Vol. 114, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 18, 1983 Pages 331-338

CHARACTERIZATION OF PHOSPHORYLASE KINASE ACTIVITIES IN YEAST

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Received May 26, 1983

Two phosphorylase kinase activities were resolved by DEAE-cellulose chromatography. The main activity peak was enriched 2800-fold, the minor appeared to be an aggregate of the enzyme. Phosphorylase kinase also phosphorylated histone and casein with no changes in phosphorylation ratios throughout the preparation steps but was most active on yeast phosphorylase. The molecular weight was 29000 ± 2000. ATP, UTP, GTP served as substrates while CTP was inactive. Mg-ions activated the kinase without inhibition at high concentrations (30 mM). In addition to this cAMP-independent kinase, cAMP-dependent protein kinase also phosphorylated phosphorylase. The catalytic subunit and phosphorylase kinase were not identical since the latter was not inhibited by yeast cAMP binding protein.

Yeast is the only microorganism where phosphorylase was shown to be regulated by phosphorylation and dephosphorylation (1). No interconverting enzymes have yet been purified and characterized, although phosphorylase kinase activity has already been demonstrated in crude preparations of casein kinase (2). Most yeast protein kinase have been found by using nonphysiological substrates such a casein, histone and protamine. In regard to the evolution of the phosphorylation-dephosphorylation regulatory mechanism and to other interconvertible enzymes in yeast (3, 4), it would be of interest to understand the physiological role of this class of enzymes. Therefore, we have prepared highly enriched phosphorylase kinase and present evidence that this kinase is not identical to any yeast kinase previously described.

Material and Methods

The enzymes were prepared from commercial baker's yeast (ABC-Rotband Hefe, Hamm, FRG). Pure yeast phosphorylase was prepared by a method developed in this laboratory (14) in different states of specific activity and content of covalently bound phosphate. Therefore, only measurements carried out with the same phosphorylase preparationcould be compared directly.

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0006-291X/83 $1.50
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Yeast cAMP-dependent protein kinase was prepared according to Hixson and Krebs (5) up to dissociation of the holoenzyme on cAMP-Agarose. DE-52 and phosphocellulose were obtained from Whatman (Springfield Mill, UK) and AcA 44 Ultrogel from LKB (Bromma, Sweden). Cibacron blue F 3 G-A Sepharose was prepared according to Böhme and Kopperschläger (6) using Sepharose 4B as carrier. Preparation of γ-P-32-ATP was according to Glynn and Chappel (7).

All solutions for the preparation of the enzyme contained 0.57 mM benzamidine-HCl, 1 mM EDTA and 20 nM phenylmethyl sulfonyl fluoride added immediately before use, except for Tris-buffer in which it is rapidly inactivated (8). The buffers Tris-10 (10 mM Tris-HCl, pH 7.0), Tris-100 (100 mM Tris-HCl, pH 7.0) and P-10 (10 mM K-phosphate, pH 6.8) contained in addition 5 % glycerol (w/v) for stabilization of the kinase. Omission of glycerol led to rapid loss of the kinase activity. All centrifugation steps were performed at 28,000 g.

Enzyme assay. For activation of yeast phosphorylase, equal volumes of yeast phosphorylase (10-20 µg) and phosphorylase kinase were incubated in 100 mM Tris-HCl, pH 8.0, 0.1 % bovine serum albumin and 1 mM EDTA (final volume 30 µl). The reaction was started by the addition of 10 µl Mg-ATP (100 mM $MgCl_2$, 50 mM ATP, pH 7.0). At time intervals, 5 µl aliquots were removed, added to 200 µl assay mixture and phosphorylase activity measured either in the direction of amylose synthesis or glycogen phosphorolysis (1). One unit of phosphorylase kinase catalyses the conversion of 1 unit phosphorylase/min at 30° C.

Measurements of incorporation of P-32 into protein. The substrate solution (80 µl) contained 30 mM $MgCl_2$, 0.3 mM γ-P-32-ATP (specific activity 300 cpm /pmol) and 160 µg of either casein (Hammarsten casein, Merck, FRG), histone (Histone IIS Sigma, USA) or 80 - 100 µg yeast phosphorylase in 100 mM Tris buffer, pH 8.0, with 0.1 % bovine serum albumin. At time intervals, 10 µl of the reaction mixture were spotted on Whatman GF/c filters. The filters were washed with trichloroacetic acid, dried and counted in toluene scintillator. One unit of kinase activity catalyzes the incorporation of 1 nmol P_i/min. Protein was determined in concentrated solutions by the biuret method (9) or estimated by absorbance at 280 nm in dilute solutions using 1 % A = 11.

