CHAPTER II

PROTEIN KINASES AND THEIR ENDOGENOUS SUBSTRATES IN YEAST

SUMMARY

Protein kinases were partially purified from two strains of yeast, namely, *Saccharomyces cerevisiae* (a non-pathogenic yeast) and *Candida albicans* (a pathogenic yeast), by using ion-exchange chromatography. Both cAMP dependent and cAMP independent protein kinase activities were detected in the two strains of yeast. In *Candida albicans* there are at least two cAMP dependent protein kinases. There appears to be atleast one protein kinase activity in *Candida albicans* which was inhibited in the presence of cAMP. In both strains endogenous substrates phosphorylated in the presence and absence of cAMP could be detected. One such protein in *Candida albicans* showed phosphorylation which was markedly stimulated in the presence of cAMP. In *Saccharomyces cerevisiae* too the endogenous substrates for the protein kinases were present.

INTRODUCTION

In fungi the role of cAMP and protein kinases is not clear. This is mainly because these are hormone insensitive organisms, hence the usual role of cAMP as a second messenger in hormone mediated responses, is not likely to be valid for these. Yet, the responsiveness of cAMP and presence of protein kinases which are cyclic nucleotide dependent, have been demonstrated.

In *Neurospora*, one cAMP dependent and another cyclic nucleotide independent protein kinase has been demonstrated (179). The cAMP dependent protein kinase used histone as its main substrate, while the cyclic nucleotide independent protein kinase used casein as substrate. Furthermore, an additional cAMP independent protein kinase was demonstrated in this system by other studies later (180). Very recently, molecular properties of *Neurospora* protein kinases have been studied. The molecular weight of the cAMP-dependent protein kinase was found to be 118,000 dalton. Two cAMP independent protein kinases with molecular weights of 56,000 and 209,000 daltons were also shown in this system (181).

Protein kinase and cAMP binding activity have been studied in a number of other lower eukaryotes. This includes organisms like *Blastocladeilla*, where again, three peaks of

protein kinase activity were resolved by DEAE cellulose chromatography. Peak I and II were cyclic nucleotide independent casein and histone kinases respectively, while peak III was cAMP dependent. Furthermore, peak II was shown to be a free catalytic subunit by inhibitor studies (182). Four cAMP independent protein kinases were earlier detected in the soluble fraction of *Dictyostelium* (183). Recently a cAMP dependent protein kinase activity has been demonstrated in *Dictyostelium discoideum* which showed a high level of cAMP dependency and appeared during the time course of development, showing it to be developmentally regulated(184). Cyclic AMP dependent protein kinases in yeast-like cells of *Mucor rouxii* showed similarities with type II protein kinases from higher eukaryotes (185).

Yeast cells possess an entire cAMP based regulatory system including adenylate cyclase and phosphodiestierases. A search for protein kinases in this system is but natural. In the early studies along these lines, a cAMP binding protein was isolated from several strains of yeast. The binding constant for cAMP at pH 7.4 was 5 nM and molecular weight of 24,000 dalton was determined. But this protein did not show any protein kinase activity, nor could a specific function be assigned to it (186). Later, a cAMP dependent protein kinase was partially purified from Baker's yeast which showed properties similar to mammalian

cAMP dependent protein kinases. An ability to activate muscle glycogen phosphorylase kinase and inactivate muscle glycogen synthetase were two such properties (187). Three protein kinases were isolated and separated from high salt wash of yeast ribosomes. Two of these were cAMP dependent protein kinases. As many as eighteen ribosomal proteins could be phosphorylated by these *in vitro*, which correspond to the phosphorylation pattern seen *in vivo* (188). An yeast protein kinase that co-purified with RNA-polymerase I during part of the purification procedure for the RNA polymerase, was partially purified and characterized. This enzyme could phosphorylate *in vitro*, one subunit of purified polymerase, which was also phosphorylated *in vivo*, without affecting its transcriptional activity (189). Solubilization of *Saccharomyces cerevisiae* mitochondrial membrane by 0.7 M sodium chloride yielded a protein kinase of 30,000 dalton molecular weight. This enzyme used casein and phosvitin as phosphate acceptor (190). A cAMP binding protein, different from those reported earlier, was found in *Saccharomyces cerevisiae* later. The molecular weight of this binding protein was shown to be 54,000 dalton, much higher than those reported earlier and suggested to be breakdown product of this high molecular weight protein (191). A homogenous purified cAMP binding protein of higher molecular weight (85,000 dalton) was reported in Baker's yeast. The enzyme

