

Contents of Nos 1–12	III–XIV	
Original Communications		
Identification of Betulin in Archaeological Tar (In German) F. SAUTER, E. W. H. HAYEK, W. MOCHE, and U. JORDIS	1151	4-(2'-Carboxyphenyl)-4-oxobutyryl Coenzyme A Ester, an Intermediate in Vitamin K ₂ (Mena- quinone) Biosynthesis R. KOLKMANN and E. LEISTNER 1207
Chemical Composition and Morphology of Epi- cuticular Waxes from Leaves of <i>Solanum tube- rosom</i> A. SEN	1153	Role of Pyrophosphate:Fructose-6-phosphate 1-Phosphotransferase in Glycolysis in Cultured <i>Catharanthus roseus</i> Cells H. ASHIHARA and T. HORIKOSI 1215
Asymmetric Reduction of 4(5)-Oxocarboxylic Acids by Baker's Yeast (In German) M. GESSNER, C. GÜNTHER, and A. MOSANDL	1159	The Separation of Two Different Enzymes Catalyz- ing the Formation of Hydroxycinnamic Acid Glucosides and Esters P. A. BÄUMKER, M. JÜTTE, and R. WIERMANN 1223
Partial Lack of N-Acetyl Substitution of Glu- cosamine in the Peptidoglycan of the Budding Phototrophic <i>Rhodomicrobium vannielii</i> U. J. JÜRGENS, B. RIETH, J. WECKESSER, C. S. DOW, and W. A. KÖNIG	1165	Protein Sequence and Structure of N-terminal Amino Acids of Subunit Delta of Spinach Photo- synthetic ATP-Synthase CF ₁ R. J. BERZBORN, W. FINKE, J. OTTO, and H. E. MEYER 1231
Accumulation of Phenolic Compounds and Phyto- alexins in Sliced and Elicitor-Treated Cotyledons of <i>Cicer arietinum</i> L. U. JAQUES, H. KESSMANN, and W. BARZ	1171	Isolation and Characterization of a Supramolecular Complex of Subunit III of the ATP-Synthase from Chloroplasts P. FROMME, E. J. BOEKEMA, and P. GRÄBER 1239
Natural Inhibitors of Germination and Growth IV Compounds from Fruit and Seeds of Mountain Ash (<i>Sorbus aucuparia</i>) U. OSTER, I. BLOS, and W. RÜDIGER	1179	The Polyphasic Rise of Chlorophyll Fluorescence upon Onset of Strong Continuous Illumination: I. Saturation Characteristics and Partial Control by the Photosystem II Acceptor Side CH. NEUBAUER and U. SCHREIBER 1246
The ν versus $\nu/[I]$ Plot A. C. BORSTLAP	1185	The Polyphasic Rise of Chlorophyll Fluorescence upon Onset of Strong Continuous Illumination: II. Partial Control by the Photosystem II Donor Side and Possible Ways of Interpretation U. SCHREIBER and CH. NEUBAUER 1255
Development of New Plate Tests for the Detection of Microbial Hydrolysis of Esters and Oxidations of 2-Hydroxycarboxylic Acids (In German) Y. YAMAZAKI and M.-R. KULA	1187	Benzofuroxan as Electron Acceptor at Photosystem I B. LOTINA-HENNSEN, A. GARCIA, M. AGUILAR, and M. ALBORES 1265
Enzymatic Synthesis of 4'- and 3',4'-Hydroxylated Flavanones and Flavones with Flower Extracts of <i>Sinningia cardinalis</i> K. STICH and G. FORKMANN	1193	CARS Investigation of Changes in Chromophore Geometry of C-Phycocyanin from <i>Mastigocladus laminosus</i> Induced by Titration with <i>p</i> -Chloromer- curibenzenesulfonate S. SCHNEIDER, F. BAUMANN, and U. KLÜTER 1269
Purification and Properties of Chalcone Synthase from Cell Suspension Cultures of Soybean R. WELLE and H. GRISEBACH	1200	Inhibition by Sethoxydim of Pigment Accumulation and Fatty Acid Biosynthesis in Chloroplasts of <i>Avena</i> Seedlings H. K. LICHTENTHALER, K. KOBEC, and K. ISHII 1275

Continued overleaf

Bioenergetics Studies of the Cyanobacterium <i>Anabaena variabilis</i> S. SCHERER, H. SADOWSKI, and P. BÖGER	1280	Thermal Lability of Membrane Proteins of Age Separated Erythrocytes as Studied by Electron Spin Resonance Spin Label Technique G. BARTOSZ, G. CHRIST, H. BOSSE, R. STEPHAN, and H. GÄRTNER	1343
Radiochemical Methods for Studying Lipase-Catalyzed Interesterification of Lipids R. SCHUCH and K. D. MUKHERJEE	1285	Lipids in the Gular Gland Secretion of the American Alligator (<i>Alligator mississippiensis</i>) P. J. WELDON, A. SHAFAGATI, and J. W. WHEELER	1345
cAMP-Dependent Protein Kinase Activity in Yeast Mitochondria G. MÜLLER and W. BANDLOW	1291	Identification of the Sex Pheromone of Eggplant Borer <i>Leucinodes orbonalis</i> Guenée (Lepidoptera: Pyralidae) Z. PINGCHOU, K. FANLEI, Y. SHENGDI, Y. YONGQING, J. SHUPING, H. XINHUA, and X. JIANWEI	1347
Twisted Fibrils are a Structural Principle in the Assembly of Interstitial Collagens, Chordae Tendineae Included W. FOLKHARD, D. CHRISTMANN, W. GEERCKEN, E. KNÖRZER, M. H. J. KOCH, E. MOSLER, H. NEMETSCHKE-GANSLER, and T. NEMETSCHKE	1303	(Z)-5-Dodecen-1-ol, Another Inhibitor of Pheromonal Attraction in <i>Coleophora laricella</i> E. PRIESNER	1349
Lysine Decarboxylase from <i>Hafnia alvei</i> : Purification, Molecular Data and Preparation of Polyclonal Antibodies H. BEIER, L. F. FECKER, and J. BERLIN	1307	(Z)-3-Tetradecenyl Acetate as a Sex-Attractant Component in Gelechiinae and Anomologinae (Lepidoptera: Gelechiidae) E. PRIESNER	1352
Recognition of HLA Class II Molecules by Antipeptide Antibodies Elicited by Synthetic Peptides Selected from Regions of HLA-DP Antigens A. CHERSI, R. A. HOUGHTEN, M. C. MORGANTI, and E. MURATTI	1313	Mammalian Pheromone Studies. VI. Compounds from the Preorbital Gland of the Blue Duiker, <i>Cephalophus monticola</i> B. V. BURGER and P. J. PRETORIUS	1355
Active Cyanogenesis – in Zygaenids and Other Lepidoptera K. WITTHOHN and C. M. NAUMANN	1319	Structural Features and Biological Functions in Blue Copper Proteins Y. NISHIDA	1358
Efficacy of Sustained-Release Radioprotective Drugs <i>in vivo</i> J. SHANI, S. BENITA, M. ABDULRAZIK, and A. YERUSHALMI	1323	Inhibition of the Acetyl-CoA Carboxylase of Barley Chloroplasts by Cycloxydim and Sethoxydim M. FOCKE and H. K. LICHTENTHALER	1361
MHC-Antigens: Constituents of the Envelopes of Human and Simian Immunodeficiency Viruses H. GELDERBLOM, H. REUPKE, T. WINKEL, R. KUNZE, and G. PAULI	1328	Formation of Large Thioredoxin <i>f</i> Accompanies Chloroplast Development in <i>Scenedesmus obliquus</i> P. LANGLOTZ and H. FOLLMANN	1364
Evidences for Circadian Rhythmicity in the <i>per</i> ⁰ Mutant of <i>Drosophila melanogaster</i> CH. HELFRICH and W. ENGELMANN	1335	Cellular Spin Resonance of Yeast in a Frequency Range up to 140 MHz R. HÖLZEL and I. LAMPRECHT	1367
Notes		Melting Pressure, Volume and Stability of Blood at High Pressure (In German) A. KLUGE and H. LENTZ	1370
Conformational Changes in Proteins Induced by Low Temperatures: an Infrared Study H. L. CASAL, U. KÖHLER, H. H. MANTSCH, F. M. GOÑI, and J. L. R. ARRONDO	1339	Subjekt Index	1373
		Authors Index	1403

cAMP-Dependent Protein Kinase Activity in Yeast Mitochondria

Günter Müller* and Wolfhard Bandlow

Institut für Genetik und Mikrobiologie der Universität München, Maria-Ward-Straße 1a,
D-8000 München 19, Bundesrepublik Deutschland

