PROPERTIES AND LOCALIZATION OF A cAMP-INDEPENDENT PROTEIN KINASE (CASEIN KINASE) ISOLATED FROM YEAST MITOCHONDRIA

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1. Introduction

One of the first protein kinases to be described was isolated from yeast [1]; a yeast protein kinase active on casein and phosvitin was purified to homogeneity in [2]. Little is known about the subcellular distribution and the biological involvements of yeast protein kinases. Recently multiple forms of such enzymes active also toward ribosomal proteins have been obtained in yeast cytosol [3]; a casein kinase bound to yeast mitochondrial membranes has been reported [4]. These results, obtained under conditions minimizing ATP disappearance due to ATPase, provide definite evidence that a mitochondrial protein kinase activity distinct from the microsomal and cytosolic ones, can be readily detected using casein as artificial phosphate acceptor. Such an activity is mainly localized in the inner membranes, where it is accessible however to externally added trypsin. Mitochondrial 'casein kinase' is only incompletely solubilized by 0.7 M NaCl. It is drastically inhibited in a reversible though not competitive way by pyrophosphate at possibly physiological concentrations and it affects the same residues of casein fractions also phosphorylated by rat liver 'casein kinase S' [5].

2. Experimental

2.1. *Isolation and purification of yeast mitochondria*

Saccharomyces cerevisiae haploid strain D41 was grown in a medium containing ethanol as a carbon source, as in [4]. Mitochondria were obtained by enzymatic lysis of the cell wall by snail enzyme and purified by repeated washings as in [6], the only modification being the addition of 0.05 mM phenylmethyl sulfonyl fluoride (Sigma) to all the solutions used to isolate and purify the mitochondria. Alternatively, the digestion of the cell wall was performed by incubating for 40 min at 37°C 20 g yeast cells (wet wt) in 100 ml solution containing 1 M sorbitol, 0.05 M citric acid, 0.15 M K_2HPO_4, 0.015 M 2-mercaptoethanol, 0.01 M EDTA and 30 mg zymolyase 60 000 (Kirin Brewery Laboratories, Takasaki, 370-12, Japan) (pH 7.5). The isolation and purification of mitochondria was the same in both procedures. The purity of the mitochondria was checked by layering them on the top of a sorbitol gradient and collecting the different fractions after centrifugation as in [4]. The different fractions were assayed for protein content, succinate dehydrogenase [7], malic dehydrogenase [7], NADPH-cytochrome *c* reductase [7] and protein kinase activity. Mitochondria constantly gave a single particulate band at ~3.2 M sorbitol, exhibiting the marker enzymes specific activities expected for pure mitochondria [8] (see table 1).

Microsomes and cytosol were prepared by centrifugation at 105 000 × *g* for 60 min of the 15 000 × *g* supernatant of the first mitochondrial sediment.

2.2. *Separation of the mitochondrial membranes*

The purified mitochondria were washed once with a hypotonic solution containing 0.01 M phosphate (pH 8.2). They were then suspended in the same solution at 30 mg protein/ml and left to swell at 0°C. After 30 min 1 vol. ice-cold shrinking buffer containing 2.5 M sucrose, 4 mM ATP, 4 mM $MgSO_4$ and 0.2% bovine serum albumin was added and the suspension was sonicated for 10 s (Labsonic Braun 1510, 30 W). The mixture was diluted to 1 M sucrose final conc. and the membrane fractions were separated by

differential centrifugation according to [9]. The 15 000 × *g* and 105 000 × *g* pellets were assayed for succinate dehydrogenase [7], kynurenine hydroxylase [9] and protein kinase activity.

2.3. *Partial purification of mitochondrial casein kinase*

Casein kinase activity was partly solubilized from mitochondria by repeated extractions with 20 mM Tris–HCl (pH 7.5) containing 0.7 M NaCl. The extract was dialyzed overnight against 20 mM Tris–HCl (pH 7.5) containing 6 mM 2-mercaptoethanol, 0.1 mM EDTA and 10% glycerol (buffer A) and submitted to a purification procedure similar to that in [3]; after filtration through a DEAE-cellulose column (1.5 × 8.0 cm) equilibrated and washed with buffer A and eluted with a linear gradient consisting of 150 ml buffer A and 150 ml buffer A containing 0.4 M KCl (see fig.1) the main peak of casein kinase activity, not retained by DEAE-cellulose (fraction I), was applied to a P-cellulose column (1.5 × 6.0 cm) equilibrated with 0.1 M Tris–acetate (pH 7.5) with the same additions of buffer A and eluted as a single peak emerging with ~0.5 M Tris–acetate of a linear gradient of 0.1–1.0 M Tris–acetate with 150 ml in each chamber. The final preparation displaying a rather broad pH optimum at 7–9 was routinely at pH 7.5 and was fairly stable for several weeks at −18°C.

2.4. *Determination of casein kinase activity*

Three critical modifications were introduced into the procedure for testing casein kinase activity in [4]:

(i) The [γ-^{32}P]ATP concentration was raised to 200 μM, spec. radioact. ~10 μCi/μmol;
(ii) The particulate fractions were solubilized with 1.0% Triton X-100, omitting any sonication before the assay;
(iii) A powerful ATPase inhibitor (AMP-PNP, Boehringer) was added at 1.5 mM.

