A cyclic AMP-independent protein kinase from *Candida albicans*

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A cyclic AMP-independent protein kinase which phosphorylates casein was purified to homogeneity from *Candida albicans* by affinity and ion-exchange chromatography. This protein kinase exhibits maximal activity with casein as substrate and is not stimulated by cyclic AMP or cyclic GMP. The M_r of the purified enzyme is 115000, as determined by h.p.l.c. It migrates as a single band on gel electrophoresis and has three non-identical subunits, of M_r 44000, 28500 and 26000, as determined by SDS/polyacrylamide-gel electrophoresis. This enzyme is insensitive to heparin, but is inhibited by polyamines. Furthermore, it is sensitive to thermal denaturation and to thiol reagents.

INTRODUCTION

The abundant distribution of cyclic AMP-independent protein kinases has generated growing interest in their function as controlling factors in cellular metabolism. Although these enzymes have been implicated in assembly of myosin into filaments, in actin-activated ATPase activity (Scholey *et al.*, 1980; Adelstein & Eisenberg, 1980), in insulin action (Kasuga *et al.*, 1982), in neoplastic transformation (Levinson *et al.*, 1978; Collett *et al.*, 1980) and in protein synthesis (Ochoa & de Haro, 1979), the physiological role of these kinases is still obscure. A common feature of these enzymes is their preference for acidic proteins such as casein and phosvitin as substrates, but not for histones and protamines. Casein-phosphorylating enzymes have been isolated from cytosol and nuclei of many types of eukaryotic cells (Hathaway & Traugh, 1982), including a lower eukaryote such as *Saccharomyces cerevisiae* (Lerch *et al.*, 1975; Rigobello *et al.*, 1980, 1982). In order to understand the role of cyclic AMP-independent protein kinases in lower eukaryotes, we have purified and characterized such an enzyme from the pathogenic yeast *Candida albicans*.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: casein, phosvitin, histone type II AS, protamine, cyclic AMP, cyclic GMP, ATP, heparin, spermine, spermidine, *p*-chloromercuribenzenesulphonic acid, *N*-ethylmaleimide, and Tris (Sigma, St. Louis, MO, U.S.A.); Sepharose 6B (Pharmacia, Uppsala, Sweden); *NN'*-methylenebisacrylamide (BDH, Poole, Dorset, U.K.); GF/C filters (2.4 cm diam.) and DE-52 DEAE-cellulose (Whatman, Maidstone, Kent, U.K.).

Organism and growth conditions

Candida albicans 3100, obtained from the National Chemical Laboratory, Pune, India, was originally *Candida albicans* N.C.Y.C. 597 from the National Collection of Yeast Cultures, Nutfield, Surrey, U.K. Cells

were grown to mid-exponential phase by the procedure of Singh & Datta (1979).

Purification of protein kinase by affinity chromatography

Cells, suspended in 20 mM-Tris/HCl buffer (pH 7.5) containing 1 mM- β -mercaptoethanol (buffer A), were vortex-mixed for 6 min with glass beads (0.45–0.5 mm diam.) and centrifuged at 3000 *g* for 5 min (Sorvall SS34 rotor) to remove whole cells and glass beads. The supernatant was collected and centrifuged at 14000 *g* for 40 min, and the supernatant obtained is termed 'crude extract'. All steps were carried out at 0–4 °C. The crude extract was applied on to a casein-Sepharose (Thornburg & Lindell, 1977) column (1.5 cm $\times$ 9 cm) previously equilibrated with buffer A. After the column was washed with 250 ml of buffer, the bound proteins were eluted with buffer A containing 0.5 M-KCl, in 2 ml fractions.

The fractions containing enzyme activity were pooled (approx. 60–70 ml total) and dialysed overnight (approx. 10 h) against buffer A and concentrated by ultrafiltration on an Amicon apparatus with a PM-20 filter. The concentrated sample was then applied on to a DEAE-cellulose DE-52 column (2.5 cm $\times$ 6 cm), previously equilibrated with the same buffer. After the column was washed, the enzyme was eluted with a 100 ml linear gradient of 0–0.6 M-KCl in buffer A.