Z. Naturforsch. **42c**, 1291–1302 (1987); received May 29/August 3, 1987

cAMP-Dependent Protein Kinase, Protein Phosphatase, Yeast, Mitochondria, Sub-Mitochondrial Location

Two different cAMP-binding proteins have been identified in yeast mitochondria by photoaffinity labelling and based on the occurrence of cAMP-binding activity in two different sub-mitochondrial fractions. One protein (M_r 45–46000) is tightly bound to the inner mitochondrial membrane whereas the other (M_r 42000) is found in the soluble intermembrane space. With endogenous substrate cAMP-dependent protein kinase activity could not be demonstrated with sufficient clarity. However, using acidic heterologous substrates, like casein and phosphotyrosine, one cAMP-dependent protein kinase was identified in the intermembrane space. Only low phosphate incorporation was found using histone fractions as substrate. cAMP-dependent modification of proteins appears to be very shortlived in mitochondria. Its physiological significance remains unknown, since neither mitochondrial transcription, translation, respiration nor import of cytoplasmically synthesized precursors into mitochondria appear to be influenced by exogenous cAMP either *in vivo* or *in vitro*. It is shown that cAMP is not actively transported into the inner mitochondrial compartment but rather binds to a receptor(s) localized outside the permeability barrier provided by the inner membrane.

Introduction

Based on the existence of Mg^{2+} -dependent, guanine nucleotide sensitive adenylate cyclase [1], phosphodiesterase [2], cAMP-binding protein [3–5] and cAMP-dependent protein kinase activity [6] in yeast, it has been postulated that in this ascomycete, like in mammalian tissues, cAMP exerts its physiological effects through the activation of a cAMP-dependent kinase. In turn, this enzyme controls the activity of several proteins by phosphorylation/dephosphorylation. Apart from the well documented involvement in the control of storage carbohydrate metabolism [7, 8] and of the cell division cycle [9–11], a possible role of cAMP was also discussed in relation to carbon catabolite repression [12–17]. Namely it was reported that cAMP, added to glucose-grown yeast cells, effects a partial release from carbon catabolite repression of several mitochondrial functions, *e.g.* of porphobilinogen synthetase activity [18], of the synthesis of mitochondrially encoded

subunits of cytochrome oxidase [19] and of mitochondrial respiration [20]. But the validity of this approach is uncertain, since it has been observed that catabolite repression in yeast is not associated with low levels of cAMP [14, 15, 21]. Consequently a role in the adaptation of mitochondrial function to the carbon source supply in the growth medium could not be assigned to cAMP. On the other hand, recent findings unequivocally prove that cAMP plays *some* part in the biogenesis and/or function of mitochondria. Ras proteins – the yeast analogs to the p21 products of mammalian ras protooncogene products [22] – were shown to control adenylate cyclase activity in yeast *in vivo* [23, 24] and after cloning in *E. coli* [25]. Destroying RAS2 gene function in yeast by disruption of the respective structural gene (one of the two RAS loci found in yeast), leads to an extremely low level of cAMP. Among other pleiotropic effects (concerning utilization of storage carbohydrates, mitosis and sporulation) these mutants exhibit a respiratory deficient phenotype [23, 26]. In addition, it was found that yeast RAS2^{val19} mutants, altered at a position analogous to the transforming viral or cellular ras^{val12} oncogene product of mammalian cells, synthesize increased levels of cAMP and show exactly the opposite phenotype as ras2 disruptions [24, 27]. These cells have giant mitochondria and exhibit enhanced mitochondrial respiration (J. Mattoon, unpublished results). These latter findings suggest that

* Present address: Institut für physiologische Chemie, physikalische Biochemie und Zellbiologie der Universität München, Goethestraße 33, D-8000 München 2.

Reprint requests to Dr. Wolfhard Bandlow, Institut für Genetik und Mikrobiologie, Maria-Ward-Straße 1a, D-8000 München 19.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/1100–1291 \$ 01.30/0

cAMP plays an important role in the biogenesis of mitochondria – although possibly not in the mediation of their response to glucose repression. But as yet it is completely unknown whether this control is exerted solely by the soluble cytoplasmic cAMP-dependent protein kinase or if mitochondria themselves possess a certain autonomy in this respect. An indication of the latter possibility was recently provided by the documentation of a cAMP-binding protein inside yeast mitochondria [28, 29]. Here we present some evidence that cAMP binds to two different mitochondrial cAMP-binding proteins. One (M_r 45–46000) is tightly bound to the inner membrane and the other (M_r 42000) is a soluble protein of the intermembrane space. We report that both endogenous and exogenous (acidic) substrates are phosphorylated in a cAMP-dependent manner, indicating that at least one cAMP-dependent protein kinase resides in the soluble intermembrane space as well. A physiological role could not yet be assigned to this kinase activity as mitochondrial respiration, transcription, translation or import into mitochondria of precursors for mitochondrial proteins synthesized on cytoplasmic ribosomes are not significantly influenced by cAMP either *in vivo* or in isolated mitochondria.

Materials and Methods

Growth of strains and preparation of mitochondria and mitochondrial subfractions

Cells of strains D273-10B (ATCC 25657) or DH1 op₁ were grown on lactate (2%), glycerol (2%), glucose (6%) or galactose (1.8%) as indicated, harvested and converted to spheroplasts using Zymolyase 100000 (Miles, Frankfurt) and lysed osmotically [30]. Alternatively, cells were broken by careful shaking with glass beads by hand [31]. Mitochondria, isolated by differential centrifugation ($3000 \times g$, 10 min, to $9750 \times g$, 15 min), were further purified by centrifugation on isokinetic Urographin gradients (Schering AG, Berlin) prepared by two rounds of freezing and thawing of 40% Urographin in 20 mM NaP_i, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.0. The fraction containing intact mitochondria (banding at 42% Urographin) was sedimented after twofold dilution ($25000 \times g$, 20 min). Subfractionation into intermembrane space, matrix and inner and outer mem-

branes was achieved in the same way as described [28].

Enzyme assays, photoaffinity labelling and protein determination

The assays of specific subcellular and sub-mitochondrial markers followed published procedures [28]. cAMP-binding was alternatively measured by a filter binding assay [32] or by equilibrium dialysis [28].

Protein kinase activity was measured as follows: 100 µg of dialyzed protein of each fraction was incubated in 100 µl (final volume) of buffer (20 mM Tris, 5 mM MgCl₂, 10 mM dithiothreitol, 50 µM EDTA, 50 µM phenylmethylsulfonyl fluoride, pH 6.8) at 30 °C either in the presence or absence of 2 µM cAMP. Protein kinase activity with exogenous substrates was assayed with 50 µg mitochondrial protein plus 50 µg of partly dephosphorylated casein, phosphovitin, or of bovine serum albumin or histone (Type V and Type VIII, Sigma). In partial reconstitution experiments the incubation mixture contained 50 µg protein, each of the intermembrane space and of inner membranes. The phosphorylation reaction was started by the addition of 5 µCi γ-[³²P]ATP in a volume of 5 µl (3.35 Ci/mmol, final concentration 15.2 µM) and terminated after 30 min or at times indicated by the addition of 10 µl of 50% trichloroacetic acid, 5% sodium dodecylsulfate, 100 mM ATP.

