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Tyrosine-89 is important for enzymatic activity of *S. cerevisiae* inorganic pyrophosphatase

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7-Chloro-4-nitro-benzofurazan selectively modifies one PPase Tyr residue per subunit and lowers the enzyme activity. Hydrolysis of the modified protein by trypsin and then by chymotrypsin produces the 82–89 peptide which possesses modified Tyr-89. Substrate analog ($CaPP_i$) and the product of the enzyme reaction, MgP_i, protect the enzyme against inactivation. Ions of metal-activators (Mg^{2+}, Zn^{2+}) exert no influence on the inactivation rate. On the contrary, the Ca^{2+}-inhibitor of the enzyme accelerates the reaction by binding to the high-affinity site, and effectively decreases it when Ca^{2+} binds to both sites. Mg^{2+} competes with Ca^{2+} for one binding site, which is the low affinity site for Mg^{2+} and the high-affinity site for Ca^{2+}. The Ca^{2+} saturation of the high-affinity site decreases the pA'_2 of Tyr-89, probably due to direct coordination between Tyr and Ca^{2+}. The observed properties of Tyr-89 modification enable us to propose that Tyr-89 serves as a proton donor for phosphate releasing during enzymatic hydrolysis of pyrophosphate. The Ca^{2+} inhibitory effect on the enzyme activity may be due to the existence of a Tyr-89 bond in the Ca^{2+} pyrophosphatase complex.

Inorganic pyrophosphatase; Chemical modification; Essential groups; Me^{2+} affinity; *S. cerevisiae*

1. INTRODUCTION

Nowadays chemical modification remains the most appropriate method to determine essential amino acids of *S. cerevisiae* inorganic pyrophosphatase and to elucidate their roles in the active site. The enzyme catalyzes hydrolysis of inorganic pyrophosphate in the presence of divalent metal ions. A catalytically active complex maintains a pyrophosphate molecule and three metal ions, two of which bind to the enzyme before PP_i addition. Mg^{2+} and Zn^{2+} are the most effective activators, whereas Ca^{2+} inhibits the enzyme activity. The amino acid sequence and three-dimensional structure have been described.

The chemical modification method has allowed to reveal that Arg [1], Glu [2], and Lys [3] residues participate in the substrate catalytic conversion. Pyrophosphatase contains eleven Tyr residues, three of them are located in the active site [4]. It is important that the residues are invariant for all pyrophosphatases with known amino acid sequences [5,6]. However, the role of Tyr residues in the catalysis is still obscure.

In the present work the modification of a Tyr residue in *S. cerevisiae* inorganic pyrophosphatase (PPase) by 7-chloro-4-nitrobenzofurazane was studied. Also the influence of the substrate, enzyme reaction product, metal-activator and metal-inhibitor on this modification was investigated.

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2. MATERIALS AND METHODS

Inorganic pyrophosphatase with a specific activity of 700 IU/mg was isolated from *S. cerevisiae* [7]. The enzymatic activity was assayed as in [8].

7-Chloro-4-nitrobenzofurazan was obtained from Serva, Germany; acetonitrile from Merck, Germany; PP_i, β-mercaptoethanol, trypsin from Sigma, USA; triethanolamine, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$, KH_2PO_4, EGTA from Fluka, Switzerland. All other chemicals and biochemicals were home-produced.

The reaction of 0.25 mM PPase with 1–5 mM benzofurazan, dissolved in acetone, was carried out in 50 mM triethanolamine buffer pH 8.0 at 22°C. In the protection experiment, 1 mM $CaCl_2$ and 0.5 mM PP_i were added to enzyme solution before benzofurazan. At various times, aliquots were picked out and assayed for PPase activity. In some experiments the reaction mixture contained either $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$ or simultaneously $CaCl_2$ and $MgCl_2$, $CaCl_2$ and $ZnSO_4$, $MnCl_2$ and KH_2PO_4, or EGTA. The control experiments were performed without benzofurazane. The apparent rate constants were calculated from the dependence of $\ln(C_0/C)$ on time, where C was the residual activity.

To obtain modified peptides, 0.36 mM PPase was treated for 20 min with 3 mM reagent dissolved in acetone. The modified enzyme was separated from the reagent excess and reduced by dithionite [9]. The cysteinyl residues of the modified enzyme were pyridylethylated with 4-vinylpyridine [10]. Enzymatic cleavage with trypsin was carried out at 37°C for 12 h in 0.1 M ammonium bicarbonate at the enzyme to *S*-pyridylethylated protein ratio 1:100 (w/w). The digestion of the peptide with chymotrypsin was carried out at the enzyme to peptide ratio 1:50 (w/w). Peptides of enzymatic digests were fractionated by high-performance liquid chromatography on Ultrasphere Octyl 4.6 × 255 mm with a gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid over 60 min at the flow rate 0.7 ml/min. The peptides were visualized by measuring the absorbance at 214, 254 and 365 nm.

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The N-terminal sequences of the peptides were determined by automatic Edman degradation using an Applied Biosystem (USA) model 470A/120A sequencer.