showed a single subunit of 50,000 dalton molecular weight on denaturing gels, which indicated it to be a dimer of identical subunits. This was judged to be a regulatory subunit of cAMP dependent protein kinase since it inhibited purified catalytic subunit of mammalian cAMP dependent protein kinase in the absence of cAMP (192). Studies in *Schizosaccharomyces* (*Schizosaccharomyces pombe*) have revealed the presence of a 64,000 dalton molecular weight cAMP binding protein, which was the regulatory subunit of cAMP dependent protein kinase. A 37,000 dalton molecular weight protein was also present, which was derived from the regulatory subunit. There was only one cAMP dependent protein kinase which phosphorylated protamine and histone equally, but was inactive on casein and phosvitin. The molecular weight of this enzyme was determined to be 230,000 dalton. The 37,000 dalton molecular weight peptide could be obtained by a limited tryptic digestion of the larger subunit, and this core of the regulatory subunit was found to be trypsin resistant (193). Some very interesting studies have been conducted in yeasts regarding cAMP control and protein kinases using mutants and relating to mating type pheromone action. Cyclic AMP and cAMP dependent protein kinases have been implicated in the mode of action of pheromones of *Saccharomyces cerevisiae* in the mating of 'a' and 'α' mating types. Pheromones of 'a' type haploid cells caused G_1 arrest of 'α' cells and

vice versa. Studies with certain temperature sensitive mutants and other *in vitro* studies indicated that these pheromones inhibit adenylate cyclase and hence, lower cAMP concentration (194, 195). This would presumably lower the level of phosphorylation of certain target proteins, which need to be phosphorylated to allow progression through G_1 phase, by lowering the level of cAMP dependent protein kinase. Mutants which required cAMP for growth (cyr 1), were used to obtain revertants which could grow in the absence of cAMP. These revertants were double mutants (cyr 1 bcy 1) which showed no detectable adenylate cyclase activity (due to cyr 1 mutation). Strikingly, these double mutants were shown to be lacking in a fully functional cAMP binding regulatory subunit of cAMP dependent protein kinase. This was determined by radio labelling with photoactivatable affinity label - 8-azido (^{32}P) cAMP, which showed the level of R subunit to be low as compared to the parent strain. Furthermore, total protein kinase activity in the mutants was only slightly stimulated on the addition of cAMP (196). Hence, the fact that adenylate cyclase deficiency of the cyr 1 strain and its inability to grow in the absence of exogenously supplied cAMP could both be overcome by the presence of an altered protein kinase in cells and that it no longer requires cAMP for activity, suggest that cAMP dependent protein kinases are the mediators of cAMP action in yeast.

It is always interesting to attempt to understand the mechanisms operative under *in vivo* conditions, on the basis of information gathered from *in vitro* analysis. But any extrapolation of *in vitro* results to *in vivo* conditions can only be done with caution since it may not be perfectly valid. Protein kinases have been established to be present in various organisms and organelles. The activities of these enzymes have mostly been tested on non-physiological substrates under *in vitro* conditions. But the substrates, *in vivo*, are expected to be different and a study of these may elucidate the significance and mechanism of action of the protein kinase far better. Though protein kinases from different sources appear to be similar, the physiological action of cAMP or other signals for these enzymes are diverse. The specificity of these actions are, hence, thought to reside in the phosphorylation of specific endogenous substrates.

Primary investigations into the endogenous substrates involved the determination of the number of such substrates and their isolation. Later, the effect of these substrates in phosphorylated and dephosphorylated states were studied. Phosphorylation of endogenous proteins of synaptic membrane have been reported in synaptic membrane fraction of rat brain by one kind of cAMP dependent protein kinase (197). A novel protein kinase, designated PK 380 from bovine brain, was found to phosphorylate a specific 120,000 dalton

polypeptide which was endogenously present (198). Major endogenous substrates for cAMP dependent protein kinases have been identified from bovine brain. Five such specific proteins and some other non-specifically phosphorylated proteins were seen. One of these was identified to be a microtubule association protein and two others were regulatory subunits of type II cAMP dependent protein kinase (199). Cell surface proteins of HeLa cells have been shown to be phosphorylated by a cell surface located protein kinase (200). Two endogenous substrates of 21,000 dalton and 8,000 dalton molecular weight were shown to be phosphorylated by a cAMP dependent protein kinase from cardiac sarcolemmal vescicle, which were then shown to be identical to each other when the membranes were treated with SDS prior to electrophoresis (201).

