YEAST VOL. 3: 161–174 (1987)

Protein Phosphorylation in Yeast Mitochondria: cAMP-Dependence, Submitochondrial Localization and Substrates of Mitochondrial protein Kinases

GÜNTER MÜLLER* AND WOLFHARD BANDLOW†

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

Received 13 February 1987; revised 2 April 1987

We describe the identification and submitochondrial localization of four protein kinases and of their target proteins in derepressed yeast mitochondria. The activity of one of the kinases depends on the presence of cyclic AMP (cAMP). It is soluble and localized in the mitochondrial intermembrane space. Its natural target is a polypeptide of 40 kDa molecular mass, which is bound to the inner membrane. Besides this natural target this kinase phosphorylates acidic heterologous proteins, like casein, with high efficiency. The other protein kinases identified so far are cAMP-independent. At least one is localized in the matrix having its natural substrates (49 and 24 kDa) in the same compartment. Two others are firmly bound to the inner membrane phosphorylating target proteins in the inner membrane (52·5 kDa) and in the intermembrane space (17·5 kDa), respectively.

KEY WORDS — *Saccharomyces cerevisiae*; mitochondria; cAMP-dependent protein kinase; submitochondrial localization; topology.

INTRODUCTION

Phosphorylation of proteins has been found to control various cellular processes in yeast. Protein kinases—both soluble and membrane-bound—have been amply documented in several cellular compartments including mitochondria (Hixson and Krebs, 1980; Kitagawa and Racker, 1982; Linn *et al.*, 1969; Mahler *et al.*, 1981; Pall, 1981; Rigobello *et al.*, 1980; for review see Krebs and Beavo, 1979). In yeast only one of them was found to be activated by cAMP. This enzyme is localized in the cytosol (Hixson and Krebs, 1980) and effects the modulation of various cellular processes by phosphorylation/dephosphorylation of key enzymes of a variety of metabolic pathways such as storage carbohydrate metabolism (Chock *et al.*, 1980; van der Plaat and van Solingen, 1979). cAMP-dependent phosphorylation of ribosomal proteins (Lastick and McConkey, 1981) has been observed, suggesting a role of the cAMP-dependent kinase also in the regulation of translation. More recently it was found that cAMP also has a key role in the cell cycle control in yeast (De Vendittis *et al.*, 1986; Matsumoto *et al.*, 1983, 1985).

Phosphorylation of proteins has repeatedly been observed after labeling whole cells (Steinberg and Coffino, 1979; Wang and Sy, 1985). For mitochondrial phosphoproteins no such *in vivo* studies exist. In a few cases mitochondrial protein kinases have been enriched due to their ability to phosphorylate exogenous substrates like casein (Kitagawa and Racker, 1982; Rigobello *et al.*, 1980). They were found to be membrane-bound. A correlation of the kinases and their natural targets and an attribution to one of the four submitochondrial compartments has, however, been achieved solely in the case of bovine pyruvate dehydrogenase (PDH) kinase (Linn *et al.*, 1969; Barrera *et al.*, 1972), which was shown to be a matrix enzyme, loosely associated with the inner membrane. Apart from the case of PDH kinase, an *in vivo* function has not yet been established for any of the other mitochondrial protein kinases. None of them was found to be activated by cAMP. To this point it is also completely

*Present address: Institut für physiologische Chemie, physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, D-8000 München 2, Federal Republic of Germany.

†Address for correspondence.

0749–503X/87/030161–14 $07.00

unclear whether mitochondria play any role of their own in the regulation processes underlying carbon catabolite repression/derepression of respiratory functions, or whether they exclusively respond, in a passive way, to signals transmitted to them from the nucleo-cytoplasmic system. The discussion is still controversial whether cAMP has any effect on mitochondria and whether it could act as one of the signals exchanged between the two compartments of the cell (Chandrasekaran and Jayaraman, 1978; Eraso and Gancedo, 1984; Fang and Butow, 1970; Kim and Kikuchi, 1972; Mahler and Lin, 1978; Matsumoto *et al.*, 1982).

In a first attempt to elucidate a possible role of cAMP in the control of mitochondria, we recently studied whether cAMP could be recognized by yeast mitochondria at all. This work succeeded in the identification of a mitochondrial cAMP receptor protein which has a molecular mass of 45 kDa and is tightly bound to the inner membrane (Rödel *et al.*, 1985). Since the only mode of action for cAMP-binding proteins found in eukaryotes so far consists in the activation of protein kinases, we have now undertaken a systematic examination of mitochondrial protein kinases. We report here the identification of one cAMP-dependent protein kinase in yeast mitochondria. We have determined its submitochondrial localization as well as its natural target protein, a single polypeptide of the inner mitochondrial membrane (molecular mass 40 kDa). In addition, three cAMP-independent protein kinases, their topology and their natural substrates are described.

MATERIALS AND METHODS

Strains and material

Cells of the strain D273-10B (ATCC 25657) were used throughout this work. Urographin was obtained from Schering A.G., Berlin. Radioactive isotopes were purchased from New England Corp., Dreieich, at the following specific activities: [^{32}P]P_i, carrier-free, 80 Ci/mg; [γ-^{32}P]ATP, 3·35 Ci/mmol. Soluene and Instagel were from Packard Instruments, Frankfurt, Nonidet P40 from Serva, Heidelberg, phenyl methyl sulfonyl fluoride, tosyl lysyl chloromethyl ketone and proteinase K from Merck, Darmstadt, casein (4765) from Sigma, Munich, and Zymolyase 100000 from Miles, Frankfurt. All other substances were obtained from Boehringer, Mannheim.

