Vol. 85, No. 4, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 29, 1978 Pages 1400-1406

ISOLATION AND PARTIAL CHARACTERIZATION OF A MEMBRANE BOUND PROTEIN KINASE FROM MITOCHONDRIA OF SACCHAROMYCES CEREVISIAE

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Received October 31, 1978

SUMMARY: Saccharomyces cerevisiae mitochondria, isolated by enzymatic lysis of the cell wall and purified by gradient centrifugation are able to phosphorylate serine residues of exogenous phosphoproteins in the presence of added [γ^{32}P] ATP. Most of the protein kinase activity is bound to the mitochondrial membranes from which it can be partially solubilized by 0.7 M NaCl. The solubilized protein kinase, whose M.W. is approximately 30,000, has been partially purified by Phosphocellulose chromatography: it displays its activity toward "acidic" phosphoproteins (α_{s2}-casein$>\alpha_{s1}$-casein = phosvitin$>\beta$-casein) while it does not phosphorylate histones even in the presence of cAMP. The enzyme requires Mg^{2+}, which cannot be replaced by Mn^{2+}, and is strongly inhibited by inorganic Phosphate.

INTRODUCTION

A ^{32}P incorporation into serine residues of proteins, occurring in yeast mitochondria respiring in the presence of ^{32}Pi has been reported several years ago (1). However nothing was known about the mechanism and the precise localization of such a process, evidenced in crude preparations obtained by mechanical disruption and presumably contaminated by other subcellular fractions. The improvements of the procedures for the isolation of yeast mitochondria and the more and more frequent reports about protein kinase activity associated with many different biological membranes prompted us to look for protein kinases (E.C. 2.7.1.37) in purified preparations of yeast mitochondria.

The results reported in the present paper provide the first clear-cut evidence that mitochondrial membranes from Saccharomyces Cerevisiae are able to phosphorylate very actively foreign acidic protein substrates, like casein and phosvitin, while displaying negligible activity toward histones. The "casein kinase(s)" responsible for such a phosphorylation has been partially purified and submitted to a preliminary characterization including its substrate specificity, M.W. evaluation and cofactors requirements.

EXPERIMENTAL

Isolation and purification of yeast mitochondria. Saccharomyces cerevisiae haploid strain D41 (α ura his ρ^+) was used throughout this work. The strain was obtained by Dr. D.Wilkie. The yeast strain was grown in Ethanol medium (2% v/v

0006-291X/78/0854-1400$01.00/0

Ethanol, 5.0 g/l Yeast Extract, 5.0 g/l Bacteriological Peptone, 1.0 g/l KH_2PO_4, 1.2 g/l $(NH_4)_2SO_4$, 0.5 g/l NaCl, 0.7 g/l $MgSO_4 \cdot 6H_2O$ and 0.4 g/l $CaCl_2$) until late exponential phase, when 20 to 25 g of cells per experiment were harvested. Mitochondria were obtained by enzymatic lysis of the cell wall using snail enzyme, essentially as described by Faye et al. (2). The only modification was that the mitochondrial pellet obtained after the first centrifugation at 13,000 rpm was washed once with 0.5 M Sorbitol containing 50 mM Tris-HCl and 0.1 mM EDTA (pH 7.4) and resuspended in the same medium at the concentration of 20 mg protein/ml (=crude preparation). Aliquots of 1.7 ml were then layered onto several tubes of a discontinuous sorbitol gradient composed of 4 ml of 70% and 2 ml each of 60, 57.5, 55, 52.5, and 50% sorbitol in 0.02 M Tris-HCl pH 7.4, as described by Henson et al. (3). After 4.5 hrs centrifugation in the SW 27 rotor of the Beckmann L5-50 ultracentrifuge the tubes were divided into six fractions (see Fig.1) and the protein content, malic dehydrogenase (4) and protein kinase activity were determined. In particular fraction 5 was collected and referred to as purified mitochondria. The approximate yield was of 50 mg of mitochondrial proteins per experiment.