Localization of phosphorylase kinase activity on polyacrylamide gels. Gel electrophoresis was carried out on 8 % polyacrylamide disc-gels according to Davies et al. (10). Gels were sliced in 2 mm discs and extracted with 50 µl of Tris-100 for 20 min at 30° C and phosphorylase kinase activity measured.

Determination of molecular weight was performed by gel filtration on an Ultrogel AcA 44 column (1.6 x 84 cm) equilibrated with Tris-100. Kinase and marker proteins were applied in a volume of 1 ml, eluted at a flow rate of 6 ml/h and fractions collected.

Results

Purification of phosphorylase kinase

Yeast (2 kg) were broken by one passage through a continuous French pressure cell (Manton Gaulin), centrifuged and pH of the supernatant brought to 5.75 with NaOH. After streptomycin sulfate (final concentration 1.5 %) precipitation at 42 % saturation ammonium sulfate precipitation followed. The exhaustively dialyzed ammonium sulfate pellet (200-250 ml) in buffer Tris-10 was applied to a DE-52 column (6.5 x 40 cm) equilibrated with the same buffer, washed with Tris-10 and eluted with a linear 0 - 0.4 M NaCl gradient at a

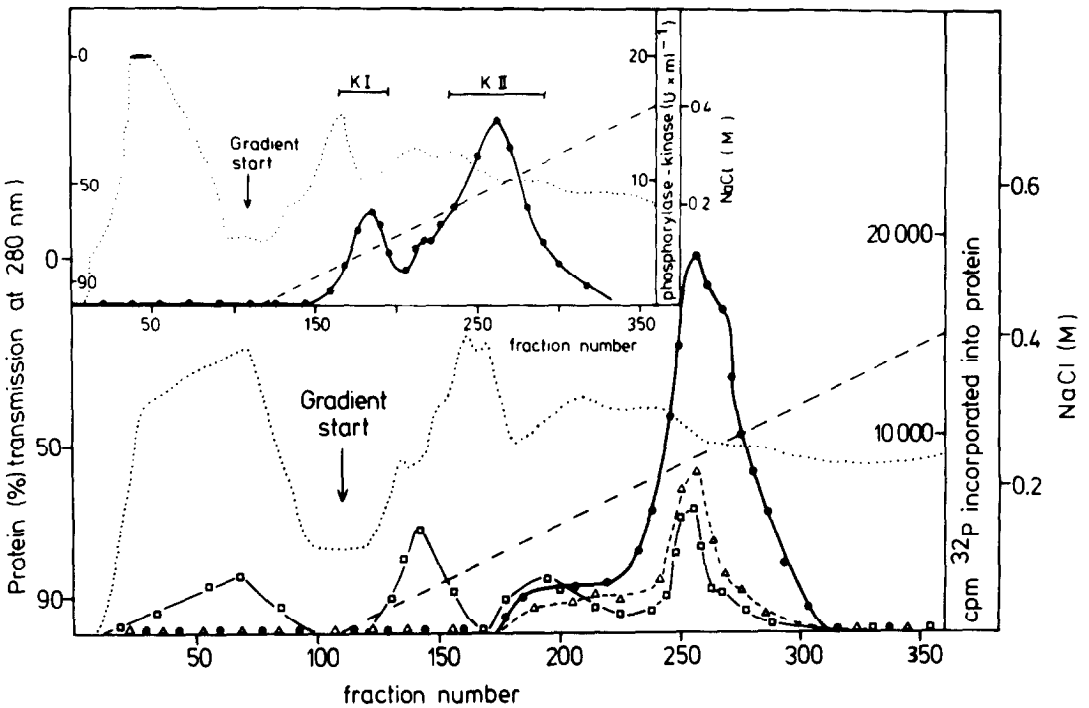

Fig. 1. Chromatography of the sediment precipitated at 40 % ammonium sulfate saturation.

For details see text. Phosphorylase kinase and protein kinase activities were assayed under standard conditions by incorporation of ^{32}P into yeast phosphorylase (●—●), histone (△—△) and casein (□-□). Transmission at 280 nm (dotted line) was recorded continuously. The dashed line shows the NaCl-gradient. The lower ammonium sulfate concentration was chosen to test the co-purification of the several kinase activities.