Cell growth and preparation of mitochondria

Cells were grown on lactate (2%) (Daum *et al.*, 1982), glycerol (2%) or glucose (12%) as indicated, harvested and converted to spheroplasts using Zymolyase 100000. After osmotic lysis, mitochondria, isolated by differential centrifugation (3000 × *g*, 10 min, to 9750 × *g*, 15 min), were further purified by centrifugation on 20–60% linear Urographin gradients as described previously (Rödel *et al.*, 1985). The fraction containing intact mitochondria (banding at 42% Urographin; Rödel *et al.*, 1985) was sedimented after two-fold dilution (10 000 × *g*, 1 h) and used for subfractionation.

Subfractionation of mitochondria

Mitochondria were resuspended in 0·1 M-sorbitol, 20 mM-KP_i, 1 mM-EDTA, 50 μM-phenyl methyl sulfonyl fluoride, pH 6·8, at 1 mg protein/ml and stirred gently for 15 min at 0°C, in order to disrupt outer membranes (Rödel *et al.*, 1985; Daum *et al.*, 1982).

Intermembrane space proteins were recovered from the supernatant after centrifugation (20 000 × *g*, 20 min) and concentrated by ethanol (80%) precipitation, if required. Mitoplasts with outer membranes still attached (shocked mitochondria) were resuspended in 20 mM-KP_i, 1 mM-EDTA, 50 μM-phenyl methyl sulfonyl fluoride, for swelling (0°C), and shrunk by the addition of 2 mM-ATP, 2 mM – $MgCl_2$ and 0·6 M-sucrose, pH 6·8 (final concentration), and matrix proteins extruded by a combination of gentle ultrasonic oscillation (3 × 5 s, 0°C, microtip) and teflon-in-glass-homogenization. After 1:1 dilution with 20 mM-KP_i, 1 mM-EDTA, 50 μM-phenyl methyl sulfonyl fluoride (pH 6·8), soluble matrix proteins were recovered from the supernatant after centrifugation (105 000 × *g*, 90 min, Beckman 70·1 Ti rotor). Inner and outer membranes were then separated after careful homogenization of the 105 000 × *g* pellet by centrifugation on a 18 ml linear 20–50% Urographin gradient (24 000 rpm, 15 h, Beckman SW 27 rotor). Fractions containing inner and outer membranes, respectively, were diluted with KP_i buffer as above and sedimented by centrifugation (105 000 × *g*, 2 h, Beckman 70·1 Ti rotor). Aliquots of the subfractions were stored at −80°C.

Topology of the cAMP-dependent mitochondrial protein kinase

Topology was determined by assaying the resistance of the protein kinase to protease treatment.

Protein (2 mg) of each intact mitochondria, mitochondria having disrupted outer but intact inner membranes (shocked mitochondria, prepared as above), and mitochondria lysed by ten strokes in a teflon-in-glass homogenizer in the presence of 0·5% Nonidet P40 was incubated with 40 μg of trypsin in a total volume of 1 ml and aliquots were withdrawn at the times indicated in the figures. The reaction was terminated by adding 40 μg/ml soy bean trypsin inhibitor and 1 mM-tosyl lysyl chloromethyl ketone. The fractions were assayed for activities of cAMP-dependent protein kinase with casein (50 μg) as substrate and for markers of known topology (Rödel *et al.*, 1985). Controls were performed by incubating mitochondrial fractions for 30 min with 40 μg/ml trypsin which had been inactivated by 50 mg/ml soy bean trypsin inhibitor prior to the incubation. Fractions were stored at −80°C until measurement.

Assay of protein kinases in vivo *and* in vitro

For labeling yeast cells with [^{32}P]P_i, a 50 ml culture was grown in phosphate-free medium, pH 4·4, containing either 2% glycerol (A), or 12% glucose (B, C), to a titer of 4×10^7 before 1·8 mCi carrier-free [^{32}P]P_i was added. Culture C received 5 mM-N^6-monobutyryl cAMP 20 min prior to the label. After 30 min of incubation cells were harvested, mitochondria prepared, purified, lysed and incubated with a mixture containing 5 mM-$MgCl_2$, 50 μM-EDTA, 50 μM-phenyl methyl sulfonyl fluoride and 50 μg of each pancreatic DNase, RNase and lipase in order to reduce high molecular weight components containing radioactive label other than protein, and finally dissolved in sodium dodecyl sulfate and electrophoresed (Douglas and Butow, 1976), fixed with trichloroacetic acid (5%) and autoradiographed.

