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Properties and Possible Functions of the Adenylate Cyclase in Plasma Membranes of *Saccharomyces cerevisiae*

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We have examined the possible role of adenosine 3',5'-phosphate (cAMP) in functions associated with the plasma membranes of *Saccharomyces cerevisiae*. Purified membranes from this source contained an adenylate cyclase which was insensitive to activation by fluoride or guanine nucleotides, only weakly responsive to changes of carbon source in the growth medium, and strongly stimulated by vanadate. They also contained at least two classes of receptor proteins for guanine nucleotides (as measured by binding of labeled 5'-guanylyl methylene diphosphate) with apparent dissociation constants equal to 1.0×10^{-7} and 3×10^{-6} M, a protein kinase capable of phosphorylating added histones, the activity of which was stimulated by cAMP, and cAMP receptors that may function as regulatory subunits for this kinase. Membrane proteins were also susceptible to phosphorylation by endogenous kinase(s), with polypeptides of apparent molecular weights equal to 160×10^3, 135×10^3, 114×10^3, and 58×10^3 as the major targets. Of these, the 114,000-molecular-weight polypeptide was probably identical to the proton-translocating ATPase of the membranes. However, the cAMP-dependent protein kinase did not appear to be involved in these reactions. Intact (*rho*$^+$ or *rho*^{0}) cells responded to dissipation of the proton electrochemical gradient across their plasma membranes by rapid and transient changes in their intracellular level of cAMP, as suggested earlier (J. M. Trevillyan and M. L. Pall, J. Bacteriol., **138**:397–403, 1979). Thus, although yeast plasma membranes contain all the essential components of a stimulus-responsive adenylate cyclase system, the precise nature of the coupling device and the targets involved remain to be established.

We are interested in the response of yeast cells to the imposition of or release from carbon catabolite repression (glucose effect; for recent reviews and citations see references 7, 31, and 41). In most bacteria, e.g. *Escherichia coli*, it is generally believed that this cue is transduced into changes of intracellular levels of adenosine 3',5'-phosphate (cAMP) brought about by altering the activity of adenylate cyclase (EC 4.6.1.1), an enzyme located in bacterial plasma membranes (2, 10, 23, 46, 47). The possible operation of an analogous mechanism in yeasts and in fungi in general has been recently reviewed by Mahler et al. (31) and by Pall (41), respectively. Fungal cells are certainly capable of transducing external stimuli into changes in intracellular cAMP. For instance, agents that affect *Neurospora crassa* plasma membrane permeability or integrity produce a transient rise in intracellular cAMP (41), and the presence of a functional adenylate cyclase appears implicated in the effect (42). Preliminary results suggest that *Saccharomyces cerevisiae* may respond in an analogous fashion to such perturbations (31, 59a). Furthermore, α factor, a peptide pheromone produced by cells of the α mating type, has been shown to inhibit the adenylate cyclase in isolated plasma membranes (28). Although formally analogous to the regulation of the enzyme by certain hormones in vertebrates (29, 38, 50, 51), the mechanism of this interaction is not yet clear (59a).

The evidence for the possibility that catabolite repression affects cAMP levels in a similar direct fashion and, in particular, through regulation of the adenylate cyclase, is equivocal. Some studies suggest that the addition of glucose to derepressed cells lowers the intracellular level of cAMP, whereas a shift from glucose to a derepressing medium or the addition of cAMP has the converse effect (31, 32, 40, 55), coincident with the synthesis of several catabolite-sensitive enzymes in the cytosol and mitochondria (30, 31, 41). In contrast, the kinetics of increase in intracellular cAMP upon a shift of *S. carlsbergensis* from glucose to galactose or maltose have

been shown to be inconsistent with positive regulation of the synthesis of α-glucosidase or succinate dehydrogenase (61). Furthermore, for both the wild-type and a mutant of *S. cerevisiae* with levels of several enzymes partially resistant to catabolite repression, the intracellular concentrations of cAMP in organisms grown on glucose are identical and are increased by 70% upon growth on galactose (54). Finally and most convincingly, in another mutant highly permeable to exogenous cAMP, the addition of cAMP at up to 5 mM does not alleviate the repression of galactokinase synthesis by glucose (36). These results suggest that elevated levels of cAMP are not required for the expression of catabolite-repressible genes. Another major question concerns the possible primary target(s) of cAMP function in unicellular eucaryotes. In bacteria, the cAMP receptor functions as a positive regulator of transcription, whereas in metazoans, it regulates the activity of a variety of susceptible proteins by affecting their state of phosphorylation (13, 24, 38). This effect is commonly believed to be mediated by binding of cAMP to the regulatory subunit (R) of cAMP-dependent protein kinases (4, 15) located both in the cytosol and in the plasma membrane (52, 53), thereby liberating the catalytic subunit (C) from an inhibited R_2C_2 complex. However, recent studies of mammalian cells in cultures suggest an additional and possibly more direct activation of C by cAMP (15). This type of kinase has been identified in the cytosol of both *S. cerevisiae* (20, 59) and *Kluyveromyces fragilis* (58). In mutants of the former, a defect in adenylate cyclase (structural gene *bcy1*) can be corrected by a second mutation that decreases the ability of R (structural gene *cyr1*) to bind cAMP (35).

