Isolation and characterization of a Type II casein kinase ('casein kinase-TS') from *Saccharomyces cerevisiae*

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1. INTRODUCTION

Among the several classes of protein kinases described up to now the so called 'casein kinases' are characterized by their insensitivity to any of the known effectors of other protein kinases, like cyclic nucleotides, Ca^{2+}, calmodulin and phospholipids and by their remarkable activity in vitro toward casein and phosvitin but not toward histones and protamines (for a review see [1]). Although the physiological involvements of casein kinases are still obscure they have been described in a variety of organisms and tissues and in some instances their site specificity has been thoroughly investigated by using artificial substrates, like purified casein fractions, as phosphate acceptor proteins [2–6]. Thus it has been clearly evidenced that casein kinases can be subdivided into two quite distinct classes, often termed Type I and II, according to their different molecular structure and specificity [1]; Type I casein kinases are monomeric enzymes, using only ATP as phosphate donor, rather insensitive to heparin and affecting only Ser residues of casein (thereafter also termed casein kinases S). Type II casein kinases, on the other hand, are oligomeric enzymes, using also GTP besides ATP, drastically inhibited by heparin and affecting both Thr and Ser residues of casein (casein kinases-TS).

While casein kinases exhibiting the properties of Type I have been described in virtually every class of eukaryotic organisms, including plants and yeasts, casein kinases unambiguously belonging to Type II had been isolated only from mammalian and avian tissues [1]. In yeast however a casein kinase fraction rather tightly adhering to DEAE-cellulose ('casein kinase-3') has been described which utilizes GTP as phosphate donor [7] and affects Thr residues of casein [8]. Such a fraction, which is lacking in mitochondria, is relatively abundant in the soluble cytosol [9].

In this paper the purification to near homogeneity and the characterization of the soluble yeast casein kinase affecting Thr residues of casein are described. Its properties are strikingly similar to those of liver casein kinase-TS, a typical casein kinase-II, and are sharply different from those of another soluble yeast casein kinase which is probably identical to the mitochondrial enzyme previously identified as a Type I (or S) casein kinase [9]. In particular the newly described Type II casein kinase from yeast cytosol exhibits an oligomeric structure with M_r = 130 000 giving rise to autophosphorylatable subunits of M_r = 37 000 and 25 000 by PAGE–SDS; it affects the Thr_{41} residue of β-casein which is also phosphorylated by animal Type II casein kinases [2,3,5] and is inhibited by roughly the same heparin concentration affecting the previously described Type II casein kinases.

2. EXPERIMENTAL

Saccharomyces cerevisiae haploid strain D41 (*α ura his rho*$^+$) and the mutant D41/50 (*α ura his rho*°), which lacks mitochondrial DNA, were used throughout this work. Cells were grown in a medium containing 20 g/l glucose, 5 g/l yeast extract, 5 g/l peptone, 1 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 $CaCl_2$. Some casein kinase preparations were obtained from soluble cytosol prepared as previously

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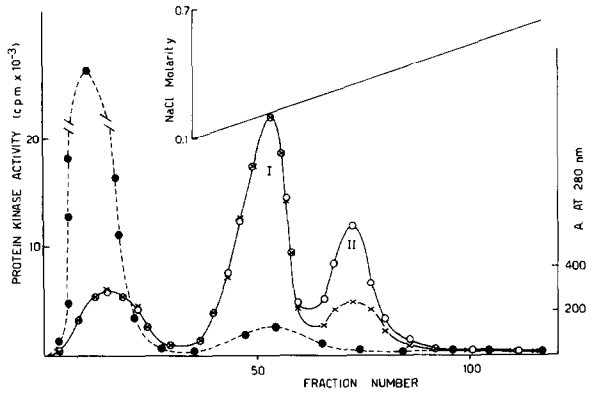

Fig.1. Phosphocellulose column chromatography of yeast cytosolic casein kinase activities tested in the absence (○—○) and in the presence (×—×) of heparin (1 μg/ml). Yeast soluble extract from strain D41 was prepared and precipitated with $(NH_4)_2SO_4$ prior to chromatography as described in the experimental section. (•—•) Absorbance at 280 nm.

described [9]. Routinely however cells were disrupted for 2 min in a Braun Cell homogenizer MSK in a medium containing 20 mM Tris–HCl (pH 7.5), 50 μM PMSF and 0.5 M NaCl. The suspension was centrifuged at 105 000 × *g* and the clear supernatant collected.

