Half-of-the-sites reactivity of inorganic pyrophosphatase from yeast is the result of induced asymmetry

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Inorganic pyrophosphatase *Half-of-the-sites reactivity*

1. INTRODUCTION

Baker's yeast pyrophosphatase (EC 3.6.1.1) displays half-of-the-sites reactivity with respect to the inhibitors, phosphoric acid monoesters [1,2]. The enzyme catalyzes the hydrolysis and synthesis of inorganic pyrophosphate. It is a dimeric protein which consists of identical subunits, each containing an active centre. This enzyme is one of a growing list of proteins which display half-of-the-sites behaviour.

The half-of-the-sites reactivity in proteins can be explained on the basis of a number of mechanisms [3]. Taking into account that the two subunits of the pyrophosphatase molecule are identical in primary structure and that the catalytic sites are distant from each other [4], the half-of-the-sites behaviour in pyrophosphatase is best explained in terms of the so-called pre-existing asymmetry or induced asymmetry models. In the former model, the oligomer consists of two conformationally different classes of subunits, which differ in their reactivity towards a modifying agent. According to the simple version of this model, whatever happens at one subunit has no influence on events that might occur on the neighbouring subunit. In the model of induced asymmetry, the subunits are conformationally identical in the native enzyme but modification of any subunit induces a conformational change in its neighbour with a resultant alteration in reactivity or other properties of the neighbour.

Here we present evidence to support the view that the half-of-the-sites reactivity observed with inorganic pyrophosphatase in the reaction with affinity inhibitors involves the induction of a conformational change in the subunit adjacent to one which undergoes the modification.

2. MATERIALS AND METHODS

Inorganic pyrophosphatase with a specific activity of 650–700 IU/mg at pH 7.2 and 25°C was isolated from baker's yeast as in [5]. Methyl phosphate, *O*-phosphoethanolamine, phosphoglycollic acid and subtilisin were the products of Sigma; *O*-phosphopropanolamine was synthesized as in [6], *N*-acetylphosphoserine was synthesized as in [7]. Enzymic activity was estimated as in [1] and [2].

The preparation of the pyrophosphatase half-of-the-sites modified with methyl phosphate, phosphoglycollic acid, *N*-acetylphosphoserine, *O*-phosphoethanolamine and *O*-phosphopropanolamine (table 1, compounds I–IV) was carried out at 25°C by the incubation of 10^{-7} M enzyme with 10^{-2} M reagent in 0.05 M Tris–HCl (pH 7.2) for 30 min. Enzyme derivatives were isolated by gel filtration through a Sephadex G-50 fine column.

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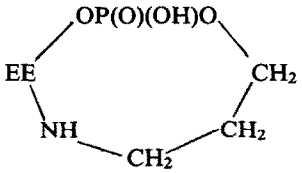
Table 1

The effect of the affinity modification on the subtilic digestibility of inorganic pyrophosphatase

Compound	Enzyme derivative: Designation	Activity (%)	T (min)
I	$CH_3O(OH)(O)P$-O-EE	50	25
II	COOH-$CHCH_2O(OH)(O)P$-O-EE $\vert$ $NHCOCH_3$	50	16
III	$NH_2CH_2CH_2O(OH)(O)P$-O-EE	50	10
IV	$NH_2CH_2CH_2CH_2O(OH)(O)P$-O-EE	50	10
V		50	15
VI	EE-$NHCH_2CH_2CH_2OP(O)(OH)_2$	100	10
VII	EE	100	10

E, enzyme subunit

The preparation of the pyrophosphatase modified with *O*-phosphopropanolamine (table 1, compounds V–VI) was carried out similarly for a 2 h incubation time. Thereafter 10^{-3} M PP_i was added and the enzyme derivatives were isolated by gel filtration for 30 min (compound V) or 3 h (compound VI) incubation.

Pyrophosphatase proteolysis was studied in 0.1 M Tris–HCl buffer (pH 7.8) at 37°C, the concentration of the enzyme and subtilisin being 10 μg/ml and 250 μg/ml, respectively.

3. RESULTS AND DISCUSSION

To distinguish between induced and pre-existing asymmetry as underlying causes of the apparent half-of-the-sites reactivity in the reactions of inorganic pyrophosphatase with affinity inhibitors, a comparative study has been carried out of the conformational states of the free subunits in pyrophosphatase half-sites modified by a series of phosphoric acid monoesters. If the half-of-the-sites reactivity behaviour is due to the induction effect, the magnitude of the induction could be expected to depend on the nature of the covalent modification. On the other hand, if the half-of-the-sites reactivity behaviour is due to the pre-existing asymmetry and there is no communication between the catalytic sites, the properties of unmodified subunits should be independent of the nature of the covalent derivative formed on the modified subunits. According to the induced asymmetry hypothesis, the conformational states of the free subunits of the half-sites-modified pyrophosphatase might be different for different covalent modifications, whereas the pre-existing asymmetry hypothesis implies that the conformational state of the free subunits of the half-sites-modified enzyme should be the same regardless of the modifying group.

