The role of inorganic pyrophosphatase regulatory centres in subunit interactions

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A baker's yeast inorganic pyrophosphatase derivative has been obtained in which one carboxyl group of each regulatory centre is converted to hydroxamate. This enzyme retains the capacity to hydrolyze the substrate while remaining a dimer. However, introduction of ligands to the active centre of one subunit of such enzyme does not affect the conformation of the other as is the case with native pyrophosphatase, i.e., the regulatory centre-modified enzyme behaves as a monomer. Thus, selective modification of the regulatory centres results in the disappearance of subunit interactions.

Inorganic pyrophosphatase *Regulatory center* *Subunit interaction*

1. INTRODUCTION

Baker's yeast inorganic pyrophosphatase (EC 3.6.1.1) catalyzes inorganic pyrophosphate hydrolysis in the presence of Mg^{2+}. The enzyme consists of two identical subunits, each containing an active and regulatory centre [1]. It is known that the monomer and dimer are comparable in activity [2]. Pyrophosphatase is characterized by the different behavior of the subunits in the modification of the active centres by phosphoric acid monoesters [3,4] or by hydroxylamine [5], and in a reaction of the regulatory centres with phosphate [6].

The aim of this work is to elucidate the role of the regulatory centres for subunit interactions. A modified enzyme has been studied in which one carboxyl phosphate acceptor group of each regulatory centre is converted to hydroxamate [7]. The data obtained support the suggestion about the involvement of the regulatory centres in subunit interactions.

2. MATERIALS AND METHODS

Baker's yeast inorganic pyrophosphatase with a specific activity of 650–700 IU/mg was isolated as in [8]. The enzymatic activity was assayed as in [4,5].

The preparation of pyrophosphatase with modified regulatory centres was carried out at 30°C by incubating 15–20 μM enzyme with 2 mM NaH_2PO_4 and 5 mM $MgCl_2$ in 0.05 M Tris-HCl (pH 7.2) for 15 min, and with 2 M hydroxylamine or *N*-[^{3}H]methylhydroxylamine for 50 min. The modified enzyme was isolated as in [7]. The molecular mass was determined according to [9].

3. RESULTS AND DISCUSSION

The pyrophosphatase with modified regulatory centres was obtained in a reaction of the phosphorylated protein with hydroxylamine in conditions at which this reagent cannot modify the active centres groups [7]. This derivative contains a hydroxylamine residue in each regulatory centre which is confirmed by incorporation of *N*-[^{3}H]methylhydroxylamine. Modified pyrophosphatase retains the capacity to hydrolyze pyrophosphate while remaining a dimer. A set of facts presented here demonstrates that selective modification of enzyme regulatory centres leads to

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disappearance of subunit interactions, i.e., this derivative exhibits properties similar to those of a monomer.

(i) The subunit interactions are clearly manifested under hydroxylamine-induced inactivation of the enzyme. Hydroxylamine, in the absence of Mg^{2+}, reacts with the active centre carboxyl group which is a protein ligand of the activator cation [10]. The inactivation does not exceed 50%, i.e., half of the enzyme centres exhibit their reactivity (fig.1). Modification of the regulatory centres changes the course of the reaction with hydroxylamine. In this case the inhibition is complete; both subunits react at the same rate (fig.1).

(ii) The non-identical behaviour of the native enzyme subunits is revealed in a reaction of pyrophosphatase with the affinity inhibitors, phosphoric acid monoesters. Some of them inactivate the enzyme as much as 50%, i.e., only half-of-the-sites reactivity is displayed in this case as well. Other inhibitors cause a total inactivation, but the two subunits react at different rates as a result of their interaction [3,4]. The protein quaternary structure is essential for the enzyme-irreversible inhibition by phosphoric acid monoesters, therefore the monomer can bind the inhibitor but reversibly [11].

Modification of the regulatory centres alters the reactivity of phosphoric acid monoesters: they lose their ability to inhibit irreversibly the enzyme and operate only as competitive inhibitors in substrate hydrolysis as it takes place with the monomer (table 1). Besides, the enzyme with modified

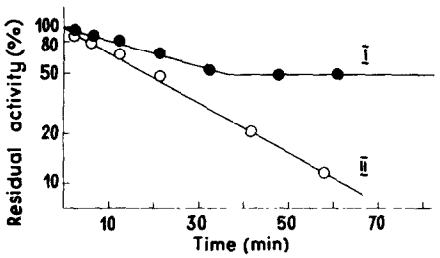

Fig.1. Inactivation of the native (I) and the regulatory centre-modified (II) pyrophosphatase (10^{-7} M) by 0.01 M hydroxylamine. Conditions: 0.05 M Tris-HCl (pH 7.2), 30°C.

regulatory centres is also distinguished, compared with the native pyrophosphatase, for a deteriorated affinity with respect to the inhibitor, i.e., as a monomer versus a dimer [11].

(iii) The different behaviour of the two identical subunits of the dimer as noted in (i) and (ii) is caused by conformational changes in one subunit as a result of the ligand being introduced into the other one. Proteolysis by subtilisin can be a convenient control over the free subunit conformation in the enzyme containing the ligand in the active centre of the neighbouring subunit. This approach was used for comparing the properties of the native pyrophosphatase and the regulatory centre-modified enzyme.

Modification by hydroxylamine of the active centre of one subunit of the native protein destabilizes the second subunit, and the proteolysis rate increases compared with the native

Table 1

Interaction of the native and regulatory centre-modified pyrophosphatase with affinity inhibitors

Reagent	Native enzyme		Modified enzyme	
	Inactivation (%)	K_i (mM)	Inactivation (%)	K_i (mM)
O-Phosphoethanolamine	100	1.37	0	4.3
Methyl phosphate	50	7.04	0	16.0
Phosphoglycollic acid	50	2.80	0	6.3

The effect of phosphoric acid monoesters (0.5–4.0 mM) on the rate of enzyme-caused substrate hydrolysis ([$MgPP_i$] = 10–100 μM) was studied in the presence of 1 mM Mg^{2+} in 0.1 M Tris-HCl (pH 7.2), at 25°C. Inhibition constants were determined from K_M^{app} plots versus inhibitor concentration

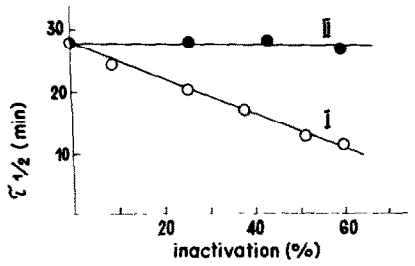

Fig.2. Proteolysis of the native (I) and the regulatory centre-modified (II) pyrophosphatase (8 μg/ml) by subtilisin (0.24 mg/ml) vs hydroxylamine-caused inhibition. Conditions: 0.1 M Tris-HCl (pH 7.8), 37°C. $\tau_{1/2}$ = the observed inactivation half-time.

pyrophosphatase. Indeed, the higher the degree of hydroxylamine-induced inhibition, the faster the loss of enzyme activity caused by subtilisin (fig.2).

On the other hand, the regulatory centre modification desensitizes pyrophosphatase, and introduction of the reagent into the active centre of one subunit does not change the stability of the other. As a result, the subtilisin-induced inactivation rate of the enzyme with modified regulatory centres remains constant irrespective of the reaction with hydroxylamine (fig.2).

Thus, the regulatory centre carboxyl group of baker's yeast inorganic pyrophosphatase has a key position in subunit interactions.

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