In this study, we describe some of the properties of purified *S. cerevisiae* plasma membranes of potential relevance to the questions posed above: the presence and possible regulation of adenylate cyclase in the membranes, the presence and nature of their receptors for GTP and cAMP, and the presence and nature of the substrates of an endogenous protein kinase.

MATERIALS AND METHODS

Abbreviations used. CCCP, carbonylcyanide-*m*-chlorophenyl-hydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl)*N,N'*-tetraacetic acid; [^{3}H]GppCH$_2$p, 5'-[8-^{3}H]guanylyl methylene diphosphonate; Gpp(NH)p, guanylyl-β,γ-imidodiphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; α-methylglucoside, α methyl-D-glucoside; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; TEMED, *N,N,N',N'*-tetramethylethylenediamine; IBMX, 3-isobutyl-1-methylxanthine; MES, 2[*N*-morpholine]ethanesulfonic acid.

Yeast strains and growth conditions. We used a number of laboratory strains of *S. cerevisiae* for preliminary studies. However, most of the results described here were obtained with the strain D243-4A (haploid *rho*^{0} *MAT ade1 lys2*; 17) and its wild-type (*rho*$^+$) parent, obtained from R. Criddle (University of California, Davis) (18), and KM91 (diploid *rho*$^+$ *ade1/ade1*), obtained from M. Claisse (Gif-sur-Yvette, France). For most of the experiments, we used cells grown at 30°C in YPD medium (1% yeast extract, 1% peptone, 2% glucose) or the same medium with 2% galactose or 2% α-methylglucoside to an absorbancy at 600 nm of 0.5 (corresponding to a cell density of 1.6 × 10^7 cells × ml^{-1}; Zeiss PMQ spectrophotometer).

Isolation of purified plasma membranes. For isolation of purified plasma membranes, we used two methods. The first (preparation A), applied to cells derived from D243-4A grown on a variety of carbon sources, was modified slightly from the method of Duran et al. (9). Protoplasts were prepared by the method of Cabib (5), with the exception that DTT (20 mM) was substituted for 2-mercaptoethanol, and the spheroplasts were suspended in 0.8 M sorbitol to a final concentration of 1 g (wet weight) per ml. The plasma membranes were then isolated on Renografin gradients, with the addition of RNase A (100 μg × ml^{-1}) to the spheroplast lysis buffer (9). Purified membranes were stored as frozen pellets at −27°C. Preparation B was used principally with KM91 cells; it was based on the method of McDonough et al. (37) and included an additional washing step before the collection of crude membranes by centrifugation as well as the use of a self-generating Percoll gradient for the separation of plasma membranes from mitochondrial inner membranes (J. P. McDonough and H. R. Mahler, J. Biol. Chem., in press). After centrifugation for 30 min, the gradient yielded two prominent bands, one at a density of 1.345 ± 0.001 g × ml^{-1} that contained the mitochondrial contaminants and one at a density of 1.349 ± 0.001 g × ml^{-1} that contained predominantly plasma membranes.

Enzyme assays. Marker enzyme assays used to determine the amount of contaminating activities in the plasma membrane fraction included CN^--sensitive cytochrome *c* oxidase, antimycin A- and CN^--sensitive NADH oxidase, antimycin-sensitive NADH-cytochrome *c* reductase (mitochondrial inner membrane), L_s-isocitrate dehydrogenase (mitochondrial matrix), monoamine oxidase (mitochondrial outer membrane), β-D-fructofuranoside fructohydrolase (cytosol), and glucose-6-phosphatase (endoplasmic reticulum) (9, 14, 22, 44, 45). Assays for oligomycin and efrapeptide-sensitive and -insensitive Mg^{2+}-ATPase are described in McDonough et al. (37).

Protein kinase activity was determined with histone as the protein substrate followed by adsorption on phosphocellulose paper (67). Membranes (5 mg of membrane protein per ml) were suspended in a medium containing the following (final concentrations): 50 mM sodium acetate (pH 6.5), 20 mM $MgCl_2$, 0.5 mg of histone per ml, and 1.0 μM cAMP as shown in Table 3. The reaction was initiated by the addition of [γ-^{32}P]ATP (25 Ci/mmol; final concentration, 1.0 μM). The reaction (final volume, 0.1 ml) was terminated by transferring 25-μl aliquots onto phosphocellulose P51 paper (1 by 2 cm) and immediately immersing the paper in water (25 to 50 ml per sample). Samples were

washed three times in fresh changes of water (total wash time, 5 min), and each paper disk was transferred to acetone (2 ml) and then petroleum ether (2 ml) and finally air dried. The amount of radioactivity was counted in 10 ml of Redi Solve HP (Beckman Instruments, Inc.) with an LS-7000 scintillation counter.

