Cyclic AMP Receptor Protein from Yeast Mitochondria: Submitochondrial Localization and Preliminary Characterization

GERHARD RÖDEL,* GÜNTER MÜLLER, AND WOLFHARD BANDLOW

Institut für Genetik und Mikrobiologie der Universität München, D-8000 München 19, Federal Republic of Germany

We have identified and characterized a cyclic AMP receptor protein in mitochondria of the yeast *Saccharomyces cerevisiae*. The binding is specific for cyclic nucleotides, particularly for cyclic AMP which is bound with high affinity (K_d of 10^{-9} M) at 1 to 5 pmol/mg of mitochondrial protein. The mitochondrial cyclic AMP receptor is synthesized on cytoplasmic ribosomes and has an apparent molecular weight of 45,000 as determined by photoaffinity labeling. It is localized in the inner mitochondrial membrane and faces the intermembrane space. Cross-contamination of mitochondrial inner membranes by plasma membranes or soluble cytoplasmic proteins is excluded.

In eucaryotes, cyclic AMP (cAMP) receptors are ubiquitous. In all cases where they have been characterized in greater detail, they have been identified as regulatory subunits of protein kinases (12, 24, 26). In the yeast *Saccharomyces cerevisiae*, the search for cAMP receptors resulted in the detection of at least five polypeptides. Four of them are located in the plasma membrane fraction, whereas one is found in the cytoplasm (15, 16). (All other cytoplasmic cAMP receptors described are likely to be degradation products, as discussed by Hixson and Krebs [15].) Although no definite function could yet be assigned to the plasma membrane-bound receptors (with apparent molecular weights of 58,000, 46,000, 34,000, and 25,000), the soluble cytoplasmic cAMP-binding protein (M_r, ≈50,000) has been shown to constitute the regulatory subunit of a protein kinase. Kinases of this kind were found to modulate the activities of a variety of target enzymes, e.g., glycogen synthetase, phosphorylase *b* kinase, trehalase, lipase, and ribosomal proteins (15, 18, 31, 35, 37, 38).

cAMP added to yeast cells in vivo also results in a stimulation of mitochondrial functions, e.g., of mitochondrial respiration (10), levulinic acid dehydratase activity (25), and synthesis of the mitochondrially encoded subunits of cytochrome *c* oxidase (6). Whether these effects of cAMP on mitochondrial activities are mediated by one or another of the extramitochondrial cAMP receptors described above is still unclear (24). On the other hand, no data are yet available on whether the organelle itself is able to receive and transmit signals in the form of cAMP. Although cAMP-independent protein kinases have amply been documented in mitochondria (14, 17, 21, 29, 30), no cAMP-dependent kinase or other cAMP receptor has been characterized in this organelle. Therefore, we started a systematic search for cAMP receptors in mitochondria, which resulted in the identification of a cAMP-binding protein in the inner mitochondrial membrane. We present results on the main characteristics of this protein and its submitochondrial localization.

MATERIALS AND METHODS

Preparation of cellular subfractions. Cultures of the *S. cerevisiae* strains SM202 and D273-10B were grown at 30°C in 1% yeast extract–1% peptone–3% glycerol, 1% yeast

extract–1% peptone–2% dextrose, or lactate medium (7) to a cell density of about 3×10^7 cells per ml. A cell homogenate was obtained from a lysate of spheroplasts by centrifugation at 1,000 × *g* for 10 min at 0 to 4°C. Crude mitochondria were pelleted from the homogenate (25,000 × *g*, 10 min). Crude mitochondria and post-mitochondrial supernatant were further purified by centrifugation on linear 20 to 60% Urographin (Schering AG, Berlin) gradients. The resulting bands were recovered, pelleted after fourfold dilution, and washed once with 0.6 M Sorbitol–10 mM Tris–1 mM EDTA buffer (pH 7.4) to remove Urographin.

Submitochondrial particles were prepared from purified mitochondria by extensive homogenization by 20 strokes at 800 rpm in a Teflon-in-glass homogenizer at 0°C in 20 mM sodium acetate (pH 6.5)–50 μM EDTA–5 mM MgCl$_2$–1 μM phenylmethylsulfonyl fluoride.

