Regulation of yeast phosphorylase by phosphorylase kinase and cAMP-dependent protein kinase

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Yeast phosphorylase is phosphorylated and activated by a cyclic AMP-independent protein kinase (called phosphorylase kinase) and a cyclic AMP-dependent protein kinase. Only in the presence of both kinases is phosphorylase fully activated and phosphorylated. No evidence was found for the presence of two phosphorylation sites as an identical phosphopeptide pattern of phosphorylase is obtained after phosphorylation by either one or both kinases. The kinases probably phosphorylate identical sites but recognize different subunits of phosphorylase. Phosphorylase kinase phosphorylates the high-M_r subunit while cAMP-dependent protein kinase phosphorylates the low-M_r subunit.

Yeast *Phosphorylase* *Phosphorylase kinase* *Cyclic AMP-dependent protein kinase*

1. INTRODUCTION

Yeast phosphorylase activity is modulated by reversible phosphorylation of a threonine residue [1]. Activation of the phosphorylase by a cAMP-independent kinase (termed phosphorylase kinase because of a low K_m for yeast phosphorylase) varies from preparation to preparation per P_i incorporated. However, P_i incorporated never exceeds one phosphate per dimer. This paradox was resolved upon the discovery that phosphorylase is also a substrate for yeast cAMP-dependent protein kinase which phosphorylates and activates the enzyme in a manner analogous to phosphorylase kinase. If both enzymes are simultaneously present in the in vitro assay, total phosphate incorporation is additive, while the activation of phosphorylase is synergistic. These observations are indicative of two phosphorylation sites in yeast phosphorylase. However, peptide mapping of phosphorylase activated by either of the kinases revealed an identical pattern of phosphopeptides carrying phosphate exclusively on a threonine residue.

These puzzling observations can be explained by the recent finding that yeast phosphorylase consists of two enzymatically active subunits differing slightly in molecular mass. Phosphorylase kinase incorporates phosphate into the high-M_r subunit (l-subunit) while cAMP-dependent protein kinase phosphorylates the low-M_r subunit (s-subunit).

2. MATERIAL AND METHODS

2.1. *Activation of phosphorylase*

The reaction mixture for activation contained phosphorylase (1 mg/ml), phosphorylase kinase and/or cAMP-dependent protein kinase (30 μg/ml, 0.1 mM [γ-^{32}P]ATP (3×10^8 cpm/μmol) [2], 10 mM $MgCl_2$ in 20 mM Mes (pH 6.2), 1 mM EDTA, 5% glycerol. In the presence of cAMP-dependent protein kinase, 1.25 μM cAMP was added. Controls without phosphorylase were run under identical conditions to correct for autophosphorylation of cAMP-dependent protein kinase. Aliquots of the reaction mixture were removed and phosphate incorporation determined as in [3] and phosphorylase activity as in [1]. Phosphate content of phosphorylase was measured as in [4].

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2.2. *Peptide mapping*

Yeast phosphorylase (1–2 mg), ^{32}P-labelled by either phosphorylase-kinase, cAMP-dependent protein kinase or both, was incubated with 0.2–0.4 mg trypsin for 3 min at 30°C. Reaction was terminated by addition of trichloroacetic acid (10% w/v). The supernatant after centrifugation, containing 90–100% of the radioactivity, was washed with ether and dried by rotary evaporation. The residue was dissolved in water, and subjected to two-dimensional peptide mapping [5] on Polygram Cel 300 sheets (Machery and Nagel, Düren) at 500 V, 15 mA on a Pharmacia flat bed apparatus for 3 h, and 5 h in the second dimension. Phosphopeptides were located by autoradiography and quantified by liquid scintillation counting.

2.3. *Gel electrophoresis*

SDS gel electrophoresis was performed as in [6] on 7.5–15% polyacrylamide gradient gels.

