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Mg^{2+} activation of *Escherichia coli* inorganic pyrophosphatase

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Abstract Further refinement of X-ray data on *Escherichia coli* inorganic pyrophosphatase [Oganessyan et al. (1994) FEBS Lett. 348, 301–304] to 2.2 Å reveals a system of noncovalent interactions involving Tyr^{55} and Tyr^{141} in the active site. The pK_a for one of the eight Tyr residues in wild-type pyrophosphatase is as low as 9.1 and further decreases to 8.1 upon Mg^{2+} binding, generating characteristic changes in the absorption spectrum. These effects are lost in a Y55F but not in a Y141F variant. It is suggested that the lower-affinity site for Mg^{2+} in the enzyme is formed by Tyr^{55} and Asp^{70}, which are in close proximity in the apo-enzyme structure.

Key words: Inorganic pyrophosphatase; Tyrosine; Mg^{2+} binding; Differential spectrophotometry; X-ray analysis

1. Introduction

Inorganic pyrophosphatase (EC 3.6.1.1; PPase) hydrolyzes pyrophosphate (PP_i), providing thus a driving force for the synthesis of important biopolymers. PPase requires four Mg^{2+} ions per active site for activity. Two of them are bound directly to the enzyme and the remaining two are bound in the presence of the substrate PP_i [1,2]. The active site cavity contains fifteen polar groups, which presumably provide ligands for PP_i and Mg^{2+} and fulfill other catalytic roles [3,4]. However, the information on the role of Mg^{2+} in catalysis and the mode of its binding to protein is quite limited because the three-dimensional structure has been determined only for the apo-enzyme [3,4]. Estimates of the binding stoichiometry and affinities for Mg^{2+} (K_d of 0.08 and 1.7 mM) were obtained using equilibrium dialysis in combination with atomic absorption spectroscopy at pH 7.2 [2], one unit below the pH optimum of PPase activity.

In this paper, we present a new, spectrophotometric method for measuring Mg^{2+} binding to PPase, which in combination with the X-ray data refined to 2.2 Å allowed us to probe for the role of Tyr^{55} and Tyr^{141} residues in Mg^{2+} binding to the active site of *E. coli* PPase.

2. Experimental

Recombinant *E. coli* PPase was prepared as described previously [3]. Modified genes carrying the Y55F and Y141F substitutions were obtained using a two-step PCR-based targeted mutagenesis technique [5]. The synthesized fragments were treated by *Bam*H1 and *Eco*R1 to produce 0.8 kb fragments. The *Bam*H1–*Eco*R1 fragments were ligated into plasmid *pUC19*. The resulting recombinant plasmids were transformed into *E. coli* strain *JM109*. The mutations were checked by sequencing using the dideoxy-chain-termination method. Transformants were grown at vigorous stirring at 37°C in 2.5 l of the M9 medium containing ampicillin (50 μg/ml). The cells were pelleted, resuspended in 50 ml of 50 mM Tris-HCl (pH 7.5) containing 10% sucrose and disrupted by lysozyme. Nucleic acids were precipitated by slow addition of a one-third volume of 5% streptomycin sulfate. After a 20 min incubation at 0°C the precipitate was pelleted and discarded. The solution was placed on an AH-agarose column with 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM Mg^{2+}. The column was washed with the same buffer containing 0.2 M NaCl, and elution was carried out with 0.3 M NaCl; wild-type PPase left the column at 0.4 M NaCl.

Difference spectra were recorded in a Hitachi 254 double-beam spectrophotometer. Protein solution (0.2–1.2 mg/ml, 10–60 μM subunit) was in both sample and reference cells. $MgCl_2$ (0.01–1 M) or NaOH (0.4 M) solutions were added to the sample, and the same volume of the buffer solution was added to the reference cell. Volume changes during the titration were negligible.

PPase crystallization, X-ray data collection, processing and structure determination at 2.5 Å resolution were described earlier [3]. The structure was further refined using the PROLSQ program in the CCP4 suite, to the extension of resolution to 2.2 Å which allowed to reveal the parts invisible in the initial electron density. About 500 cycles of refinement with 'O' sessions after each 20 cycles were done. The final R-factor was 16% in the resolution range of 10–2.2 Å. Standard deviations for 1–2 and 1–3 bond lengths were 0.012 and 0.028, respectively. The model consists of 1,380 nonhydrogen atoms of the enzyme and of 115 solvent molecules per subunit.