For assays of adenylate cyclase, plasma membranes (1.5 mg of protein $\times$ ml^{-1}) were incubated for 20 to 30 min at 30°C in a medium containing the following (final concentrations): 100 mM PIPES-KOH buffer (pH 6.2), 10 mM $MnCl_2$ unless stated otherwise, 10 mM phosphoenolpyruvate (potassium salt), 0.25 mg of pyruvate kinase per ml, 2 mM ATP, and 1 mM IBMX, a phosphodiesterase inhibitor. The reaction was terminated by boiling the sample for 3 min. The denatured protein was pelleted at 4°C for 5 min (1,000 $\times$ g_{ave}), the supernatant solution was removed, and cAMP measured by the protein binding assay described by Gilman (13) with the minor modifications described by Rubin et al. (52). The reaction mixture was incubated for 60 min at 0°C in a volume of 400 μl containing the following (final concentrations): 50 mM sodium acetate-acetic acid buffer (pH 4.0), 5 μg of protein kinase, 14 μg of protein kinase inhibitor, and 15 pmol of [^{3}H]AMP (50 Ci/mmol). The reaction was initiated by the addition of the supernatant solution to be assayed and terminated by a 10-fold dilution with 20 mM K_2HPO_4 buffer (pH 6.0). The reaction mixture was filtered, and each filter was rinsed with 10 ml of 20 mM K_2HPO_4 buffer and then dried and counted. Known amounts of cAMP added to each incubation mixture were used as internal standards for all experiments, thus controlling for all nonspecific binding (13, 52, 63, 64).

Cell permeabilization. Cell permeabilization was achieved with DMSO (1), chitosan (21), or toluene–Triton X-100 (18) with similar results. Toluene–Triton X-100 was used routinely. Cells (0.4 g [wet weight]) were suspended in 10 ml of medium containing the following (final concentrations): 8 mM imidazole buffer (pH 6.5), 10% sorbitol, 100 mM KCl, and 5 mM $MgCl_2$ (or $MnCl_2$). Permeabilization was initiated by the addition of toluene–ethanol–10% Triton X-100 (1:4:1). After 30 s, the mixture was diluted threefold with ice-cold buffer, and the cells were pelleted for 5 min (1,000 $\times$ g_{ave}) at 4°C and washed three times with 10 ml of ice-cold buffer.

Binding of GTP analogs to membrane proteins. Membranes (2 mg of protein per ml) were suspended in a medium containing the following (final concentrations): 20 mM HEPES buffer (pH 7.4), 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 2 mM *p*-aminobenzamidine, and 3 mM $MgSO_4$ (48). Binding was initiated by the addition of [^{3}H]GppCH_2p (9.7 Ci/mmol) and completed by incubation at 30°C. After 30 min, the mixture was diluted 10-fold with ice-cold buffer, and the membranes were immediately collected on filters. The filters were washed twice with 6-ml aliquots of ice-cold buffer, dried, and counted. The addition of 1 mM unlabeled GTP to the reaction mixture reduced the radioactivity to background levels.

Membrane perturbation and measurements of intracellular cAMP. Cultures of *S. cerevisiae* D243-4A *rho*$^+$ and D243-4A *rho*^{0} were grown on synthetic medium (8) plus 100 μg of Na_2SO_4 per ml and 2% glucose. Cells were harvested at an absorbancy at 600 nm of 1.0 (Zeiss PMQ spectrophotometer), corresponding to a cell density of 4.0 $\times$ 10^7 ml^{-1}. After centrifugation, cells were washed once with fresh growth medium and resuspended in this medium to an absorbancy at 600 nm of 5. All of these procedures were carried out at 25°C. Cells (25 ml) were challenged with a variety of effectors, and 3.0-ml aliquots were quickly filtered on Whatman GF/A glass fiber filters and dropped into 2 ml of cold 5% trichloroacetic acid. Sampling was rapid, requiring less than 10 s from culture flask to acid. Cells were extracted for 30 min at 0°C and centrifuged for 5 min at 0°C and 26,000 $\times$ g_{ave}, and the supernatant was immediately passed through 0.5-ml alumina columns (60) prepared from neutral aluminum oxide (activity grade I; Woelm) suspended in water. Extracts were placed on the columns, and the columns were washed twice, each time with 2 ml of distilled water. cAMP was then eluted with 2 ml of 0.1 M ammonium acetate. All experiments were performed in triplicate. In a control experiment, samples of [^{3}H]cAMP were added to trichloroacetic acid-extracted samples, and 88 $\pm$ 4% of the label applied to the column was eluted with ammonium acetate as described above.

Preparation of samples and electrophoresis. Phosphorylated membranes were solubilized by addition of an equal volume of 2$\times$ Laemmli sample buffer (25), incubated for 15 min at 37°C, and separated on 7.5% SDS-polyacrylamide gels with a discontinuous buffer system (25).

For electrophoresis, the system developed by Laemmli (25) was adapted for slab gel analysis. The separating gel consisted of acrylamide (with a 7.5 or 10% linear or 10 to 15% exponential gradient), 0.1% bisacrylamide, 4.5% Tris base, 0.1% SDS, 0.05% TEMED, and 0.05% ammonium persulfate (pH 8.8). The gel was overlaid with water-saturated 2-methyl-1-propanol to minimize surface convection during polymerization. The alcohol was removed after 2 h and replaced by a buffer solution containing 4.5% Tris base and 0.1% SDS (pH 8.8). The following morning, a 3.75% stacking gel was poured 1 h before the gel was loaded. Electrophoresis was performed (20 mA per slab) until the dye in the upper reservoir buffer had migrated to within 1 cm of the bottom of the gel. The gel box was cooled with circulating tap water. The gels were then fixed (45 ml of methanol–10 ml of acetic acid–45 ml of water).