Most of the casein kinase activity was precipitated with $(NH_4)_2SO_4$ between 20% and 70% saturation. The precipitate was dissolved with the minimum volume of buffer A (0.1 M Tris–HCl (pH 7.5), 0.1 M NaCl, 6 mM 2-mercaptoethanol, 10% glycerol and 50 μM PMSF) and dialyzed against the same buffer. Aliquots of this preparation, corresponding to 10 g of yeast cells (wet weight) were applied to a phosphocellulose column (4 × 6 cm) equilibrated with buffer A. After washing the column with the same buffer until the 280 nm absorbance of the efflux was negligible, a linear gradient was started consisting of 200 ml of buffer A and 200 ml of the same buffer containing 0.7 M NaCl. 4.3 ml fractions were collected and analyzed for their absorbance at 280 nm and their casein kinase activity. The more retarded peak of casein kinase activity (fraction II, see fig.1) was submitted to further purification by DEAE-cellulose column chromatography as previously described [9] and by gel filtration through a Sepharose 6B column (2 × 140 cm) equilibrated and operated with buffer A including also 0.5 M NaCl and 0.01% Brij 35 (Serva). Its purification factor ranged between 1000 and 1300 and it resulted nearly homogeneous when submitted to polyacrylamide gel electrophoresis in the presence of SDS.

Casein kinase activity was determined by 10 min incubation with whole casein (5 mg/ml), 50 μM [γ-^{32}P]ATP and 12 mM $MgCl_2$, essentially as previously described [10].

Gel electrophoresis of the autophosphorylated casein kinase II was run at pH 8.9 on vertical plates of 10% polyacrylamide prepared essentially according to [11], as detailed elsewhere [12].

The identification of the residues of β-casein A^2 affected by casein kinases-I and II was accomplished by tryptic digestion followed by Sephadex G50 gel filtration and high voltage paper electrophoresis, using as reference markers the authentic fragments 32–48 and 1–25 phosphorylated by rat liver casein kinases-TS and S respectively [2,4].

3. RESULTS

Unlike the mitochondrial extracts whose casein kinase activity is totally accounted for by a single Type I (or S) enzyme [9], yeast cytosol from both the wild type strain D41 and the *rho*° mutant D41/50 give rise to two distinct peaks of casein kinase activity once subjected to phosphocellulose column chromatography. As shown in fig.1 the first peak, eluted by 220 mM NaCl, is quite insensitive to heparin, whereas the more retarded one, eluted by 350 mM NaCl, becomes almost undetectable if 1 μg/ml heparin is added to the casein kinase assay. Such a heparin inhibition, as shown in fig.2, A and B, is half maximal at a concentration of about 100 ng/ml and it appears to be competitive with respect to casein, as it has been shown to be the case of animal Type II casein kinases [13,14].

The purification of the casein kinase fraction II from phosphocellulose could be further improved by DEAE-cellulose column chromatography and Sepharose 6B gel filtration (see methods). By this latter procedure the molecular weights of both the casein kinase peaks from phosphocellulose were determined: as shown in fig.3 they resulted to be 30 000 and 130 000 respectively. While the former value is the same calculated for the mitochondrial casein kinase [10] and is expectable for a Type I

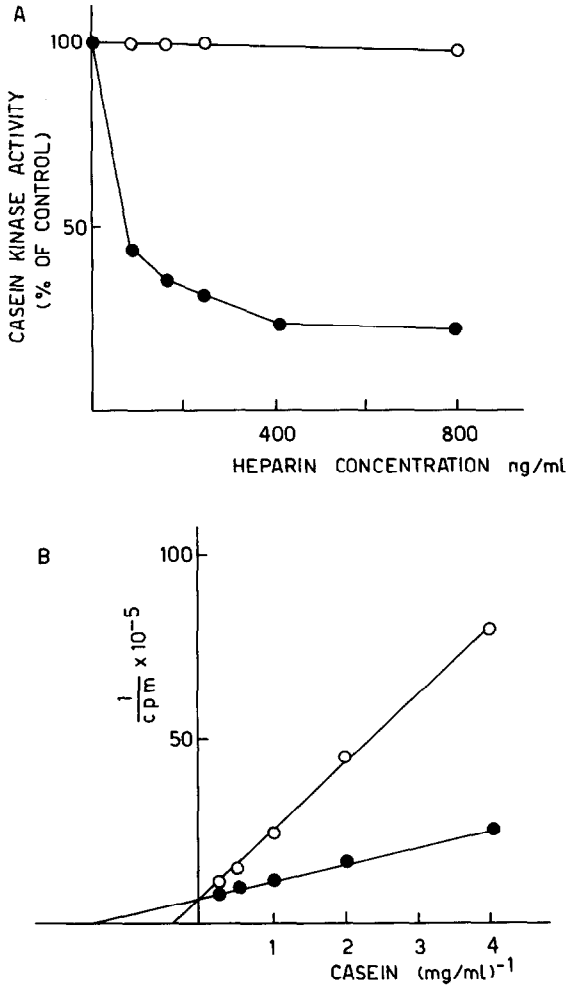

Fig.2. Inhibition of casein kinase-II by heparin. (A) Effect of increasing heparin concentrations on the activities of casein kinases-I (○—○) and II (●—●). (B) Double reciprocal plot of casein kinase-II activity versus casein concentration in the absence (●—●) and presence (○—○) of heparin (0.1 μg/ml).

casein kinase, the high molecular weight of the more retarded phosphocellulose fraction together with its sensitivity to heparin strongly supported its identification as a Type II casein kinase.