3. Results and discussion

Three-dimensional structures of *E. coli* PPase previously published were of 2.5–2.7 Å resolution [3,4], which was not enough to identify interactions between all the amino acid side chains in the active site. In this work, we were able to extend the resolution to 2.2 Å by refinement of the previously collected data [3]. Fig. 1 presents a view of the active site with distances between polar groups. There are three Tyr residues, 141, 55 and 51, in the active site. Tyr^{55} and Tyr^{141} are involved into a branched system of noncovalent interactions formed by a great number of carboxyl groups – potential metal ligands. Tyr^{51} is somewhat more distant, and its hydroxy group seems to have no interactions with other groups, in accord with a small effect of its substitution by Phe on enzymatic activity [6]. Basing on the structure shown in Fig. 1, one would expect the acidity of the hydroxy groups of the two Tyr residues, Tyr^{55} in particular, changes in PPase.

Ionization of the tyrosine side chain can be monitored by measuring an increase in absorbance at 292 and 243 nm [7]. *E. coli* PPase contains eight Tyr residues in each of its six subunits. The difference spectrum of the enzyme at pH 11.8 versus pH 6.5 (Fig. 2) is characteristic of Tyr ionization, and

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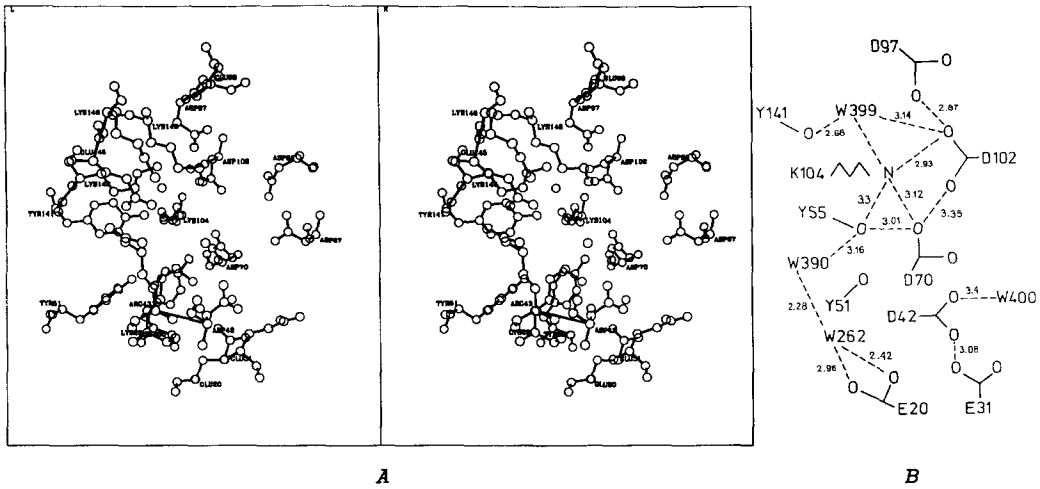

Fig. 1. (A) Stereo drawing of the active site cavity of *E. coli* PPase. (B) Scheme of the active site region; the figures indicate the distances less than 3.5 Å between groups.

the heights of the peaks at both 292 and 243 nm are consistent with the number of Tyr residues (only small contribution from the two SH-groups present in each subunit has to be taken into consideration for 243 nm) [7]. This observation is in contrast to the widely accepted opinion that a number of protein groups contribute into absorbance at low wavelengths and show that one can quantitate the number of Tyr residues from measurements at either 292 or 243 nm.

The acid-base titration curve shown in Fig. 3 indicates that in the absence of Mg^{2+} the first macroscopic pK_a for Tyr ionization in wild-type PPase is 9.1. An analysis of derivative spectra indicated that this pK_a belongs to one Tyr residue rather than being a consequence of a system of eight Tyr, each with a normal microscopic pK_a (data not shown). In the presence of 30 mM Mg^{2+}, the titration curve changes dramatically (Fig. 3), indicating a further decrease in the first pK_a value to 8.1. A clear shoulder on the titration curve obtained in the presence of Mg^{2+} confirms that this effect is caused by perturbation of only one Tyr residue. A pK_a value of 8.1 was also obtained in another titration, in which only one cuvet contained Mg^{2+} and alkali being added to both.