Under such conditions [γ-^{32}P]ATP was only partially hydrolyzed during the protein kinase assay of mitochondria, thus making possible a reliable comparison with the other subcellular fractions exhibiting much lower ATPase activities. The specific activity of mitochondrial casein kinase obtained by this modified procedure is 3–5-times higher than that in [4]. Probably however it is still underestimated since the recovery of casein kinase in mitochondrial extracts devoid of ATPase, was >100% (see table 2) in spite of the fact that part of the activity could not be solubilized.

Whole acid casein and purified casein fractions (α_{s1}, α_{s2} and βA^2) were kindly provided by Dr Ribadeau-Dumas. Other chemicals were either from Sigma or Merck.

3. Results and discussion

3.1. *Mitochondrial localization of casein kinase activity*

As shown in table 1, under the modified conditions of section 2, a remarkable 'casein kinase' activity, specifically associated with the purified mitochondrial fraction can be seen. The low NADPH-cytochrome *c* reductase activity of our preparations, quite consistent with the values reported for pure mitochondria [8,10] rules out any significant contamination by microsomes, whose protein kinase, furthermore, exhibits a DEAE-cellulose pattern markedly different from that of mitochondria (see next paragraph). On the other hand any appreciable contamination from the soluble cytosol is very unlikely since repeated washings with isotonic and diluted saline solutions cannot remove the mitochondrial casein kinase activity and the cytosolic casein kinase fraction III is not detectable in mitochondria (see next paragraph). Moreover mitochondrial membranes repeatedly extracted with 0.7 M NaCl retained 40–50% of their original casein kinase activity which could be solubilized with 0.5% Triton X-100.

The submitochondrial compartmentation of casein kinase is also shown in table 1: though the outer membranes display a significant activity, most of casein kinase activity is accounted for by the inner compartment, whose contamination by outer membranes is quite negligible according to the absence of kynurenine hydroxylase, a specific marker for outer membranes [9]. Most of casein kinase is still insoluble after sonication in hypotonic media, suggesting that it is bound to the inner membrane rather than located within the matrix. Moreover ~70% of such casein kinase activity is lost upon preincubation with trypsin, which is consistent with the exposure of the kinase on the outer surface of the inner membrane. Accordingly the trypsin treatment after sonication, which is supposed to produce a majority of inside out vesicles [12], is less effective.

3.2. *Partial purification and characterization*

Apparent purification of mitochondrial casein

Table 1
Casein kinase activity of yeast mitochondria and submitochondrial fractions

	Casein kinase (cpm . min^{-1} . mg^{-1})	Succinate dehydrogenase (nmol . min^{-1} . mg^{-1})	Malate dehydrogenase (μmol . min^{-1} . mg^{-1})	NADPH-cyt. *c* reductase (nmol . min^{-1} . mg^{-1})	Kynurenine hydroxylase (nmol . min^{-1} . mg^{-1})
Expt. 1					
Microsomes (105 000 × *g* pellet)	2560	2.1	–	108	–
Purified mitochondria	1800	196	6.2	18.5	–
after gradient	1825	192	5.5	18.0	–
Expt. 2					
Outer membrane (7 mg) (105 000 × *g* pellet)	733	8.72	–	–	10.8
Inner compartment (40.5 mg) (15 000 × *g* pellet)	1340	222	–	–	0.0
Inner compartment sonicated (insoluble)	1053	220	–	–	–
trypsin treated[a]	402	190	–	–	–
sonicated, trypsin treated[a]	713	60	–	–	–

[a] According to [11]

Conditions for the isolation of microsomes, mitochondria and submitochondrial fractions and for the assays of casein kinase and marker enzymes are in section 2. The casein kinase activity of soluble cytosol (105 000 × *g* supernatant) in expt. 1 was 720 cpm . min^{-1} . mg^{-1}

kinase by >100-times was achieved by submitting to DEAE- and phospho-cellulose column chromatography the 0.7 M NaCl mitochondrial extracts (see table 2). The DEAE-cellulose profiles of mitochondrial extracts, soluble cytosol and microsomal extracts are different from each other (fig.1). In particular the cytosolic profile very closely resembles that in [3], exhibiting a third retarded peak of activity which is absent in the mitochondrial extract. The microsomal pattern on the other hand, though exhibiting the peak III, is almost completely accounted for by the peak II, which is a minor fraction both in cytosol and mitochondria. These findings support a fairly specific distribution of the enzymatic equipment for protein phosphorylation within the different yeast subcellular compartments.