Gels labeled with ^{32}P were dried in a slab gel dryer (Bio-Rad Laboratories) and exposed to Kodak SB-5 X-ray film. Autoradiographs were scanned with a Beckman DU-8 spectrophotometer equipped with a gel scanning accessory. Apparent molecular weights were determined with a set of standard proteins (Bio-Rad) run in parallel (see Fig. 3).

Chemicals. Concanavalin A, histone, DTT, protein kinase, protein kinase inhibitor, GppCH_2p, neutral aluminum oxide, and Percoll were obtained from Sigma Chemical Co. [γ-^{32}P]ATP was purchased from New England Nuclear Corp., and [^{3}H]GppCH_2p was purchased from Amersham Corp. SDS was purchased from BDH Chemicals, and polyacrylamide gel electrophoresis reagents were purchased from Bio-Rad Laboratories. Ampholine carrier ampholytes were from LKB Instruments, Inc. Renografin was purchased from E. R. Squibb & Sons. All other reagents were of the highest grade available commercially.

TABLE 1. Enzymatic activities in membrane fractions of preparation B[a]

Enzyme marker	Location[b]	Enzymatic activity in[c]:		
		Crude membranes	Band A	Band B
NADH oxidase (antimycin A-sensitive)	IM		1.38	0.05
Monoamine oxidase	OM		<0.01	<0.01
Glucose-6-phosphatase	ER		<0.01	<0.01
Mg^{2+}-ATPase				
Without efrapeptin	PM + IM	0.52	0.45	0.83
With efrapeptin (75 nM)	PM	0.44	0.05	0.75
Adenylate cyclase		10	0.5	11
Protein (% of crude membrane)		100	36	36

[a] All fractions were from KM91 cells grown on YPD medium (see text) to mid-exponential phase ($1.75 \pm 0.25 \times 10^7$ cells $\times$ ml^{-1}).

[b] IM, Inner mitochondrial membrane; OM, outer mitochondrial membrane; ER, endoplasmic reticulum; PM, plasma membranes (see text).

[c] All values are expressed as micromoles per minute per milligram of protein, except for values for adenylate cyclase, which are picomoles per minute per milligram, and protein values. Bands A and B of the Percoll gradient are described in the text; they contain predominantly IM and PM, respectively.

RESULTS

Properties of purified plasma membranes. Preparation A, a slight modification of the method of Duran et al. (9), resulted in plasma membranes of satisfactory purity (<10% contamination of mitochondrial inner membranes, mitochondrial matrix, or cytosol by marker assay). These observations corroborate conclusions reported previously (9); the utility of this preparation for investigating membrane-bound adenylate cyclase has been demonstrated by Liao and Thorner (28). We used it for this purpose as well as for studies on binding of nucleotides and their analogs and to obtain protein kinase with exogenous acceptors. However, it proved unsatisfactory for investigating kinase activity with endogenous substrates and for comparisons of the adenylate cyclase with membrane ATPase which is inhibited by residual Renografin. To this end, we utilized an alternate procedure (procedure B) which made use of a Percoll gradient. Some of the characteristics of this preparation are summarized in Table 1. These data show that contamination of the plasma membranes by mitochondrial inner membranes was <10%, as determined on the basis of both the levels of cytochrome *c* oxidase and the levels of efrapeptin-sensitive Mg^{2+}-ATPase. The distribution of the ATPase and the adenylate cyclase which results from this isopycnic centrifugation indicates that both these activities are located in the same membrane, which we know to be the plasma membrane in the case of the ATPase (McDonough and Mahler, in press).

Properties of adenylate cyclase in plasma membranes. Although we have shown that adenylate cyclase was present in highly purified plasma membranes, we elected to use the cruder starting material to establish its properties. The rationale for this choice was based on the possibility that extensive purification and handling might result in the removal of or inactivation of a potential regulatory subunit (47, 48, 50, 51) from the enzyme. Even with this relatively crude enzyme, there was no indication of possible regulatory effects; the enzyme used Mg-ATP as the substrate but required Mn^{2+} for maximal activity and was unaffected by fluoride, GTP, the non-hydrolyzable GTP analog $GppCH_2p$, or kirromycin, which inhibits the activity of GTP-binding proteins of bacteria (3, 6). The enzyme was also, not unexpectedly, insensitive to the addition of agents that affect intact membranes by altering their integrity (e.g., nystatin [60]), their permeability (e.g., gramicidin S [42, 47]), or the transmembrane electrochemical proton gradient (e.g., CCCP [11, 27, 60]). It resembled some of the enzymes from mammalian cells in being activated by vanadate (16, 56). Although its specific activity was increased by growth of cells on galactose or α-methylglucoside rather than on glucose, we did not establish explicitly whether this enhancement was real or simply due to a lower level of contamination (see below).