For labeling whole mitochondria with [γ-^{32}P] ATP the incubation mixture contained 0·7 M-sorbitol, for lysed mitochondria 0·5% Nonidet P40. In subfractions protein kinase activity was measured as follows: 100 μg of dialyzed (20 mM-Tris, 5 mM-$MgCl_2$, 10 mM-dithiothreitol, 50 μM-EDTA, 50 μM-phenyl methyl sulfonyl fluoride, pH 6·8) protein of each fraction was incubated in 100 μl of the same buffer at 30°C either in the presence or absence of 2 μM-cAMP. Casein phosphorylation was assayed with 50 μg mitochondrial protein plus 50 μg of partly dephosphorylated casein. In partial reconstitution experiments the incubation mixture contained 50 μg protein of each of two neighboring compartments. The phosphorylation reaction was started by the addition of 5 μCi [γ-^{32}P]ATP and terminated at times indicated by the addition of 10 μl of 50% trichloroacetic acid, 5% sodium dodecyl sulfate and 100 mM-ATP. The reaction was linear with time for at least 40 min. In triplicate 10 μl of each assay was spotted on filters, washed for 16 h at 4°C with several changes of 5% trichloroacetic acid (containing in addition 2 mM-sodium pyrophosphate, 2 mM-adenine sulfate) and twice with ethanol. Dry filters were counted in 2 ml toluene-based scintillation cocktail. The residual incubation mixture was precipitated with eight volumes of ethanol at −80°C, centrifuged at 4°C (10 min, Eppendorf centrifuge), the pellet rinsed successively with 70% and absolute ethanol in the cold, solubilized and electrophoresed (Douglas and Butow, 1976).

cAMP binding and miscellaneous assays

cAMP-binding was measured by equilibrium dialysis in a total volume of 0·6 ml per half cell in a Dianorm dialysis apparatus as described previously (Rödel *et al.*, 1985). Alkaline phosphatase, cytochromes c_1 and b_2 and porin were assayed as described in Rödel *et al.* (1985) and aconitase as in Racker (1950). Published procedures were also used for electroblotting of proteins (Towbin *et al.*, 1979), and for quantitative evaluation of immune decorations (Suissa, 1983). Protein was determined both by the methods of Bradford (1976) and Heil and Zillig (1970).

RESULTS

Phosphorylation of mitochondrial proteins in vivo

Yeast cells were labeled with [^{32}P]P_i under different growth conditions and mitochondria, purified by gradient centrifugation, were analyzed by polyacrylamide gel electrophoresis in an attempt to identify mitochondrial target proteins of protein kinases. As it has been reported repeatedly (Mahler *et al.*, 1981; Chandrasekaran and Jayaraman, 1978; Fang and Butow, 1970; Mahler and Lin, 1978) that cAMP releases yeast cells from glucose repression, the intention of this experiment was to find out whether cAMP effects a change in the pattern of mitochondrial phosphoproteins from that of glucose-repressed cells to that of derepressed cells.

Figure 1A shows the labeling patterns found. It reveals mainly quantitative differences between mitochondria isolated from derepressed (lane 1) and repressed cells (lane 2). For example, bands with molecular weights of 57 000, 38 500, 29 000, 22 500,