We have here attempted to isolate the detect protein kinase activities in the yeasts, *Candida albicans* and *Saccharomyces cerevisiae*. Our interest in these enzymes was generated by the fact that in our previous studies we observed cAMP inhibiting induction of enzymes and also total protein synthesis in both these strains of yeast at an optimum concentration of 3 mM. We have shown here the presence of protein kinases, both cyclic AMP dependent and independent, in these yeasts. Though yeasts are hormone insensitive organisms, the presence of cAMP dependent protein kinases indicate that the stimulus for cAMP action may be different

but still the mechanism of cAMP action may be primarily through the activation of protein kinases. In addition to the protein kinases, we have also detected endogenous substrates for protein kinases in crude preparations from both [illegible] and [illegible]. The presence of these endogenous substrates is indicative of the fact that protein kinases probably control cellular activity through the phosphorylation of these peptides.

RESULTS

(A) Protein Kinases in [illegible]:

As in Fig. 14, cytosolic extracts were fractionated on a DE 52 column equilibrated with extraction buffer. After complete washing of the column to remove all the unbound protein, the bound proteins were eluted in 4 ml fractions by a linear gradient of 0 to 0.5 M potassium chloride. On assaying the enzyme activity, two peaks of cAMP dependent activity eluting at 0.02 M and 0.03 M KCl were detected. The activity of these enzymes were stimulated upto 2-3 fold in presence of cAMP. At 0.25 M KCl concentration, there appears to be a cAMP independent protein kinase activity. In fraction no. 27 (at 0.12 KCl concentration) a cAMP independent protein kinase activity was detected, which was inhibited in the presence of cAMP. The activity was reduced to 25% in presence of cAMP. The occurrence of this type of protein kinase has not yet been reported in many organisms.

A protein kinase activity detected in the disrupted cilia of *Paramaecium* (202) which showed peak activity at pH 8.0, was slightly inhibited by both cAMP and cGMP, between 10^{-7}M and 10^{-4}M cAMP, but here the inhibition was much more pronounced.

(B) Protein Kinase Activity in *Saccharomyces cerevisiae* :

Fig. 15 shows the separation of protein kinases of *Saccharomyces cerevisiae* on DE 52 column. Two protein kinase activities have been reported in Baker's yeast, one of which was cAMP dependent (using histone as substrate) and other was cAMP independent (using casein as substrate) (203). In our studies, however, we could not distinctly demonstrate the cAMP dependent activity. This could be due to overlapping of the proteins present in the fractions between 12 and 15. However, a cAMP independent protein kinase activity is clearly shown in fraction number 40.

(C) Endogenous Substrates for Protein Kinases in *Candida albicans* :

The number of phosphorylated products in the presence and absence of cAMP was studied by using SDS-polyacrylamide gel electrophoresis. In Fig. 16 the result of such an experiment is shown. There are two distinct peaks of phosphorylation. One such peak shows equal degree of phosphorylation in the presence and absence of cAMP, which presumably is a substrate for the cAMP independent protein kinase.

FIGURE 14 : PARTIAL PURIFICATION OF PROTEIN KINASES OF [illegible] by DE-52 COLUMN CHROMATOGRAPHY.

Cells from 3-4 litres of exponential culture were used to make cell extract in 20 mM Tris-HCl buffer (pH 7.6) containing 1 mM DTT. The cell extract was loaded on a DE-52 column (2.5x10 cm) previously equilibrated with the same buffer and washed with 5 column volumes of the same buffer to remove unbound proteins. The bound proteins were eluted with a linear gradient of KCl 0-0.5 M in the same buffer. 4 ml fractions were collected and the protein kinase activity was assayed in each fraction as described in 'Materials and Methods'.