Presence of guanine nucleotide-binding proteins. In higher eucaryotes, sensitivity of adenylate cyclase to hormones or other ligands frequently requires the presence of a member of a class of proteins (G, G/F, or N protein; 29, 48, 50, 51, 57) capable of interaction with and bind-

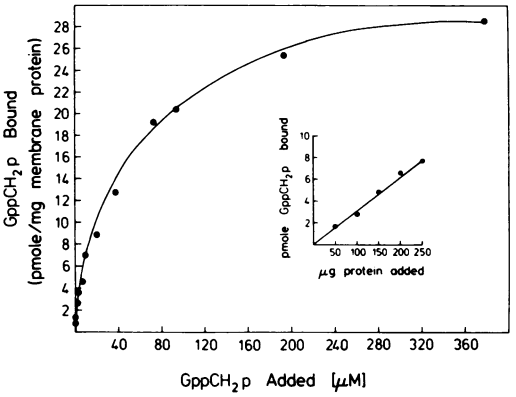

FIG. 1. Binding of labeled $GppCH_2p$ to plasma membrane proteins from glucose-grown cells. Insert, Extent of binding versus amount of glucose membrane protein added. See text for conditions.

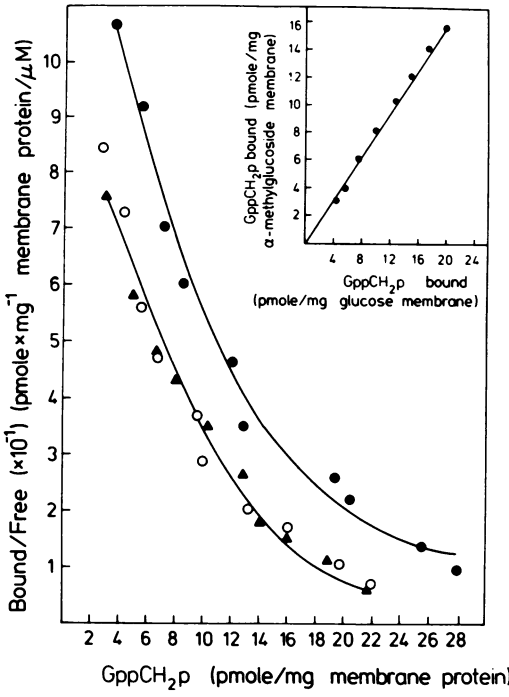

FIG. 2. Scatchard plot of $GppCH_2p$ bound to membranes isolated from cells grown on glucose (●) and α-methylglucoside (○). Insert, Amount of ligand bound per milligram of membrane protein (glucose) versus membrane protein (α-methylglucoside) at different ligand concentrations. The slope of the curve in the insert equals 0.79. The results for membranes from glucose-grown cells multiplied by 0.79 are also plotted (▲). For other details see the text.

ing of GTP or its analogs. The same proteins are also implicated in activation by fluoride, and a member of this class in *Dictyostelium discoideum* has recently been identified (26). Therefore, the lack of response of the adenylate cyclase to these agents might be due to the absence, inactivation, or loss of an analogous GTP-binding protein even in the crude membrane preparations used. We therefore investigated the capacity of more highly purified plasma membranes for reversible binding of $[^3H]GppCH_2p$, a ligand frequently used for the identification and characterization of G proteins. The results (Fig. 1 and 2; Table 2) demonstrate the presence of receptors for this nucleotide on the plasma membranes of cells grown on glucose (preparation A) and show the constancy of both characteristic parameters: $\bar{K}_D$, the apparent dissociation constant, and β, the number of binding sites in the membranes of cells grown either on glucose or on α-methylglucoside. The observed $\bar{K}_D$ is of the same order of magnitude as that characteristic of active G proteins associated with cyclases of higher eucaryotes (48, 50, 51, 57). These observations provide a necessary, although by no means sufficient, demonstration that yeast plasma membranes contain essential components of a GTP-requiring adenylate cyclase system.

Responses of adenylate cyclase in more complex systems. The presence of GTP-binding proteins in the isolated membrane, taken together with the complete insensitivity of the adenylate cyclase to modulation by GTP, may be due either to the absence of a functional coupling device or to the irrelevance of GTP receptors to the regulation of the adenylate cyclase. To study regulation of the adenylate cyclase in a more native environment, permeabilization of intact *E. coli* cells by treatment with toluene has permitted the demonstration of the coupling of the adenylate cyclase to sugar transport systems (10, 18, 23). We elected to pursue this approach with yeasts. With these organisms, permeabilization has

TABLE 2. Apparent dissociation constants ($\bar{K}_D$) and binding capacities for $GppCH_2p$ on plasma membranes[a]

Carbon source used	Binding site affinity	$\bar{K}_D$ (M)	Binding capacity (pmol × mg of membrane protein^{-1})
Glucose	High	1.0×10^{-7}	16 ± 3
	Low	2.5×10^{-6}	48 ± 4
α-Methylglucoside	High	1.0×10^{-7}	14 ± 2
	Low	2.5×10^{-6}	30 ± 3

[a] These values are the averages of two experiments done in duplicate.

been achieved by treatment with such diverse agents as protamine or cytochrome *c* (62), chitosan (21), DMSO (1), toluene-ethanol (62), and toluene–Triton X-100 (P. Jaynes and H. R. Mahler, Fed. Proc. 37:1394, 1978). We tried the last three agents and got the same results. Permeabilization was accomplished successfully as indicated by the demonstration of adenylate cyclase activity, which could not be measured in intact cells. With toluene–Triton X-100, adenylate cyclase levels equalled 1.6 to 3.3 pmol × min^{-1} × mg of cell $protein^{-1}$, levels which were unaffected by $GppNH_2p$ (100 μM), F^- (2 to 10 mM), Ca^{2+} (2 mM), EGTA (100 μM), or glucose (0.2 to 5%). However, although maximal activity could be attained by the addition of Mg^{2+} (10 mM) in the complete absence of Mn^{2+}, enzyme activity remained insensitive to modulation by all the potential effectors described above.