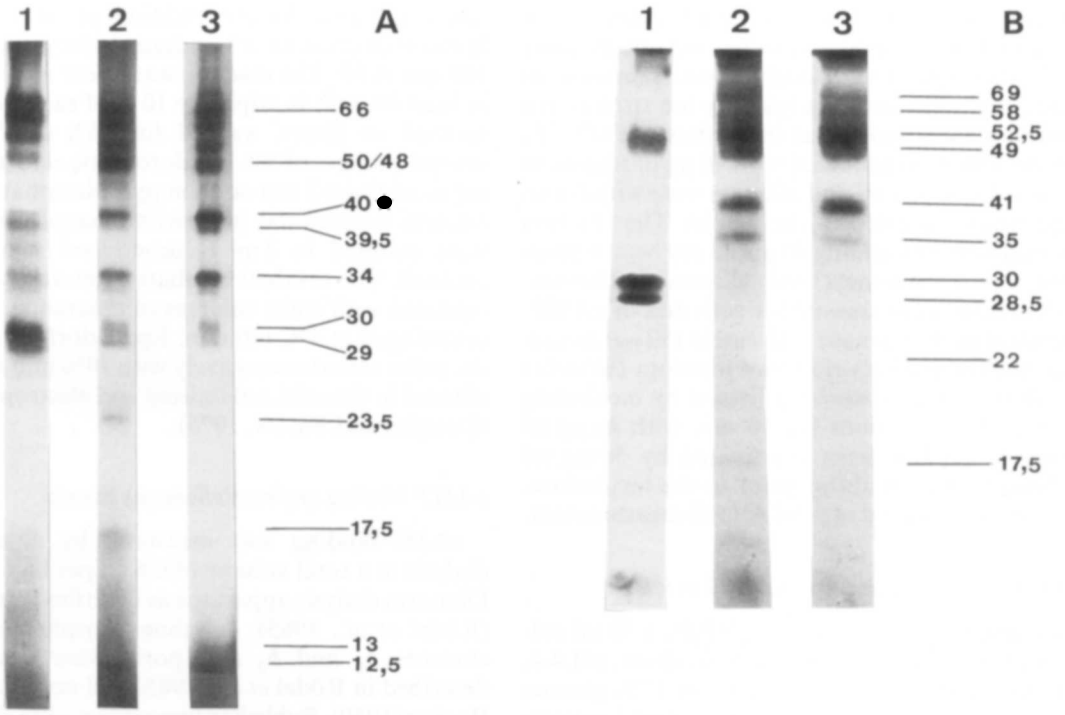

Figure 1. (A) Autoradiogram of mitochondrial phosphoproteins labeled with [^{32}P]P_i *in vivo* (see Materials and Methods). The lanes contain protein from purified mitochondria from glycerol-grown cells (lane 1); from glucose-grown cells (lane 2); from glucose-grown cells labeled in the presence of 2 mM-N^6-monobutyryl cAMP (lane 3). Mitochondrial protein (80 μg) was loaded on each lane of the gel. The molecular weight standards used were bovine serum albumin, 67 000; ovalbumin, 45 000; carbonic anhydrase, 28 000; chymotrypsinogen, 25 000; myoglobin, 17 800; and cytochrome *c*, 12 400. A candidate for the phosphorylated α-subunit of PDH is marked by an asterisk. (B) Autoradiogram of mitochondrial phosphoproteins obtained after labeling purified mitochondria (Rödel *et al.*, 1985) with [γ-^{32}P]ATP. The lanes contain mitochondrial protein from glycerol-grown cells (lane 1); from glucose-grown cells (lane 2). The cells of lane 3 were grown in glucose medium and received 5 mM-cAMP (at pH 4·4) during the last 90 min before harvesting. 2 mM-cAMP was also present during the labeling. The same molecular weight standards were used as in (A).

22 000 and 16 000 become primarily labeled in derepressed and those with molecular weights of 40 000, 34 000, 23 500, 13 000 and 12 500 in repressed mitochondria. Some bands, e.g. those found with molecular weights of 66 000, 58 000, 54 000, 48 000 and 17 500, are labeled equally well under either condition. cAMP has no striking effects on the labeling pattern, except that a band with molecular weight 39 500, which is not prominent under either condition, is labeled a little stronger in its presence (lane 3). Altogether 23 bands (10 major and 13 minor) can be detected, into which phosphate label had been incorporated (summarized in Table 1). It is not known which of these proteins had been phosphorylated by mitochondrial kinases and which might have been imported into mitochondria from the cytosol in a phosphorylated state.

Phosphorylation in isolated mitochondria

In order to be able to correct the number of phosphoproteins obtained after labeling *in vivo* for imported phosphoproteins and to give a minimum number of proteins phosphorylated by intramitochondrial protein kinases, we incubated mitochondria with [γ-^{32}P]ATP (Figure 1B). After labeling isolated and purified mitochondria from derepressed and repressed cells, similar patterns of radiolabeled proteins were obtained as after *in vivo* labeling. Again, only minor differences can be observed between glucose-repressed mitochondria labeled in the presence and absence of cAMP. After labeling *in organello*, a total number of 19 phosphoproteins can be detected including minor bands not visible in the reproduction (summarized in Table 1).

Table 1. Synopsis of mitochondrial phosphoproteins observed after labeling under various conditions.

Labeling condition		Molecular masses of observed mitochondrial phosphoproteins (kDa)																						
In vivo	Repressed	(75)	66	58	—	54	50	48	**40***	—†	(38·5)	—†	34	—†	(30)	(29)	—	(23·5)	—	—	(17·5)	—	(13)	(12·5)
	Repressed + cAMP	(75)	66	58	—	54	50	48	**40**	(39·5)	(38·5)	(36·5)	34	(32·5)	(30)	(29)	—	(23·5)	—	—	(17·5)	—	(13)	(12·5)
	Derepressed	—	66	58	(57)	54	50	48	(40)	(39·5)	38·5	(36·5)	—	(32·5)	30	29	(25)	—	(22·5)	(22)	(17·5)	(16)	—	—
In purified mitochondria	Repressed	82	69	60	—	56	52	49	**41**	—	—	—	**35**	—	—	—	—	(24)	—	—	(18)	—	(14)	13
	Repressed + cAMP	82	69	60	—	56	52	49	**41**	—	—	—	**35**	(33)	—	—	(26)	(24)	—	—	(18)	—	(14)	13
	Derepressed	82	—	—	—	—	52	49	—	—	—	—	—	(33)	**30·5**	**28·5**	—	—	(22)	(21)	(18)	(16·5)	—	13
In subfractions derepressed	Matrix							49											24					
	Inner membranes						52·5			40														
	Intermembr. space																				17·5			

Weak signals are in brackets, strong signals are bold. *Possible candidate for phosphorylated α-subunit of pyruvate dehydrogenase. †Candidates for cAMP-stimulated phosphorylation.

The fewer bands of phosphoproteins found after labeling *in organello* as compared to the *in vivo* situation may be primarily due to the absence of mitochondrial phosphoproteins modified by cytoplasmic protein kinases *in vivo* and subsequently imported into mitochondria (Steinberg, 1984).

Phosphorylation in mitochondrial subfractions

No striking influence by cAMP could be observed after incorporation of label into mitochondrial proteins *in vivo* and *in vitro*, although a cAMP-binding protein has been identified in yeast mitochondria (Rödel *et al.*, 1985) and although it has been shown previously that the effector is, in fact, taken up (Bandlow, 1979). This failure could be due to one of two reasons: (i) either the cAMP receptor identified in mitochondria (Rödel *et al.*, 1985) has a function other than a regulatory subunit of a protein kinase. However, no such other function has as yet been observed in eukaryotes. On the other hand, (ii) the turnover of the phosphorylated target protein of a possible cAMP-dependent protein kinase is so rapid that it remains undetected in the assays applied.