Enzyme assay

Protein kinase was assayed by the method of Datta *et al.* (1977). In an assay volume of 0.05 ml, 50 μ g of casein, 25 mM-Hepes/KOH (pH 7.2), 4 mM-magnesium acetate and 40–80 μ M- $[\gamma$ - 32 P]ATP (approx. 40 c.p.m./pmol), were used. The reaction was started by adding 30 μ l of enzyme fraction and carried out at 30 °C for 15 min. Then 1 ml of cold 10% (v/v) trichloroacetic acid was added. The hot-trichloroacetic-acid-precipitable proteins were collected on a glass-fibre filter (Whatman, GF/C), washed with 50 ml of 5% trichloroacetic acid, dried and counted for radioactivity in a toluene-based scintillation fluid.

Protein was assayed by the method of Bradford (1976).

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RESULTS

Enzyme purification

A cyclic AMP-independent protein kinase was purified from *Candida albicans* by column chromatography on casein-Sepharose and DEAE-cellulose DE-52. The cytosolic crude extract of the yeast was applied on to a casein-Sepharose column, and bound proteins were eluted with buffer A containing 0.5 M-KCl. As shown in Fig. 1, a single protein peak having enzyme activity was eluted. The active fractions from four such purifications were pooled and used for DEAE-cellulose DE-52 column chromatography. In the wash, a very minor protein peak without any enzyme activity appeared. The bound proteins were eluted as one major peak (0.2–0.3 M-KCl) and one minor peak (0.02 M-KCl), as shown in Fig. 2. Interestingly, the same pattern was obtained when the crude extract without purification was chromatographed on DEAE-cellulose DE-52 (results not shown). The specific activity of the minor peak was very low, and therefore, for all further work, the major peak of enzyme activity was used. The specific activity of this enzyme was 38400 pmol of ^{32}P transferred/min per mg of protein. The enzyme was stable for at least 15 days at 0–4 °C and was stable to dialysis, but was unstable to freezing and thawing. Furthermore, enzyme activity was completely lost on heating at 70 °C for 10 min (Table 1).

M_r and subunit composition

The homogeneity of the protein kinase was determined by electrophoresis under non-denaturing conditions, which showed a single protein band (Fig. 3a). Polyacrylamide-gel electrophoresis in the presence of SDS revealed

Fig. 1. Purification of protein kinase by casein-Sepharose column chromatography

Crude extract obtained from 4 g wet wt. of cells was chromatographed on casein-Sepharose as described in the Materials and methods section. One unit of enzyme activity is defined as the transfer of 1 pmol of ^{32}P to casein under the assay conditions.

three bands (Fig. 3b). Individual protein fractions from the DEAE-cellulose DE-52 column having enzyme activity were used for both experiments, and each showed

Fig. 2. Purification of protein kinase by DEAE-cellulose DE-52 column chromatography

Fractions from the casein-Sepharose column with enzyme activity were pooled and chromatographed on a DEAE-cellulose DE-52 column as given in the Materials and methods section. Enzyme activity is defined as in Fig. 1 legend.

Table 1. Substrate specificity and effect of inhibitors on protein kinase activity

Protein kinase activity was assayed on the purified enzyme, with different substrates, in the presence and absence of cyclic nucleotides and with different concentrations (as indicated) of inhibitors, as described in the Materials and methods sections.

Component in assay	Enzyme activity (pmol of ^{32}P transferred/ assay)	Inhibi- tion (%)
Substrate (5 μg)		
Casein	37.00	—
Phosvitin	28.44	—
Histone	0.94	—
Protamine	0.65	—
None	0.08	—
Heat-treated enzyme (70 °C/10 min)*	0.48	
+ cyclic AMP*	31.9	—
+ cyclic GMP*	38.06	—
Inhibitors		
<i>p</i> -Chloromercuribenzenesulphonic acid (mM)		
0.0	39.51	0.0
0.5	4.0	89.0
1.0	2.0	94.0
1.5	1.6	95.0
2.0	0.8	97.0
<i>N</i> -Ethylmaleimide (mM)		
0.0	39.51	0.0
0.5	10.8	72.0
1.0	6.8	82.0
1.5	6.9	82.0
2.0	6.4	83.0

* Casein was used as substrate.

the same pattern. The M_r values of the three non-identical subunits shown by SDS/polyacrylamide-gel electrophoresis were 44000, 28500 and 26000.

The M_r of the protein kinase determined by h.p.l.c. on an LKB Ultropac TSK G3000 SW column (7.5 mm $\times$ 600 mm) was 115000.

