtration of the protein employed in pH 8.5 experiments. Portions (0.5 ml) of the same enzyme solutions were placed into both cells of the spectrophotometer, the base line was adjusted, 2 μl of stock metal solutions and water were added into the measuring and reference cells, respectively, and spectra recorded. The lines shown in the figure are means of three independent measurements.

constant of 6 ± 2 mM was derived from the dependence of the effect size on $[Mg^{2+}]$. The fluorescence spectra responded to Mg^{2+} similarly. At a concentration of 10 mM, Mg^{2+} caused further quencing at pH 8.5 compared to that at 0.5 mM Mg^{2+} with no change in the maximum wavelength. The quenching was not the result of absorption spectrum changes since the signs of the two effects were opposite. No similar change in fluorescence was observed at high Mg^{2+} level at pH 7.2. The values of quenching at saturation and dissociation constant were determined at pH 8.5 graphically and found to be 5% and 8 ± 2 mM, respectively. The identity of the dissociation constants for the enzyme-metal complexes derived from the fluorescence and absorption spectroscopy at high metal concentrations means that the same site is involved.

Analysis of the Mg^{2+} effect on the absorption spectrum revealed that sites μ_2 and μ_3 are vacant in the F^--stabilized enzyme-substrate compound (Fig. 2b). The change in 285—300 nm range accompanying addition of Mg^{2+} to the enzyme-substrate compound at pH 7.2 is quite characteristic of μ_2 site. Here again, the spectra at 0.5 and 10 mM Mg^{2+} were identical. The effect observed at high Mg^{2+} concentration at pH 8.5 was not so characteristic but its mere existence indicated that one more site, presumably μ_3, is free.

The fluorescence of the enzyme-substrate compound at pH 7.2, on the contrary, did not change on addition of 10 mM Mg^{2+} or Ca^{2+} and 0.2 mM Zn^{2+} or Mn^{2+}. Since these concentrations were high enough to saturate the enzyme at site μ_1 (see Table I), one can conclude that this site is occupied in the inactivated enzyme. At low concentrations ($\leqslant$0.5 mM) Mg^{2+} did not affect either the enzyme-substrate compound fluorescence at pH 8.5. Further increase in the metal level, however, caused quenching, which indicates that site μ_3 is free. The value of the dissociation constant governing the binding at this site was determined from this effect to be approximately 14 mM.

TABLE I

FLUORIMETRICALLY DETERMINED DISSOCATION CONSTANTS AND QUENCHING DEGREES FOR METAL (II) COMPLEXES OF NATIVE PYROPHOSPHATASE AND PYROPHOSPHATASE-SUBSTRATE COMPOUND

Conditions: 25°C, 0.1 M (pH 7.2) or 0.2 M (pH 8.5) Tris-HCl, 0.04—0.06 mg/ml enzyme. The enzyme-substrate compound was prepared by fluoride treatment as described in Methods with the only difference that the gel filtration of the enzyme used in pH 8.5 experiments was performed in 0.2 M Tris-Cl buffer (pH 8.5 when measured at 25°C). Protein fluorescence was recorded for 2—3 min after addition of increasing amounts of metals in portions of 2 μl to 1 ml of the enzyme. Mean results of 3—5 titrations were corrected for dilution and plotted according to Benesi and Hildebrand [19] to determine the dissociation constants and degrees of quenching at saturation. The values of these parameters for the second site (second line from bottom) were obtained similarly but in this case the size of the effect was corrected for the first site contribution by subtracting the quenching value at 0.5 mM $MgCl_2$.

Metal	pH	K_d (μM)	Quenching at saturation (%)
Mg	7.2	20 ± 5 (16) *	3.0 ± 0.3
Ca	7.2	1 400 ± 300 (800) *	8.0 ± 0.3
Zn	7.2	3.2 ± 1 (11) *	2.3 ± 0.5
Mn	7.2	2.1 ± 0.4 (2) *	4.0 ± 0.8
Mg	8.5	11.5 ± 1	5.0 ± 0.5
		8 000 ± 2000	5.0 ± 1.5
Mg **	8.5	14 000 ± 4000	3.7 ± 1.1

* The values in parenthesis were reported by Ridlington and Butler [4] (30°C, 0.24 M Tris-Cl, pH 7.4).
** For enzyme-substrate compound.