Based on these observations, a new method was developed to study Mg^{2+} interaction with PPase. In this method, increasing amounts of Mg^{2+} are added to the enzyme and difference spectra are recorded versus metal-free enzyme (Fig. 4A). The dissociation constant for Mg^{2+} binding to PPase can be determined from the hyperbolic dependence of the absorbance

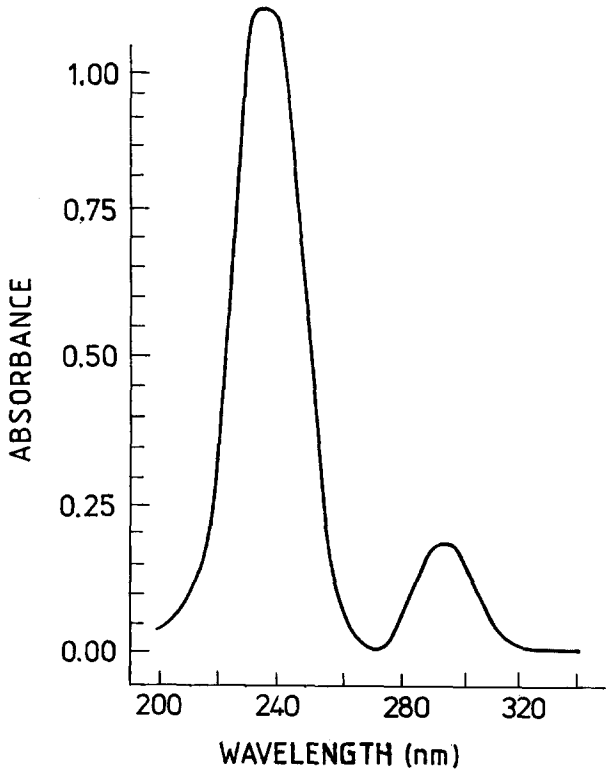

Fig. 2. Differential spectrum of *E. coli* PPase at pH 11.8 versus pH 6.5. Conditions: 10 μM enzyme subunit, 0.05 M bis-tris-propane, 23°C.

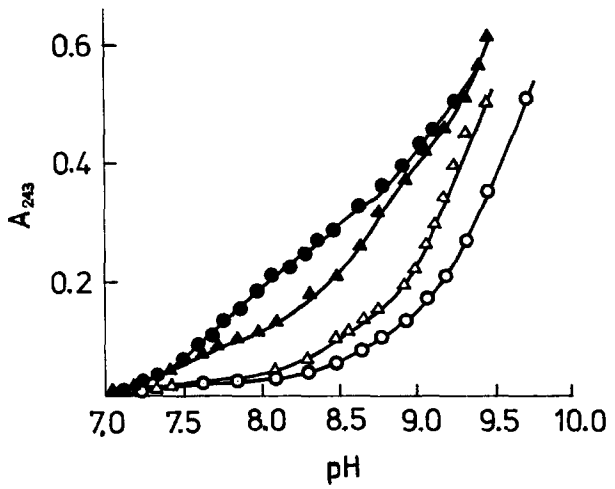

Fig. 3. Absorbance at 243 nm as a function of pH for wild-type PPase (circles) and Y141F PPase (triangles). The open and closed symbols refer to data collected in the absence and in the presence of 30 mM $MgCl_2$, respectively. pH was measured with glass electrode in a parallel larger-volume experiment. Conditions: 50 μM enzyme subunit; 0.05 M bis-tris-propane, 24°C.