Mitochondrial subfractionation. Purified mitochondria were treated hypotonically with 0.1 M mannitol–10 mM Tris–1 mM EDTA–50 μM phenylmethylsulfonyl fluoride (end concentrations), pH 6.8, to disrupt outer membranes. From these "shocked mitochondria" the soluble intermembrane space, matrix, outer membrane, and inner membrane were prepared successively as described previously (1, 7). The following modification was applied as compared with reference 7: To separate inner and outer membranes, a 25 to 55% linear Urographin gradient was used instead of a sucrose gradient.

Topology of the mitochondrial cAMP receptor protein. Intact mitochondria, shocked mitochondria, and mitochondria lysed with 0.5% Nonidet P-40 were treated with various concentrations of trypsin for 30 min at 0°C. Digestion with protease was terminated by the addition of a fourfold molar excess of soybean trypsin inhibitor and 5 mM tosyl lysyl chloromethyl ketone. cAMP-binding proteins and various mitochondrial marker proteins of known topology (porin, outer membranes [11]; cytochrome *c* peroxidase, intermembrane space [7]; fumarase, soluble matrix [33]) were assayed.

cAMP binding assay. A 117-μg sample of mitochondrial particles was incubated with 0 to 100 nCi of [^{3}H]cAMP (52 Ci/mmol; Amersham, Braunschweig) in a total volume of 225 μl for 15 min and centrifuged at 100,000 × *g* for 10 min in a Beckman Airfuge, and 150 μl of the supernatant was counted with 5 ml of Instagel (Packard Instrument, Frankfurt). Alternatively, a filter binding assay was used (39). In

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* Corresponding author.

this case purified mitochondria were lysed in MTP buffer (5 mM $MgCl_2$, 1% Tween 80, [Serva, Heidelberg], 100 mM KH_2PO_4, pH 7.2). A 100-μg sample of the protein (0.5 mg ml^{-1}) was incubated with 0 to 100 nCi of [^{3}H]cAMP for 15 min at room temperature, filtered through cellulose ester filters (Millipore, Neu-Isenburg), and washed three times with 6 ml of MTP buffer. Filters were dried and counted in 10 ml of toluene-based scintillation cocktail. cAMP binding assays were performed in triplicate. In later experiments, cAMP binding was routinely assayed by equilibrium dialysis as indicated. Each half cell of a Dianorm dialysis cell, separated by a Visking dialysis membrane, contained 0.6 ml of 0.5% Nonidet P-40–25 mM KH_2PO_4–5 mM $MgCl_2$–15% glycerol–1 mM dithiothreitol–50 μM phenylmethylsulfonyl fluoride (pH 7.0). To one of the half cells 100 μg of protein was added, and to the other half cell 20 nCi of [^{3}H]cAMP (52 Ci $mmol^{-1}$) was added. Dialysis was performed at 4°C for 3 h. Bound cAMP was calculated from the difference of counts per minute values obtained by counting 0.45-ml samples of each half cell in 2 ml of Instagel.

Photoaffinity labeling. A 100-μg sample of lysed purified mitochondria (1% Tween 80) was labeled with 10 μCi of 8-N_3-[^{3}H]cAMP (New England Nuclear Corp., Dreieich) (27) in the presence of 5 mM AMP as a competitor. Labeled proteins were separated on exponential 10 to 15% sodium dodecyl sulfate–polyacrylamide gels (9); the gels were dried and fluorographed (4).

Other assays. Published procedures were used for the determination of the following enzyme activities: succinate:cytochrome c oxidoreductase (19), adenylate cyclase (32), alkaline phosphatase and catalase (3), aconitase and fumarase (28), and cytochrome c peroxidase (8). Porin was assayed after electroblotting (36) by immunodecoration with specific antibodies and radioiodinated protein A (7) and quantified either by integration of densitometric tracings of autoradiograms or by elution and spectrophotometric evaluation of autoradiographic bands (34). Since Urographin interferes with the protein determination by the Folin reagent (23), it was removed by washing or dialysis. Alternatively, two other protein assays were employed (5, 13) which essentially gave identical results.