2.4. *Enzymes and material*

Yeast phosphorylase (pure) [7], phosphorylase kinase (2800 times enriched) and cAMP-dependent protein kinase (same issue) were purified from commercial yeast (DHW, Hamm) by methods developed in this laboratory. Trypsin (TPCC-treated) was a product of Serva (Heidelberg).

3. RESULTS AND DISCUSSION

3.1. *Activation of phosphorylase by phosphorylase kinase and cAMP-dependent protein kinase*

The final achieved phosphate incorporation and activation of phosphorylase by phosphorylase kinase varied between different phosphorylase preparations. Phosphorylase of low specific activity contained <0.1–0.2 mol P_i/subunit; <0.5 mol P_i/subunit was incorporated by the kinase even if a large excess was used for activation. These observations implied that some phosphorylation sites of phosphorylase were not accessible to phosphorylase kinase. This idea was supported by the discovery of a cAMP-dependent protein kinase which phosphorylates and activates yeast phosphorylase. The enzyme was purified and separated from cAMP-independent phosphorylase kinase. In the absence of cAMP, the enzyme was totally inactive.

As in the case of phosphorylase kinase, cAMP-dependent protein kinase was not able to achieve maximum phosphorylation and activation of phosphorylase. In the experiment presented in fig.1, phosphorylase kinase activates phosphorylase up to a spec. act. of 80 units/mg and incorporates 0.52 mol P_i/subunit while cAMP-dependent protein kinase incorporates 0.2 mol P_i/subunit with a concomitant increase in activity of 20 units/mg. With both kinases present, the maximum spec. act. of 115 units/mg and incorporation of 0.7 mol

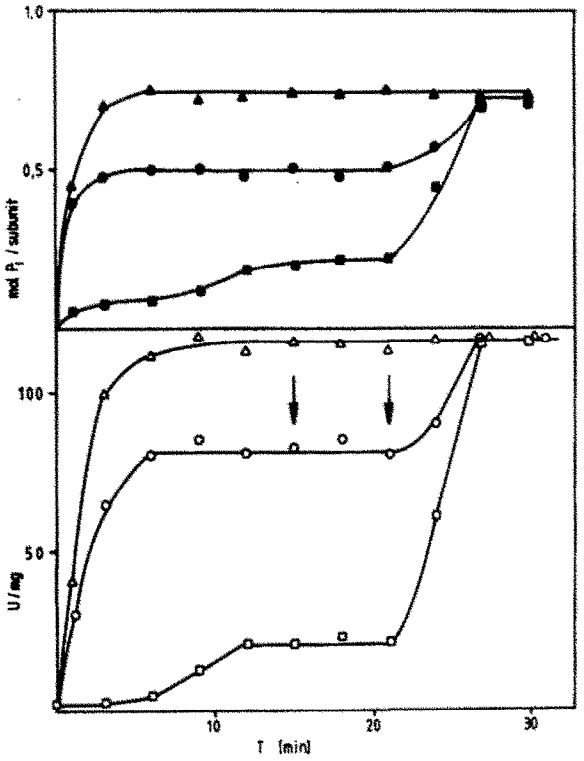

Fig.1. Activation and phosphorylation of phosphorylase by phosphorylase kinase and/or cAMP-dependent protein kinase. Yeast phosphorylase was activated by phosphorylase kinase (○), cAMP-dependent protein kinase (□) or both (△) as described in the text. Every 3 min aliquots of the reaction mixture were removed for determination of phosphate incorporation (filled symbols) and specific activity of phosphorylase (open symbols). After 15 min additional phosphorylase kinase or cAMP-dependent protein kinase were added (first arrow) and the reaction continued for 6 min. Following this time, cAMP-dependent protein kinase was added to the phosphorylase kinase-containing reaction and phosphorylase kinase to the cAMP-dependent protein kinase-containing reaction (second arrow).