o——————o with cAMP

●——————● without cAMP

Δ——————Δ KCl concentration in buffer

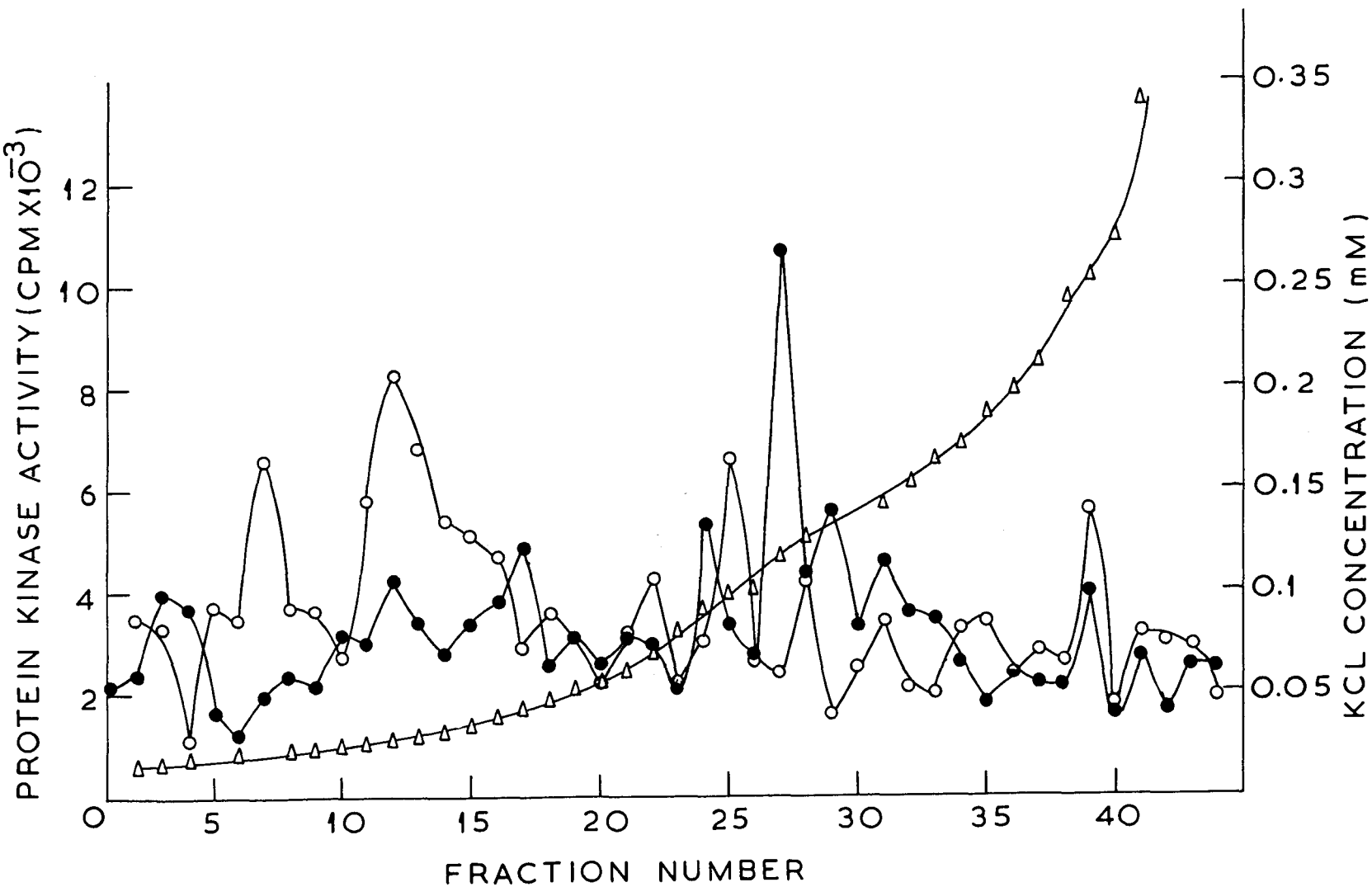

PROTEIN KINASE ACTIVITY (CPM X10^{-3})
KCL CONCENTRATION (mM)
FRACTION NUMBER
0
2
4
6
8
10
12
0.05
0.1
0.15
0.2
0.25
0.3
0.35
5
10
15
20
25
30
35
40

FIGURE 15 : PARTIAL PURIFICATION OF PROTEIN KINASE OF *Dictyostelium discoideum* BY DE-52 COLUMN CHROMATOGRAPHY.