Solubilization and Partial Purification of Mitochondrial Membrane bound Protein kinase. 50 mg of yeast mitochondria purified by sorbitol gradient centrifugation, previously sonicated in 25 ml of 20 mM Tris-HCl buffer pH 7.5 and precipitated in order to remove the soluble proteins, were resuspended with 15-20 ml of the same buffer containing 0.7 M NaCl and extracted for 30 min at 0-2° by continuous stirring. The clear supernatant was collected by 100,000 x g centrifugation and the precipitated mitochondrial membranes were extracted twice more by the same procedure. Aliquots of the pooled supernatants were dialyzed overnight against 0.05 M Tris-acetate pH 7.5 and submitted to Phosphocellulose chromatography through a 2x1 cm column equilibrated with the same buffer. After washing of the column with 15 ml of the equilibrium buffer, the elution was started with a 200 ml gradient ranging from 0.05 M to 0.5 M of Tris-acetate pH 7.5. The flow rate was about 50 ml/hr and 4.5 ml fractions were collected and immediately tested for their protein kinase activity. The 3 fractions displaying the maximal activity eluted by a Tris-acetate molarity of about 0.27-0.30 were pooled, if necessary concentrated by ultrafiltration (Diaflo UM 10 Membranes), and used within 10 hrs for the characterization of the enzyme.

Determination of Protein kinase Activity. 0.3-0.4 mg of mitochondria or mitochondrial membranes were incubated for 10 min at 35° in 0.25 ml of a medium containing: Hammarsten whole Casein 0.5 mg, 100 mM Tris-HCl buffer pH 7.5, 12 mM $MgCl_2$, 40 μM ATP containing 0.5 μC as [γ^{32}P] ATP and 0.1% Triton X100. The incubation was stopped by addition of 4 ml of 10% Trichloroacetic acid and the precipitated proteins, collected by centrifugation, were washed with 5 ml of 10% Trichloroacetic acid, three times at room temperature and once at 100° for 5 min, prior to be dissolved with 6 ml of Instagel scintillation liquid and counted in a liquid scintillator. Control experiments were constantly run in which either casein or the enzyme preparation were omitted. In the experiments with soluble extracts and purified enzyme Triton X100 was omitted from the incubation medium and the additions of the enzymatic preparations ranged between 20 and 80 μg. In some experiments whole casein was replaced with 0.25 mg of purified casein fractions or other protein substrates.

Evaluation of Protein Kinase Molecular Weight. A M.W. evaluation of mitochondrial protein kinase was performed by layering 0.3 ml of the NaCl crude extract additioned with standard protein markers (0.8 mg), onto 4.2 ml of a preconstructed linear 5-20% sucrose gradient in 50 mM Tris-HCl, 2 mM $MgCl_2$ and 150 mM NaCl (pH 7.5). The tubes were centrifuged in the SW39 rotor of the Beckmann L ultracentrifuge at 39,000 rpm for 20 hrs. They were than punctured at the bottom and fractions of 0.28 ml collected and analysed for absorbance at 230 nm and protein kinase activity.

Isolation and determination of ser-^{32}P and Thr-^{32}P from phosphorylated protein substrates were performed by pH 1.9 paper electrophoresis after 4 hrs hydrolysis in 6N HCl at 105°, as previously described (5).

Phosphorylable Substrates and other Chemicals. Whole casein was Hammarsten casein from Merck. Purified α_{s1}-, α_{s2}-, β-, and k-caseins were gifts from Dr. B. Ribadeau-Dumas (INRA, Jouy-en-Josas). Partial dephosphorylation of caseins was performed with potato acid phosphatase as described by Brignon et al. (6). Phosvitin was prepared according to Mecham and Olcott (7). Histone type IIA and Salmine sulfate were from Sigma, while Histone F1 was prepared from calf thymus according to Johns (8). Soybean Trypsin Inhibitor, DNAse I and Bovine Serum Albumin were from Serva, Worthington and Sigma respectively. All other proteins and reagents were from Merck.