Response of adenylate cyclase to membrane perturbations. The observations reported above raised the question whether the adenylate cyclase of our particular strains grown under the conditions described was susceptible to any form of regulation. Trevillyan and Pall (60) made the important observation that in *N. crassa*, treatments that interfere with the structural and functional integrity of the plasma membrane produce transient increases in intracellular cAMP levels; these workers also reported preliminary observations that this phenomenon is probably characteristic of fungi in general, including *S. cerevisiae*. Whereas this study implicated adenylate cyclase by inference only, a more recent report from the same laboratory (42) showed that two *N. crassa* gene (*cr-1* and *fr*) mutations that result in a low level of adenylate cyclase activity or in an aberrant adenylate cyclase prevent the increases in cAMP seen in wild-type cells. Since the *cr-1* mutants also exhibited exceedingly low levels of intracellular cAMP, the inference that the wild-type allele of this gene is directly concerned with the function or structure of the enzyme appeared warranted, and therefore, the latter is responsible for the phenomenon under discussion (41).

We therefore undertook a study of the response of cAMP levels to membrane perturbations in our experimental system. Our earlier results (31) have already demonstrated that the response is not restricted to wild-type (*rho*$^+$) cells but is elicited equally well in respiration-deficient (*rho*^{0}) cells. Furthermore, the changes in cAMP levels (and, as shown by the argument just presented, the adenylate cyclase levels) appear to be regulated by the electrochemical proton gradient across the membrane (60). This gradient is generated by the proton-translocating ATPase in the membrane (33, 37, 43, 64) and can be dissipated by ion movements. This dissipation can be either partial, if it involves the membrane potential or the pH gradient separately, or complete, if both components are implicated (11, 27). We have confirmed these predictions in recent experiments (data not shown). The maxima in the transient increase of intracellular cAMP were observed within ~2 min of the exposure of intact cells to various agents, with the uncoupler 2,4-dinitrophenol (300 μM) giving the greatest increase (ca. fivefold; 31, 42). Ca^{2+} ions (5 mM) were ineffective when added alone, but mobilization of endogenous Ca^{2+} by means of the Ca^{2+} ionophore A23187 (100 μM) produced a response about 50% of that generated by the uncoupler; this is interpreted as an ionophoretic transport of the ion, thereby dissipating the membrane potential without affecting the pH gradient. In contrast, the simultaneous addition of K^+ (1 mM instead of the 600 mM present in the synthetic medium used), nigericin, and valinomycin (1 μM each), which facilitates efficient counter-transport of K^+ (inward) and H^+ (outward) and should result in a complete collapse of the electrochemical proton gradient, is as effective as 2,4-dinitrophenol in generating the transient increase of intracellular cAMP but does so more slowly (~2 min).

Protein kinases and action of cAMP. As mentioned above, in the model currently favored for the mode of action of cAMP in higher eucaryotes, cAMP functions as a ligand for the R of an inhibited R_2C_2 complex of a protein kinase, thereby releasing C in an active form. For this model to be valid in the present instance, it would be necessary to demonstrate the presence in the membrane of two (classes of) proteins, one capable of functioning as a potentially regulatory cAMP receptor and the other capable of functioning as a catalytically active protein kinase.

We have already shown that membranes contain a set of cAMP-binding proteins (22, 31). With 8-[^{3}H]azidoadenosine 3′,5′-phosphate as a photoaffinity ligand, the M_rs of the most prominent species were 56,500, 46,000, and 26,000, whereas labeling with 8-azidoadenosine [^{32}P]3′,5′-phosphate disclosed prominent species with M_rs of 58,000, 46,000, and 25,000. The two largest species may be candidates for Rs of types II and I (4, 12, 41, 52, 53), respectively. The apparent overall dissociation constant of 0.5 μM (determined in the dark) for these complexes is in agreement with this supposition. Very recently, Sy and Roselle (58) have identified a cAMP-binding protein with an M_r of 64,000 as the R of a soluble cAMP-dependent protein kinase in *K. fragilis*, as well as a second such protein with an M_r of 37,000 as a fragment of the first, derived from it by proteolysis.

It was therefore of interest to determine

TABLE 3. Plasma membrane protein kinase activity with histones as the acceptors

Assay addition	Protein kinase activity[a]
No membrane (control)	5 ± 4
Membranes only	180 ± 12
Membranes +	
cGMP (1.0 μM)	148 ± 6
cAMP (1.0 μM)	412 ± 16
Ca^{2+} (5 mM)	195 ± 7
ADP (1.0 μM)	172 ± 12
Ca^{2+} + cAMP	382 ± 19

[a] Protein kinase activity was measured with 0.5 mg of histones per ml and is expressed as femtomoles of γ-^{32}P incorporated per milligram of membrane protein per minute. Membrane preparation A was used throughout. Assays (see text) were run in triplicate. Values obtained with membranes but without added histones were equal to background values (<2 fmol/mg).

whether plasma membrane preparations contain protein kinases capable of phosphorylating either added soluble proteins or endogenous proteins in the membrane. The latter appear of particular interest since phosphorylation of either the R itself (4, 12, 19, 53) or adenylate cyclase (23, 46, 49) may be an essential feature of a possible regulatory system involving cAMP.