Cells from 3-4 litres of exponential culture were used to make cell extract in 20 mM Tris-HCl buffer (pH 7.6) containing 1 mM DTT. The cell extract was loaded on DE-52 column (2.5x10 cm) previously equilibrated with the same buffer, washed with 5 column volumes of the same buffer to remove unbound proteins. The bound proteins were eluted with a linear gradient of KCl 0-0.5 M in the same buffer. 4 ml fractions were collected and the protein kinase activity was assayed in each fraction as described in 'Materials and Methods'.

●——● with cAMP

○——○ without cAMP.

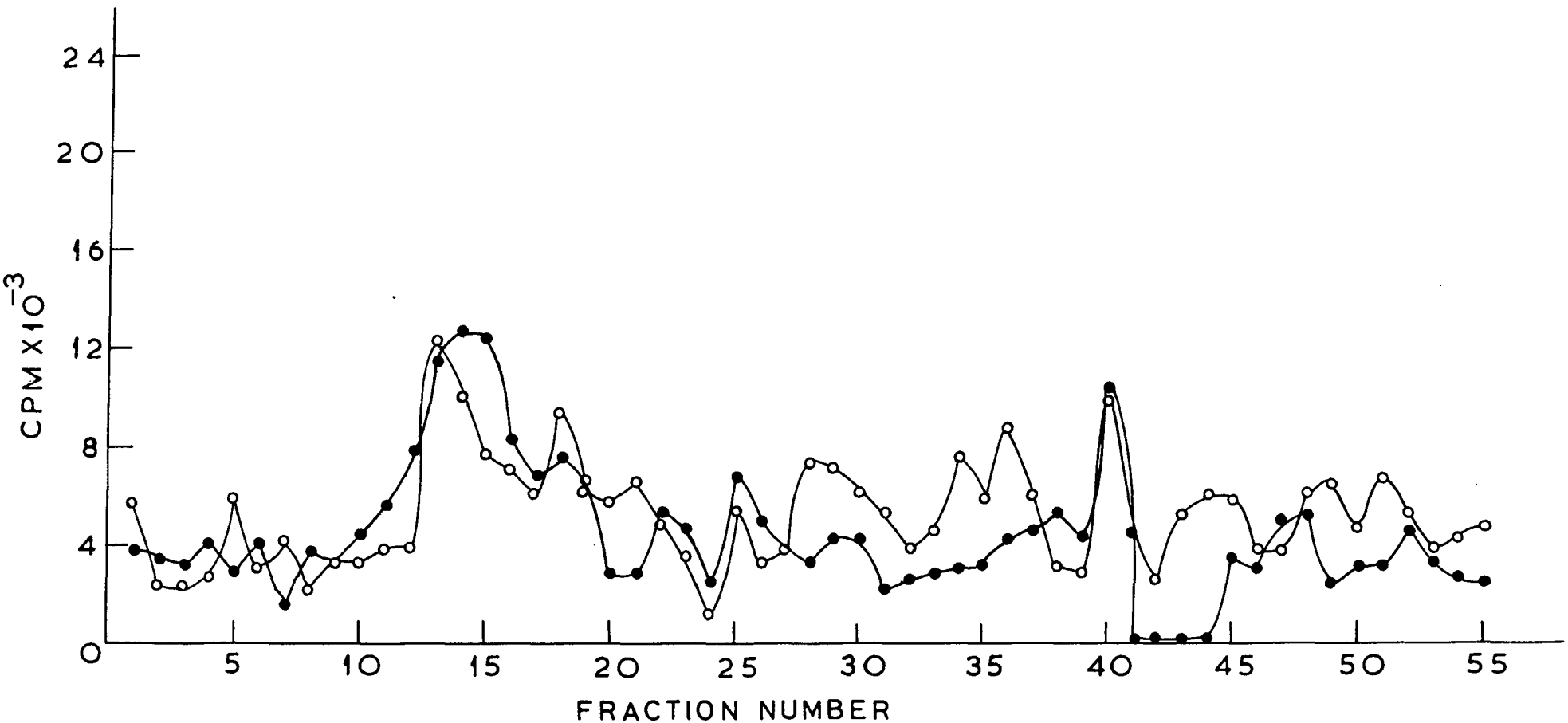

24
20
16
12
8
4
0
CPM X10^{-3}
5
10
15
20
25
30
35
40
45
50
55
FRACTION NUMBER

FIGURE 16 : DETECTION OF ENDOGENOUS SUBSTRATES OF PROTEIN KINASES IN [illegible] BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Crude extract of midlog phase cells, in 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, was incubated with 50 mM PIPES buffer (pH 7.0) and 5 mM magnesium chloride in the presence and absence of cAMP, for 4 min at 30°C. The reaction was stopped and samples were electrophoresed on SDS-polyacrylamide gels as described in 'Materials and Methods'.

o———o with cAMP

●———● without cAMP.