P_i/subunit are obtained. After 15 min incubation, additional phosphorylase kinase or cAMP-dependent protein kinase were added to the reaction mixtures with no changes in activation or phosphorylation. Only if cAMP-dependent protein kinase was added to phosphorylase kinase or vice versa was a further increase in activity observed. These results strongly suggest that yeast phosphorylase is phosphorylated and activated by cAMP-dependent and a cAMP-independent protein kinase. Hence two different kinases are able to regulate phosphorylase.

3.2. *Peptide mapping of the phosphopeptides*

These results suggested the presence of two phosphorylation sites. However, the phosphopeptide patterns observed were independent of the kinase used for activation and phosphorylation of phosphorylase, (fig.2); namely a major one (80% of the radioactivity) and two minor ones (15 and 5%). Identification of the phosphoamino acid revealed, in both cases, phosphothreonine. Most likely both kinases phosphorylate the same site of the phosphorylase molecule. It can not be excluded that different threonine residues in the same peptide are phosphorylated if phosphorylation at one threonine prevents phosphorylation at the others.

3.3. *Differences in phosphorylation of the phosphorylase subunits*

Recently it was observed that yeast phosphorylase is composed of two different subunits differing slightly in molecular mass [7]. Each of the kinases was able to phosphorylate only one of the

