Vol. 94, No. 1, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1980 Pages 16-22

IDENTIFICATION OF cAMP BINDING PROTEINS ASSOCIATED WITH THE PLASMA MEMBRANE OF THE YEAST *SACCHAROMYCES CEREVISIAE*

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Received February 18,1980

SUMMARY

We have investigated the major 3':5'-adenosine monophosphate (cAMP) binding proteins associated with the plasma membrane of *Saccharomyces cerevisiae*. At least four proteins with apparent molecular weights of 25,000, 34,000, 46,000 and 58,000 have been identified using the photoaffinity probe 8-azidoadenosine[^{32}P]3':5'-monophosphate (8-N_3-[^{32}P]cAMP). Together, they constitute a homogeneous population of binding sites interacting with the photoaffinity ligand in a non-cooperative fashion.

INTRODUCTION

The existence in both pro- and eucaryotic cells of regulatory sequences mediated by cAMP has been documented extensively (reviews in 1,2,3,). Some of these events are elicited by changes in growth conditions. For example, fluctuations in the intracellular levels of cAMP have been observed in unicellular eucaryotes, such as *Neurospora crassa*, *Mucor racemosus* and *S. cerevisiae*, and transient rises in cAMP concentrations have been correlated with membrane depolarization in these organisms (4,5). More persistent changes have been found in *Tetrahymena pyriformis*, where the addition of glucose to stationary phase cells results in an increase in cAMP (6). In *S. cerevisiae*, the addition of glucose to cells grown in a medium containing ethanol and therefore free of catabolite repression results in a decrease in the level of intracellular cAMP (David Allmann, personal communication), while transfer of glucose-grown cells to ethanol has the converse effect (7). What is

* Recipient of a Research Career Awark K06 05060 from the Institute of General Medical Sciences; research supported by Research Grant PCM 78-18-393 from the NSF.

+ Publication No. 3361

0006-291X/80/090016-07$01.00/0
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the significance of these observations, and in particular, do they suggest a common mechanism (2,3)? Soluble cAMP binding proteins (8,9) and cAMP-dependent protein kinases (10-12) have been reported to be present in *S. cerevisiae*. However, in contrast to higher eucaryotes, in which such cAMP-binding proteins as regulatory subunits of cAMP-dependent protein kinases (2,3,13-16) are also known to be associated with plasma membranes, no comparable entities have so far been reported in Ascomycetes. We now show that, using the cAMP photoaffinity analog 8-N_3-[cAMP] (14-18), purified plasma membranes of *S. cerevisiae* contain a population of high affinity receptors for this molecule, distributed among four polypeptides with apparent molecular weights of 25,000, 34,000, 46,000 and 58,000.

MATERIALS AND METHODS

Preparation of plasma membranes: Cultures of *S. cerevisiae* (strain A-364A from L. Hartwell) were grown at 30°C in YPD medium (1% yeast extract, 1% peptone, 2% dextrose) to an absorbancy of 0.5 (600 nm, Zeiss PMQ), corresponding to a cell density of 1.6×10^7/ml. After harvesting by centrifugation, spheroplasts were obtained as described by Cabib (19), except for the use of dithiothreitol (20 mM) instead of β-mercaptoethanol. Purified plasma membranes were isolated as the concanavalin A (Con A) conjugates by rate density centrifugation on Renografin gradients (20,21).

Photoaffinity labeling: (All initial operations at 4°C). 8-N_3-[^{32}P]cAMP (68 Ci/mmole, ICN), at the indicated concentrations, was added in the dark to a medium containing (final conc.): 50 mM HOAc/NaOAc buffer (pH 4.0), 100 µg/ml BSA, 0.5 µM phenylmethylsulfonyl fluoride (PMSF), 0.5 µM pepstatin. The reaction was initiated by the addition of 200 µg of membrane protein and samples were incubated for 60 min in the dark and then irradiated for 5 min with a short wave UV ultraviolet lamp (Mineralight, Model SL 2537, at a distance of 8 cm). The reaction was terminated by ten-fold dilution with buffer A (50 mM HOAc/NaOAc, pH 4.0; 100 µg/ml BSA and 0.5 mM cAMP). The mixture was filtered through 0.45 µm Millipore filters prewashed with buffer A, and then rinsed with 5 ml of buffer A. The filters were dried and counted in 10 ml of Aquasol (New England Nuclear) using a Beckman LS-7000 scintillation counter.