RESULTS

As outlined above, it is not known whether the cAMP-dependent effects on mitochondrial functions (6, 10, 25) are mediated by extra-mitochondrial events (24) or by the organelle itself. Therefore we started a systematic search of whether mitochondria contain a cAMP-binding protein(s). In doing so, particular care was taken to control cross-contaminations of mitochondrial preparations by other cellular structures that might interfere with an unambigious mitochondrial assignment of cAMP binding.

Purification of mitochondria. Crude mitochondria, prepared from spheroplasts, were further purified by centrifugation on continuous Urographin gradients (20 to 60%). The resulting particulate bands were removed and either dialyzed or sedimented and washed. Both specific and total activities of a variety of marker enzymes were determined as shown in the DeDuve plots (Fig. 1). Alkaline phosphatase and adenylate cyclase were chosen as marker enzymes for the plasma membrane. 5'-Nucleotidase (E.C. 3.1.3.5), a highly selective marker for plasma membranes in mammals, was found to be absent in yeast. Mitochondrial markers were succinate:cytochrome c oxidoreductase, aconitase, fumarase, and porin. Catalase was taken as a vacuolar marker enzyme.

Two fractions exhibited high rates of succinate:cytochrome c oxidoreductase activity (Fig. 1). They banded at 33 and 43% Urographin, respectively. As deduced from the higher specific activities of both aconitase and fumarase, the latter (denser) is considered to contain mitochondria that are more intact than those in the lighter fraction. The denser fraction was essentially free of catalase activity (Fig. 1), nuclear DNA (as shown by CsCl isopycnic centrifugation, data not shown), and cytoplasmic rRNA (as shown by agarose gel electrophoresis [22], data not shown). Contamination by plasma membranes was extremely low as judged from the retention of 0.12% of adenylate cyclase activity relative to that found in the homogenate. The higher value for alkaline phosphatase activity may be due to a mitochondrial phosphatase(s) active at the pH of the assay. In contrast to the deprivation of plasma membrane markers, an enrichment of cAMP-binding activity as compared with that observed in the homogenate was found. About 13.7% of the
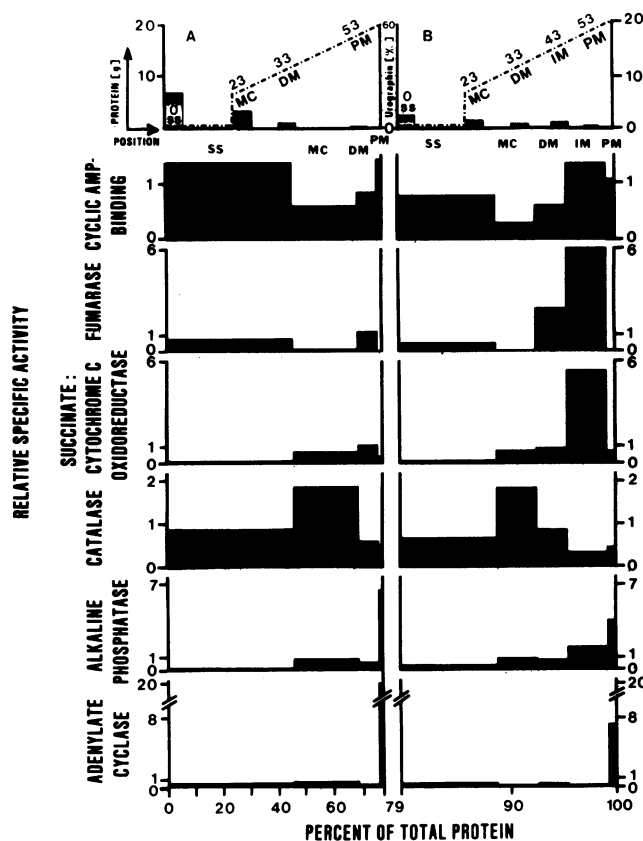

