Phosphorylation of the regulatory subunit of yeast cAMP-dependent protein kinase

(Kluyveromyces fragilis/photoaffinity labeling/gel electrophoresis)

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ABSTRACT In vitro phosphorylation of the regulatory subunit of yeast cAMP-dependent protein kinase was studied. The cAMP-binding regulatory subunit (R subunit) can be multiply phosphorylated. Three distinct phosphorylation sites were inferred from the different ATP concentrations required for phosphorylation and from the presence of two discrete mobility shifts in NaDodSO4/polyacrylamide gel electrophoresis of the R subunit on phosphorylation. Limited trypptic digestion of the phosphorylated R subunit showed that a Mr 37,000 cAMP-binding peptide contained one of the phosphorylation sites and that a separate Mr 12,000 peptide contained another phosphorylation site. The yeast R subunit is therefore similar to the type II R subunit of mammalian origin, although it has a larger Mr (64,000 vs. 58,000) and is multiply phosphorylated. In vivo, both phosphorylated and unphosphorylated forms of the R subunit were found in cells grown in lactate or to stationary phase in 1.5% glucose, while cells grown in 5% glucose contained the unphosphorylated form.

Modification of a protein by phosphorylation and dephosphorylation is an important regulatory mechanism in cellular metabolism. Many of these phosphorylation reactions are carried out by the cAMP-dependent protein kinases (1). The kinase holoenzyme contains two catalytic subunits and two cAMP-binding regulatory subunits (R subunits). On binding cAMP, the R subunits separate from the holoenzyme, thereby freeing and activating the catalytic subunits. In mammalian systems, there are two types (I and II) of cAMP-dependent protein kinase that differ only in their respective R subunits. Both of these enzymes, which catalyze protein phosphorylation, are themselves phosphorylated (2, 3, 4, 5). Phosphorylation of type II cAMP-dependent protein kinase has been extensively studied because the purified enzyme can catalyze a self-phosphorylation reaction whereas the type I enzyme does not (3, 4, 5). The self-phosphorylation reaction can occur in the absence of cAMP and the site of phosphorylation has been found to be in the R subunit. Phosphorylation of the R subunit has been shown to affect cAMP binding and the association of regulatory and catalytic subunits (3, 5).

Recently, we have shown that cAMP inhibits the outgrowth of Saccharomyces fragilis (Saccharomyces) fragilis from the lag phase and that the inhibition can be prevented by methionine or S-adenosylmethionine (6). In studying the underlying biochemical mechanism of cAMP inhibition, we have isolated a cAMP-dependent protein kinase from this yeast (7) and found that, in contrast to earlier reports of a low molecular weight enzyme in baker's yeast (8), our yeast enzyme has a molecular weight similar to that of the type II protein kinase present in animal tissues. The cAMP-binding R subunit of our yeast enzyme has a Mr of 64,000, which is somewhat higher than that (57,000) of type II protein kinase. By using 8-azidoadenosine 3',5'-[32P]phosphate (8-N3-[32P]cAMP) photoaffinity labeling techniques, we found a slower migrating (Mr ~68,000) cAMP-binding band in NaDodSO4/polyacrylamide gel electrophoresis. The intensity of the band varies from preparation to preparation. We report here that this slower migrating band is the phosphorylated form of the R subunit and that the degree of phosphorylation depends on the metabolic carbon source.

MATERIALS AND METHODS

K. fragilis (ATCC 10022) was grown in yeast extract/peptone medium (9) supplemented with 5% or 1.5% glucose or 2% lactate. The cells were harvested at midlogarithmic phase or as indicated and used the same day for preparation of cAMP-dependent protein kinase. We have found that freezing and thawing of cell paste led to formation of a low molecular weight cAMP-binding protein (7).