10 µl of each assay were spotted on filters in triplicate, washed for 16 h with several changes of 5% trichloroacetic acid (containing, in addition, 2 mM sodium pyrophosphate, 2 mM adenine sulfate), which removed acid-labile phosphate adducts, and two times with ethanol. Dry filters were counted in 2 ml toluene-based scintillation cocktail.

Cellular respiration was measured [33] in a gas-tight thermostated vessel (2.5 ml) equipped with a Clarke-type polarographic electrode at 25 °C, O₂ saturation 0.253 µmol/ml.

Photoaffinity labelling experiments were performed essentially as described [34]. 100 µg of mitochondrial protein were incubated in a final volume of 100 µl of a buffer containing 50 mM MOPS, 50 mM NaCl, 10 mM MgCl₂, 0.1 mM 5'-AMP, 50 µM EDTA, 1 mM isobutylmethyl xanthin, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.2, and 0.5 µCi 8-N₃[³²P]cAMP (50 Ci/

mmol) in the absence or presence of 100 μM unlabeled cAMP as a competitor (for demonstration of specificity of the binding) in the dark for 15 min at 0 °C. Then the sample was exposed to UV light illumination (Hanau NN 15/44 V) at a distance of approximately 5 cm for 4 min at 0 °C. The reaction was terminated by adding 50 μl 3 \times SDS sample buffer, followed by heating to 95 °C for 2 min, before samples were subjected to SDS polyacrylamide gel electrophoresis.

Protein was determined both by the methods of Bradford [35] and Heil and Zillig [36].

In vivo mitochondrial transcription and translation

For measuring mitochondrial macromolecular syntheses *in vivo*, cells were grown over night in synthetic medium in the presence of 0.1 $\mu\text{Ci/ml}$ of either [^{14}C]isoleucine or [^{14}C]adenine as a prelabel. After the application of a chase each culture (titer 2–4 $\times 10^7$ cells/ml) was divided into two portions for pulse labelling. One of these received 2 mM N⁶-monobutryl cAMP. A single-labeled sample was kept for the determination of the spill-over. 4 ml aliquots were pulsed for 15 min after 1 h of incubation with the cAMP derivative.

For labelling mitochondrially made proteins the cells received 100 $\mu\text{g/ml}$ cycloheximide and, 90 sec later, 50 μCi [^3H]leucine. To compensate for possible cAMP-induced changes in the size of the cellular leucine pool, the label was diluted to a specific radioactivity of 80 mCi/mmol (160 μM , which was determined to correspond to about 90% pool saturation for leucine in yeast (W. Bandlow, unpublished data). The incorporation was terminated after 15 min by a 10 min chase. Labeled cells were broken by vortexing with glass beads in an Eppendorf cup, debris were removed by low speed centrifugation and mitochondria sedimented and purified by centrifugation through a 0.4 ml cushion of 40% Urographin for 20 min in an Eppendorf centrifuge. The supernatant was removed by suction, the pellet dissolved in buffer containing 2% sodium dodecylsulfate and the suspension spotted on glass fiber filters, previously soaked with 5% trichloroacetic acid, 5 mm in each, leucine and isoleucine. The filters were washed three times with the same solution, once with ethanol and once with ethanol/ether (1:1). Dry filters were counted as above. ^3H -counts incorporated during the pulse were normalized to 1000 ^{14}C -counts of the prelabel.

For measuring mitochondrial transcription 4 ml of a similar cell culture, pre-labeled with [^{14}C]adenine, received 50 μCi [^3H]uracil, diluted to a specific radioactivity of 140 mCi/mmol (90 μM), for the same reasons mentioned before for leucine (W. Bandlow, unpublished results). The incorporation was chased, cells broken and mitochondria prepared as described above. The pellet containing purified mitochondria was resuspended in 0.15 ml 10 mM Tris, 1 mM EDTA, pH 7.0, and lysed at 60 °C by the addition of 0.2% sodiumdodecylsulfate (final concentration). An equal volume of phenol (pH 7.0) was immediately added for deproteinization, followed by 0.15 ml of chloroform. The extraction was repeated two times. Nucleic acids were then precipitated from the aqueous phase by 2.5 vol ethanol and dissolved in 10 mM Tris, 1 mM EDTA, pH 7.0. Unlabelled mitochondrial DNA was prepared separately, purified by NaJ gradient centrifugation, denatured, and 8 μg of the DNA bound to nitrocellulose paper discs. After drying and baking at 80 °C under reduced pressure for 2 h the discs were prehybridized in 0.1 ml of 0.9 M NaCl, 0.09 M sodium citrate (6 \times SSC), 1 \times Denhardt's solution, 0.1% sodium dodecylsulfate, 50% deionized formamide and 100 $\mu\text{g/ml}$ of sonified, deproteinized and denatured herring sperm DNA at 35 °C for 4–6 h and then hybridized with the labeled RNA over night under the same conditions. After washing (two times 2 \times SSC, 60 °C, vortexing for 1 min, once in 0.1 \times SSC at room temperature, once with ethanol) dry filters were counted and evaluated as described above. A blank filter was used for background determination.

Transcription and translation in isolated mitochondria

For measuring macromolecular syntheses in isolated mitochondria, spheroplasts were prepared as described above and granted a recovery phase of 60 min in complete medium stabilized with 1 M sorbitol [30]. Spheroplasts were lysed by osmotic shock and mitochondria purified by differential centrifugation.

Measurement of mitochondrial protein synthesis *in organello* employed an endogenous ATP-regenerating system, similar to the one described by Grivell [37], which additionally contained 150 μM GTP. Labelling was performed with 50 μCi [^3H]leucine in a final volume of 0.1 ml with 160 μg mitochondrial protein in the presence or absence of

25 μM cAMP. It was terminated after 20 min by a chase of 10 min. (The reaction is insensitive to cycloheximide, sensitive to uncoupler, oligomycin, and chloroamphenicol and is linear for at least 30 min [33].) Mitochondria were spotted on glass fiber filters, washed and counted.

The same mitochondria as above were used for measuring mitochondrial transcription. The incubation conditions described previously [29] were used and transcripts labeled by the addition of 1 μCi [^3H]UTP in the presence or absence of 25 μM cAMP. Both, run-off transcription in intact mitochondria and poly(dA·dT)-directed RNA synthesis in mitochondrial lysates were assayed. In the first case the medium contained 0.6 M mannitol, in the latter 1% Nonidet P40 and 0.1 μg poly(dA·dT). The incorporation was terminated after 10 min (for which the reaction is linear with time) by the addition of a chase for 10 min. Mitochondrial suspensions were spotted onto glass fiber filters, washed and counted as above, except that the 5% trichloroacetic acid contained 2 mM sodium pyrophosphate and 2 mM uridine. The reaction is insensitive to 100 $\mu\text{g}/\text{ml}$ α -amanitin, but completely inhibited by 25 $\mu\text{g}/\text{ml}$ actinomycin D or 15 $\mu\text{g}/\text{ml}$ acriflavin.

A crude preparation of mitochondrial RNA polymerase was obtained from mitochondria lysed in 1% Nonidet P40 after successive chromatography on Sephacryl S300 and heparin Sepharose as described [29, 38]. Transcription was measured in the presence or absence of 25 μM cAMP with 0.1 μg of poly(dA·dT) as a template as described above for mitochondrial lysates.