FIG. 1. DeDuve plots of cAMP-binding activity and various markers in cellular subfractions. A post-mitochondrial supernatant (A) and crude mitochondria (B) (79 and 21% of the protein of the homogenate, respectively) were centrifuged on 20 to 60% Urographin gradients. The particulate fractions were assayed for cAMP-binding and various marker enzyme activities. The lines (– · – · – ·) indicate the density of Urographin (numbers refer to the densities of the particulate fractions). The specific activities in the homogenate (which are set at 1) were as follows: cAMP binding, 0.93 pmol mg^{-1}; fumarase, 3.2 U; succinate:cytochrome c oxidoreductase, 25.9 nmol mg^{-1} min^{-1}; catalase 0.20 μg^{-1} min^{-1}; alkaline phosphatase 30.5 nmol mg^{-1} min^{-1}; and adenylate cyclase 52.4 pmol mg^{-1} min^{-1}. Different scales for percentage of total protein were chosen in (A) and (B) because of better presentation. Abbreviations: SS, soluble supernatant; MC, microsomes; DM, disrupted mitochondria; IM, intact mitochondria; PM, plasma membranes.

total cAMP-binding capacity of the homogenate was retained in mitochondrial fractions. Thus, this binding activity cannot solely be accounted for by plasma membrane contamination. It rather suggests an additional mitochondrial localization of cAMP-binding activity.

The bulk of alkaline phosphatase and adenylate cyclase activities found in crude mitochondria was observed to band separately from mitochondria-containing fractions at 53% Urographin. An identical banding density of plasma membrane marker enzymes was observed after centrifugation of the post-mitochondrial supernatant, which is enriched in plasma membranes, on Urographin gradients (Fig. 1A) (2).

Sub-mitochondrial localization of the cAMP receptor protein. To prove unequivocally that a cAMP receptor is contained in mitochondria, we decided to determine the sub-mitochondrial localization and topology of the cAMP-binding activity. Shocked mitochondria were prepared from purified mitochondria (7) and further fractionated into intermembrane space, soluble matrix, and outer and inner membranes as described above. All four resulting fractions were characterized by using the activity of succinate:cytochrome c oxidoreductase as a marker for the inner membrane, the activity of aconitase and fumarase for the matrix, and the activity of cytochrome c peroxidase for the intermembrane space. Quantitative immune blotting with antiserum raised against porin was performed to specify the outer membrane. The results, which are summarized in the DeDuve plots (Fig. 2), show that the subfractionation by the method in reference 7 efficiently separated the various mitochondrial compartments. Moreover, the cAMP-binding activity exactly copurified with that of the inner membrane marker enzymes. (The fact that the specific enrichment was less than that of succinate:cytochrome c oxidoreductase is due to the instability of the cAMP-binding activity during the preparation procedures; see below). Only marginal cAMP-binding activity was observed in the other mitochondrial compartments. By contrast, only negligible activities of both adenylate cyclase and alkaline phosphatase were recovered in the inner membrane fraction, indicating the almost complete absence of plasma membrane contamination. Hence, the cAMP-binding activity in this fraction is a self-contained activity and cannot be attributed to contamination by plasma membranes.

Topology of the cAMP receptor. The orientation of the cAMP receptor within the inner mitochondrial membrane was determined by testing its accessibility to protease treatment. The loss of activity was determined in parallel in (i) purified whole, (ii) shocked, and (iii) lysed mitochondria and compared with that of marker enzymes of known topology (Fig. 3). Whereas the cAMP binding was protected in whole mitochondria, it was readily lost in shocked and in lysed mitochondria. The inactivation characteristics of the cAMP-binding activity resemble those of cytochrome c peroxidase, lactate dehydrogenase (data not shown), and cytochrome c_1 (data not shown), but not those of F_1 ATPase (data not shown) and fumarase. Combined with the results of the previous section this shows that the cAMP receptor of the inner mitochondrial membrane is oriented toward the intermembrane space.