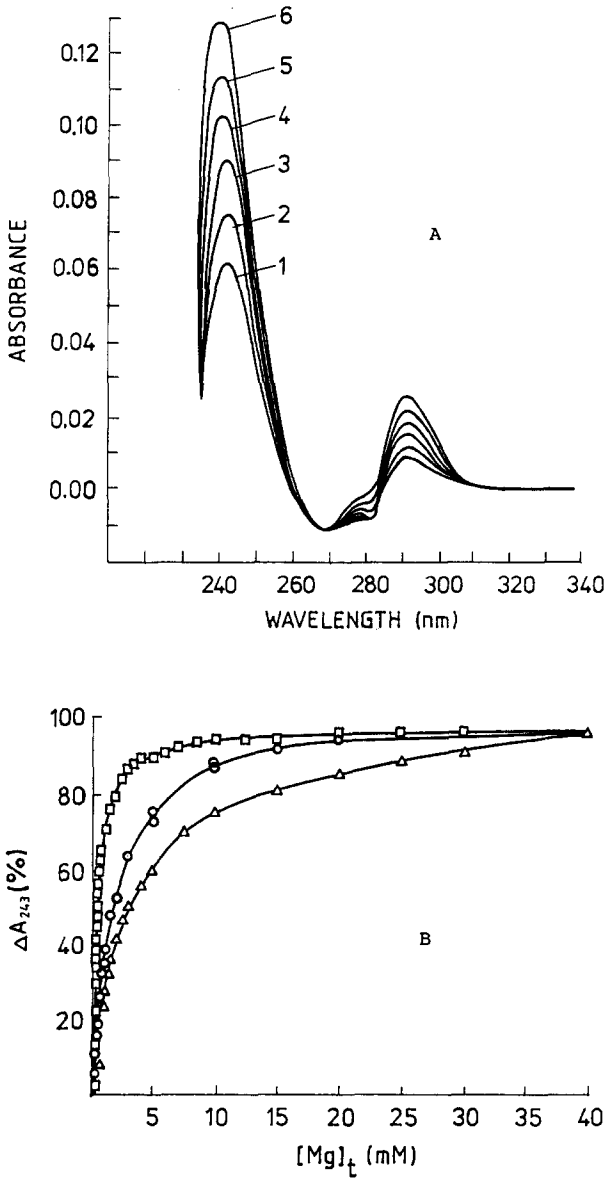

Fig. 4. (A) Typical difference spectra of PPase generated by $MgCl_2$: 1, 3 mM; 2, 5 mM; 3, 7.5 mM; 4, 15 mM; 5, 25 mM (5); 6, 50 mM. Conditions: 50 μM enzyme subunit, 0.1 M Tris-HCl, pH 8.0, 17.5°C. (B) The change in PPase absorbance at 243 nm as a function of $MgCl_2$ concentration added. 100% refer to the maximal value of ΔA_{243} at each pH value. Conditions: 20–60 μM enzyme subunit, 0.1 M Tris-HCl, pH 8.0 (triangles), 8.5 (circles) or 9.0 (squares).

change at 243 nm on Mg^{2+} concentration (Fig. 4B). Due to a large spectral effect (A_{243} increases up to 25% upon metal binding), accurate estimation of the binding constant is possible with only small amounts of enzyme which can be regenerated after measurements.

The values of the dissociation constant obtained in this way from the data shown in Fig. 4 are 2.42 ± 0.43, 1.46 ± 0.09 and 0.71 ± 0.04 mM at pH 8.0, 8.5 and 9.0, respectively. Based on these values, we believe that the spectral effect observed reports on the occupancy of the second, less tightly binding site for metal ions in PPase. The metal ion bound at this site is believed to activate a water molecule for a nucleophilic attack on PP_i [8]. Mg^{2+} binding to the high-affinity site does not markedly affect A_{243} at pH < 9. However, the presence of such site is supported by the clear sigmoidicity of the titration curves at low (<0.1 mM) metal ion concentrations, i.e., under conditions where a significant fraction of added metal is enzyme-bound.

To identify the Tyr residue whose pK_a changes on metal binding, two substitutions were made in *E. coli* PPase by site-directed mutagenesis, resulting in Y55F and Y141F variants. The titration behavior of Y141F PPase only slightly changed compared to wild-type PPase and exhibited same metal ion dependence (Fig. 3). By contrast, the Y55F variant exhibited much decreased A_{243} values at pH 7.0–8.7, which were absolutely Mg^{2+}-insensitive (data not shown). It is therefore concluded that Tyr^{55} is the residue that changes its pK_a on Mg^{2+} binding.

Stabilization of ionized Tyr^{55} is most likely attained via its coordination with Mg^{2+}. This explains why the maximum increase in absorption at 243 nm is always lower than expected for an unliganded Tyr [7]. The X-ray data indicate that the hydroxyl of Tyr^{55} is hydrogen bonded only to the carboxylate of Asp^{70} (Fig. 1). Therefore the participation of Asp^{70} as the second ligand for Mg^{2+} in the low-affinity site is quite possible. Coordination to two protein ligands, Tyr^{55} and Asp^{70}, would suffice to ensure a dissociation constant in the millimolar range observed for this site. Kinetic studies showed that substrate binding enhances enzyme affinity for Mg^{2+} essentially due to additional coordination of Mg^{2+} with the substrate oxygen atoms [1,2]. Addition of methylenediphosphonate, an unhydrolyzable substrate analog, to the Mg^{2+}-saturated PPase completely abolishes the Mg^{2+}-induced increase in Tyr absorption at 243 nm, suggesting that the Mg^{2+}–Tyr^{55} bond is lost in the enzyme-substrate complex.

At present, we use the new method to characterize the metal-binding properties of a number of variant PPases wherein active site carboxyls are replaced by their amides.

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