To resolve these discrepancies and to gain some more insight into the nature of the mitochondrial cAMP receptor, we subfractionated intact, highly purified mitochondria into their four component fractions: inner membranes (IM), outer membranes (OM), intermembrane space (IS), and matrix (MA) and assayed for protein kinase activity. The subfractions were analyzed by sodium dodecyl sulfate gel electrophoresis. The success of the subfractionation procedure was controlled by Western blotting followed by immune decoration of markers of known submitochondrial localization (Figure 2). Porin is considered to be a marker of the outer membranes, cytochrome b_2 of the intermembrane space, cytochrome c_1 of the inner membrane and aconitase of the mitochondrial matrix, respectively. Alkaline phosphatase is thought to indicate plasma membrane contaminations (Rödel *et al.*, 1985). Figures 2 and 3 demonstrate that the mitochondrial subfractions are sufficiently pure to allow the attribution of a protein kinase, which co-fractionates with one of the markers, to the corresponding mitochondrial compartment.

Endogenous phosphorylation

Figure 3 shows the distribution of protein kinase activity with endogenous substrate in mitochondrial subfractions. Only two of the purified subfractions, namely matrix and inner membranes, exhibit

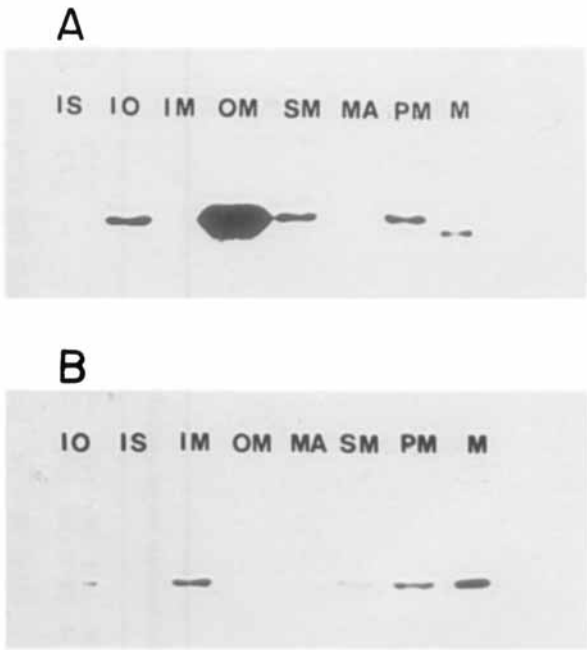

Figure 2. Detection of mitochondrial marker proteins by immune decoration (Towbin *et al.*, 1979). The bands were quantified as described (Suissa, 1983). Examples given are (A) immune decoration with an antiserum directed against porin, (B) immune decoration with serum directed against cytochrome c_1. Abbreviations used: PM, purified mitochondria; SM, shocked mitochondria; IM, inner membranes; IO, inner plus outer membranes; OM, outer membranes; IS, intermembrane space; MA, matrix; M, marker mix (bovine serum albumin, ovalbumin, carboanhydrase, chymotrypsinogen, myoglobin and cytochrome *c*). The molecular weight standard used was radioiodinated carbonic anhydrase, 29 000.

protein kinase activity (Figure 3, second panel, and Table 2), indicating that these substrates must occur in one compartment together with their kinases. Neither kinases are stimulated by cAMP (Table 2). Since after gel electrophoresis and autoradiography intermembrane space proteins and outer membranes fail to exhibit labeled bands (not shown) the little radioactivity incorporated into these fractions is obviously aspecifically bound. Figure 3 includes also the cAMP-binding for comparison. It is predominantly enriched with inner membranes and with the intermembrane space (first panel in Figure 3).

Phosphorylation of intrinsic substrates by combined subfractions

To test whether substrate and enzyme might have been separated during the subfractionation, each two neighboring fractions were recombined and assayed for endogenous phosphorylation. This was