For preparation of cAMP-dependent protein kinase, yeast cells grown in 5% glucose were broken in buffer A (20 mM Tris·HOAc, pH 7.5/10 mM KCl/10 mM Mg(OAc)2, 2 mM diethiothreitol/0.1 mM EDTA) containing the proteolytic inhibitors phenylmethylsulfonyl fluoride (2.0 mM) and p-aminobenzamide (10 mM). cAMP-dependent protein kinase was purified as described (7). Photoaffinity labeling of cAMP-binding proteins with 8-N3-[32P]cAMP was as described (7) except that, in some experiments, ATP or trypsin was added. Phosphorylation experiments were carried out at 4°C for 30 min in 40 µl of 15 mM NaOAc, pH 5.5/6 mM Mg(OAc)2 containing 1 µCi of [γ-32P]ATP and diluted with unlabeled ATP to give final ATP concentrations of 1 mM to 0.1 mM as indicated and, if present, 1 µM cAMP. The reaction was stopped by addition of NaDodSO4 (final concentration, 1%/20 mM diethiothreitol/sucrose. The mixture was then boiled for 4 min and subjected to electrophoresis in 5.7–16.5% polyacrylamide gels according to the method of Laemmlmi (10). The gels were then stained, dried, and autoradiographed.

[γ-32P]ATP was obtained from Amersham and 8-N3-[32P]cAMP was a product of ICN.

RESULTS

The cAMP-binding R subunit of yeast cAMP-dependent protein kinase can be identified in NaDodSO4/polyacrylamide gel electrophoresis as a Mr 64,000 protein by covalently labeling the enzyme with the photoaffinity agent 8-N3-[32P]cAMP (ref. 7; Fig. 1, lane 2). When photoaffinity labeling of cAMP-dependent protein kinase was done in the presence of 20 µM ATP, a slower migrating band with an apparent Mr of 68,000 was found instead of the Mr 64,000 protein (Fig. 1, lane 1). The

Abbreviations: 8-N3-[32P]cAMP, 8-azidoadenosine 3',5'-[32P]phosphate; R subunit, regulatory subunit of cAMP-dependent protein kinase.
mobility of a minor cAMP-binding protein ($M_0, 37,000$), previously identified as a proteolytic product of the R subunit (7), was not affected by incubation with ATP (Fig. 1, lanes 1 and 2). Furthermore, limited trypsin hydrolysis of both ATP-treated and untreated R subunits yielded a similar $M_0, 37,000$ cAMP-binding peptide (lanes 4 and 5). These results indicate that the $M_0, 64,000$ and $68,000$ cAMP-binding proteins were the same and that incubation of the protein kinase with ATP has modified the $M_0, 64,000$ R subunit, leading to a shift in mobility.

The enzyme was incubated with 1 $\mu$M [y-32P]ATP to determine whether phosphorylation had led to the mobility shift of the R subunit. Fig. 2 (lane 2) shows that, in the absence of cAMP, 32P-O$_4$ label was found with the $M_0, 64,000$ protein band, indicating self-phosphorylation of the yeast enzyme that is similar to the type II protein kinase. Incubation in the presence of cAMP and a moderate ATP concentration (1 $\mu$M), however, led to two slower migrating 32P-labeled bands (lane 1). The slower migrating of the two bands comigrated with the $M_0, 68,000$ band detected by 8-N$_3$-[32P]cAMP labeling. The phosphorylated $M_0, 68,000$ band was the only form detected when the enzyme was incubated in the presence of cAMP and an ATP concentration higher than 10 $\mu$M. This result suggests that phosphorylation has led to the mobility shifts of the R subunit. Only serine was found to be phosphorylated in the presence or absence of cAMP (data not shown). The detection of 32P label in the $M_0, 64,000$ and $68,000$ bands and the finding of 32P-labeled intermediate-migrating band (lane 1) imply the presence of multiple phosphorylation sites in the R subunit and further suggest that the degree of mobility shift may correspond to the extent of phosphorylation; i.e., incorporation of the first phosphate group does not lead to a shift in mobility while introduction of the second and the third phosphate groups led to the two slower migrating bands. Further proof that the incorporation of the first phosphate group into the R subunit does not lead to the mobility shift is shown in Fig. 3. When cAMP-dependent protein kinase was incubated in the presence of cAMP, with a very low (32 nM) concentration of [y-32P]ATP, the $M_0, 64,000$ band was heavily labeled with 32P without a concomitant mobility shift (lane 2). The mobility shift of the 32P-labeled $M_0, 64,000$ band can be demonstrated by the pulse-chase experiment shown in Fig. 3 (lanes 4 and 5). The enzyme was first incubated with 32 nM [y-32P]ATP in the presence of cAMP to label the $M_0, 64,000$ band and, after 30 min, it was chased with high (23 $\mu$M) concentration of unlabeled ATP. The amount of unlabeled ATP added was enough to dilute the specific activity of [y-32P]ATP so that new incorporation of 32P was not detectable (lane 5); lane 4 shows that the 32P-labeled $M_0, 64,000$ band now migrated as the $M_0, 68,000$ band. These results also indicate that the phosphate group incorporated does not significantly turn over under our incubation condition.