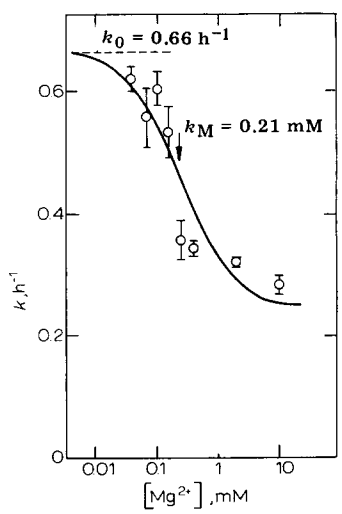

Fig. 3. Rate constant of F^--treated pyrophosphatase reactivation as a function of Mg^{2+} concentration. Conditions: 25°C, 0.1 M Tris-Cl (pH 7.2), 3 μg/ml enzyme. Inactive enzyme was prepared by fluoride treatment. Aliquots (0.3 ml) of the inactive enzyme solution were placed into small tubes and kept at 4°C. Calculated amounts of stock $MgCl_2$ solution in 0.1 M Tris-Cl (pH 7.2) were added to give a final volume of 0.8 ml, and the tubes were transferred to a water bath at 25°C. After temperature equilibration for 3 min (this moment was taken as zero time point in further calculations), reactivation progress was followed for 4—5 h by taking 50 μl aliquots and assaying them for enzymic activity. The rate constant, k, was computed from the equation $x - x_0 = (x_\infty - x_0)(1 - e^{-kt})$, where x_0, x and x_∞ refer to enzyme activity at time 0, t and infinity, respectively. The values of x_0 were usually 5—10% of x_∞ and the number of points was 8—12. Cross bars indicate the range of variation in 2—3 parallel runs. The solid line was calculated from Eqn. 3.

These results support the above suggestion that sites μ_1 and μ_2 are different and indicate that site μ_1 is occupied by one of the two Mg^{2+} per subunit in the F^--stabilized enzyme-substrate compound. Since two other metal-binding sites (μ_2 and μ_3) were free and there are no more such sites on the enzyme [6] one is bound to assume that the second metal ion in the enzyme-substrate compound originated from the $MgPP_i$ complex.

Reactivation of F^--treated pyrophosphatase in the presence of metals

The rate at which F^--inactivated pyrosphosphatase restores its activity after removal of inhibitor excess had been found previously [12] to depend on Mg^{2+} concentration. Here, this phenomenon was studied in more detail to obtain additional information about the nature of the vacant metal-binding sites in the enzyme-substrate compound.

Fig. 3 shows the dependence of the first-order reactivation rate constant on Mg^{2+} concentration at pH 7.2. These data may be interpreted in terms of a typical saturation scheme:

$$\overset{k_0}{\leftarrow} E_i \overset{K_M}{\rightleftharpoons} E_iM \overset{k_M}{\rightarrow} \quad (1)$$

were E_i refers to inactive enzyme, K_M is the dissociation constant for its complex with a metal and k_0 and K_M are the rate constants for the reactivation of the free and complexed enzyme, respectively. Similar dependences were obtained for two other activating cations, Mn^{2+} and Zn^{2+}, and an inhibitory cation, Ca^{2+}. It was found, however, that although all the activating metals slowed down the reactivation, the inhibiting one accelerated it. The observed rate constant for reactivation, k, may be calculated from the following equation [20]:

$$k = \frac{k_0 K_M + k_M[M]}{K_M + [M]} \quad (2)$$

The rate constant k_0 could be obtained at $[M] = 0$. The constants k_M and K_M could be evaluated from the kinetic data by regression analysis [21] using a rearranged form of Eqn. 2:

$$\frac{k}{k_0}[M] = K_M + \frac{k_M}{k_0}[M] - \frac{k}{k_0}K_M \quad (3)$$

The dissociation and rate constants for the four metals obtained in this way are given in Table II. The values of K_m for Mg^{2+}, Ca^{2+} and Zn^{2+} compared favourably with the corresponding dissociation constants for site μ_2 derived from absorption spectroscopy (110, 18 and less than 20 μM, respectively [9]). In case of Mn^{2+}, the spectroscopically determined binding constant was much higher (13–45 μM [6,9]). This may mean that addition of this metal to site μ_2 is sensitive to the presence of substrate in the enzyme active center. It is also likely that the constant obtained from spectral studies had been overestimated because the binding at site μ_1 with the dissociation constant of 2 μM (Table I) had not been considered in those experiments [6,9].

The existence of one more vacant metal-binding site on the enzyme-substrate compound stabilized by F^- could be inferred from analysis of a series of k

TABLE II

RATE AND DISSOCIATION CONSTANTS FOR THE REACTIVATION OF F^--TREATED PYROPHOSPHATASE IN THE PRESENCE OF VARIOUS METAL IONS

Conditions: 25°C, 0.1 M Tris-Cl (pH 7.2), 3 μg/ml enzyme. Reactivation rate measurements and data treatment were performed as described in Fig. 3 and text.