RESULTS AND DISCUSSION

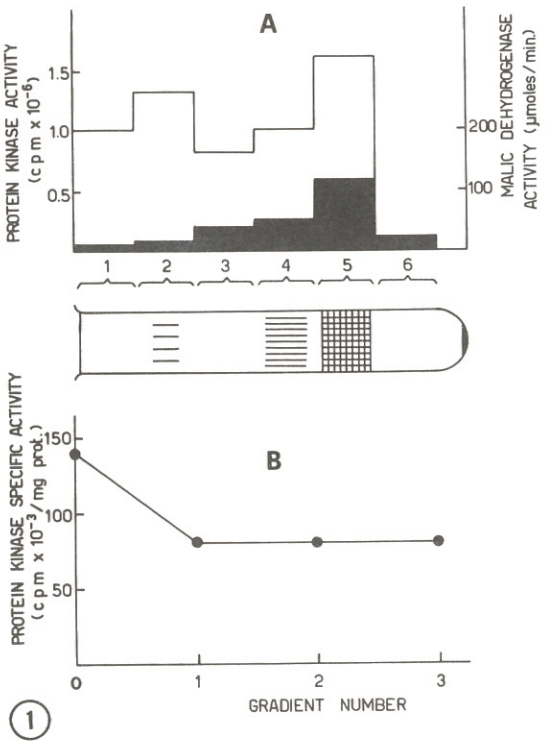
As shown in fig. 1A, by testing the protein kinase activity of crude mitochondrial preparations submitted to sorbitol gradient centrifugation, a broad and heterogeneous pattern is obtained: however the mitochondrial fraction still retains quite a lot of protein kinase activity, which can not be furtherly removed, as shown in fig. 1B, by repeating the purification procedure.

Sonication of the purified mitochondria in hypotonic solutions of 20 mM Tris-HCl pH 7.5 results in the solubilization of only about 20-30% of their casein kinase activity, while sebsequent extractions with 0.7 M NaCl solubilize about 60% of the activity. The remaining activity, accounting for 10-20% of total, is not solubilized even by repeating the NaCl extractions, while it can be easily evidenced in the extracted membranes from which it is partially solubilized by 0.1% Triton X100.

No significant differences concerning the substrate specificity, the cofactors requirements and the pH optimum (7.0-7.5) could be evidenced between the NaCl extracted and the membrane bound, NaCl insoluble, casein kinase activities. They also display quite superimposable curves of thermic inactivation, as shown in Fig.2, suggesting that the membrane bound and the NaCl solubilized caseinkinase activities are due to the same enzyme(s).

As shown in fig.3 the NaCl extracted casein kinase(s) can be efficiently purified by Phosphocellulose column chromatography from which its major peak of activity is eluted with 0.27 M Tris-acetate buffer, free of most of the contaminating proteins. Such a purified preparation however completely inactivates within 20-30 hrs at 2°. The molecular weight of mitochondrial casein kinase has been therefore evaluated by submitting the crude extract to sucrose gradient centrifugation, in parallel with standard proteins: as shown in fig.4 the peak of activity overlaps the DNAse band, thus displaying a M.W. of about 30,000.

A preliminary characterization of the partially purified yeast mitochondria protein kinase, concerning its substrate specificity, divalent cations requirements and its response to some effectors is reported in table I. The activity toward histones is quite negligible, while phosvitin and casein fractions are actively phosphorylated through a mechanism apparently insensitive to cAMP:in particular α_{s2}-casein behaves as the most suitable substrate; α_{s1}-casein is