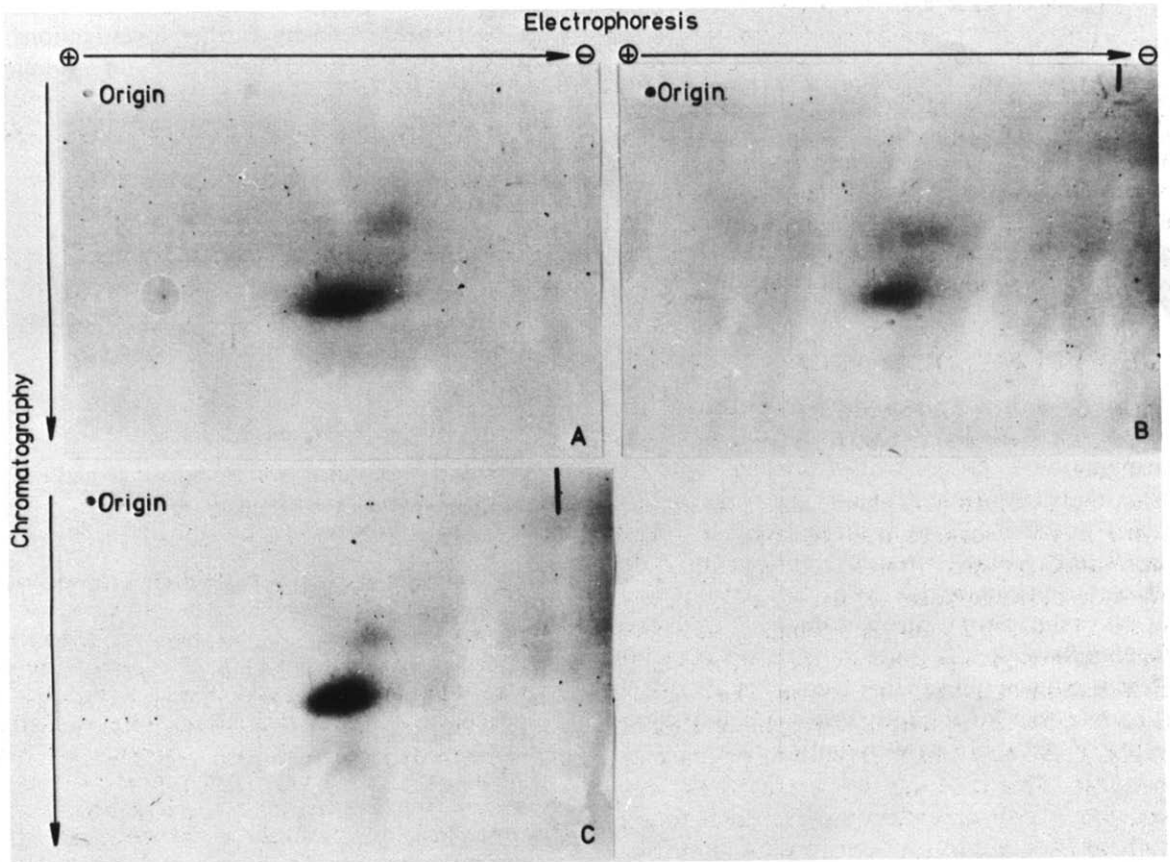

Fig.2. Peptide mapping of phosphopeptides. Phosphorylase was ^{32}P-labelled with both kinases (A), cAMP-dependent protein kinase (B) and phosphorylase kinase (C) and digested by trypsin as described in the text. The peptides were separated by two-dimensional peptide mapping [5] and the phosphopeptides located by autoradiography.

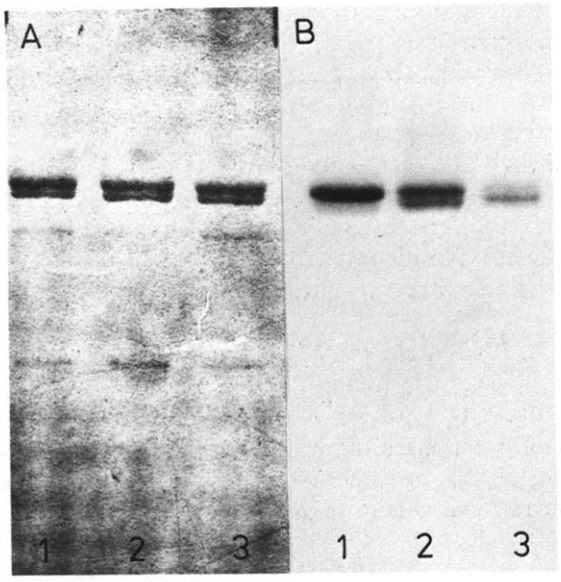

Fig.3. Phosphorylation of phosphorylase subunits. Phosphorylase was ^{32}P-labelled with phosphorylase kinase [1], both kinases [2] and cAMP-dependent protein kinase [3], and the reactions stopped by boiling aliquots of the mixtures in the presence of 1% SDS and 2 mM mercaptoethanol. Samples (10 μg protein) were subjected to SDS electrophoresis on 7.5–15% polyacrylamide gradient gels [6] and the dried gels autoradiographed (B), protein staining of the gels (A).

subunits. While the l-subunit was substrate for phosphorylase kinase (fig.3A), the s-subunit was phosphorylated by cAMP-dependent protein kinase (fig.3C). Both subunits were phosphorylated if both kinases were present (fig.3B). This observation reconciles the different action of the kinases on phosphorylase with the fact that only one phosphorylation site is found. The kinases phosphorylate identical sites which are located on different polypeptide chains. Hybridization of the chains may lead to the observed synergism in activation of phosphorylase by both kinases.

3.4. *Evidence of the generation of the small subunit from the large subunit by limited proteolysis*

The small subunit is most probably generated from the large one by limited proteolysis since they share a common phosphorylation site (vide infra) and an identical carboxy-terminus, but differ in their amino-terminus [7]. Transition of large to small-subunit can be mimicked in vitro by incubation of ^{32}P-labelled phosphorylase with trypsin. This treatment initially lowers the molecular mass of l-subunit to that of the small one retaining the phosphorylation site which is subsequently lost without any further detectable change in molecular mass [7].

If limited proteolysis changes the susceptibility of an enzyme towards different kinases as suggested by the results, this may constitute an interesting novel regulatory system. However, this has to be substantiated by further investigations addressing the question of whether the s-subunit is really produced from the large one and whether this transition occurs in the living cell.

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