Insert: Chromatography of the sediment after precipitation at 42 % ammonium sulfate saturation as employed in the standard purification protocoll. Phosphorylase kinase activity was measured by activation of yeast phosphorylase (see Material and Methods). K I and K II denote the two activity peaks emerging under these conditions.

flow rate of 185 ml/h. Two peaks, K I and subsequently K II, were eluted from the column (Fig. 1 insert). K II always contained the bulk of the activity while the amount and appearence of K I depended largely on concentration of ammonium sulfate used for the precipitation. Fig. 1 shows the nearly complete disappearence of K I if ammonium sulfate was used at 40 % of saturation. For further purification, K II was pooled, concentrated and applied to a phosphocellulose column (2 x 30 cm) equilibrated with P-10. The enzyme was eluted with a linear 0.01 - 1 M KH_2PO_4 gradient (flow rate 45 ml/h), concentrated and applied to a Cibacron blue F 3 G-A Sepharose column (2 x 10 cm), equilibrated with Tris-100. After washing the column with 0.5 M NaCl in the

Table I: Purification of phosphorylase kinase from yeast.

Purification step	Volume ml	Total protein mg	Total activity units	Spec. activity units/mg	Yield %	Purification -fold
Crude extract	2 600	108 680	(10 700)	(0.098)	100	-
Streptomycin	2 330	87 608	-	-	-	-
42 % $(NH_4)_2SO_4$ saturation sediment,dialyzed	220	11 836	9 600	0.8	90	8
DEAE-cellulose pool (concentrated)	45	1 000	6 060	6.06	57	62
Phosphocellulose pool (concentrated)	10	74	4 541	61.4	43	630
Cibacron blue I pool (concentrated)	4	14	2 220	159	21	1 623
Cibacron blue II pool	30	7.5	2 055	274	19	2 800

Enzyme activity was determined from the ammonium sulfate precipitation step onward by incorporation of ^{32}P into yeast phosphorylase (0.9 mg · ml^{-1}) under standard conditions. In the crude extract and streptomycin sulfate supernatant this method could not be used because these fractions contained a high amount of endogenous phosphate acceptors. Therefore, the standard activation assay was used for these purification steps. Numbers in parentheses represent the total enzyme activity calculated by multiplication of the value of the 42% $(NH_4)_2SO_4$ sediment with the purification factor derived from the activation assay. Protein was determined by the biuret method (9) except for the last two steps where it was measured by absorbance at 280 nm.

same buffer, activity was eluted with a linear gradient of 0.5 - 2 M NaCl (flow rate 25 ml/h). Kinase fractions were collected and after concentration applied to a second Cibacron blue F 3 G-A column (1 x 10 cm) in Tris-100 and the chromatogram developed with a linear 0 - 0.2 M NaCl gradient at a flow rate of 5 ml/h without previous salt wash of the column.

The purification procedure is summerized in Table 1. Phosphorylase kinase was enriched 2800-fold with a yield of ca. 20 % of total activity. Gel electrophoresis of the native enzyme on 8 % polyacrylamide gels exhibited several protein bands which did not migrate with phosphorylase kinase activity. The kinase activity appeared as two bands and a part of the activity did not penetrate the gel, likely due to enzyme aggregation.

This observation raised the question whether K I from the DEAE-cellulose column may also constitute an aggregate form of phosphorylase kinase. To test

this, K I was pooled and rechromatographed on DE-52 under otherwise identical conditions but in the presence of 0.1 % Triton X-100 to dissociate aggregates. A smaller peak of kinase activity emerged at the position of K I and a second peak at the position of K II, indicating that K I constituted an aggregate of K II.

Properties of the enzyme

As can be seen in Fig 1, phosphorylase-, histone- and casein kinase activity copurify closely with K II. A distinctly different casein kinase activity without activity on other substrates emerges in the breakthrough of the DE-52 chromatography. During purification, the substrate specificity of K II was measured by determining the phosphorylation ratios for phosphorylase, histone and casein under standard conditions. When expressed as percent activity with phosphorylase as 100 %, the ratio of phosphorylation rates is 100 : 11 : 7.3 for phosphorylase, histone and casein, respectively. This relation did not change significantly during purification. The assumption that phosphorylase, histone and casein kinase activities in K II represent the same enzyme was supported by kinetic studies with different concentrations of Mg^{2+}. None of the activities was inhibited by concentrations of Mg^{2+} as high as 30 mM. In fact, all activities were stimulated by increasing Mg^{2+} concentrations. A broad pH optimum was observed at pH 8 with 70 % activity at pH 7.2 and 9.0 in the standard activation assay. The K_m values for ATP, GTP and UTP with yeast phosphorylase as substrate were 0.018, 0.6 and 1.4 mM, respectively, while CTP had a K_m value too high to be measured in the assay. Molecular weight of the enzyme was 29,000 ± 2,000 determined by gel filtration. If highly purified kinase was used an additional peak emerged at the void volume again pointing to aggregation of the enzyme.