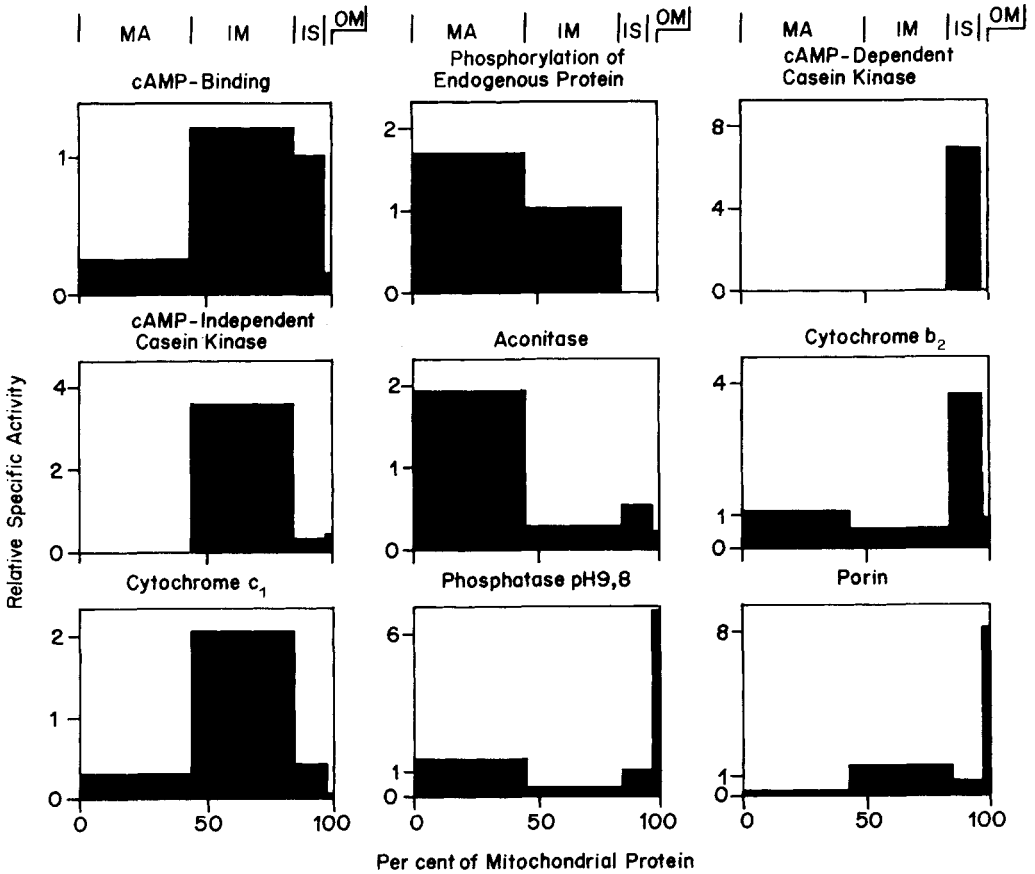

Figure 3. De Duve plots of cAMP binding, protein kinase activity and various markers of mitochondrial subfractions. Mitochondria were subfractionated into matrix (MA), inner membranes (IM), intermembrane space (IS), and outer membranes (OM). The abscissa reflects the respective contribution of each fraction to whole mitochondria based on the protein content. Specific markers for the matrix (aconitase), the inner membrane (cytochrome c_1), the intermembrane space (cytochrome b_2), and the outer membrane (porin) were determined together with cAMP binding, total protein kinase with endogenous substrate, casein kinase (activity with endogenous substrate subtracted) and cAMP-dependent casein kinase (cAMP-independent casein kinase activity subtracted). The specific activities in whole mitochondria, lysed with 0·5% Nonidet P40 (which were set at 1) were as follows: cAMP binding, 1·3 pmol mg^{-1}; phosphorylation of endogenous proteins, 110 pmol ^{32}P mg^{-1} h^{-1}; cAMP-independent casein kinase, 10 pmol ^{32}P mg^{-1} h^{-1}; cAMP-dependent casein kinase, 8 pmol ^{32}P mg^{-1} h^{-1}; aconitase, 6900 units as defined by Racker (1950); alkaline phosphatase (pH 9·8), 45·9 nmol mg^{-1} min^{-1}. Cytochromes c_1 and b_2 and porin were determined by immune decoration (Suissa, 1983; Towbin *et al.*, 1979, and cf. Figure 2).

then compared to that found in the separate fractions. The results, given in Table 3, show that partial reconstitution of inner membranes with intermembrane space proteins (i) exhibits phorphorylation activity which exceeds that of the separate fractions (cf. Table 2, lines 3 plus 4) and (ii) that this phosphorylation is enhanced by cAMP. The stimulation factor is 1·8, when only that kinase is regarded which is active in the reconstituted system but inactive in the separate fractions, (i.e. when cAMP-independent activities, observed in the separate fractions, are subtracted; the difference between Table 3, line 2, and Table 2, lines 3 plus 4). These data document that (i) inner membranes or intermembrane space contain at least one protein kinase active in the absence of cAMP, whose substrate is localized in the respective other fraction, and (ii), in addition, that a cAMP-dependent activity is present in these two fractions as well. This would suggest that there might be two kinases, whose enzyme and substrate become separated during the subfractionation, one of them cAMP-dependent.

Table 2. Activity of protein kinases and stimulation by cAMP in various mitochondrial subfractions.

Mitochondrial fraction	Specific radioactivity (cpm × 10^{-3}/100 µg) −cAMP	+cAMP	Stimulation by cAMP (%)
Intact mitochondria	64·5	65·8	+2
Matrix	110·0	111·2	+1
Inner membrane	66·0	62·7	−5
Intermembrane space	2·5	2·6	+4
Outer membrane	2·1	2·0	−5
Ribosomes			
repressed	3·0	2·2	−27
derepressed	2·2	1·2	−45

After subfractionation, phosphorylation of endogenous mitochondrial proteins by kinases present in the respective fractions was measured as incorporation of ^{32}P from [γ-^{32}P]ATP into acid-insoluble material in intact mitochondria and various mitochondrial subfractions. Ribosomes were prepared directly from labeled mitochondria in a separate experiment. Mean value for two experiments.

Table 3. Partial reconstitution by combination of each two neighboring compartments, stimulation by the combination relative to the phosphorylation in the constituent fractions and influence of cAMP.