Membrane preparations of type A contain a cAMP-dependent protein kinase, capable of phosphorylating calf thymus histones, a set of soluble proteins commonly used as a substrate for this reaction (Table 3; 41, 59). However, probably for reasons described above, we were unable to demonstrate the phosphorylation of endogenous membrane proteins in this preparation. We therefore made use of the alternate procedure, developed to provide purified membrane preparations (preparation B) with high activity levels of the efrapeptin-insensitive Mg^{2+}-ATPase, an intrinsic protein of this membrane (Table 1). With this preparation, we were readily able to show the phosphorylation of a number of membrane proteins, the major ones having M_rs of 160,000, 135,000, 114,000, and 58,000 (Fig. 3). Of the three most prominent phosphoprotein bands, the one with an apparent molecular weight of 114,000 has been identified as the ATPase (34, 66; McDonough and Mahler, in press). However, the phosphorylation of all of these entities was not stimulated by the addition of cAMP, nor was it inhibited by a specific inhibitor of the C of mammalian cAMP-dependent kinase, which is known to be effective against fungal enzymes (41). As already mentioned, Rs in higher eucaryotes and *Kluyveromyces lactis* exhibit molecular weights of ~56,000 and of ~50,000 or 64,000, respectively, whereas the value of this parameter for the adenylate cyclase in animal cells is probably of the order of 90,000 to 150,000 (50). Thus, phosphorylation of an R type II by a cAMP-independent kinase or a similar modification of the C of adenylate cyclase remains a possibility.

DISCUSSION

On the basis of the experiments presented here, there can be little remaining doubt that the plasma membrane of *S. cerevisiae* contains an active adenylate cyclase, as suggested earlier (28, 31). This localization of the enzyme appears to be a common feature extending from cells of *E. coli* (10, 23, 46, 47) to those of vertebrates (50,

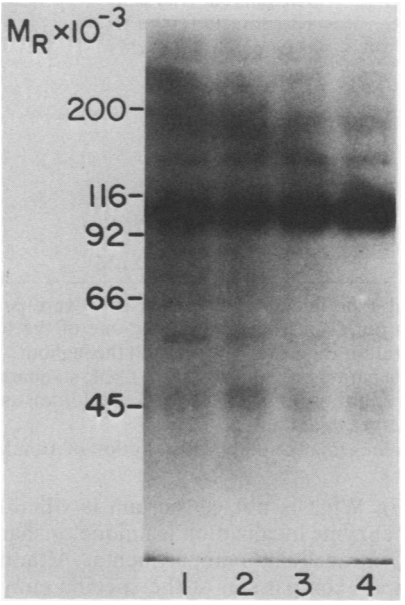

FIG. 3. Effect of cAMP on phosphorylation of plasma membrane proteins by endogenous protein kinase. Samples of purified plasma membrane (preparation B, 50 μg of protein per reaction) were incubated in a final volume of 40 μl containing 50 mM MES-KOH (pH 6.0), 1 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (~0.2 mCi/ml), and 1 mM IBMX plus cAMP (10 μM), cAMP-dependent protein kinase inhibitor (Sigma; 0.4 mg per reaction mixture), or both. After incubation for 10 min at 24°C, an equal volume of 2× sample buffer was added, and the samples were incubated for 15 min at 37°C before electrophoresis on 7.5% SDS-polyacrylamide gels as described in the text. Lane 1, Control (no cAMP or protein kinase inhibitor); lane 2, with protein kinase inhibitor; lane 3, with cAMP; lane 4, with cAMP plus protein kinase inhibitor. Numbers on the left are the relative molecular weights of the standard proteins.

TABLE 4. Effectors of membrane-bound adenylate cyclase[a]

Part	Addition	Relative amt of enzyme activity of cells grown in:		
		Glucose	Galactose	α-Methyl-glucoside
1	None	100 (25.1)	100 (28.3)	100 (35.3)
2	Mg^{2+} (10 mM)[b]	2	3	
	Mn^{2+} (10 mM)	25		
	Mn^{2+} (10 mM) + Mg^{2+} (0.5 mM)	60		
	Mn^{2+} (10 mM) + Mg^{2+} (1.5 mM)	100		
	Mn^{2+} (10 mM) + Mg^{2+} (2.5 mM)	105		
	Mn^{2+} (10 mM) + Mg^{2+} (5.0 mM)	58		
	Mn^{2+} (10 mM) + Mg^{2+} (7.5 mM)	32		
3	Efrapeptin (150 μM)	97		
	CCCP (50 μM)	105		
	Nystatin (4 μg/ml)	100		
	Gramicidin S (20 μM)	80	92	86
	DCCD (400 μM)	110	105	101
	GppNHp (100 μM)	100		
	NaF (10 mM)	98		
	Kirromycin (100 μM)			90
	Kirromycin (100 μM) + GppNHp (100 μM)			95
4	VO_4^{3-} (50 μM)	125		
	VO_4^{3-} (150 μM)	150	140	145
	VO_4^{3-} (200 μM)	180		
	VO_4^{3-} (400 μM)	230		
	VO_4^{3-} (150 μM) + $GppNH_{2p}$ (100 μM)	149		
	VO_4^{3-} (150 μM) + gramicidin S (20 μM)	225		

