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Regulation of enzymatic activity by kinase-free phosphorylation

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Inorganic pyrophosphatase activity is regulated by kinase-free phosphorylation. Phosphorylation by ATP activates the enzyme and that by P_i eliminates the activating effect of ATP. Acyl phosphate formed in the reaction with ATP is a covalent intermediate of ATP hydrolysis in the regulatory site of the enzyme. Therefore, kinase-free phosphorylation shares the properties of both regulatory and catalytic phosphorylations.

Protein phosphorylation; Kinase-free phosphorylation; Inorganic pyrophosphatase; Acyl phosphate; NMR, ^{31}P-

1. INTRODUCTION

Protein phosphorylation is an essential constituent of numerous biochemical conversions. So far, two types of protein phosphorylation, catalytic and regulatory, have been distinguished. The former includes formation of a phosphorylated intermediate in active sites of enzymes catalyzing the phosphoryl group transfer [1]. The latter is the most common type of reversible post-translational modification of proteins controlling almost all cellular processes [2,3]. Special enzymes, the protein kinases, serve for the phosphoryl transfer from high-energy phosphorus-containing compounds, mainly ATP, to proteins; phosphatases are responsible for dephosphorylation. This type of phosphorylation is called regulatory because such modifications often alter the catalytic activity of enzymes or functional properties of the proteins not involved in catalytic processes. However, activation of the yeast and *E. coli* inorganic pyrophosphatases (EC 3.6.1.1) by ATP phosphorylation without protein kinases [4-6] challenged the conventional classification.

Baker's yeast inorganic pyrophosphatase consists of two identical subunits of ~32 kDa [7], each having an active site catalyzing pyrophosphate (PP_i) hydrolysis. It was established that phosphate (P_i) phosphorylates the Asp residue [8] at an enzyme site distinct from the active site, the enzymatic activity being unchanged [9]. Moreover, ATP, ADP and other monosubstituted derivatives of pyrophosphoric acid phosphorylate and activate the enzyme, while PP_i and AMP do not modify it [5]. Therefore, the question arises about the nature of pyrophosphatase phosphorylation. This paper demonstrates that kinase-free phosphorylation of the yeast inorganic pyrophosphatase possesses the properties of both regulatory and catalytic phosphorylation.

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2. MATERIALS AND METHODS

ATP and Pipes were obtained from Sigma, D_2O from Merck, and [γ-^{32}P]ATP from Amersham. Inorganic pyrophosphatase with a specific activity 680 IU/mg (pH 7.2, 25°C) was isolated from baker's yeast as described [10]. All experiments were performed at 20°C in 0.1 M Pipes buffer, pH 6.4, containing 80% D_2O in the case of ^{31}P-NMR samples.

The enzyme was phosphorylated by incubation with 0.1-0.8 mM [γ-^{32}P]ATP and 1 mM $MgCl_2$ for 1 min and subjected to centrifugal gel filtration [5]. In some experiments NaH_2PO_4 was added up to 2, 5 and 6 mM after 1 min incubation with 0.8 mM [γ-^{32}P]ATP, and samples were subjected to gel filtration 7 min later. The protein content [11], radioactivity and pyrophosphatase activity [12] were determined after gel filtration.

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To investigate enzyme dephosphorylation, inorganic pyrophosphatase phosphorylated by [γ-^{32}P]ATP was incubated with 0.7 mM ATP and 1.0 mM $MgCl_2$; at particular time intervals aliquots were taken for gel filtration and ^{32}P bound to the enzyme was determined.

Hydrolysis of 0.7 mM ATP was examined in the presence of 1 mM $MgCl_2$ or 1 mM $MgCl_2$ and inorganic pyrophosphatase (0.3 mg/ml). P_i formation was estimated in aliquots as in [13].

^{31}P-NMR spectra were recorded at 121.41 MHz on a Varian VXR-300 spectrometer. The data were expressed as positive down-field from H_3PO_4 (85%).