Characterization of the cAMP receptor protein. (i) Nucleotide-binding specificity. To test the specificity of nucleotide ligands, [^{3}H]cAMP binding assays were performed in the presence of a 10-fold molar excess of unlabeled nucleotides. Nucleotide di- and triphosphates as well as 5'- and 3'-noncyclic monophosphate esters did not compete with the labeled cAMP for the binding (Table 1). However, unlabeled
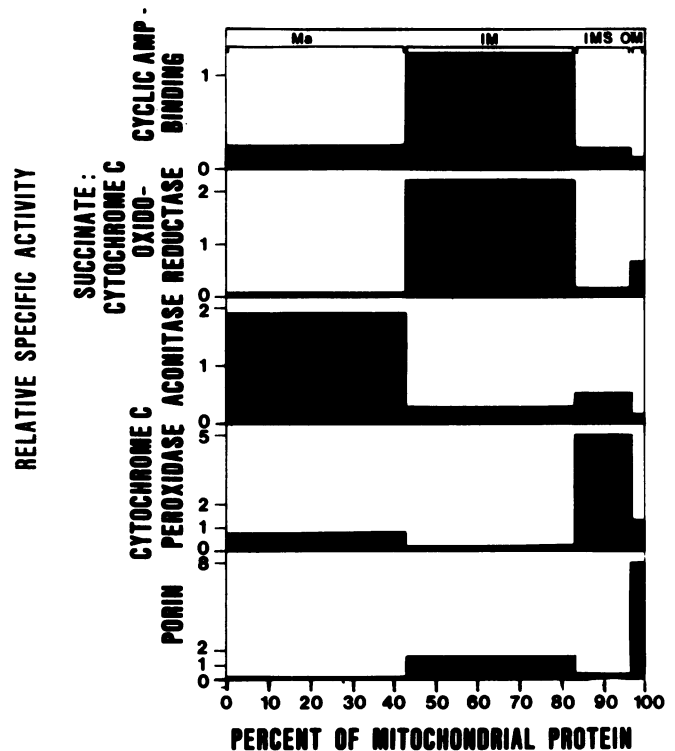

FIG. 2. Mitochondrial subfractionation. Marker enzymes and cAMP-binding activities were assayed in mitochondrial subfractions. The specific activities in intact mitochondria (which were set at 1) were as follows: cAMP binding, 1.30 pmol mg^{-1}; succinate:cytochrome c oxidoreductase, 170.1 nmol mg^{-1} min^{-1}; aconitase, 6,900 U; cytochrome c peroxidase, 56.7 nmol mg^{-1} min^{-1}; porin, 0.80 relative units mg^{-1}. Recoveries were in the range of 65.4 to 78.9%, except for cAMP binding (47.2%; see the text for explanation). Abbreviations: Ma, matrix; IM, inner membrane; IMS, intermembrane space; OM, outer membrane.

cGMP and especially cAMP and the synthetic analog N-6-monobutyryl cAMP diminished the [^{3}H]cAMP binding to the expected value of the control. Thus the cAMP receptor exhibited a pronounced specificity for cyclic nucleotides, especially for cAMP and N-6-monobutyryl cAMP.

(ii) Influence of pH, ions, and storage on cAMP binding. cAMP-binding activity was slightly stimulated by the addition of MgCl$_2$ (maximally at a concentration of 8 mM) and reduced by the addition of EDTA (data not shown). This might be an indication for a dependence of the cAMP binding on divalent cations. A further characteristic of the binding activity is its extreme lability: it was readily lost upon freezing and thawing, storage at room temperature, extensive dialysis, or mild sonication, even in the presence of 15% glycerol, 1 mM dithiothreitol, and 50 μM phenyl-methylsulfonyl fluoride.

(iii) Binding constant and concentration of the cAMP receptor. Scatchard plot analysis of the cAMP binding was performed with purified mitochondria lysed either hypotonically or with 1% Tween 80 (Fig. 4). In both cases an apparent K_d for the ligand of about 1 nM, and a binding capacity in the range of 1 to 5 pmol of cAMP per mg of protein was observed in many repeated experiments. Due to the instability of the binding activity, the concentration of binding sites must be interpreted as a minimum value. The low K_d is in line with the rapid time course of the binding reaction, which is nearly complete within 3 min (data not shown).