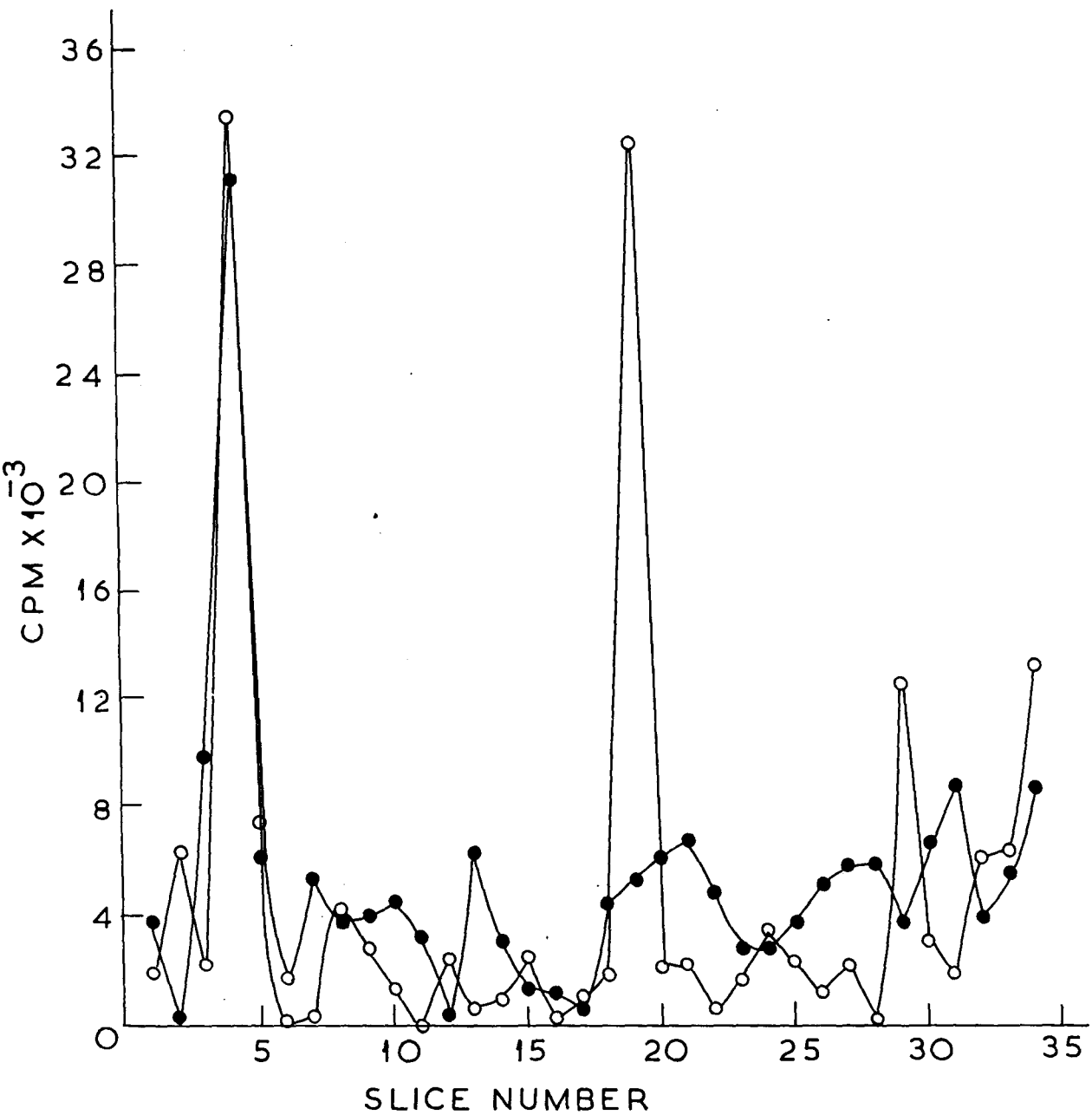