3. RESULTS

The yeast inorganic pyrophosphatase, homogeneous according to electrophoresis and terminal amino acid sequence analysis, undergoes rapid phosphorylation by ATP in the presence of Mg^{2+} (fig.1). Phosphorylation activates the enzyme, the activity attaining 200% of the initial value upon incorporation of 1 mol phosphoryl group per mol protein. The enzyme site, distinct from the active site, is modified, since pyrophosphatase phosphorylated by [γ-^{32}P]ATP preserves radioactivity in PP_i hydrolysis [5]. It is essential that P_i decreases the degree of enzyme phosphorylation by ATP and eliminates the ATP activating effect (fig.1). Thus, the activity of inorganic pyrophosphatase is regulated by the action of ATP, a precursor of PP_i in biosynthetic processes, and P_i, a product of the enzymatic reaction.

The bond between the protein and phosphoryl group formed by ATP phosphorylation is labile in acidic and alkaline media and is readily cleaved by hydroxylamine [5]. These properties are typical of an acyl phosphate link. To obtain proof of the formation of acyl phosphate the interaction of the enzyme with ATP was examined by ^{31}P-NMR. Fig.2a shows a ^{31}P-NMR spectrum of ATP in the presence of Mg^{2+}. Addition of inorganic pyrophosphatase to such a solution drastically changes the spectrum (fig.2b). The enzyme hydrolysed all of the ATP (the signal of the ATP β-phosphate group disappeared at -21.4 ppm and that of P_i emerged at 1.4 ppm) in less than 2 h (while the spectrum was monitored). Moreover, the ADP formed (two broad signals with maxima at -6.1 and -9.3 ppm) was partially hydrolysed and AMP appeared (2.3 ppm). The phosphorylated enzyme produced a signal (-18.6 ppm) corresponding to the incorporation of 0.85 mol phosphoryl group per mol enzyme. This signal indicates an acyl phosphate bond

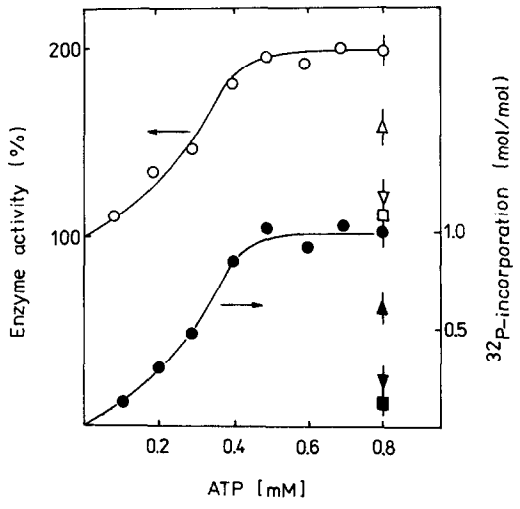

Fig.1. Dependence of phosphorylation and activation of yeast inorganic pyrophosphatase on ATP concentration in the absence of P_i (●,○) and presence of 2 mM (▲, △), 5 mM (▼, ▽) and 7 mM (■, □) P_i.

in phosphorylated pyrophosphatase, since no other bond in phosphorylated proteins has such an upfield chemical shift [14]. Acyl phosphates in transport ATPases and the model compound L-seryl-L-phosphoaspartate have chemical shifts of -17.5 and -17.4 ppm, respectively [15]. One should take into account that the latter were obtained at pH 7.4, whereas the spectrum in fig.2 was recorded at pH 6.4, and protonation of low molecular mass acyl phosphates with pK_a about 4.9 leads to upfield signal displacement by 5 ppm [16]. Thus, ^{31}P-NMR demonstrates acyl phosphate formation in pyrophosphatase phosphorylation. In addition, the marked hydrolysis of ATP by inorganic pyrophosphatase from yeast in the presence of Mg^{2+} was first discovered. ATP is hydrolyzed in the enzyme regulatory site, phosphoenzyme being an intermediate in the reaction. The experiments described below confirm this.