Solubilization by detergent had no influence on the binding constant, but lowered slightly the concentration of binding sites, presumably because of the instability of the receptor (see above). The constant slopes in the Scatchard plots suggest the existence of only one major type of binding site. Interestingly, the concentration of binding sites also varied with the growth conditions, being two to three times higher in derepressed cells as compared with repressed cells (data not shown). The dissociation constant remained unchanged.

(iv) Molecular weight determination and biosynthetic origin of the mitochondrial cAMP receptor. The molecular weight of the cAMP receptor was determined by the photoaffinity

TABLE 1. Ligand specificity of the mitochondrial cAMP receptor[a]

Unlabeled nucleotide added in 10-fold molar excess	[³H]cAMP retained on filter	
	cpm	%
None	854	100
ATP	875	102.5
ADP	835	97.8
2'-AMP	765	89.6
3'-AMP	749	87.7
5'-AMP	831	97.3
cGMP	205	24.0
Monobutyryl cAMP	60	7.0
cAMP	58	6.8

[a] Lysed purified mitochondria (150 μg of protein) were assayed for [³H] cAMP binding by the filter procedure (39) as described in the text in the absence or presence of a 10-fold molar excess of unlabeled nucleotides (1.9 × 10^{-8} M).

labeling method described previously (27) with radioactive 8-N₃-cAMP as a ligand. Unspecific binding was reduced by the addition of 5 mM 5'AMP to the assay. One faint band of a photolabeled polypeptide exhibiting an apparent molecular weight of 45,000 was detected after analysis in sodium dodecyl sulfate-polyacrylamide gels (Fig. 5). This band was observed neither when the assay was performed in the dark (data not shown) nor when a 100-fold molar excess of unlabeled cAMP was added (Fig. 5, lane 3). The labeled smear seen at the top of the running gel (Fig. 5, lane 2), which disappeared in the presence of an excess of unlabeled cAMP (lane 3), may result from high-molecular-weight aggregates due to incomplete reduction or solubilization (or both) of the membraneous protein sample before electrophoresis.

No other labeled polypeptide was found, which again indicates that the mitochondrial preparation was essentially free of contamination by cytoplasm and plasma membranes.
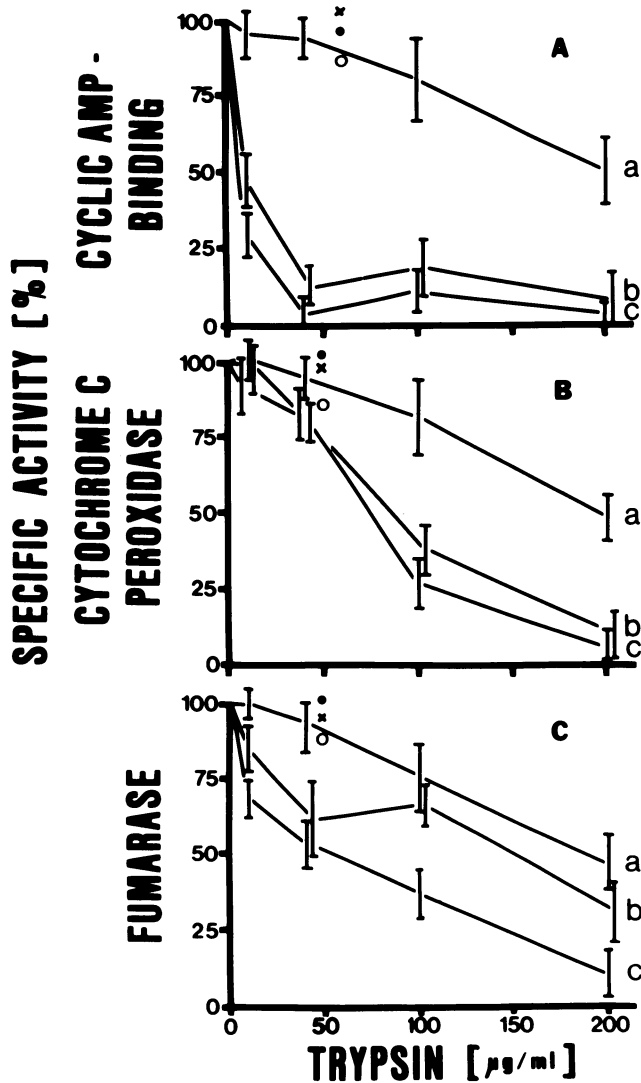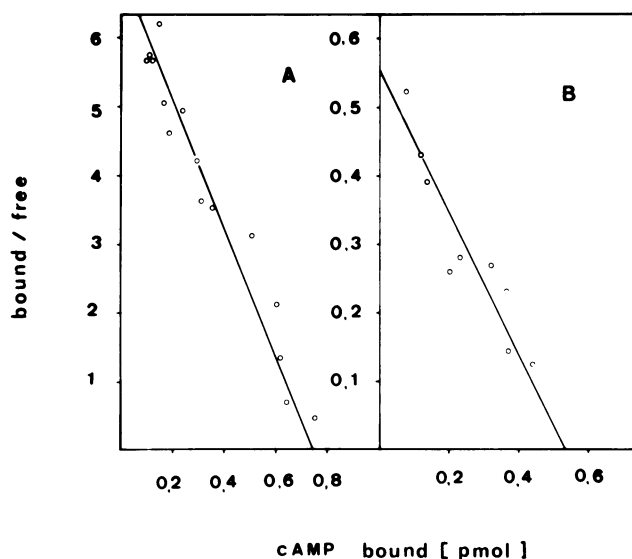