The peptide M_r value assay was carried out on mass spectrometer MX3303 (Russia, Snt Peterburg). Ionization was performed by extraction of soluble ions at atmospheric pressure.

3. RESULTS AND DISCUSSION

The treatment of PPase with 7-chloro-4-nitrobenzofurazan leads to inactivation with simultaneous incorporation of the reagent into the enzyme. The processes are rather rapid and, for example, in the presence of 5 mM reagent the enzyme loses 90% of its activity in 13 min. Protection against the inactivation occurred upon the addition of $CaPP_i$.

It is known that benzofurazan can modify Tyr, Cys, Lys protein residues [11]. Derivatives of Tyr and Cys are labelled and their treatment with SH-compounds regenerates amino acids. The evidence for PPase tyrosine modification is based on two experimental facts. First, the inactive enzyme recovers its activity after β-mercaptoethanol action. Secondly, PPase, with its cysteine residues completely blocked by $HgCl_2$ [12], reacts with benzofurazan with the same rate as the native one.

To localize modified Tyr in the amino acid sequence, the inactive protein was digested with trypsin. Only one peptide with the bound reagent was registered after hydrolysate separation. It was absent in the case of PPase modification in the presence of $CaPP_i$. The high absorption of the modified peptide at 254 nm supported the presence of a Trp residue. The amino acid sequence of PPase contained six Trp residues, which were localized in six tryptic peptides (Fig. 1). After modification, one of them, containing the reagent, changed its time retention. As deduced from the primary structure, only one peptide of tryptic digestion has both Trp and Tyr residues. This peptide spans from Asn-82 to Lys-111: AsnCysPheProHisHisGlyTyrIleHisAsnTyrGlyAlaPheProGlnThrTrpGluAspProAsnGluSerHisProGluThrLys.

The peptide structure was confirmed by molecular mass assay and N-terminal sequence analysis. Two Tyr residues (Tyr-89 and Tyr-93) were observed in the peptide. But it was difficult to choose between them since tyrosine derivatives were not stable under N-terminal sequences determination [9]. Therefore peptide 82–111 was hydrolyzed by chymotrypsin, resulting in a modified new peptide 82–89, containing only one Tyr-89, separated and its structure proved by the sequence and M_r value assay using the mass-spectrometry method. Thus, we have shown that Tyr-89 is an essential residue of PPase. Addition of Mg^{2+} or Ca^{2+} to the reaction mixture during modification did not change the peptide composition.

Investigation of the effects of pH, P_i, metal-activators and metal-inhibitor on the reaction of PPase with benzofurazane has revealed some interesting features. The

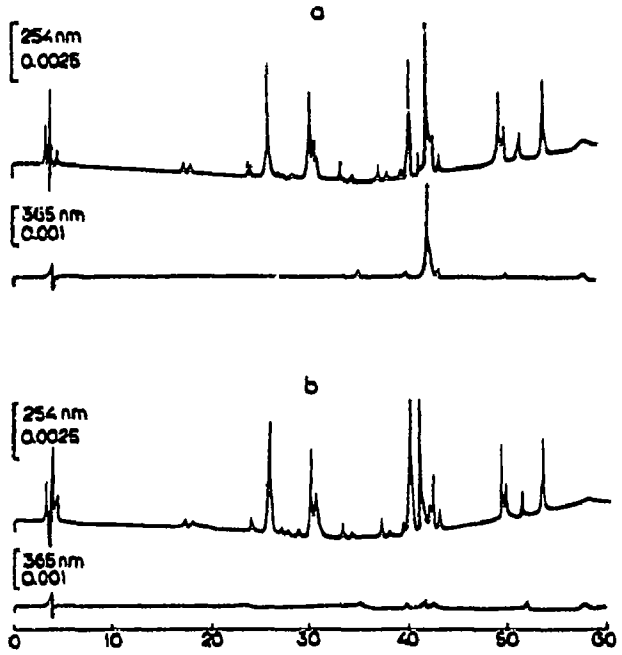

Fig. 1. Peptide separation after tryptic digestion. PPase modified without addition of $CaPP_i$ (a), in the presence of $CaPP_i$ (b).

values of the apparent rate constant are summarized in Table I.

(i) The ionic activators (Mg^{2+}, Zn^{2+}) did not protect the enzyme under the conditions of either low or high concentration (Exp. 2, 3, 10 and 11). Protection was observed only with Mn^{2+} which is the weakest activator of the enzyme. The opposite effect was found in the presence of Ca^{2+}. At a low concentration of Ca^{2+} during its binding to the high-affinity site, the inactivation rate clearly increased (Exp. 4). Increasing the Ca^{2+} level led to the saturation of the second binding site and at the same time decreased the inactivation rate, which be-