The yeast mitochondrial casein kinase activity is inhibited by several salts, among which phosphate proved especially effective [4]. However, a much more dramatic inhibitory effect is induced by PP: which is effective at only 2–3 mM. Its effect cannot be accounted for by the binding of Mg^{2+} as it is inde-

Table 2
Purification of the mitochondrial casein kinase

Fraction	Protein[a] (mg)	Total activity (cpm . min^{-1}) . 10^{-5}	Specific activity (cpm . min^{-1} . mg^{-1}) . 10^{-3}	Purification factor
Mitochondria	210	4.2	2	–
0.7 M NaCl extract	100	8.13	8.13	4
DEAE-cellulose (fraction I)	27	6.23	23.1	11.3
Phosphocellulose	0.5	1.11	222.2	108.5

[a] Determined with the Bio-Rad protein assay reagent (Bio-Rad Labs. [13]

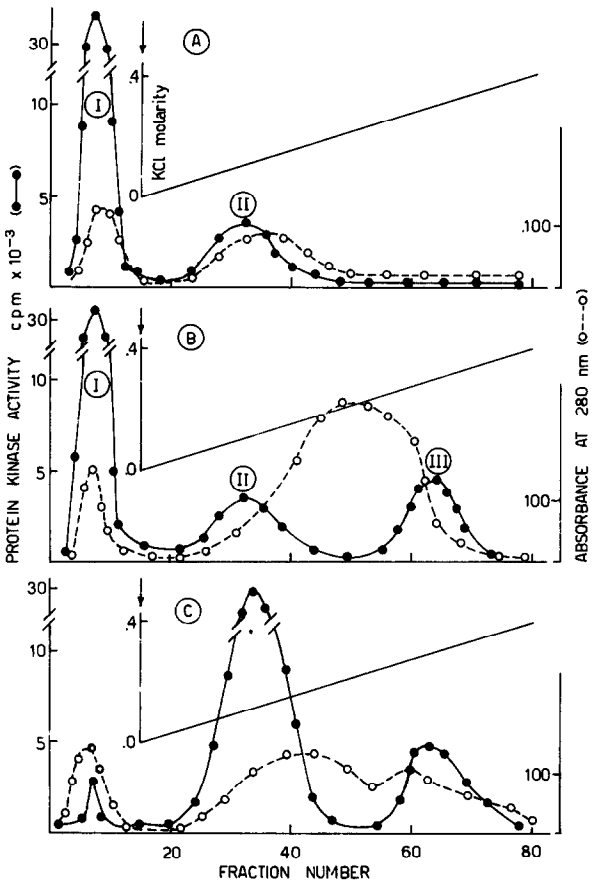

Fig.1. DEAE-cellulose profiles of yeast casein kinases from mitochondria (A), cytosol (B) and microsomes (C): 100 mg protein of mitochondrial and microsomal extracts and 150 mg protein of soluble cytosol were submitted to DEAE-cellulose column chromatography as in section 2.

pendent of $[Mg^{2+}]$. Such a pyrophosphate inhibition is inexplicable as a competitive effect toward ATP or casein since, as shown in fig.2, the V_{max}-values are drastically reduced by 3 mM pyrophosphate, whereas the app. K_m-values are unchanged. However the inhibition induced by preincubating the enzyme with 3 mM pyrophosphate can be reversed by a brief dialysis. These data are still consistent with an allosteric inhibition by pyrophosphate resulting in a decrease of the phosphorylation efficiency though not affecting the affinity for the substrates.

As reported in [4] the mitochondrial protein kinase is actually insensitive to cAMP and inactive toward basic proteins like histones and protamines, its activity being readily detected with phosvitin and casein fractions. The sites affected in β- and α_{s2}-casein

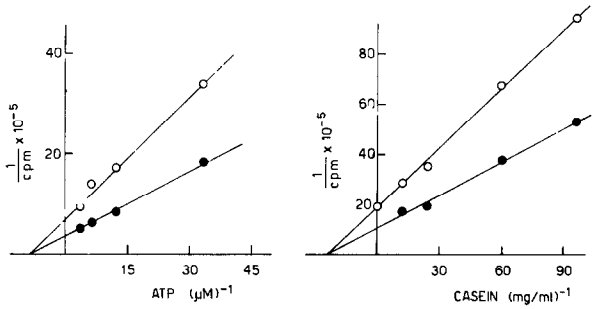

Fig.2. Double reciprocal plots of casein kinase activity as a function of ATP (left) and casein (right) concentrations. Effect of pyrophosphate: (●——●); (o——o) + 3 mM Na-pyrophosphate.

have been now identified following the procedures described for liver protein kinases [5]. The only residue phosphorylated in β-casein in ser_{22}, included in the sequence:

–SerP–SerP–SerP–Glu–Glu–$SerP^{32}$–Ile Thr–Arg

while the two residues phosphorylated in α_{s2}-casein are the seryl residues 13 and 135 included in the following sequences:

SerP–SerP–SerP–Glu–Glu–$SerP^{32}$–Ile–Ile–SerP–

and

SerP–Thr–SerP–Glu–Glu–Asn–$SerP^{32}$–Ile Thr–Arg–

These sites, none of which is phosphorylated in native caseins, are the same affected by rat liver 'casein kinase S', which fully confirms our suggestion [4] that the yeast mitochondrial enzyme belongs to the class of socalled casein kinase S, supposed to require an acidic cluster on the N-terminal side of the target residues [5].

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