Metal	K_M (μM)	k_M (h^{-1})
Mg	210 ± 50	0.25 ± 0.03
Ca	7 ± 2	1.3 ± 0.15
Zn	10 ± 2	0.29 ± 0.03
Mn(II)	0.5 ± 0.2	0.29 ± 0.06

versus [M] plots obtained at several pH values (Fig. 4). The binding of Mg^{2+} to this site caused marked enhancement of reactivation, in contrast to what was observed for site μ_2. A similar dependence of the reactivation rate constant on $[Mg^{2+}]$ at pH 8.5 was observed in earlier kinetic studies of fluoride effect on pyrophosphatase reaction [11]. The instability of the reactivated enzyme in the absence of Mg^{2+} at all pH values, except for pH 8.5, and complexity of corresponding equations made it impossible to determine the kinetic and thermodynamic parameters for reactivation from Fig. 4 in the same way as for

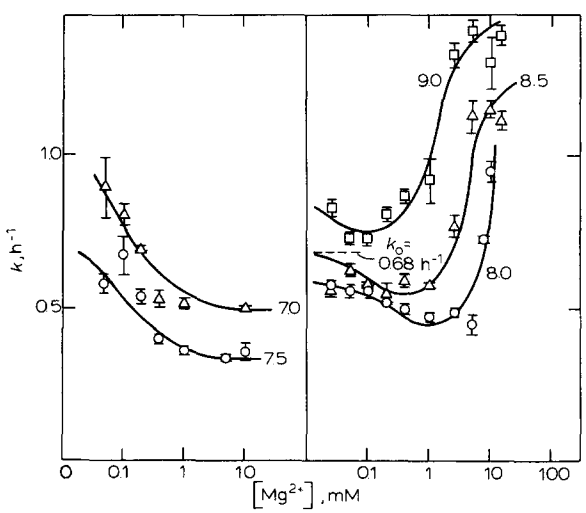

Fig. 4. Rate constant of F^--treated pyrophosphatase reactivation as a function of Mg^{2+} concentration at several pH values. Conditions: 25°C, 10 mM Tris + 10 mM 2-(*N*-morpholino)ethanesulfonic acid, pH as indicated at the curves, 0.1 M ionic strength (tetramethylammonium iodide), 3 μg/ml enzyme. Inactive enzyme was prepared by fluoride treatment as described in Methods, except that the mixture of 10 mM Tris and 10 mM 2-(*N*-morpholino)ethanesulfonic acid (pH approx. 7) was substituted for the Tris-Cl buffer for chromatography. Aliquots (0.3 ml) of the inactive enzyme solution were placed into small tubes and kept at 4°C. Calculated amounts of stock $MgCl_2$ and tetramethylammonium iodide solution in the same buffer were added and the pH was adjusted to a value corresponding to desired pH at 25°C using microliter quantities of KOH solution. A GK 2320C combination glass electrode and PHM 26 pH-meter (Radiometer, Copenhagen) were used for pH measurements. The tubes containing 0.8 ml fluid were transferred to a water bath at 25°C. Activity assay and the computation of rate constants were performed as described in Fig. 3. Changes in pH in the course of reactivation were less than 0.02 unit. The solid lines were drawn by eye. The value of k_0 was measured and shown for pH 8.5 only.

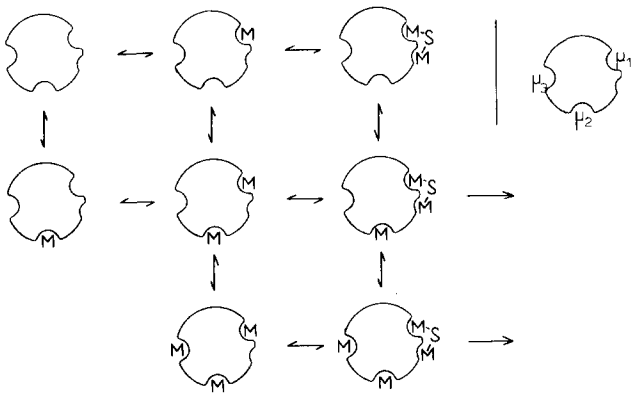

Fig. 5. A scheme of inorganic pyrophosphatase interactions with substrate and activator showing relative localization of substrate and metal sites on enzyme subunit. On the right hand side, the sites are designated as in the text.

Fig. 3. It appeared, however, that the dissociation constant for the second site lay in a millimolar range and increased when pH was decreased. This suggested the involvement of site μ_3 found on the native enzyme by spectral methods. It should be mentioned that the reactivated pyrophosphatase was stable if even only 25 μM Mg^{2+} were added to the reactivation mixture. This indicated that the occupancy of site μ_1 afforded protection against thermal inactivation of pyrophosphatase.