Hydrolysis of ATP by inorganic pyrophosphatase in the presence of Mg^{2+} is represented in fig.3. Taking into account that phosphorylation proceeds to completion within a few seconds it may be supposed that the rate of the ATPase reaction is determined by that of decomposition of the phosphorylated enzyme (E-P) and can be expressed as $v = k$[E-P]. As follows from the data in fig.3, k is equal to 0.048 min^{-1}, the enzyme being entirely phospho-

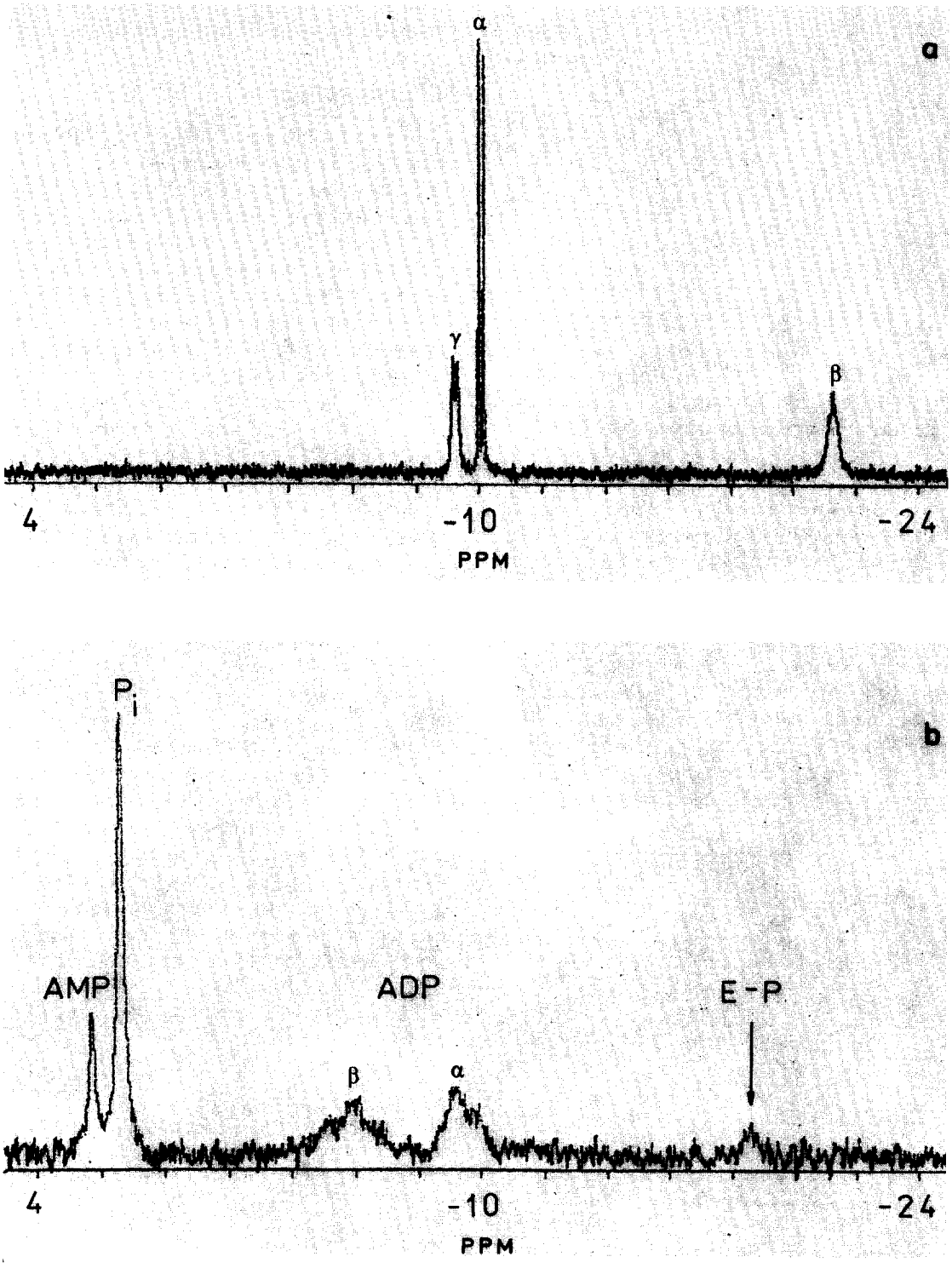

Fig.2. ^{31}P-NMR spectra of samples containing 2.5 mM ATP and 1.4 mM $MgCl_2$ (a); 2.5 mM ATP, 2.0 mM $MgCl_2$ and 0.62 mM inorganic pyrophosphatase (b).