In order to strengthen such a conclusion the specificity and self phosphorylation of yeast casein kinase-II have been studied and compared with those of rat liver casein kinase-TS, a Type II casein kinase. Both enzymes were found to display rather high affinity for GTP (K_i = 70 μM) and to affect mainly Thr residues of whole casein and β-casein

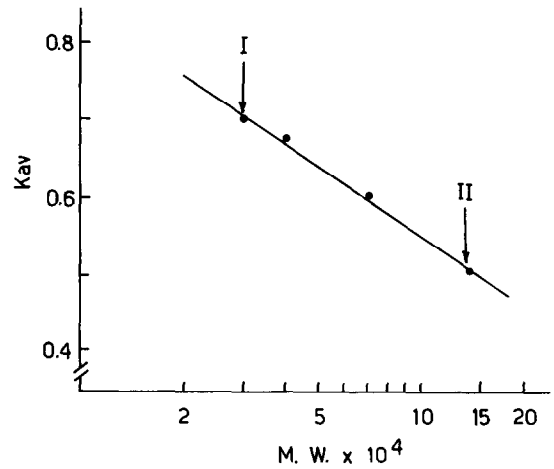

Fig.3. Determination of the molecular weights of yeast soluble casein kinases by Sepharose 6B gel filtration. The column, operated as described in the experimental section, was calibrated with: lactate dehydrogenase (M_r = 140 000), malate dehydrogenase (M_r = 70 000), ovalbumin (M_r = 45 000) and DNase (M_r = 31 000).

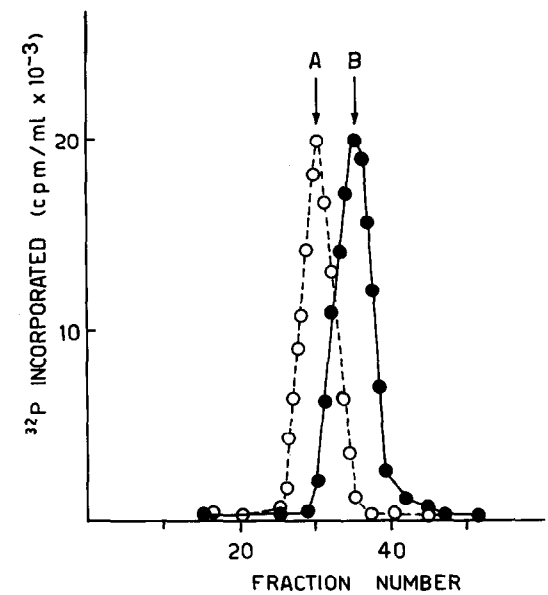

Fig.4. Co-elution of radioactive tryptic peptides from [32P]β-casein A2 phosphorylated by yeast casein kinases I (○- - -○) and II (●—●) with fragments 1–25 and 33–48 labeled by rat liver casein kinases-S and TS respectively. The elution positions of fragments 1–25 and 33–48 are indicated by arrows A and B respectively. Only [32P]Ser could be detected in the tryptic fragment labeled by casein kinase-I, while [32P]Thr accounted for more than 90% of the radioactivity incorporated in the fragment labeled by casein kinase-II.

(not shown). The identification of the Thr residue affected in β-casein A^2 by yeast casein kinase-II is outlined in fig.4. It can be seen that the whole radioactivity incorporated into β-casein by the heparin sensitive fraction II is accounted for by a single tryptic fragment containing only [^{32}P]Thr and eluted from Sephadex G50 at the same position as the tryptic fragment labeled by rat liver casein kinase TS. After high voltage paper electrophoresis such a ^{32}P-labeled peptide has been analyzed for its aminoacid content and identified as the predicted tryptic fragment 33–48, including a single Thr at the 41 position. Thus the target of yeast casein kinase II in β-casein is the same Thr_{41} residue, included within the sequence: Gln-Gln-Gln-*Thr*-Glu-Asp-Glu- which has been shown to be specifically affected by rat liver casein kinase TS [2], reticulocyte casein kinase 2 [3] and pig liver protein kinase NII [5], all of which are Type II casein kinases.