[a] All experiments except for part 2 were performed under standard conditions (see text) on D243-4A cells grown on YP medium containing one of the three carbon sources (20 g/liter) indicated; crude membranes of preparation B (see text) were used throughout. Values are means of triplicate samples except for part 1, in which three separate preparations were used; standard deviations were ≤5% from the mean. The values expressed are relative to those of samples without addition as 100. Actual values (shown in parentheses) are in picomoles per minute per milligram.

[b] Values unaffected by the addition of 10 mM fluoride or 10 μM GppNHp.

51, 57). What is not yet certain is whether the yeast enzyme localization is unique or shared by other intracellular compartments. Although in principle, comparison of the specific enzymatic activity of the purified membrane with that of the starting homogenate combined with its recovery from this source can provide an answer, the ready solubilization of the membrane-bound enzyme during homogenization and fractionation discovered by Varimo and Londesborough (62) introduces such a serious ambiguity as to invalidate this approach.

All available studies (28, 62, 65) with either commercial or genetically defined yeast cells agree that the bulk of the enzymatic activity is in particulate fractions, and Liao and Thorner (28) claim that the activity found in the plasma membrane accounts for the total cellular activity. These studies, in which a preparation virtually identical with our preparation A was used, show a specific activity of 10 pmol × min^{-1} × mg^{-} of $protein^{-1}$ (reference 28, Fig. 1), compared with values of this parameter in crude membranes (preparation B) of various strains: 25 to 35 pmol × min^{-1} × mg^{-1} (Table 4) or 11 pmol × min^{-1} × mg^{-1} after isopycnic centrifugation of strain KM91 membranes (Table 1). Comparisons of these values with those obtained from permeabilized cells either by us or by Varimo and Londesborough (63) from commercial yeast cells or by Matsumoto et al. (36) from whole homogenates of genetically defined strains (7.8 to 15.5 pmol × min^{-1} × mg of $protein^{-1}$) make it clear that the membrane-bound form of the enzyme must have been subject to some inactivation or other modification during isolation.

In agreement with the results obtained by other investigators (28, 31), we found that the membrane-bound enzyme required Mn^{2+} and was relatively insensitive to activation by fluoride, GTP, and the nonhydrolyzable GTP ana-

logs $GppCH_2p$ and Gpp(NH)p and to inhibition by glucose. These results confirm our earlier published report (31) but differ in some respects from those reported by us in a preliminary study (P. Jaynes and H. R. Mahler, Fed. Proc. 37:1394, 1978). We have not explored this discrepancy and are therefore at a loss to account for it. If, as it appears likely, the enzyme turns out to conform to the pattern established for higher eucaryotes and perhaps for *D. discoideum* (26), then its properties in the isolated membranes suggest that it is present in these membranes in a fully uncoupled state, i.e. as free C (29, 48, 50, 51). Correspondingly, in the permeabilized cell preparation studied here, unlike in the preparation of Varimo and Londesborough, the enzyme is of the potentially regulatable NC type (50).

Two other properties of the enzyme appear of interest: its specific activity is elevated when the cells are grown on relatively nonrepressible carbon sources, and it is activated by vanadate. Although the former property might simply be due to a lowered extent of contamination by other membranes in the relatively crude preparations used, this is not likely, since the specific activity of the Mg^{2+}-dependent ATPase, an authentic marker for the plasma membrane, remained unaffected by this change in carbon source (31). The effect of vanadate is probably intrinsic to the enzyme and unrelated to inhibition of the ATPase in the same membranes, since DCCD at concentrations sufficient to inhibit the ATPase by 90% has no effect on the adenylate cyclase (31). It is of interest that gramicidin S, which generates cation channels in membranes (39), is capable of antagonizing the stimulation by vanadate.

Purified membranes also contain receptors for cAMP and a non-hydrolyzable analog of GTP as well as a protein kinase(s) capable of phosphorylating either soluble or endogenous membrane-bound acceptor proteins. However, although the reaction with added substrate is dependent on exogenous cAMP, we were unable to demonstrate such a requirement in the case of endogenous membrane proteins. In addition, substantial transient changes in intracellular cAMP levels were demonstrated when cells were exposed to agents known to bring about specific perturbations in the transmembrane electrochemical proton gradient. Similar changes are known to be mediated through the adenylate cyclase in both *N. crassa* (41) and *E. coli* (47).

Thus, as also independently suggested by Thorner (59a), yeast plasma membranes contain the essential elements of a stimulus-responsive adenylate cyclase system analogous to that of higher eucaryotes. We have not succeeded, however, in establishing a precise correspondence between the yeast and metazoan systems. Nevertheless, it is hoped that the testing of the hypothesis that the yeast adenylate cyclase functions in a manner similar to that of higher eucaryotes will provide a more detailed picture of the evolution and role of adenylate cyclases throughout the eucaryotic world.

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