FIG. 3. Topology of the mitochondrial cAMP receptor. Intact (a), shocked (b), and lysed (c) mitochondria were treated with trypsin at 0°C for 30 min, and cAMP-binding activity (A), cytochrome c peroxidase activity (B), and fumarase activity (C) were determined. Controls were performed by incubating intact (×), shocked (○), and lysed (●) mitochondria with 50 μg of trypsin per ml in the presence of a fourfold molar excess of inhibitor from soybeans. Intact mitochondria were suspended in 10 mM Tris–1 mM EDTA (pH 7.2) containing 0.6 M mannitol. After hypotonic treatment in 0.1 M mannitol (same buffer), the osmolarity of the buffer for shocked mitochondria was increased by the addition of sucrose (final concentration, 0.45 M). The incubation buffer (same buffer) for lysed mitochondria contained 0.5% Nonidet P-40.

FIG. 4. Scatchard plot analysis of cAMP-binding by isolated mitochondria. Purified mitochondria were lysed hypotonically (A), or with 1% Tween 80 (B) and assayed for cAMP binding as described in the text. The K_d values and the concentrations of sites calculated from the Scatchard plots are 5 × 10^{-8} M and 4.8 pmol mg^{-1} (A) and 2.6 × 10^{-9} M and 1.5 pmol mg^{-1} (B), respectively.

Thus it is likely that the observed cAMP-binding activity in mitochondria is primarily, or even exclusively, mediated by a single protein. This is in line with the constant slopes in the Scatchard plots.

To determine the site of synthesis of the receptor protein, we analyzed coretention of [^{3}H]cAMP and of mitochondrial proteins labeled with ^{35}S in the presence of either chloramphenicol or cycloheximide. Coretention of ^{3}H and ^{35}S labels is only to be expected if the labeling conditions allow the synthesis of the receptor; e.g., in case of a mitochondrially synthesized receptor, coretention is only expected after labeling in the presence of cycloheximide, but not of chloramphenicol. A concomitant increase of ^{35}S protein label and [^{3}H]cAMP label on cellulose ester filters was only observed when chloramphenicol had been present during the in vivo labeling (Table 2). Thus cAMP enhanced only the retention of cytoplasmically, but not of mitochondrially, synthesized proteins on the filters. We therefore conclude that the cAMP receptor protein is synthesized on cytoplasmic ribosomes.

DISCUSSION

In this paper, we have described the identification of a cAMP receptor protein in yeast mitochondria. We have shown its intra-mitochondrial localization by comparing marker enzyme activities: whereas specific activities of plasma membrane enzyme are reduced in fractions containing inner mitochondrial membranes, those of mitochondrial markers are enriched, as is the one of cAMP binding. The observation that the enrichment and yield of cAMP-binding activity are less than those of other mitochondrial marker enzymes is explained by the extreme lability (i.e., loss of activity) of this protein during the fractionation procedure (e.g., mild sonication).