The intermediate-migrating phosphorylated band can also be
demonstrated by labeling the R subunit with 8-N_2[^32P]cAMP and then incubating the enzyme with ATP. Fig. 4 shows the results of photoaffinity labeling the R subunit in the presence of various ATP concentrations. At ATP concentrations of <0.1 μM, only the M_4, 64,000 band was detected. The intermediate-migrating band began to form at 0.1 μM ATP and became the predominant form at 0.5 μM ATP. The fully phosphorylated M_4, 68,000 band started to form at 0.5 μM ATP and became the predominant form at 5 μM ATP. No further shift in mobility was detected when the ATP concentration was increased to as high as 0.1 mM. The finding of two discrete mobility shifts of the R subunit following phosphorylation further indicates the presence of multiple phosphorylation sites on the R subunit.

Limited trypsic digestion of the phosphorylated R subunit was used to show the presence of distinct phosphorylation sites. Two types of ^32P-labeled R subunits were digested with trypsin (Fig. 5). In one, ^32P labels were introduced by incubating at a low (9.3 nM) concentration of [γ-^32P]ATP followed by a chase with unlabeled 2 μM ATP. Tryptic digestion led to the formation of a M_4, 37,000 peptide containing most of the ^32P label (Fig. 5A). This result indicates that the M_4, 37,000 peptide containing the cAMP binding site (Fig. 1) has a phosphate acceptor site. The second type of ^32P-labeled R subunit was made by incubating the enzyme with a low (1 nM) concentration of unlabeled ATP and then chasing it with a higher (1 μM) concentration of [γ-^32P]ATP. Tryptic digestion of this ^32P-labeled R subunit leads to the formation of a M_4, 12,000 peptide containing the majority of the ^32P label and the M_4, 37,000 peptide as a minor constituent. This result indicates the presence of a distinct phosphorylation site outside the M_4, 37,000 cAMP-binding core peptide.

Fig. 6A shows that the fully phosphorylated R subunit was found in yeast grown in lactate and in yeast grown in 1.5% glucose but harvested at stationary phase. The fully phosphorylated R subunit was not detected in yeast grown in 5% glucose harvested at midlogarithmic phase (Fig. 6B).

DISCUSSION

The cAMP-binding R subunit of yeast cAMP-dependent protein kinase closely resembles the extensively studied R subunit of type II cAMP-dependent protein kinase of mammalian origin. Both protein kinases can phosphorylate the R subunit leading to a mobility shift in NaODS/SO_4/polyacrylamide gel electrophoresis. The type II R subunit is phosphorylated at one site, which leads to a single mobility shift (11, 12), while the yeast R subunit can be phosphorylated at three sites, leading to two mobility shifts. Similar to the type II R subunit (13, 14) the yeast R subunit contains a cAMP-binding, phosphorylated, trypsin-resistant core peptide of M_4, 37,000. These results indicate the highly conserved nature of the cAMP-binding domain in the R subunits. In contrast to the type II R subunit, the yeast R subunit contains a second trypsin-resistant peptide (M_4, 12,000) containing an additional phosphorylation site. At present, we are not certain where the third phosphorylation site is, although the trypsic-digestion results in Fig. 5B suggest that both sites phosphorylated at higher ATP concentrations are present in the M_4, 12,000 fragment.

The M_4, 64,000 and 68,000 forms of the R subunit were detected in extracts of cells grown in lactate as a carbon source.
Similar results were obtained with extracts of stationary phase cells grown in 1.5% glucose. In contrast, cells grown to mid-logarithmic phase in 5% glucose contained only the $M_r$ 64,000 band, indicating no or single-site phosphorylation of the R subunit. These data are consistent with the finding of a low cellular cAMP concentration in glucose-grown cells and a high cAMP concentration in stationary phase or lactate-grown cells (15). Our findings also suggest that the in vivo phosphorylation state of cAMP-dependent protein kinase is under metabolic control. The mammalian type II R subunit has been found to be mainly in the phosphorylated form (11) and, in contrast to the yeast R subunit, variation in phosphorylation state has not been reported with metabolic changes. The yeast enzyme should therefore be a good system to study the biological significance of phosphorylation of protein kinase.

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