Import of precursor protein into mitochondria in vivo and in vitro

For measuring import of precursor protein into mitochondria *in vivo* and *in vitro*, cells were grown in either 12% glucose or 2% lactate as the carbon source. At a titer of 3×10^7 cells/ml cells were converted to spheroplasts. After a recovery phase of 1 h in the same medium containing 1 M sorbitol [30], spheroplasts were re-collected by centrifugation, washed and resuspended in synthetic medium containing 1 M sorbitol. Import was then impaired by destroying the membrane potential by 200 μM carbamyl cyanide *m*-chlorophenyl hydrazine, thus allowing the accumulation of mitochondrial precursor proteins in the cytoplasm [39]. After a preincubation

period of 10 min the total products of cytoplasmic protein synthesis were labeled after the addition of 2 μCi [^{35}S]methionine. The labelling was terminated after 10 min by a chase together with the addition of 50 mM β -mercaptoethanol to restore energy coupling and initiate import into mitochondria [40]. The culture was immediately divided into two portions, one of which received 5 mM cAMP. Spheroplasts were harvested after 30 min, washed, broken by osmotic shock, mitochondria prepared and freed from labeled material adhering to the outer surface by incubation with 20 $\mu\text{g}/\text{ml}$ trypsin at 0 $^\circ\text{C}$ [40]. This digestion was terminated after 15 min by the addition of 50 $\mu\text{g}/\text{ml}$ of soybean trypsin inhibitor and 2 mM tosyl lysyl chloromethyl ketone. 100 μg of twice washed and re-sedimented mitochondria were either dissolved in 0.5 ml Soluene (Packard Instruments, Frankfurt) and counted in 2 ml scintillation fluid, or immunoprecipitated with a mixture of sera (directed against cytochrome *c*, cytochrome c_1 , subunits α and β of F_1 -ATPase, cytochrome *c* peroxidase, the cytoplasmically made subunits IV–VIII of cytochrome oxidase and citrate synthase), washed and counted.

For assaying *in vitro* import of precursor protein into mitochondria, 5 μg of total yeast poly A⁺ RNA [41] was translated in 100 μl of nuclease-treated rabbit reticulocyte lysate [42], supplemented with 100 μCi [^{35}S]methionine. After 60 min synthesis at 37 $^\circ\text{C}$, 25 μl of the post-ribosomal supernatant ($100000 \times g$, 10 min, Beckman Airfuge) were freed from unincorporated label by a passage over a Sephadex G50 column. 3×10^7 cpm equivalent of freshly prepared mitochondria were incubated with 50 μg of precursors were incubated with 50 μg of freshly prepared mitochondria for 60 min at 25 $^\circ\text{C}$ in the presence of 0.6 M sorbitol with or without 20 μM cAMP. After re-isolation mitochondria were treated with trypsin (120 $\mu\text{g}/\text{ml}$, 0 $^\circ\text{C}$, 30 min). 1.2 mg/ml of soybean trypsin inhibitor and 2 mM tosyl lysyl chloromethyl ketone terminated the digestion. Then the re-isolated and washed (three times) mitochondria were either spotted on filters, washed and directly counted or immunoprecipitated with a mixture of antisera as above, washed and counted.

Exchange of cAMP by isolated mitochondria

Forward exchange rates by isolated mitochondria of labeled cAMP and other nucleotides was determined by the silicon oil layer centrifugal filtration technique essentially as described by Klingenberg and Pfaff [43]. Mitochondria were prepared from

spheroplasts [30] of strain D273-10B grown on 2% lactate or DH1 op_1 grown on 1.8% galactose. Mitochondria were washed twice with 0.6 M mannitol, 25 mM Tris-maleate, pH 6.5, 1 mM EDTA, 0.1% bovine serum albumin and resuspended at 2.0 mg protein per ml in storage buffer containing 0.35 M mannitol, 25 mM Tris-maleate, pH 6.5, 0.2 mM EGTA, 1 mM $MgCl_2$, 0.1% bovine serum albumin. In a siliconized 3.5 ml pyrex tube (DuPont-Sorvall) the following components were successively layered on top of each other: 0.35 ml $CsCl$ ($\rho = 1.10$ g/ml), 0.6 ml silicon oil $\rho = 1.078$ g/ml (AR200 Wacker Chemie, Burghausen), 0.1–2.1 ml incubation layer, containing in storage buffer additionally 18 mg/ml dextrane T40 and 0.1 $\mu Ci/ml$ [^{14}C]-labeled compound (either ATP, ADP, cAMP, sucrose or polyethylene glycol 60000, 500 μM final concentration, each), 0.2 ml separation layer, containing in storage buffer additionally 12 mg/ml dextrane T40, 0.2 ml mitochondrial suspension (0.4 mg protein) in storage buffer and 2.0–0 ml of a balancing layer containing 0.1 M mannitol, 25 mM Tris-maleate, 0.2 mM EGTA, pH 6.5. Atractylate (100 μM), bongkrecic acid (20 μM) or carbonyl cyanide *m*-chlorophenyl hydrazone (15 μM) were used in the control experiments. The tubes were then placed in nitrocellulose tubes filled with 1 M sucrose solution for stabilization and centrifuged in a SW27 rotor until a velocity of 16000 rpm was reached. The $CsCl$ layer was removed and counted. Different incubation periods were achieved by the variation of the volume of the incubation layer from 0.1–2.1 ml. Sedimentation velocities were calculated according to [44] and corrections for adhering medium were applied by subtracting the cpm-values measured after incubation with polyethylene glycol [45], which does not penetrate mitochondrial membranes. Uptake of sucrose measured the freely permeable intermembrane space.

Back exchange of adenine nucleotides was measured with mitochondria pre-loaded with the respective [3H]-labeled carrier-free compound at 4 °C as described [46]. After sedimentation and two brief washings in an Eppendorf microcentrifuge (2 min, 4 °C), mitochondria (0.4 mg protein) were incubated in 1 ml storage buffer as above, containing 15 mM of unlabeled nucleotide, for 6 min at room temperature. Then mitochondria were sedimented and supernatant and mitochondrial pellet collected separately for counting.

Inside-out mitochondrial particles were obtained by sonication of mitochondria (about 10 mg/ml protein) in 250 mM sucrose, 10 mM Tris acetate, 1 mM EGTA, 1 mM $MgCl_2$, pH 7.2, 10 pulses of 5 sec each cooled by an ice-salt mixture. After removal of a low speed pellet (10 min, 12000 $\times g$) sonic particles were sedimented by centrifugation for 90 min at 145000 $\times g$ and resuspended in storage buffer at 2 mg protein/ml.

Results

cAMP-binding to mitochondrial proteins

Mitochondria, prepared from yeast cells either after enzymatic digestion of the cell walls and osmotic shock of the spheroplasts [30], or after carefully shaking with glass beads by hand to a fraction of 60–70% disrupted cells [31], were further purified by centrifugation on isokinetic urographin gradients [28]. Whole mitochondria with their outer and inner membranes intact were isolated, washed and subjected to mitochondrial subfractionation using porin, cytochrome b_2 , cytochrome c_1 and aconitase as specific markers for outer membranes, intermembrane space, inner membranes and soluble matrix, respectively [28].

cAMP-binding in the subfractions was measured as shown in Tables I and II. Significant cAMP-binding is associated with whole mitochondria, the specific binding exceeding that of the total cellular homogenate. The bulk of the binding activity sediments with mitochondrial membrane particles after hypotonic disruption of mitochondria, and only a minor, but significant portion is consistently found in the soluble supernatant in the absence of detergents. Zwittergent 14 (or Mega 10 or CHAPS, not shown) solubilize more than 95% of the cAMP-binding activity from the particulate fraction (Table I).

After fractionation of whole mitochondria into the four submitochondrial compartments in the complete absence of detergents, most of the cAMP-binding resides in the inner membrane (Table II). But significant ligand binding of high specific activity is coincidentally observed, with the two binding assays applied, in the soluble intermembrane space fraction. The fact that the sum of binding activities recovered in inner membranes and intermembrane space is much less than originally present in whole mitochondria is caused primarily by the low stability of the binding site even under optimal conditions.