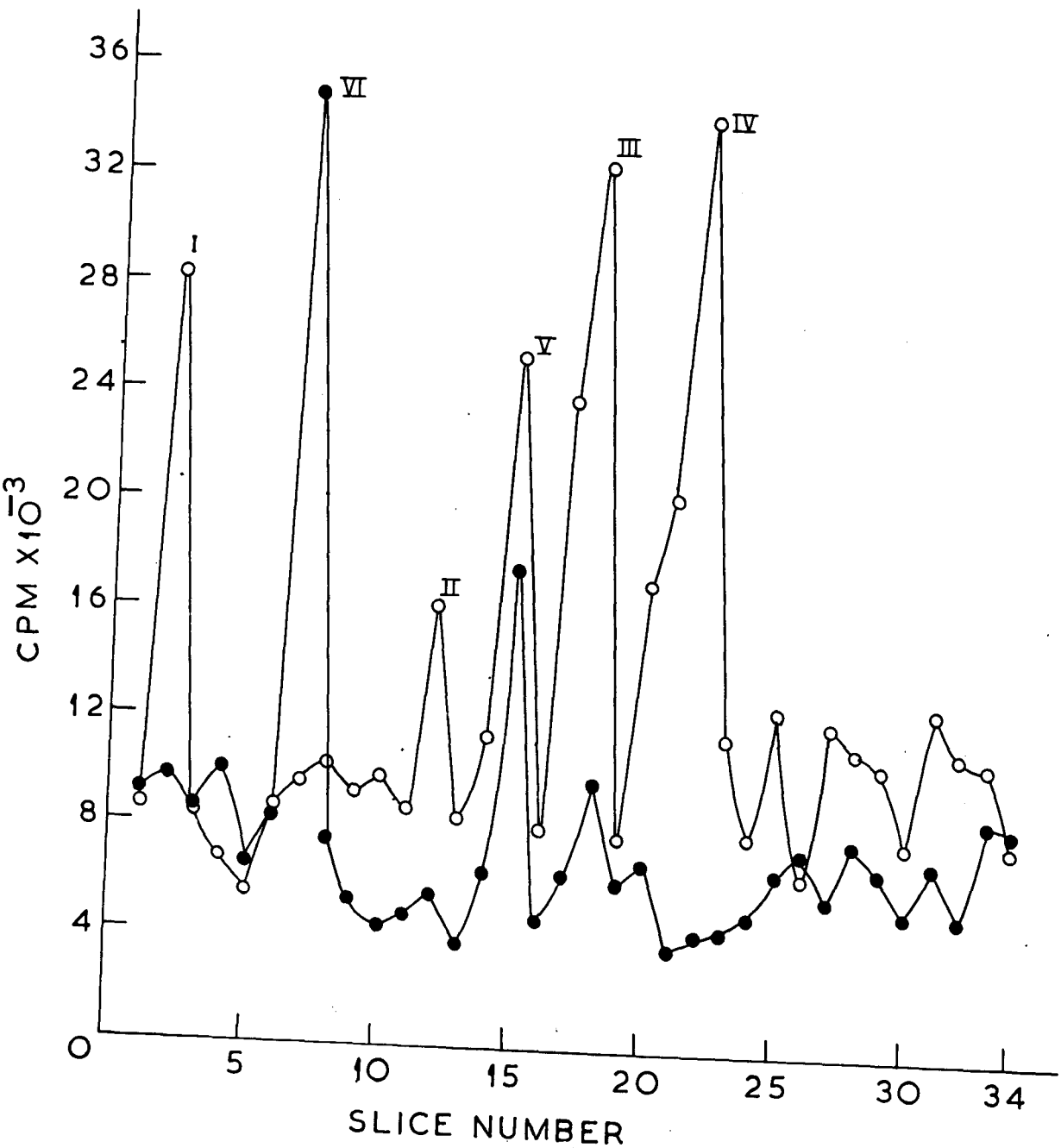