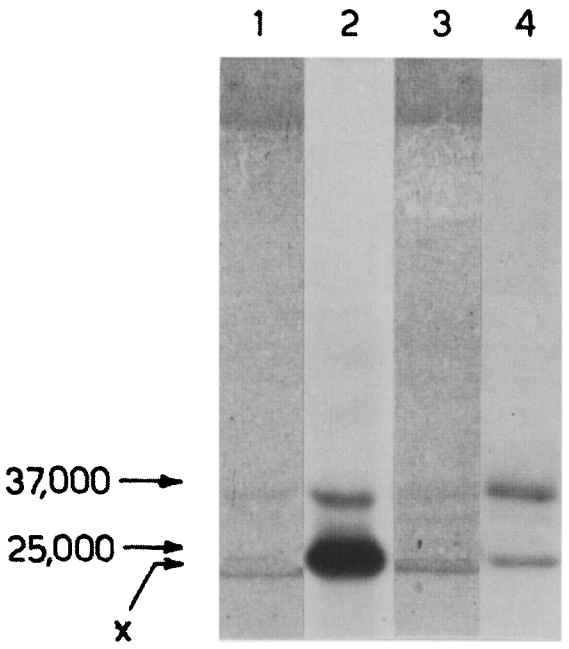

Fig.5. Co-migration of the autophosphorylation products of yeast casein kinase-II with the subunits of rat liver casein kinase TS upon polyacrylamide gel electrophoresis in the presence of SDS. Both rat liver casein kinase TS and yeast casein kinase II were incubated separately with [γ-^{32}P]ATP in the absence of any phosphate acceptor protein and subjected to 10% polyacrylamide gel electrophoresis in SDS. Processing of the gel and autoradiography were performed as in [12]. 1 and 2: rat liver casein kinase-TS; 3 and 4: yeast casein kinase-II. Odd lanes: Coomassie protein staining. Even lanes: autoradiographies. Molecular weights were estimated by calibration with ovalbumin (45 000) glyceraldehyde phosphate dehydrogenase (36 000), carboxypeptidase (34 000) and trypsinogen (24 000). The spurious band denoted (×) is an artifact of the staining procedure.

By a similar procedure, also outlined in fig.4, it was possible to demonstrate that the Ser residue affected in β-casein by the yeast casein kinase eluted first from phosphocellulose is included within a different tryptic fragment corresponding to the sequence 1 (2)–25. Such a residue could be identified as Ser_{22} following a procedure previously described [4]; the same residue is also the target of rat liver casein kinase S [4], rabbit reticulocyte casein kinase-1 [3], as well as of a casein kinase previously isolated from yeast mitochondria [9]. It is quite likely therefore that the soluble heparin-insensitive low molecular weight casein kinase and the mitochondrial one are identical.

When the high molecular weight, heparin-sensitive, casein kinase II is incubated with [γ-^{32}P]ATP in the absence of any protein substrate and subjected to polyacrylamide gel electrophoresis in the presence of SDS two radioactive bands become detectable with M_r = 37 000 and 25 000 respectively. As shown in fig.5, both bands comigrate with the subunits of rat liver cytosol Type II casein kinase-TS, the only difference consisting in the remarkable autophosphorylation of the yeast enzyme 37 000 subunit, whereas the liver kinase incorporates ^{32}P almost exclusively at its low molecular weight subunit.

4. CONCLUSIONS

This paper describes the purification from yeast and the characterization of a new soluble protein kinase exhibiting the properties of Type II (or TS) casein kinases which have been described up to now only in animal tissues. Interestingly such a yeast enzyme resembles the animal Type II casein kinases much more closely than a similar kinase isolated from cultured tobacco cells nuclei [15], whose lower molecular weight and reduced sensitivity to heparin suggest remarkable differences from 'typical' casein kinases-II.

The close analogies between the newly described yeast casein kinase and the animal Type II casein

kinases are supported by the following findings: (a) Both display high molecular weight (about 130 000) and oligomeric structure. (b) Both undergo autophosphorylation affecting the same 25 000 dalton component considered the non-catalytic subunit of animal casein kinases-II [16]. (c) Both exhibit high affinity for GTP. (d) Both affect mainly Thr residues of casein and, in particular, phosphorylate the same residue (Thr_{41}) in β-casein A[2]. (e) Both are dramatically inhibited by nanomolar concentrations of heparin.

As these studies were being completed a report was presented at the Special FEBS meeting on Cell Function and Differentiation (Athens, April 25–29, 1982) concerning an oligomeric casein kinase from yeast whose subunit composition resembles that of the enzyme described in this paper, except for its loose binding of the 25 000 dalton component which can be removed by gel filtration [17].

It should be finally mentioned that while the heparin sensitive casein kinase-II has been detected in yeast cytosol from both the glucose grown wild type strain D41 and the *rho*° mutant derived from it, any quantitative evaluation of the two casein kinases in different strains and under different growth conditions is not yet available. Such a study might be of interest considering the recent reports that glycogen synthase is to be enumerated among the physiological targets of liver and muscle Type II casein kinases [12,18].

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