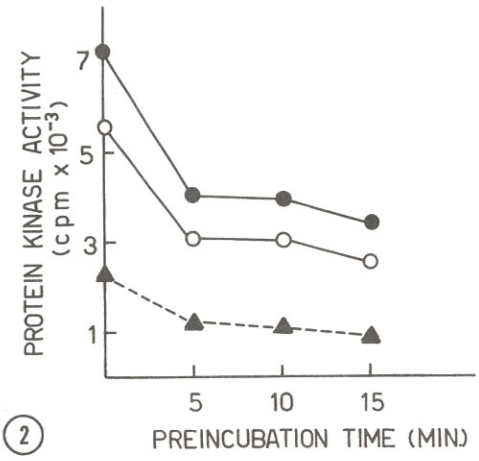


Fig. 1 - Evidentiation of Protein kinase activity in yeast mitochondria purified by gradient centrifugation.

The upper figure (A) outlines the purification of crude mitochondria by sorbitol gradient centrifugation (empty bars: protein kinase activity, tested on whole casein; black bars: malic dehydrogenase activity, expressed as μmoles of substrate oxidized/min. Both activities refer to the fractions collected from one tube, as indicated). In the lower figure (B) the specific casein kinase activities of crude mitochondria and mitochondria purified by successive sorbitol gradients are compared. Essentially the same results were obtained by replacing the sorbitol gradients with sucrose linear gradients.

Fig. 2 - Inactivation of casein kinase activity in NaCl soluble extracts (o——o) and extracted membranes (●——●) preincubated for different times at 40°.

The endogenous ^{32}P incorporation into protein membranes in the absence of casein (▲--▲) is also reported. Identical results were obtained replacing casein with phosvitin. 5 min preincubation at 45° and at 50° resulted in a 70% and 96% inactivation respectively, of both the soluble and the membrane bound protein kinases.

phosphorylated at about the same rate as phosvitin and better than β-casein, and the previous partial dephosphorylation of the protein substrates is apparently without any effect on the enzymatic reaction catalyzed by our protein kinase preparation. Such an activity spectrum, together with its low M.W. and

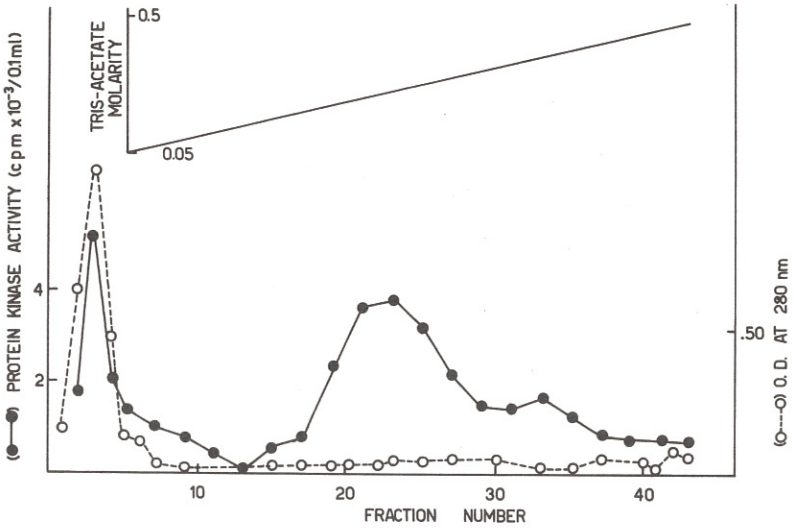


Fig. 3 - Purification of NaCl extracted mitochondrial Protein kinase activity by Phosphocellulose column chromatography.

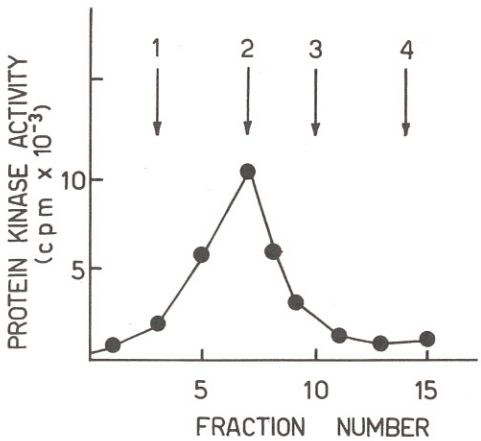


Fig. 4 - Molecular Weight evaluation of NaCl extracted mitochondrial Protein kinase by sucrose gradient ultracentrifugation.