Electrophoretic and autoradiographic analysis: Labeled membrane samples were diluted 100x with buffer A and centrifuged for 30 min at 108,000 x g_{ave} in an SW41 (Beckman) rotor. The pellet was resuspended in (final conc.): 10% glycerol, 5% β-mercaptoethanol, 3% sodium dodecyl sulfate and 50 mM Tris-HCl (pH 6.8), and heated at 100° for 4 min for complete solubilization. Sodium dodecyl sulfate (SDS) gel electrophoresis was by the system described by Laemmli (22), adapted for slab gels. Following electrophoresis, gels were fixed (45% methanol, 10% HOAc, 45% H_2O), dried in a Bio Rad slab gel dryer and exposed to Kodak SB-5 X-ray film. Autoradiographs were scanned using a Helena Quick Scan Densitometer.

cAMP binding assay: As described in (16), with minor modifications.

Materials: Pepstatin, Con A, cAMP and dithiotheitrol were all from Sigma Chemical Co., Tris HCl was from ICN, BSA was from Miles Laboratories, Renografin-76 was from Squibb Chemical Co., SDS was from BDH Biochemicals, and PMSF was from CalBiochem.

RESULTS AND DISCUSSION

Characterization of plasma membranes: Purity of the plasma membranes for the binding experiments was established by assaying for both intracellular (antimycin-sensitive NADH:cytochrome *c* reductase, cyanide-sensitive cytochrome *c* oxidase and L_S-isocitrate dehydrogenase) and cell wall-associated (β-D-fructofuranoside fructohydrolase) enzymes. Compared to whole cells the preparation retained approximately 2% of the former and 3% of the latter activities. Additional characterization, using two-dimensional polyacrylamide gel systems (23), has been published (21, Jaynes and Mahler, in preparation) and further substantiates an estimate of the maximum amount of contamination of 3%.

Photoaffinity labeling of cAMP-binding proteins: 8-N_3-[^{32}P]cAMP, a photoreactive analog of cAMP, has been successfully used previously in many systems for the identification and assay of cAMP-binding proteins (14-18). In order to extend these studies to yeast plasma membranes, these were exposed to the ligand and subjected to analysis by electrophoresis and autoradiography. A densitometer scan from such an experiment is shown in Fig. 1. The major protein components are designated A-D. Specific photoinsertion of the cAMP analog into these components was demonstrated by control experiments in which i), a 1000 fold excess of cAMP was added to the reaction mixture prior to irradiation, or ii), the reaction was carried out in the dark, neither of which produced any significant incorporation of label. Furthermore, neither ATP nor 5'AMP at concentrations as great as 20 μM had any effect on incorporation.

Characteristics of the binding reaction: Purified plasma membranes were exposed to 8-N_3-[^{32}P]cAMP in the dark and binding assayed after Millipore filtration

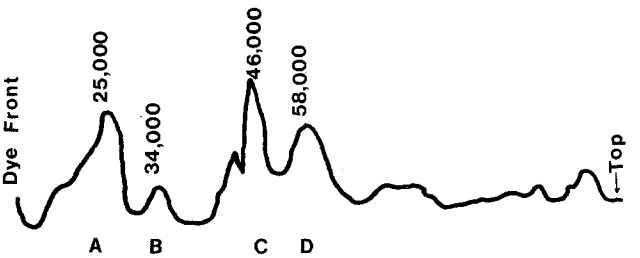

Fig. 1. Molecular weight of the membrane associated cAMP binding proteins. Proteins (60 µg per gel) were separated electrophoretically, after insertion of the photoreactive label, using a 10% acrylamide SDS gel. Molecular weights (M_R) estimated from the usual linear plots of log M_R vs relative mobility, using the following standard proteins (M_R in parenthesis): human γ-globulin - small subunit (23,500); chymotrypsinogen (25,700); *E. coli* RNA polymerase, α subunit (40,000); ovalbumin (46,000); γ globulin, large subunit (50,000); rabbit muscle pyruvate kinase (57,000); BSA (68,200); RNA polymerase, σ subunit (90,000).