Combined mitochondrial compartments	Specific radioactivity (cpm × 10^{-3}/200 µg) −cAMP	+cAMP	Sum of constituent fractions from Table 2 (cpm × 10^{-3}/200 µg) −cAMP	+cAMP	Stimulation by combination of two compartments (%) −cAMP	+cAMP	Stimulation by cAMP (%)
Matrix + inner membrane	177·3	177·8	176·0	173·9	+0·5	+2·0	0
Inner membrane + intermembrane space	110·0	144·3	68·5	65·3	+61	+128	+31
Intermembrane space + outer membrane	3·2	3·5	4·6	4·6	−30	−24	+9

Each two neighboring compartments were combined and assayed for phosphorylation of intrinsic proteins as described for Table 2. The result was compared with the sum of phosphorylation obtained in the respective two fractions (taken from Table 2). Mean values of two experiments.

Phosphorylation of exogenous substrate by combined subfractions

In a second series of experiments we provided partly dephosphorylated casein hydrolysate as an artificial substrate. Table 4 shows that the addition of casein significantly enhances phosphorylation in those fractions containing the intermembrane space and, to a much lesser extent, inner membranes. In the case of the intermembrane space this phosphorylation is again stimulated by cAMP (up to 18·5-fold), whereas it is not with inner membranes. These results are also included in Figure 3, panels 4 and 5. Almost no incorporation into casein (or other heterologous substrates such as histone or serum albumin, data not shown) or even inhibition of phosphorylation is observed with outer membranes and matrix, respectively. The data confirm the conclusions drawn from the experiments with endogenous substrate and suggest that a cAMP-dependent kinase is present in the intermembrane space together with its regulatory subunit, whereas its natural substrate must be bound to the inner membrane. Additionally, one further cAMP-independent kinase might be

Table 4. Mitochondrial casein kinases: phosphorylation of casein as compared to endogenous phosphorylation and influence by cAMP.

Mitochondrial fraction	Specific radioactivity with casein (cpm × 10^{-3}/100 μg) −cAMP	+cAMP	Stimulation by casein (%) −cAMP	+cAMP	Additional stimulation by cAMP (%)
Lysed mitochondria	80·4	94·1	+10	+16	+78
Matrix	107·8	100·0	−2	−10	0
Inner membrane	88·5	79·0	+34	+26	−28
Intermembrane space	4·5	41·6	+79	+1500	+1850
Outer membrane	2·2	2·1	+6	+2	0
Inner membrane + intermembrane space	152·9	339·5	+39	+135	+355

The phosphorylation of exogenously added casein by protein kinases present in mitochondrial subfractions, or combinations thereof, was assayed and compared with that measured in the absence of casein (Table 2 and 3). For the calculation of % stimulation by cAMP of casein phosphorylation, the value obtained for endogenous phosphorylation was subtracted. Mean from two experiments.

associated with inner membranes, which finds its substrate in the intermembrane space, and which is capable of phosphorylating casein.

Outer membranes are apparently devoid of protein kinases having their substrates either in the outer membrane or in the intermembrane space. Neither is any appreciable phosphorylation of endogenous proteins detectable in this fraction (Table 2), nor does partial reconstitution of outer membranes with the intermembrane space lead to a phosphorylation activity that exceeds that of the separate fractions (Table 3), nor does the addition of casein or other heterologous substrates (data not shown) to outer membranes enhance the incorporation of radioactivity into material insoluble in trichloroacetic acid. We cannot exclude the possibility that there are protein kinases in the outer membrane which act specifically on cytoplasmic target proteins, or that it contains the substrate for cytoplasmic kinases.

Identification of target proteins of cAMP-independent mitochondrial protein kinases in subfractions

To identify the substrates of mitochondrial protein kinases we electrophoresed mitochondrial proteins phosphorylated either in the individual or combined subfractions. As all fractions have been incubated with a mixture of RNase, DNase and lipase prior to gel electrophoresis and as the gels have been incubated with trichloroacetic acid in order to remove acid-labile adducts, the bands found in the autoradiogram are likely to represent phosphoproteins. This was confirmed by hydrolysis and thin-layer electrophoresis of casein phosphorylated by mitochondrial protein kinases. The results revealed that phosphate label was incorporated into both serine and threonine residues (data not shown). Figure 4A shows that the matrix (MA) from derepressed mitochondria contains two phosphoproteins of molecular mass 49 and 24 kDa, whereas one such band of 52·5 kDa is found in the inner membrane (IM). These three proteins are phosphorylated by kinases which obviously occur in the same compartment as their targets. Combination of inner membranes with the matrix fraction (IM + MA) does not give rise to phosphorylation of additional proteins. This is consistent with the data shown in Table 3, where the combination of the two fractions (IM + MA) did not enhance phosphorylation of endogenous proteins. It is concluded that inner membranes contain one protein kinase and the matrix at least one protein kinase and that no kinases are active in the matrix of derepressed mitochondria which have their targets in the inner membrane or *vice versa*. No phosphorylated protein band is found in the intermembrane space (IS) or in outer membranes (OM). Similarly a combination of the two (IM + OM) fails to exhibit any phosphorylated polypeptides, in accordance with Table 3. However, when inner membranes and the intermembrane space (IS + IM) are incubated together with [γ-^{32}P]ATP, one additional band of 17·5 kDa is found phosphorylated. This is interpreted as