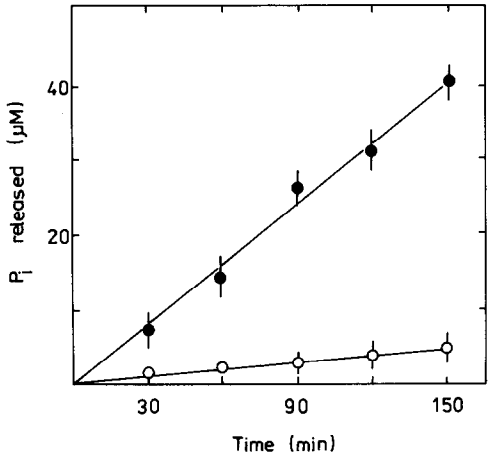

Fig.3. Hydrolysis of 0.7 mM ATP in the absence (○) and presence (●) or inorganic pyrophosphatase (0.3 mg/ml).

rylated. Dephosphorylation of pyrophosphatase containing radioactive phosphoryl group in the regulatory site (fig.4) was studied under the same conditions. The half-life of labelled phosphoenzyme was 18 min, which corresponds to the rate constant for E-P decomposition of 0.039 min^{-1}. Good agreement of this value with the above-mentioned rate constant for the ATPase reaction proves that ATP is hydrolyzed in the regulatory site of yeast pyrophosphatase in the presence of Mg^{2+} and that the phosphorylated enzyme is an intermediate of this reaction. Consequently, the regulatory site of inorganic pyrophosphatase is also the catalytic site of ATP hydrolysis on the enzyme.

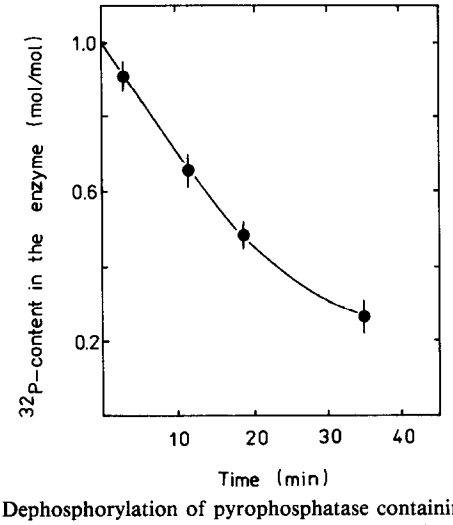

Fig.4. Dephosphorylation of pyrophosphatase containing ^{32}P-labelled phosphoryl group in the regulatory site.

4. DISCUSSION

Regulation of yeast inorganic pyrophosphatase operates by phosphorylation of the enzyme regulatory site. However, contrary to the conventional regulation mechanism via phosphorylation-dephosphorylation by protein kinases and phosphatases, pyrophosphatase activity can be regulated only by phosphorylation; here either ATP or P_i is the phosphoryl donor and no additional enzymes are required. ATP and P_i phosphorylate different residues of dicarboxylic amino acids [5]. However, these residues are coupled so that the phosphorylation of one prevents phosphorylation of the other [5] or leads to dephosphorylation of the latter (see fig.1 for an example). In reactions with P_i and ATP the phosphoryl group is covalently bound in the regulatory site of only one of two pyrophosphatase subunits. Phosphoryl groups evidently exchange via the flip-flop mechanism: phosphorylation of the free subunits leads to hydrolysis of the acyl phosphate bond in the other subunit [9]. ATP and P_i phosphorylate not only yeast but also the *E.coli* pyrophosphatase without additional enzymes [6]. Regulatory sites of the *E.coli* enzyme are also phosphorylated, while ATP activates and P_i inhibits the enzyme to some extent. The present work demonstrates that the regulatory site of yeast pyrophosphatase is also the catalytic site of ATP hydrolysis, the activation of the enzyme being a coupling of two active sites hydrolysing PP_i and ATP with rates differing by 6 orders of magnitude. Therefore, kinase-free phosphorylation is the earlier unknown type of enzymatic activity regulation effacing the formerly distinct boundary between regulatory and catalytic phosphorylation.

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