Unambiguous evidence for the intra-mitochondrial localization was obtained after subfractionation of mitochondria. cAMP binding clearly coincides with marker enzymes of the inner mitochondrial membrane banding at 46% Urographin, as compared with 53% Urographin at the position of plasma membranes. No enzymatic activity characteristic for plasma
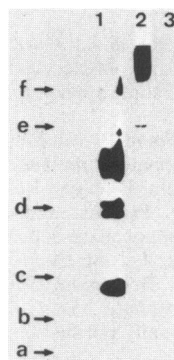
TABLE 2. Biosynthetic orgin of the cAMP-binding protein[a]

Sample	^{35}S labeling	cAMP	[^{3}H]cAMP	cpm bound		% Enhancement by cAMP of ^{35}S-labeled protein retained
				^{35}S	^{3}H	
a	CAP[b]	−	−	144	0	
b	CAP[b]	+	−	188	0	28.5
c	CAP[b]	−	+	181	357	
d	CHX	−	−	143	0	
e	CHX	+	−	151	0	6.3
f	CHX	−	+	153	365	

[a] The procedure is based on the increase of retention of cycloheximide or chloroamphenicol resistant label by cellulose ester filters in the presence of cAMP. Cells were labeled either in the presence of chloramphenicol (CAP) or cycloheximide (CHX) with [^{35}S] sulfate. Mitochondria were lysed with 1% Tween 80, and 50 μg of protein was either directly filtered through cellulose ester filters (samples a, and d; absence of cAMP, background control) or after incubation with unlabeled cAMP (samples b and e) or ^{3}H-labeled cAMP (samples c and f). The numbers given represent mean values of three independent experiments.

[b] Mitochondria labeled in the presence of chloramphenicol were diluted with unlabeled mitochondria to obtain the same specific radioactivity (140 cpm/50 μg of mitochondrial protein) as those labeled in the presence of cycloheximide.

membranes can be detected in the fraction containing inner membranes, again confirming that contamination by plasma membranes can virtually be excluded. In line with this conclusion are the results of Scatchard plot analysis and of photoaffinity labeling with [^{3}H]azido cAMP, both of which reveal the presence of only one cAMP-binding component.

Limited digestion with trypsin of shocked mitochondria (having disrupted outer membranes) is strongly indicative for a localization of the cAMP receptor outside the inner mitochondrial membrane.

Taken together, these results suggest that at least a part of the effects exerted by cAMP on mitochondrial functions may be mediated by the organelle itself. No information about the function of this cAMP receptor is available at the moment. The localization on the outer surface of the inner membrane presumably excludes a mode of action analogous to the cAMP-CAP system in Escherichia coli, i.e., a direct influence on gene expression via regulation of transcription. In line with this conclusion is the observation that cAMP does not significantly stimulate mitochondrial RNA synthesis, either in vivo or in organello or in vitro with a partially purified RNA polymerase (20) (W. Bandlow and R. Schuh, unpublished data). Preliminary results hint at the existence of a cAMP-dependent protein kinase in yeast mitochondria (G. Müller, unpublished), but no data are yet available which prove the identity of the mitochondrial cAMP receptor with the regulatory subunit of a protein kinase.

FIG. 5. Photoaffinity labeling of mitochondrial proteins with 8-N$_3$-[^{3}H]cAMP. A 100-μg sample of purified mitochondrial protein was photolabeled with 8-N$_3$-[^{3}H]cAMP in the presence of 5 mM AMP (lane 2) or 5 mM cAMP (lane 3) and analyzed by sodium dodecyl sulfate-gel electrophoresis as described in the text. Mitochondrial proteins labeled with [^{35}S]SO$_4$ in the presence of cycloheximide were run in a parallel slot (lane 1). Molecular weights were estimated by use of the following protein standards: (a) cytochrome c (13,500), (b) horse myoglobin (17,800), (c) chymotrypsinogen A (24,500), (d) carbonic anhydrase (29,000), (e) ovalbumin (45,000), (f) bovine serum albumin (67,000).

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