Discussion

Earlier kinetic studies [2,22,23] showed the yeast inorganic pyrophosphatase exists during catalysis as a mixture of several species differing in substrate and metal content. One, however, could only specular about the pathways of those species formation. This resulted from the fact that the substrate of this reaction forms complexes of two types with activating metal ions (MS and M_2S). As a consequence, steady-state kinetic experiments could only reveal the stoichiometry of catalytically-important enzyme-substrate-metal complexes formed but failed to determine if some particular metal ion in them is bound as itself or as a part of the complexes with substrate.

In the present work, the importance of various hypothetical routes leading to the reactive enzyme-substrate-metal complexes was evaluated using the unique ability of fluoride to selectively stabilize the EM_2S species to such an extent that no marked substrate and metal dissociation occurs from it during the time needed for binding experiments. Basing on the results obtained, a new interpretation can be given of earlier kinetic data [2,22,23] as shown in Fig. 5. The new scheme includes as variants the schemes proposed earlier for acidic [2] and alkaline [23] pH, in the sense it contains all complexes of different stoichiometry from the partial schemes, and is therefore consistent with the kinetic data from which they were derived. The main pecularity of the scheme in Fig. 5 is that substrate is supposed to bind only as a 1 : 1 metal complex and all ternary combinations are obtained by addition of metal ions to three

enzyme sites, of which two are essential for substrate binding (μ_1) and conversion (μ_2), respectively, and the third one (μ_3) only affects both processes.

The inertness of the uncomplexed substrate is supported by the following line of reasoning. The earlier suggestion [2,22—24] that the free substrate can interact with pyrophosphatase was based on the assumption that the EM_2S species results from EM_2 and S. If so, two metal-binding sites belonging to the enzyme must have been filled up in EM_2S. Our data, however, indicate that only one site (μ_1) is occupied, which means that the second metal in EM_2S comes from MS. In other words, EM_2S originates from EM and MS and is further converted into a reactive complex by means of metal addition to site μ_2 rather than to the bound substrate.

The inertness of M_2S, although less evident, is quite probable. First of all, it seems to be more economical to attribute the change in activity at high Mg^{2+} concentrations only to the influence of μ_3 site, insofar as spectral studies have revealed protein structure alterations upon metal binding to it. Besides, the values of the equilibrium constants for metal addition to EM_2S at pH 9.0 derived from the steady-state kinetics of pyrophosphatase activation by Mg^{2+} [25] using the scheme shown in Fig. 5 (4.7 and 5.4 mM for PP_i and tripolyphosphate as substrates, respectively) and from fluorimetry (Table I) are quite close. The independence of this constant on substrate nature, as follows from activation kinetics, is also consistent with the idea that the site other than that formed by the substrate is involved. And finally, the virtual equality of the binding constants for MS and M_2S obtained by the treatment of the kinetic data in terms of a 'two-substrate' model [23,25] is difficult to reconcile with the strict enzyme specificity. It should be pointed out that the proposed scheme does not exclude the binding of M_2S to the enzyme species in which site μ_1 is free. These routes are, however, of little importance because the proportion of such species at the metal concentrations which are necessary to produce M_2S is very small.

It should be noted that the scheme in Fig. 5 is formulated for pyrophosphatase subunit. Although this enzyme is dimeric, the subunits are identical and metal- and substrate-binding sites of different subunits seems not to interact as follows from dialysis [6] and kinetic [2,22,23] experiments, respectively. The extent of interaction, if any, between the three metal-binding sites in the same subunit is unknown because their affinities to cations differ considerably and they are virtually filled up one after another when metal concentration is increased.

The results obtained in this work shed light on the relative localization of the substrate and metal-binding site on the enzyme surface. Dramatic tightening of the bonds attaching metal to site μ_1 upon covalent binding of the substrate may mean that this site belongs to the active center. An alternative explanation involving burial of site μ_1 due to a probably conformational change in the protein seems less likely. Really, structure alterations must be quite peculiar in this case in that they must involve both the inactive center where the metal originated from MS is located and some remote metal-binding site, and virtually not affect sites μ_2 and μ_3. Small, if any, effect of the substrate upon metal binding to the latter sites suggests that they are at some distance from the active center. Thus, the dissociation constant for Mg^{2+} binding at site μ_2 is

110—140 μM in the free enzyme [6,9] and 210 μM in the enzyme-substrate compound (K_M in Table II) at pH 7.2. Besides, the equilibrium constants for Mg^{2+} binding at site μ_2 in EM and EM_2S species derived from steady-state treatment of enzyme kinetics are quite close [2,23,25]. The binding at μ_3 is also only slightly affected by the substrate (Table I and text). The dependence of enzyme activity on sites μ_2 and μ_3, which do not belong to the active center, is best explained in terms of their effect on protein conformation.

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