Table I. cAMP-binding to yeast cellular subfractions. Yeast cells were broken either by osmotic lysis of spheroplasts ^a or by careful shaking with glass beads by hand ^b and homogenate, 100000 × g cytoplasmic supernatant and purified mitochondria prepared as detailed in Materials and Methods. Whole mitochondria were homogenized by 20 strokes in a teflon in glass homogenizer in a buffer containing 20 mM NaH₂PO₄, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and separated at 100000 × g into soluble supernatant and membranes, respectively, for 10 min at 4 °C in a Beckman Airfuge. Mitochondrial membranes were solubilized by 5 mM Zwittergent 14 in the same buffer as above containing in addition 10 mM MgCl₂ and 15% glycerol and centrifuged as above. cAMP-binding was measured by the filter binding assay or by equilibrium dialysis [28, 32]. Each value represents the average from 3 (filter assay) or 5 (equilibrium dialysis) determinations. Experimental deviations are in the range of < 10%.

	cAMP-binding [pmol/mg protein]	
	Filter assay	Equilibrium dialysis
Cell homogenate	1.1 ^a	0.93 ^a
Cytoplasmic supernatant (100000 × g)	1.5 ^a	1.26 ^a
Whole purified mitochondria	1.6 ^a 2.0 ^b	1.32 ^a 1.25 ^b
Mitochondrial soluble protein	–	1.05 ^a
Mitochondrial membranes	–	1.35 ^b
Mitochondrial membranes solubilized with Zwittergent 14	–	1.40 ^a
Non-solubilizable residue	–	1.30 ^b
	–	1.22 ^a
	–	0 ^a

Table II. cAMP-binding in yeast submitochondrial fractions. Mitochondria were prepared from spheroplasts and purified by isokinetic Urographin gradient centrifugation. (The experiment is different from that shown in Table I.) Mitochondria (54 mg) were subfractionated [28] into outer membranes (1.2 mg), intermembrane space (3.0 mg), inner membranes (13.4 mg), and matrix (14.6 mg). Protein yield 59%; yield in cAMP-binding activity 33%. cAMP-binding was measured by equilibrium dialysis using 100 µg mitochondrial protein for each assay. Each value represents the mean from 3 binding assays.

	cAMP-binding	
	Total activity [pmol]	Specific activity [pmol/mg protein]
Whole purified mitochondria	87.5	1.62
Outer membranes	0.1	0.06
Intermembrane space	3.1	1.04
Inner membranes	22.8	1.70
Soluble matrix	3.0	0.21

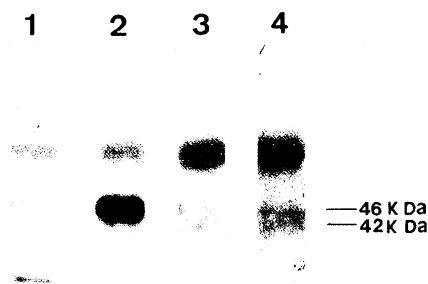


Fig. 1. Photoaffinity labelling of mitochondrial proteins with 8-N₃[³²P]cAMP. Mitochondria were subfractionated into membranes (lanes 1 and 2) and soluble fraction (lanes 3 and 4) by homogenization in hypotonic medium and centrifugation at 105000 × g (20 min). 50 µg of the membranes and 20 µg of the soluble fraction were incubated with 0.5 µCi 8-N₃[³²P]cAMP in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 10 µM unlabeled cAMP, fractionated by polyacrylamide gel electrophoresis and autoradiographed. The markers used were bovine serum albumin (67 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), chymotrypsinogen (24.5 kDa), myoglobin (17.8 kDa) and cytochrome *c* (12.5 kDa).

Fig. 1 shows that by photoaffinity labelling with N⁸-azido [³²P]cAMP two different binding proteins can be detected in yeast mitochondria, both of which are distinct from the one present in the cytoplasm. The larger cAMP-binding protein (*M_r* 45–46 kDa) is found associated with the inner mitochondrial membrane (lane 2) and is apparently identical with that previously observed in mitochondria [28]. The smaller (*M_r* 42000) is localized in the soluble intermembrane space (lane 4). Since the other bands observed after photoaffinity-labelling cannot be competed for by an excess of unlabeled cAMP, these data provide solid evidence that only two different cAMP-binding proteins occur in yeast mitochondria.

cAMP-dependent protein kinase activity in mitochondria

To test whether any of these two cAMP-binding activities is associated with a protein kinase and might represent its regulatory subunit, we assayed both the intermembrane space and the inner membranes for cAMP-dependent protein kinase activity. In the separate fractions (as well as in the matrix and

in outer membranes, not shown) no such activity was found in the absence of exogenous substrate. However, when the two neighbouring fractions were incubated together, cAMP-dependent phosphorylation was observed – beside cAMP-independent phosphate incorporation (Table III). Providing exogenous substrates, an effect by cAMP was evident also in individual subfractions, particularly with the intermembrane space. With inner membranes a stimulation by cAMP is not evident. This different capability to phosphorylate exogenous substrates by inner membranes and intermembrane space could indicate that i) the two cAMP receptors present commonly regulate one single kinase, localized in the intermembrane space, ii) that two different kinases are present, one of which is membrane-associated and, being specific, does not accept exogenous substrates, iii) that a very active protein phosphatase masks protein phosphorylation in the inner membrane, or, iv) that the cAMP-receptor of the inner membrane has a function different from a regulatory subunit of a protein kinase. Table III reveals also that acidic substrates are preferentially phosphorylated over histone fractions and bovine serum albumin, phosphovitin being an even better substrate than casein.

These properties and substrate preferences clearly distinguish the mitochondrial cAMP-dependent protein kinase from its cytoplasmic counterpart which preferentially phosphorylates histone fractions [4].

Fig. 2 shows that the time course of phosphate incorporation is linear with time under the conditions applied for at least 40 min with all fractions used, also in the presence of exogenous substrates (casein is given as an example).

Effects of cAMP on mitochondrial macromolecular syntheses and protein import

We were now interested in the role cAMP and the cAMP-dependent mitochondrial protein kinase might play in cell life. For example, it had been observed that mitochondrial RNA polymerase is a repressible activity [47] and that exogenous cAMP stimulates mitochondrial gene expression [19] and respiration [20]. As a first approach to answering the question whether mitochondria themselves can use cAMP as a signal and transmit it to the respiratory machinery in one way or another, we screened the major processes involved in the biogenesis of mitochondria, namely transcription, translation and import of cytoplasmically synthesized precursors of mitochondrial proteins for an effect of cAMP.

The results of these experiments are compiled in Table IV. It can be seen that cAMP has little influence on mitochondrial gene expression, or, rather, that it acts slightly inhibitory. This is particularly evident from an experiment using an enriched fraction of mitochondrial RNA polymerase (prepared as in [29]). These results suggest that cAMP does not stimulate the bulk of either mitochondrial transcription or translation.

Next we examined whether the stimulation of oxygen consumption observed in yeast spheroplasts after the administration of cAMP [20] could be caused by a cAMP-dependent activation of an import channel or processing pathway for mitochondrial proteins made in the cytoplasm. Such a control could satisfactorily explain cAMP-dependent effects in mitochondria. On the other hand phosphorylation of precursors

Table III. Protein kinase activity and stimulation by cAMP in mitochondria and mitochondrial subfractions with endogenous and exogenous substrates *in vitro*. Mitochondria were subfractionated as described under Table II and incorporation of ^{32}P from $\gamma\text{-}^{32}\text{P}\text{ATP}$ into acid insoluble material assayed as detailed in Materials and Methods. Protein kinase activity is given as pmol ^{32}P incorporated per mg mitochondrial protein per h. cAMP-dependent protein kinase activity is approximated by the difference between the kinase activities measured in the presence and in the absence of cAMP.