as described in Methods. The results of these experiments are presented as a double reciprocal plot in Figure 2, yielding an apparent K_D for the ligand equal to 0.5 µM, with a specific binding capacity of 2.5 fmol 8-N_3-[^{32}P]cAMP bound per µg of membrane protein. Figure 3 presents a Hill plot of these same

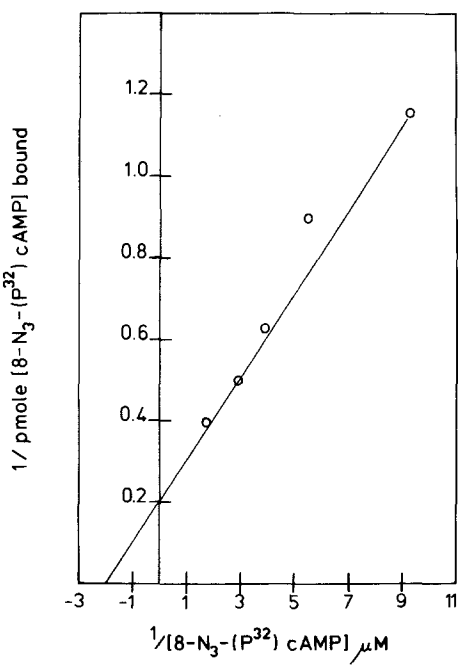

Fig. 2. Estimation of the binding constant for the affinity ligand in the dark. See text for binding conditions.

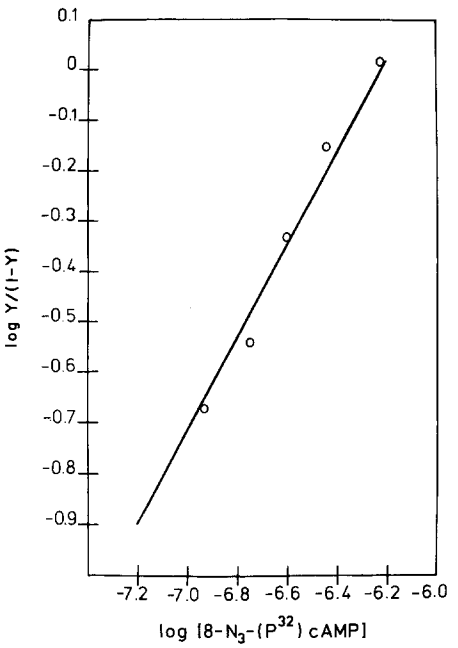

Fig. 3. Hill Plot of data presented in Fig. 2.

data: the Hill coefficient equals 0.9. In conjunction, these results indicate that the analog interacts non-cooperatively with a single population of binding sites.

These observations serve to substantiate the existence of cAMP-receptor sites in yeast plasma membranes and delineate some of their characteristics, which appear quite similar to those previously reported for these entities in higher eucaryotes. While the identity and absolute number of the proteins responsible remain in doubt, it may be significant that soluble cAMP binding proteins, with apparent molecular weights of 24,000 (8), 28,000 (10) and 50,000 (12) have been observed in yeast. Furthermore, yeast cyclic nucleotide phosphodiesterase, a potential candidate for labeling by the photoreactive probe used, has been reported to have a molecular weight of 60,000 (24). Thus, based on molecular weights at least some of the entities associated with the plasma membrane identified in the present study may be similar to their soluble analogs. Furthermore, the two species of highest molecular weight may represent regulatory subunits of Type I and II protein kinases.

This last supposition is based on the demonstration of extensive structural similarities between membrane associated and cytosolic protein kinases in bovine cerebral cortex (15,16) and human erythrocytes (25) and the presence of both types in the former (15,26). Dery *et al.* (9) have identified a major, soluble, cAMP-binding protein in yeast with a molecular weight of 54,000 and speculate that proteolytic cleavage may explain the apparent molecular weight heterogeneity found among proteins of this class by others. We find multiple species associated with membranes even in the presence of PMSF, but use of p-aminobenzamidine, an even more effective protease inhibitor in yeast (26) may reduce their number. However, final resolution of the question of the number, function and types of cAMP-binding species present in both the cytoplasm and plasma membranes of this organism must await isolation and characterization of the actual proteins implicated.

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