36
32
28
24
20
16
12
8
4
0
CPM X10^-3
5
10
15
20
25
30
35
SLICE NUMBER

FIGURE 17 : DETECTION OF ENDOGENOUS SUBSTRATES OF PROTEIN KINASES [illegible] BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS.

Crude extract of midlog phase cells, in 20 mM Tris-HCl (pH 7.5) containing 1 mM DTT, was incubated with 50 mM PIPES buffer (pH 7.0) and 5 mM magnesium chloride in the presence and absence of cAMP, for 4 min at 30°C. The reaction was stopped and samples were electrophoresed on SDS-polyacrylamide gels as described in 'Materials and Methods'.

o———o with cAMP

●———● without cAMP.


36
32
28
24
20
16
12
8
4
0
CPM X10⁻³
I
II
III
IV
V
VI
5
10
15
20
25
30
34
SLICE NUMBER

Another peak which migrated further down the gel, was phosphorylated only in the presence of cAMP, indicating that this might be the endogenous substrate for a cAMP dependent protein kinase.

(D) Endogenous Substrates for Protein Kinase in *Saccharomyces cerevisiae*:

The phosphorylated protein profile of the endogenous proteins of *Saccharomyces cerevisiae* was also studied (Fig.17). There appear to be four substrates (marked as I to IV) for cAMP dependent protein kinases in this which are clearly separated on SDS-PAGE. There was one substrate for a cAMP independent protein kinase (marked as V). In addition, one substrate (marked as VI), whose phosphorylation was inhibited by cAMP, was also detected.

DISCUSSION

Cyclic AMP dependent and independent protein kinases have been demonstrated in various organisms and the effect of cAMP and other stimuli appear to be through these enzymes. In our system, the inhibitory effect of cAMP on the induction of GlcNAc catabolic enzymes and general protein synthesis indicated the probable involvement of protein kinases. Protein kinases have not been reported in *Candida albicans*. We have here demonstrated the presence of both cAMP dependent and independent protein kinases in this yeast. In addition to

these, cAMP inhibited protein kinase activity was also found. Such activities have not been commonly found, but such inhibition by cAMP has been reported in disrupted cilia of *Paramaecium*(202). In slime mold, strong inhibition of the autophosphorylation of the regulatory subunit of type II cAMP dependent protein kinase by cAMP was reported (204). In crude extracts of *Coprinus macrorhizus*, protein kinase activity was significantly inhibited by cAMP and complete inhibition was attained with high concentrations of cAMP. The fractionation of crude extract on sepharose 6B, in this system, indicated that in addition to cAMP independent protein kinase, there were two types of protein kinases which were either stimulated or inhibited by cAMP (205). We have also found these three types of protein kinases in *Candida albicans* fractionated on DE 52.

On determining the endogenous substrate for protein kinases in the presence of absence of cAMP in *Saccharomyces cerevisiae* we found four major phosphorylated products. The phosphorylations were seen either in the presence of cAMP or independent of the presence or absence of cAMP. In addition the phosphorylation of one substrate was inhibited by cAMP. The phosphorylation of cellular proteins of the myclelia of Fis^{c} in *Coprinus macrorhizus* showed a similar pattern (135). In this report the phosphorylation of two proteins A and B was stimulated by cAMP in the

presence of magnesium, while that of one protein C was inhibited in the presence of cAMP. The concentration of cAMP used was 10^{-7}M and it was suggested that this protein was the substrate for a cAMP inhibited protein kinase(135).

Cyclic AMP dependent and independent protein kinases have been implicated in a large number of cellular mechanisms (8). The independent protein kinases have been well studied for their role in protein synthesis. These have been shown to inhibit ternary complex formation which is the first step in the initiation of protein synthesis. However, the role of cAMP in protein synthesis still remains speculative and the exact mechanism is not clear (206, 207, 208). It is, however, likely that protein kinases may be involved in some way. Our previous observation that cAMP inhibited protein synthesis in yeast (209) indicated its involvement in translation control. Whether or not protein kinases mediate this effect is an interesting question. The demonstration of protein kinases in these yeasts is but a preliminary step towards answering this question. The evidence that endogenous substrates for such enzymes are also present is also an indication of a physiological role of these enzymes in yeast. The significance and physiological role of cAMP inhibited protein kinases is perhaps least understood. This is because these enzymes have not been commonly found in most systems. The possibility of control mechanisms,

mediated by these enzymes, cannot be excluded and need to be studied. Both *Candida albicans* and *Coprinus macrorhizus* are lower eukaryotes and both appear to have such an enzyme and this may be a point of relevance. To impart a definite role to these protein kinases, it would be essential to purify them. Only purified preparations of these enzymes and their substrates can facilitate the detailed study of the mechanism of action, role and importance of these enzymes.