Exogenous substrate	Whole mitochondria			Inner membranes			Intermem. space			Inner membranes + interm. space		
	- cAMP	+ cAMP	cAMP dep.	- cAMP	+ cAMP	cAMP dep.	- cAMP	+ cAMP	cAMP dep.	- cAMP	+ cAMP	cAMP dep.
None	87	91	4	89	94	5	3.4	3.5	0.1	74	97	23
Phosvitin	129	148	19	138	142	4	8.5	71	63	120	262	142
Casein	108	126	18	119	106	0	6.0	56	50	103	228	125
Bovine s. albumin	91	96	5	90	91	1	0.3	13.6	13	0.4	31.5	31
Histone V	86	90	4	94	95	1	3.0	16.3	13	5.5	48.0	42
Histone VIII	86	89	3	95	91	0	5.5	5.5	0	0.5	31.5	31

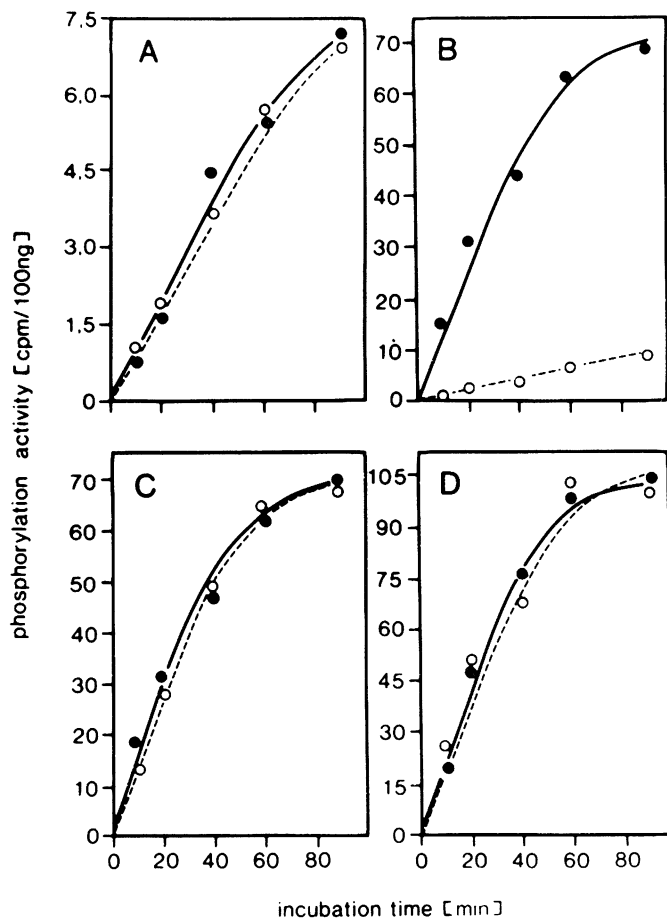


Fig. 2. Kinetics of protein kinase activity. Mitochondrial subfractions were incubated with γ [32 P]ATP in the absence (O---O) and presence (●—●) of cAMP and with endogenous (A, C) or exogenous (B, D) substrate (see Materials and Methods). A, intermembrane space proteins; B, intermembrane space plus casein; C, inner membranes; D, inner membranes plus casein.

Table IV. Influence by cAMP on mitochondrial macromolecular syntheses and on the import of precursor proteins into the organelle *in vivo* and *in vitro*. All data given represent counts per minute. ^a, Mitochondrial transcription was measured by hybridization of labeled mitochondrial RNA to filter-bound mitochondrial DNA. ^b, Protein import reflects carbamyl cyanide m-chlorophenyl hydrazone-sensitive, trypsin resistant incorporation of labeled protein into mitochondria. ^c, Immunoprecipitation was performed with a mixture of sera directed against subunits α and β of F_1 -ATPase, subunits IV-VIII of cytochrome *c* oxidase, cytochromes *c* and *c*₁, cytochrome *c* peroxidase and citrate synthetase. ^d, Values give cpm ³H-pulse label per 1000 cpm ¹⁴C-prelabel. ^e, Run-off transcription in intact mitochondria with endogenous template. ^f, Transcription in lysed mitochondria with poly[dA·dT] as template. ^g, Transcription with enriched RNA polymerase and poly[dA·dT] as template [29, 38]. ^h, Import of precursors synthesized by a reticulocyte lysate programmed with yeast poly A⁺ RNA; total *in vitro* synthesized yeast polypeptides equal 3.0×10^7 cpm in each experiment.

	Mitochondrial transcription ^a		Mitochondrial translation		Mitochondrial total import ^b		Immune precipitate ^{b,c}	
	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP	-cAMP	+cAMP
Derepressed cells	650 ^d	580 ^d	3500 ^d	4000 ^d	95200	87800	1800	2700
Repressed cells	360 ^d	350 ^d	2300 ^d	2500 ^d	32200	34500	800	1500
Derepressed mitochondria	7400 ^a 19100 ^f 3400 ^e	7000 ^a 19000 ^f 2100 ^e	7000	6100	14100 ^b	8400	1100 ^b	700 ^b
Repressed mitochondria	4400 ^a 10300 ^f	4900 ^a 10800 ^f	4600	2200	5600 ^b	5000 ^b	400 ^b	400 ^b

sors to be transported has been observed both with mitochondria (α -subunit of F_1 -ATPase, [48]) and chloroplasts (ribulose bisphosphate carboxylase, small subunit, [9]).

Table IV shows the results of two experiments. One was performed *in vivo*, the other with isolated mitochondria. Energy-dependence of this process [39] and resistance to protease of imported proteins [40] were taken as criteria for mitochondrial precursor import. Both total import into mitochondria and the import of a few individual proteins after quantitative immunoprecipitation by a mixture of sera was measured. In the *in vivo* experiment the import had been impaired by carbamyl cyanide m-chlorophenyl hydrazone during the radioactive pulse thus allowing the accumulation of mitochondrial precursors in the cytoplasm [39]. After a chase, subsequent restoration of energy coupling by addition of SH-reagents initiated the import [39]. The influence of cAMP on this import was tested.

For measuring import into isolated mitochondria a reticulocyte lysate was programmed with yeast poly A⁺ RNA and the proteins made were labeled with [³⁵S]methionine. Then purified mitochondria were added and the import of labeled precursors was as-

sayed in the presence or absence of cAMP. In both cases no pronounced influence of cAMP on total precursor import could be detected (Table IV).

Apart from total incorporation, a few proteins were assayed individually by quantitative immune precipitation (see legend to Table IV). Again no obvious influence by cAMP could be detected. Thus a control by cAMP of a general import pathway is very unlikely. But it cannot principally be excluded that for the import of yet one or a limited number of proteins (*e.g.* RNA polymerase) cAMP could play a role.

Table V reveals that in spheroplasts from glucose grown yeast cells cAMP has, in fact, a small (less than twofold) stimulatory effect on mitochondrial respiration, whereas the effector consistently acts slightly inhibitory in derepressed cells. But as non-cyclic nucleotides have similar effects – confirming earlier results of others [20] – it is concluded that the effect of cAMP on respiration is non-specific.

Does cAMP cross both mitochondrial membranes or only the outer membrane?

The results shown in Tables IV and V suggest that cAMP does not exert any effect inside the inner mitochondrial compartment. On the other hand it has been reported [50] that cAMP is taken up by isolated mitochondria. We attempted to verify these results and to test whether cAMP traverses the inner mitochondrial membrane.

Fig. 3A shows that, in fact, cAMP co-sediments with intact mitochondria in the silicon oil layer centrifugation in a similar fashion as ADP and ATP. The amount taken up considerably exceeds that measured for polyethylene glycol and sucrose known not to penetrate the outer or the inner membranes, respectively. But in strict contrast to ADP, cAMP is also taken up by mitochondria in the presence of bongkrecic acid (or carboxyatractylate (not shown), which block adenine nucleotide translocation, or of the uncoupler carbonyl cyanide m-chlorophenyl hydrazone), as well as by mitochondria from strain DH1 op₁ (Fig. 3B) blocked by mutation in the ADP/ATP translocation [51]. Under the latter conditions, uptake of ATP is reduced to the level of sucrose, which is known not to traverse the inner mitochondrial membrane, whereas that of cAMP is not significantly diminished. Inside-out particles, prepared by sonic oscillation and freed from most of the matrix,

Table V. Effects of various nucleotides on the respiration of repressed (YPD, 6% glucose) and derepressed (YPL, 2% lactate) cells of strain D273-10B after 4 h of incubation with 2 mM effector at pH 4.4. Respiratory rates are expressed as nmol O₂/min × 10⁸ cells. DB = dibutylryl, MB = monobutryl.