cAMP-dependent phosphorylase kinase activity

Figure 2 shows the cAMP-stimulated activation of phosphorylase in the presence of cAMP-dependent protein kinase. In order to test a possible identity of phosphorylase kinase with catalytic subunit of cAMP-dependent kinase,

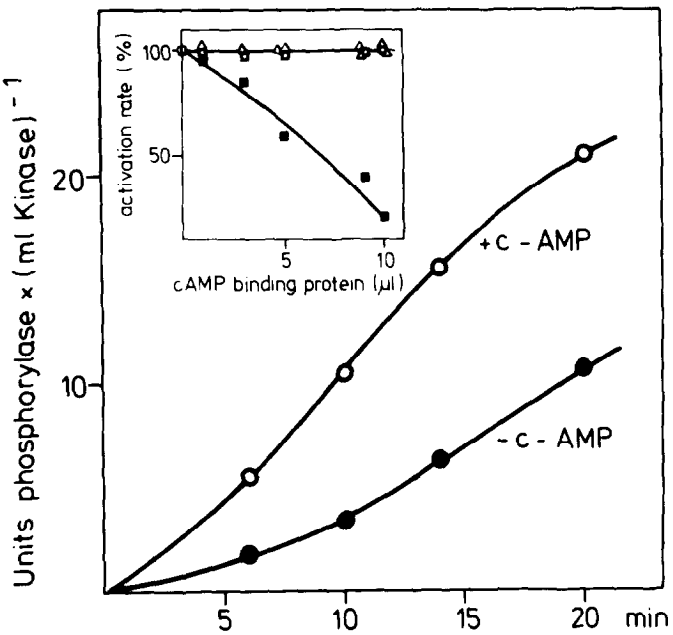

Fig. 2. Activation of yeast phosphorylase by cAMP-dependent protein kinase in the presence and absence of cAMP (2 µM). cAMP-dependent yeast protein kinase was purified according to (5) and used in the standard activation assay. In the ordinate the amount of phosphorylase activity generated by the action of phosphorylase kinase is given.

Insert: Effect of cAMP binding protein on phosphorylase kinase (Δ) and cAMP-dependent kinase catalytic subunit (□) in the absence (open symbols) and presence (filled symbols) of cAMP. Protein concentration of cAMP-binding protein was too low to be accurately determined. From gel electrophoresis a concentration of 0.1 mg/ml was estimated.

equal amounts of both kinases were incubated with cAMP-binding protein from yeast prepared and freed of cAMP as described in (5) in the absence and presence of cAMP (Fig. 2, insert). While the catalytic subunit of cAMP-dependent kinase was clearly inhibited by increasing amounts of the cAMP-binding protein in the absence of cAMP, K II was not inhibited.

Discussion

DEAE-cellulose chromatography of fractionated yeast proteins separated two peaks of phosphorylase kinase activities. One of them, K I, probably constituted an aggregate of the second, K II. K II showed a strong tendency for aggregation while K I could be partially converted into K II by treatment with detergent. Highly purified K II resembled a typical protein kinase acting also on casein and histone, albeit only at 10 % of the activity compared to a

similar concentration of yeast phosphorylase. Therefore, the enzyme is refered to as phosphorylase kinase.

Substrate specificity and effector sensitivity of phosphorylase kinase could be clearly distinguished from the ribosomal yeast protein kinases described by Becker-Ursic and Davies (11) and Kudlicki et al. (12, 13) which prefer acidic substrates and are bound to ribosomes from which they are separated only by washing with high salt. Casein kinase isolated by Lerch et al. (2) did not act on yeast phosphorylase. Phosphorylase kinase is clearly distinct from the catalytic subunit of protein kinase which also recognizes yeast phosphorylase as substrate. While the catalytic subunit of the cAMP-dependent protein kinase was inhibited by its regulatory subunit from yeast, phosphorylase kinase was not. During purification of phosphorylase kinase, the cAMP-dependent kinase is apparently destroyed as no SH-protecting reagents were used.

Recently it was discovered that the I-form of yeast glycogen synthase is converted into the D-form by phosphorylase kinase. The highly purified protein kinase contains glycogen synthase pointing to a strong affinity between these enzymes (Dr. Ch. Mishra, unpublished). This points to a role of phosphorylase kinase in regulation of glycogen metabolism in yeast which seems to be regulated quite differently than in rabbit muscle where phosphorylase kinase is a specific enzyme.

Acknowledgements

The authors thank Dr. Thomas Buckhout for critical reading of the manuscript. The work was supported in part by the Deutsche Forschungsgemeinschaft.

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