Substrate preference and other properties

Table 1 shows that the purified protein kinase preferred acidic proteins such as casein and phosvitin as substrate over basic proteins such as histones and protamines. The enzyme was not stimulated by cyclic AMP or cyclic GMP. It showed no autophosphorylation, as shown by lack of phosphorylation in the absence of added substrate. The K_m value for ATP was approx. 200 μM with a constant concentration of casein. The optimum Mg^{2+} concentration was 3–4 mM (results not shown).

As shown in Table 1, thiol reagents such as *p*-chloromercuribenzenesulphonic acid and *N*-ethylmaleimide markedly inhibited the enzyme activity, and almost 100% inhibition was observed with 2 mM of the former.

Effect of heparin and polyamines on enzyme activity

Since the activity of casein-phosphorylating protein kinase is modulated by heparin and polyamines, the effect

Fig. 3. Polyacrylamide-gel electrophoresis of protein kinase

(a) Purified protein kinase (20–40 μg) was used for electrophoresis under non-denaturing conditions by the method of Gabriel (1971), with 5% -acrylamide separating gels. (b) Purified protein kinase (40–60 μg) was used for SDS/polyacrylamide-gel electrophoresis under denaturing conditions as described by Laemmli (1970), with 10% -acrylamide separating gels. The markers are: 1, phosphorylase *b* (M_r 94000); 2, bovine serum albumin (M_r 67000); 3, ovalbumin (M_r 43000); 4, soya-bean trypsin inhibitor (M_r 20000).

of these on enzyme activity was determined. This protein kinase was insensitive to heparin up to a concentration of 40 $\mu\text{g}/\text{ml}$ (Fig. 4, inset), and was markedly inhibited by both the polyamines, i.e. spermine and spermidine (Fig. 4). Moreover, the effect of spermine was more pronounced than that of spermidine.

DISCUSSION

Casein kinases have been classified into two types, I and II (Hathaway & Traugh, 1982). Kinase I is a monomer of M_r 37000, whereas kinase II is more complex (M_r 120000–180000) and has two or three subunits. Casein kinase I is eluted from DEAE-cellulose column at a univalent-cation concentration of 0.07–0.13 M, and kinase II at 0.15–0.22 M. In the present study, DEAE-cellulose DE-52 column chromatography yields two peaks of enzyme activity (Fig. 2). The minor peak was eluted at a lower KCl concentration, like kinase I. However, further characterization was not done, because of poor yield. The protein kinase (major peak) purified in the present study shows some properties similar to those of casein kinase II: for example, it is eluted from DEAE-cellulose DE-52 at 0.2–0.3 M-KCl and its M_r is 115000.

The quaternary structure of casein kinase II is reported to be $\alpha\alpha'\beta_2$ in some systems (Meggio *et al.*, 1981; Dahmus, 1981; Dahmus & Natzle, 1977; Hathaway & Traugh, 1979) and $\alpha_2\beta_2$ in others (Glover *et al.*, 1983; Cochet & Chambaz, 1983). The M_r of the β subunit has been reported to be around 24000, whereas those of α and

Fig. 4. Effect of polyamines and heparin on enzyme activity

Purified protein kinase was used for the assay as described in the Materials and methods section. Different concentrations of spermine (○) and spermidine (●) were used. The inset shows the effect of different concentrations of heparin on enzyme activity.

α' subunits are 44000 and 35000 respectively. The M_r values of the subunits of casein kinase in the present work are 44000, 28500 and 26000. If these are taken to be α , α' and β subunits, the M_r values of α and β subunits are similar to those previously reported, whereas, the α' subunit has a much lower M_r . By scanning a Coomassie-Brilliant-Blue-stained gel, the molar ratio of the subunits was calculated to be 1.0:2.8:0.8. This neither confirms to an $\alpha_2\beta_2$ nor to an $\alpha\alpha'\beta_2$ structure.

Casein kinase II has been reported to be strongly inhibited by heparin at low concentrations (Yan & Tao, 1982; Rigobello *et al.*, 1980). However, in the present study, no such inhibition was observed at comparable or higher concentrations of heparin. Polyamines are known to stimulate casein kinase II (Mäenpää, 1977; Hara & Endo, 1982; Nakajo *et al.*, 1984), but the *Candida*

albicans protein kinase was markedly inhibited by polyamines, e.g. spermine and spermidine. A casein kinase isolated from wheat germ was also strongly inhibited by spermine. However, this casein kinase was slightly stimulated by low concentrations of spermidine (Yan & Tao, 1982). All these results suggest that the *Candida albicans* protein kinase is different from other reported kinases.

Further work is needed to understand the role of this protein kinase and to identify its endogenous substrate.

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