Growth conditions	Additions	Respiratory rates
YPD (repressed)	none	62
	N ⁶ -DBcAMP	60
	O ² -MBcAMP	72
	N ⁶ -MBcAMP	120
	3':5'-cAMP	125
	3':5'-cGMP	90
	N ⁶ -MBcAMP + 5'-AMP	90
	3':5'-cAMP + 5'-AMP	112
	ATP	84
	ADP	94
	GTP	71
	5'-AMP	98
	3'-AMP	78
2'-AMP	79	
YPL (derepressed)	none	445
	N ⁶ -MBcAMP	415
	3':5'-cAMP	395

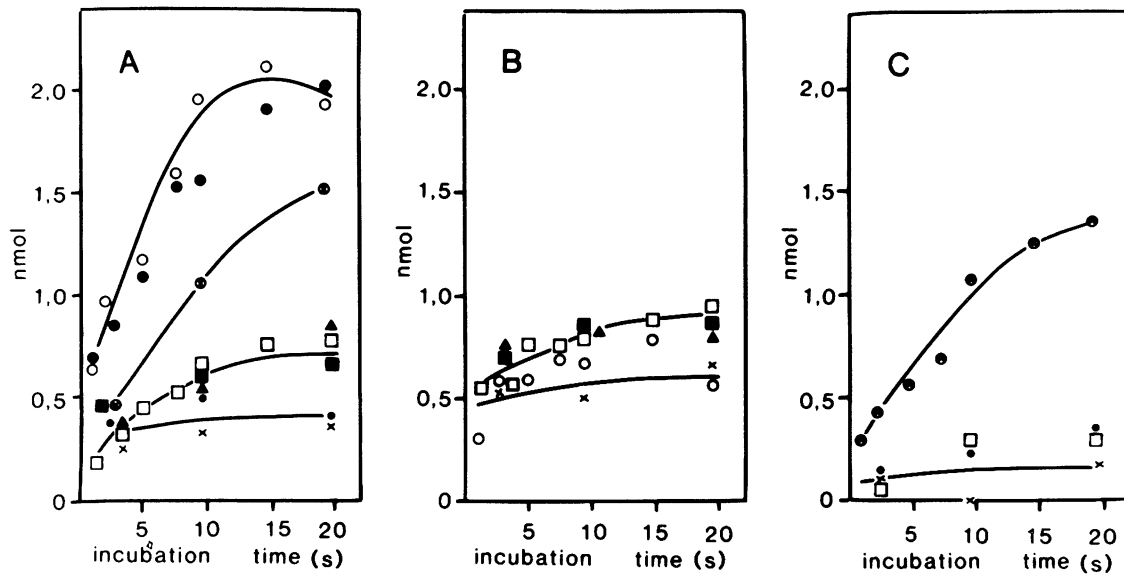


Fig. 3. Forward exchange by isolated mitochondria of cAMP in comparison to ATP and ADP. The uptake of ^{14}C -labeled compounds ($500\ \mu\text{M}$) was measured by the silicon oil layer filtration technique [43] as detailed in Materials and Methods. All values were corrected for labeled solute adhering to mitochondria during the passage through the oil layer by subtraction of the value obtained after incubation with polyethylene glycol. Mitochondria are impermeable to this polymer [45]. Uptake of labeled sucrose above this background measured the intermembrane space, which is freely permeable to low molecular weight substances. Controls were performed in the presence of $20\ \mu\text{M}$ bongkreecic acid (BA) or $15\ \mu\text{M}$ carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP). A, whole mitochondria from wild type yeast; B, mitochondria from mutant strain DH1 *op*₁, defective in adenine nucleotide translocation [51]; C, inside-out particles. \square , cAMP; \blacksquare , cAMP + BA; \blacktriangle , cAMP + CCCP; \circ , ADP; \otimes , ATP; \bullet , ADP + BA; \bullet , ADP + CCCP; \times , sucrose.

well accumulate ATP but retain only insignificant amounts of cAMP. The fraction of the latter correlates well with the portion of right side-out particles present in the preparation (Fig. 3C).

Table VI shows an experiment in which isolated wild type mitochondria were pre-loaded with either labeled cAMP, ATP or sucrose and then incubated in the presence or absence of excess unlabeled solute in order to measure the velocity of the back exchange. In the case of mitochondria pre-loaded with cAMP and with ATP in the presence of atractylate significant label is retained with mitochondria

after centrifugation and only a minor fraction found in the supernatant. The label is, however, rapidly released into the supernatant (within 6 min) in all other cases.

Taken together these experiments show that cAMP-uptake by mitochondria is independent on the adenine nucleotide translocator and on a membrane potential (Fig. 3A + B, Table VI). Since in inside-out particles (Fig. 3C) uptake of cAMP is diminished to a level close to that of sucrose (and polyethylene glycol) it is concluded that cAMP does not traverse the inner mitochondrial membrane to a significant

Table VI. Release of labeled solutes into the supernatant from preloaded mitochondria in the presence of excess unlabeled solute ("back exchange"). All data are given as cpm. Atr = atractylate. Atractylate was present also during the washing of preloaded mitochondria.

	^3H ATP	^3H ATP + Atr	^3H cAMP	^3H sucrose
Radioactivity in mitochondria	17700	22800	5800	110
Radioactivity retained	2130	21700	5380	10
Radioactivity released	16900	2900	590	115
% of compound exchanged	88.9	11.7	9.8	92

extent. Uptake of cAMP by whole mitochondria is, however, significantly higher than that of sucrose suggesting that it binds to some receptor(s) outside the permeability barrier provided by the inner mitochondrial membrane. This is consistent with earlier topological studies of cAMP-binding in mitochondria [28].

Discussion

The recent demonstration of cAMP-binding to (a) mitochondrial receptor protein(s) [28] leaves the question unanswered whether this protein participates in the glucose repression/derepression mechanism of mitochondria and whether it controls mitochondrial gene expression in a manner analogous to the positive transcriptional regulation found in *E. coli* and other bacteria. Alternatively, it could constitute the regulatory subunit of a cAMP-dependent protein kinase, so that mitochondria follow eukaryotic principles of regulation. Here we demonstrate for the first time that yeast mitochondria harbour at least one cAMP-dependent protein kinase, which hints at the latter possibility. Moreover, we provide some evidence that two different cAMP-binding proteins are present in intact mitochondria. They are clearly distinct from one another and from the cytoplasmic regulatory subunit in terms of molecular mass and substrate preference. One of the two cAMP-binding proteins (M_r 45–46000) is bound to the outer surface of the inner mitochondrial membrane and has been described earlier ([28], Müller, Bandlow, *Yeast* 3, in the press). The other (M_r 42000), too, is localized outside the permeability barrier provided by the inner mitochondrial membrane and is found in the soluble intermembrane space. For the cytoplasmic regulatory subunit a molecular weight of 50000 has been reported [4]. The mitochondrial kinase catalyzes phosphate incorporation into acidic proteins whereas the cytoplasmic counterpart preferentially phosphorylates histones.

But, nevertheless, it cannot be decided at present, whether these three cAMP-binding proteins represent differently processed forms of the same precursor protein and are encoded by one single structural gene or by different genes. Although unspecific degradation cannot completely be ruled out it appears extremely unlikely because of the presence of protease inhibitors in all buffers. It also is not yet known, whether the two mitochondrial cAMP-binding species regulate the same catalytic subunit, which appears to be localized in the soluble intermembrane space, or whether two different cAMP-dependent protein kinases are present in mitochondria. *In vivo* and *in vitro* cAMP-dependent protein kinase activity can hardly be detected with endogenous substrates. It appears very likely that cAMP-dependent phosphorylation is only transient and rapidly removed by a vicinal phosphatase.

cAMP appears to exert all of its effects outside the inner mitochondrial compartment. This finding is consistent with earlier topological studies which have shown ([28] and Müller and Bandlow, *Yeast* 3, in the press) that the cAMP receptor proteins are localized outside the permeability barrier provided by the inner membrane. Here we have demonstrated that cAMP does not traverse the inner mitochondrial membrane. It was also shown that the ligand does not influence mitochondrial transcription or translation, either *in vivo* or *in vitro* which contrasts to earlier reports [19, 20]. Also at least the bulk portion of the import of cytoplasmically synthesized precursors into mitochondria is, apparently, not influenced by cAMP, either *in vivo* or *in vitro*. Thus the physiological role of the mitochondrial cAMP-binding proteins remains to be established.