The half-sites-modified pyrophosphatase has been prepared by reaction with methyl phosphate, *N*-acetylphosphoserine, *O*-phosphoethanolamine and *O*-phosphopropanolamine. The reaction of

pyrophosphatase with phosphoric acid monoesters induced fast modification of only one subunit with 50% of the initial activity being lost [1,2].

In the resulting enzyme derivatives (table 1, compounds I–IV), the inhibitor is attached to the active centre via the phosphate moiety of the molecule. This linkage can be broken by treatment with PP_i, PPP_i or Mg^{2+} with full activity being restored.

A long-term incubation of the enzyme with methyl phosphate or *N*-acetylphosphoserine does not alter the amount of the bound inhibitor and the activity of the modified protein [1]. Affinity reagents of another type, phosphoethanolamine and phosphopropanolamine [2], modify both subunits; the reaction is biphasic. After fast modification of one subunit slow incorporation of one more mol reagent per mol enzyme occurs and a fully inactive enzyme derivative is formed. In this case the inhibitor molecule is attached to the second subunit through two points; i.e., via the phosphate moiety to the active centre and via the amino group outside it [8].

The reactivation of the modified pyrophosphatase on incubation with PP_i also proceeds in two steps. Fast restoration of 50% activity correlated with the amount of the released monoesters, and led to compound V in the case of phosphopropanolamine. Compound V has 50% activity with one free and one modified subunit. The reagent is attached to the second subunit in two points. The incubation of compound V with PP_i for a long time yields a fully active enzyme derivative VI without change of the amount of bound phosphopropanolamine. This compound contains one molecule of the covalently bound reagent outside the active centre.

The conformational states of the unmodified subunit were also assessed by subtilitic inactivation. Table 1 compares the observed inactivation half-times (*T*) for different enzyme derivatives.

Incubation of the native pyrophosphatase (compound VII) and the half-sites-modified enzyme with subtilisin results in a time-dependent inactivation, which is pseudo-first order (fig.1). The observed inactivation half-time is 10 min for the native enzyme. The rate of subtilitic digest of free subunit in the modified enzyme depends on the nature of the reagent attached to the modified subunit.

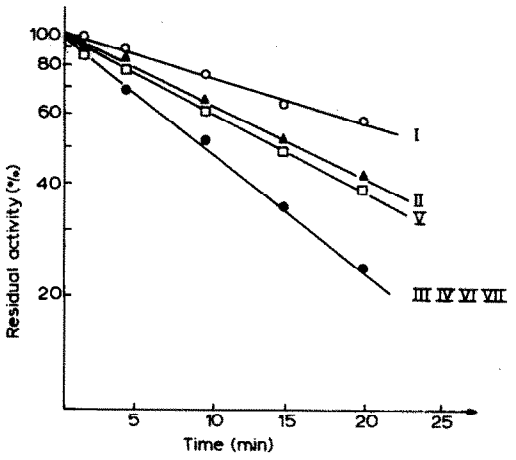


Fig.1. Time-dependence of the effects of subtilisin on native and modified pyrophosphatase.

The results show that half-sites modification by methyl phosphate or *N*-acetylphosphoserine results in a stabilization of free subunits. The inactivation of compounds I and II proceeds 2.5-times and 1.5-times slower, respectively, compared to the native enzyme. On the other hand, the reaction of one subunit of pyrophosphatase with phosphoethanolamine or phosphopropanolamine leading to compounds III and IV appears to have no effect on the subtilitic digestibility of free subunit. The observed inactivation half-times of the active subunits of the native enzyme and of compounds III and IV, which contain phosphoethanolamine or phosphopropanolamine in the active centres of the first subunit are close. In contrast to the results obtained with compounds III and IV, the modification of pyrophosphatase with phosphopropanolamine yielding compound V with the inhibitor bound via both the phosphate and amine moieties made the active subunit of the half-sites-modified enzyme more resistant to subtilitic inactivation compared to the native pyrophosphatase. The elimination of the inhibitor from the active centre leading to compound VI makes the first subunit similar to that in the native enzyme.

The results of the proteolysis studies demonstrate a significant difference in the susceptibility of the half-sites-modified pyrophosphatase to the subtilitic inactivation of which indicates a significant difference in the conformation of the free subunit of the modified enzyme depending on

the nature of the covalent modification. These observations are consistent with a model in which there is communication between two subunits of inorganic pyrophosphatase in the reaction with phosphoric acid monoesters.

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