Table I

PPase inactivation by 7-chloro-4-nitrobenzofurazan in the presence of metal ions

Exp. no.	Composition of reaction mixture	k_{app} (min^{-1})	Exp. no.	Composition of reaction mixture	k_{app} (min^{-1})
1.	1 mM reagent			5 mM reagent	
	20 mM EGTA	0.038	9.	20 mM EGTA	0.172
2.	50 mM $MgCl_2$	0.038	10.	2 mM $MgCl_2$	0.172
3.	20 mM $ZnCl_2$	0.038	11.	2 mM $ZnCl_2$	0.172
4.	5 mM $CaCl_2$,	0.086	12.	100 mM $MnCl_2$,	
	2 mM reagent		13.	1 mM P_i	$\ll$0.017
5.	20 mM EGTA	0.073	14.	100 mM $MnCl_2$	0.046
6.	5 mM $CaCl_2$,			5 mM $MgCl_2$,	$\ll$0.017
	50 mM $MgCl_2$	0.172	15.	25 mM P_i	
7.	5 mM $CaCl_2$,			1 mM $CaCl_2$	0.038
	20 mM $ZnCl_2$	0.172	16.	1 mM $CaCl_2$,	
8.	5 mM $CaCl_2$,			0.5 mM PP_i	$\ll$0.005
	500 mM $MgCl_2$	0.080			

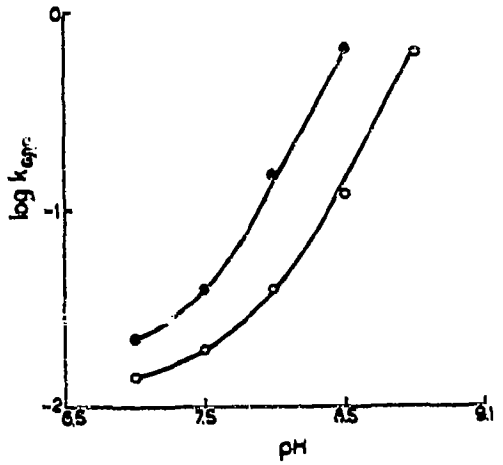

Fig. 2. Dependence of the rate constant of PPase inactivation on pH in the presence of 5 mM $CaCl_2$ (○) and without Ca^{2+} (●).

came lower than that for the holo-enzyme (compare Exp. 9 and 15). The experiments where the Ca^{2+} concentration was varied in the range of 1–2 mM allowed for the calculation of the dissociation constants of 5 mM and 500 mM, which were approximately the same as cited in the literature [13,14].

The Ca^{2+} effect did not disappear in the reaction mixture, containing $MgCl_2$ in a concentration sufficient for saturation of its high-affinity site (Exp. 6). The same was valid for Zn^{2+} (Exp. 7). It means that neither Mg^{2+} nor Zn^{2+} compete with Ca^{2+} at the high-affinity site, and that these sites are different. At the same time, the growth of Mg^{2+} concentration and its binding at the low affinity site blocked the Ca^{2+} effect (Exp. 8). This enables us to affirm that Ca^{2+} and Mg^{2+} (Zn^{2+}) have only one common binding site, for Ca^{2+} it is the site of high-affinity and of low affinity for Mg^{2+} or Zn^{2+}.

(ii) The Ca^{2+} saturation of the high-affinity site decreases the pK_a of the Tyr-89 hydroxyl group (Fig. 2), which concerts with the increasing rate of inactivation in the ion presence. The reason for this may be a direct Ca^{2+} coordination with the tyrosine protein residue. The inhibitory Ca^{2+} effect on the enzyme activity has been known for many years, but unfortunately its molecular mechanism was not understood. It cannot be excluded that Tyr-89 incorporation into the complex with Ca^{2+} prevents it from participating in catalysis.

(iii) The inorganic phosphate, the product of enzymatic pyrophosphate conversion, effectively protects the enzyme in the presence of Mn^{2+} when the concentration of P_i is sufficient for saturation of the high-affinity site (Exp. 12). A similar protective action is known for Arg-77 modification [1]. Consequently, Tyr-89 and Arg-77 are probably drawn together and located near the phosphate site.

(iv) The modified PPase possesses an absorption spectrum with λ_{max} = 345 nm, which is not typical for low molecular weight tyrosine derivatives. The hypochromic displacement by 35 nm indicates the absence of π-electron interaction between tyrosine and benzofurazane and shows a strict Tyr orientation in the active site.

(v) The PPase amino acid sequence maintains eleven Tyr residues, but PPase treatment with benzofurazane leads to modification of single Tyr-89. High Tyr reactivity demonstrates its increasing nucleophilicity, that is its high ability for deprotonation.

Combined experimental data permit to assume that Tyr-89 is strictly fixed at the active site near the P_i binding site. The ease of Tyr deprotonization allows for the formation of an H-bond between the Tyr hydroxyl group and the oxygen both of P_i and PP_i. Since phosphoric acid, liberated from the substrate is a weaker acid than pyrophosphate it can leave the active site only after binding of at least one proton. Probably Tyr-89 is a donor of this proton.

The importance of Tyr-89 for the enzyme activity is confirmed by recent results on site-directed mutagenesis of another enzyme, PPase from *E. coli* [15]. Unfortunately, the Tyr role remains undetermined. However, it should be noted that in spite of the high homology of two PPases from *S. cerevisiae* and from *E. coli*, the mechanisms of their action are similar but not identical. In any case, 7-chloro-4-nitrobenzofurazane under conditions appropriate for the modification of PPase from *S. cerevisiae* does not inhibit PPase from *E. coli*.

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