Acknowledgements

We thank A. Haid and G. Schatz, The Biocenter Basel, for the gracious donation of antisera, and G. Strobel and I. Haberl for their help with the preparation of the figures and the typing of the manuscript.

- [1] G. F. Caspersen, N. Walker, A. R. Brasier, and H. R. Bourne, *J. Biol. Chem.* **258**, 7911–7914 (1983).
- [2] K. Suoranta and J. Londesborough, *J. Biol. Chem.* **259**, 6964–6971 (1984).
- [3] C. Dery, S. Cooper, M. A. Savageau, and S. Scanlon, *Biochem. Biophys. Res. Commun.* **90**, 933–939 (1979).
- [4] C. S. Hixson and E. G. Krebs, *J. Biol. Chem.* **255**, 2137–2145 (1980).
- [5] P. K. Jaynes, J. P. McDonough, and H. R. Mahler, *Biochem. Biophys. Res. Commun.* **94**, 16–22 (1980).
- [6] Y. Takai, H. Yamamura, and Y. Nishizuka, *J. Biol. Chem.* **249**, 530–535 (1974).
- [7] P. B. Chock, S. G. Rhee, and E. R. Stadtman, *Annu. Rev. Biochem.* **49**, 813–843 (1980).
- [8] J. B. van der Plaats and P. van Solingen, *Biochem. Biophys. Res. Commun.* **56**, 580–587 (1979).
- [9] A. L. Boynton and J. F. Whitefield, *Adv. Cyclic Nucleotide Res.* **15**, 193–294 (1983).
- [10] D. L. Friedman, R. A. Johnson, and C. E. Zeilig, *Adv. Cyclic Nucleotide Res.* **7**, 69–114 (1976).
- [11] I. H. Pastan, G. S. Johnson, and V. B. Anderson, *Annu. Rev. Biochem.* **44**, 491–522 (1975).
- [12] J. P. Jost and H. V. Rickenberg, *Annu. Rev. Biochem.* **40**, 741–773 (1971).
- [13] G. M. Tomkins, *Science* **189**, 760–763 (1979).
- [14] P. Eraso and J. M. Gancedo, *Europ. J. Biochem.* **141**, 195–198 (1984).
- [15] K. Matsumoto, I. Uno, T. Ishikawa, and Y. Oshima, *J. Bacteriol.* **156**, 898–900 (1983).
- [16] G. Schlanderer and H. Dellweg, *Europ. J. Biochem.* **49**, 305–316 (1974).
- [17] R. van Wijk and T. M. Konijn, *FEBS Lett.* **13**, 184–186 (1971).
- [18] H. R. Mahler and C. C. Lin, *Biochem. Biophys. Res. Commun.* **61**, 963–970 (1978).
- [19] K. Chandrasekaran and J. Jayaraman, *FEBS Lett.* **87**, 52–54 (1978).
- [20] M. Fang and R. A. Butow, *Biochem. Biophys. Res. Commun.* **41**, 1579–1583 (1970).
- [21] K. Matsumoto, I. Uno, A. Toh-e, T. Ishikawa, and Y. Oshima, *J. Bacteriol.* **150**, 277–285 (1982).
- [22] S. Powers, T. Kataoka, O. Fasano, M. Goldfarb, J. Broach, and M. Wigler, *Cell* **36**, 607–612 (1984).
- [23] K. Tatchell, L. C. Robinson, and M. Breitenbach, *Proc. Natl. Acad. Sci. USA* **82**, 3785–3789 (1985).
- [24] T. Toda, I. Uno, T. Ishikawa, S. Powers, T. Kataoka, D. Brock, S. Cameron, J. Broach, K. Matsumoto, and M. Wigler, *Cell* **40**, 27–36 (1985).
- [25] G. L. Temeles, J. B. Gibbs, J. S. D'Alonzo, I. S. Sigal, and E. M. Scolnick, *Nature (London)* **313**, 700–703 (1985).
- [26] D. Fraenkel, *Proc. Natl. Acad. Sci. USA* **82**, 4740–4744 (1985).
- [27] T. Kataoka, S. Powers, C. McGill, O. Fasano, J. Strathern, J. Broach, and M. Wigler, *Cell* **37**, 437–445 (1984).
- [28] G. Rödel, G. Müller, and W. Bandlow, *J. Bacteriol.* **161**, 7–12 (1985).
- [29] W. Bandlow, U. Schwarz, G. Rödel, G. Strobel, and C. Wachter, *Biol. Chem. Hoppe-Seyler* **366**, 545–553 (1985).
- [30] H. T. Hutchison and L. H. Hartwell, *J. Bacteriol.* **94**, 1697–1705 (1976).
- [31] B. Lang, G. Burger, I. Doxiadis, D. Y. Thomas, W. Bandlow, and F. Kaudewitz, *Analyt. Biochem.* **77**, 110–121 (1977).
- [32] G. M. Walton and L. D. Garren, *Biochemistry* **9**, 4223–4229 (1970).
- [33] W. Bandlow, R. Metzke, A. Klein, K. Kotzias, I. Doxiadis, H. Bechmann, R. J. Schweyen, and F. Kaudewitz, *Europ. J. Biochem.* **76**, 373–382 (1977).
- [34] U. Walter and P. Greengard, *Meth. Enzymol.* **99**, 154–162 (1983).
- [35] M. M. Bradford, *Analyt. Biochem.* **72**, 248–254 (1976).
- [36] A. Heil and W. Zillig, *FEBS Lett.* **11**, 165–168 (1970).
- [37] L. A. Grivell, *Biochem. J.* **105**, 44C–46C (1967).
- [38] D. Levens, A. Lustig, and M. Rabinowitz, *J. Biol. Chem.* **256**, 1474–1481 (1981).
- [39] G. A. Reid and G. Schatz, *J. Biol. Chem.* **257**, 13056–13060 (1982).
- [40] G. Schatz, S. M. Gasser, and G. Daum, *J. Biol. Chem.* **257**, 13034–13041 (1982).
- [41] H. Aviv and P. Leder, *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412 (1972).
- [42] H. R. B. Pelham and R. J. Jackson, *Europ. J. Biochem.* **67**, 247–256 (1976).
- [43] M. Klingenberg and E. Pfaff, *Meth. Enzymol.* **10**, 680–684 (1967).
- [44] M. Klingenberg, E. Pfaff, and A. Kröger, in: *Rapid Mixing and Sampling Techniques in Biochemistry* (B. Chance, R. H. Eisenhardt, Q. H. Gibson, and K. K. Lonberg-Holm, eds.), pp. 333–337, Academic Press, New York 1964.
- [45] E. Pfaff, M. Klingenberg, E. Ritt, and W. Vogell, *Europ. J. Biochem.* **5**, 222–232 (1968).
- [46] E. Pfaff and M. Klingenberg, *Europ. J. Biochem.* **6**, 66–79 (1968).
- [47] D. J. South and H. R. Mahler, *Nature* **218**, 1226–1232 (1968).
- [48] R. A. Steinberg, *J. Cell Biol.* **98**, 2174–2178 (1984).
- [49] J. Soll and B. B. Buchanan, *J. Biol. Chem.* **258**, 6686–6689 (1983).
- [50] W. Bandlow, *Hoppe-Seyler's Z. physiol. Chem.* **360**, 228–229 (1979).
- [51] J. Kotarov, J. Šubík, and L. Kováč, *Biochim. Biophys. Acta* **267**, 465–478 (1972).