Arrows indicate the migration position (maximum $A_{230\ nm}$) of the following standard proteins: 1) Soybean Trypsin Inhibitor (M.W. 21,500); 2) DNAse I (M.W. 31,000); 3) Ovalbumin (M.W. 45,000); 4) Bovine Serum Albumin (M.W. 76,000).

the failure of Mn^{2+} to replace Mg^{2+} as an activator, would suggest that the mitochondrial protein kinase has different properties than the soluble yeast protein kinase whose purification has been described by Lerch et al. (9). The

TABLE I - Substrate specificity, cofactors requirements and inhibition by salts of partially purified yeast mitochondria Protein kinase.

Exp.	Substrate	^{32}P incorporated into substrate (cpm).
1	whole casein	21,000
	α_{s2}-casein	28,000
	α_{s1}-casein	20,400
	" 50% dephosphorylated	20,100
	β -casein	12,800
	" 30% dephosphorylated	13,100
	k-casein	550
	phosvitin	19,400
	" + cAMP (10^{-6}M)	20,000
	mixed histones (Type IIA)	350
	" + cAMP (10^{-6}M)	310
	histone F1	310
	Protamine	340
2	whole casein	15,700
	" $MgCl_2$ omitted	630
	" " + 10 mM $CaCl_2$	880
	" " + 10 mM $MnCl_2$	750
	" " + 10 mM $CoCl_2$	4,200
3	phosvitin	20,100
	" + NaCl	10,000
	" + KCl	10,500
	" + Na acetate	15,300
	" + NaH_2PO_4	4,900
	" Tris-acetate replaced by Tris-HCl	17,500
	α_{s1}-casein	21,200
	" + NaCl	16,100
	β -casein	13,000
	" + NaCl	3,100

Protein kinase extracted by 0.7 M NaCl and purified by Phosphocellulose chromatography was used throughout these experiments which were repeated at least 3 times with essentially the same results. General conditions are those described in the experimental section except for the replacement of Tris-HCl buffer with Tris-acetate. Salts concentration, where not indicated, was 50 mM.

activity of our enzyme is about 50% inhibited by 50 mM NaCl or KCl; Na acetate is less inhibitory, while 50 mM Na Phosphate almost completely prevents the phosphorylation of phosvitin indicating that inorganic phosphate, at concentrations occurring within mitochondria, might influence the activity of protein kinase. The inhibitory effect of salts however appears to be dependent also on the nature of the protein substrate.

The radioactivity incorporated into both phosvitin and casein by the mitochondrial protein kinase was found to be almost completely accounted for by ser-^{32}P, while thr-^{32}P residues are either absent or anyway negligible. This finding, together with the very similar M.W., the insensitivenessto the previous dephosphorylation of the substrates and the localization within insoluble struc-

tures, would suggest a remarkable similarity between this enzyme and the so called "casein kinase S" isolated from rat liver (10,11), where it appears to be preferentially associated with insoluble structures like mitochondria (12) and microsomes (13). As yet the biological role of such an yeast mitochondria protein kinase in unknown, though it is conceivable that it might be responsible for the phosphorylation of membrane bound proteins. Indeed an endogenous phosphorylation of mitochondrial membrane proteins has been evidenced, whose thermal inactivation, shown in fig. 2, parallels that of protein kinase activity toward foreign substrates, suggesting that the same enzyme(s) is responsible for both the processes.

ACKNOWLEDGMENTS

The Authors are indebted to Mrs. Giuliana Giungarelli and Mr. Giorgio Parpajola for their valuable cooperation in preparing the manuscript and the graphs.

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