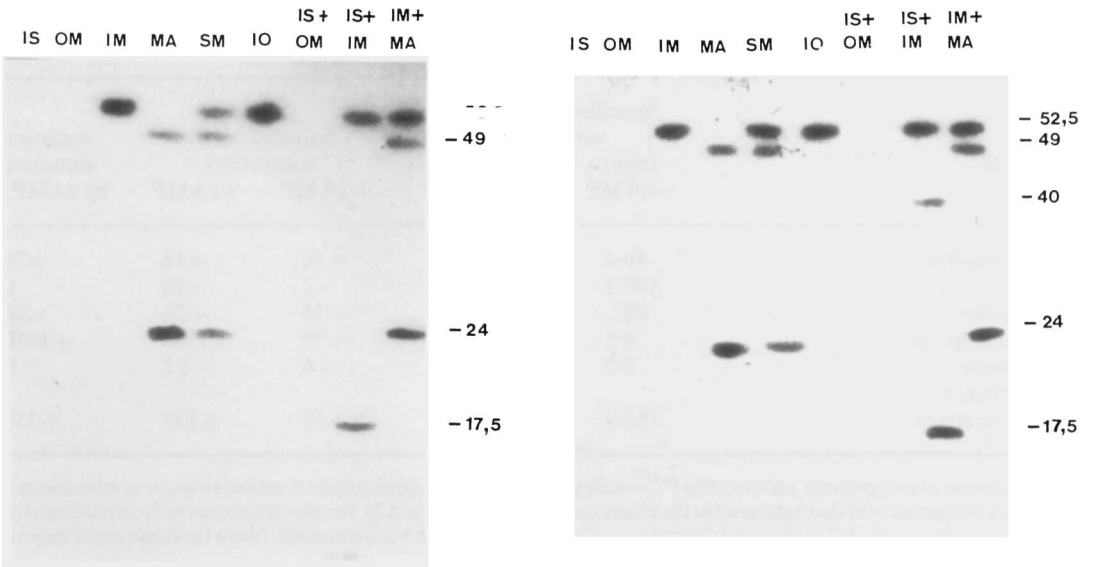

Figure 4. Autoradiogram of phosphorylated target proteins of mitochondrial protein kinases after labeling in the absence (A) and presence (B) of cAMP. Phosphorylation of intrinsic polypeptides was assayed in various subfractions of mitochondria, intermediate steps of the subfractionation procedure and partially reconstituted fractions. The abbreviations used are described under Figure 2. IM + OM, IS + IM, and IM + MA: reconstituted fractions. The same molecular weight standards were used as in Figure 1A.

indicative of a third cAMP-independent protein kinase, which is present in the inner membrane and has its natural target in the intermembrane space or *vice versa*. The significant cAMP-independent increase in ^{32}P-incorporation observed upon addition of casein to inner membranes (Table 4) suggests a localization of this kinase in the inner membrane and, correspondingly, of its target, M_r 17 500, in the intermembrane space.

The results are confirmed by those lanes of Figure 4A which contain shocked mitochondria (SM) and inner plus outer membranes (IO), as these intermediate steps of the membrane separation procedure show that the number of phosphorylated proteins observed in these lanes is exactly the combination of those found in their component fractions.

Target proteins of cAMP-dependent protein kinases

The addition of cAMP to the phosphorylation assay leads to the emergence of a single polypeptide of 40 kDa above those already phosphorylated in the absence of the ligand. This additional phosphoprotein is found in the partially reconstituted fraction containing inner membranes plus intermembrane space proteins (IS + IM, Figure 4B). Combined with the data taken from Table 4 it is concluded that the intermembrane space harbours a soluble cAMP-dependent protein kinase which finds its natural target protein (40 kDa) in the inner membrane.

These results (shown in Figure 4B and in Tables 3 and 4), which document the presence of a cAMP-dependent protein kinase in mitochondria, contrast with those obtained after labeling mitochondria *in vivo* or *in organello* (Figure 1A and B), which failed to exhibit a significant influence of cAMP on the phosphorylation pattern. In an attempt to resolve this discrepancy we assayed inner membranes for the presence of protein phosphatase activity, as Table 4 also shows that reconstitution of the intermembrane space with inner membranes drastically diminishes the radioactive label incorporated into casein. This effect is even more pronounced in the presence of cAMP. In order to test whether a hydrolase is present in the inner membrane, which would prevent a high steady-state level of cAMP-dependent incorporation of phosphate into casein or inner membrane proteins *in situ*, casein was phosphorylated with [γ-^{32}P]ATP in the presence of intermembrane space proteins and cAMP but in the absence of inner membranes. After removal of non-incorporated radioactivity by a passage over a

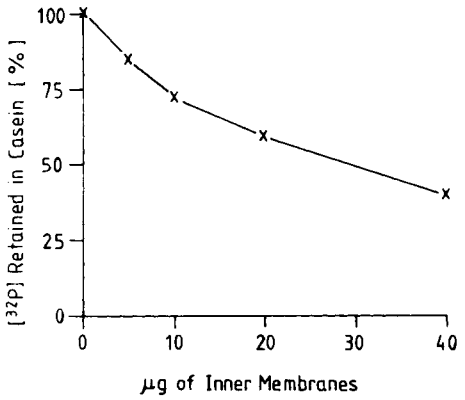

Figure 5. Assay of the stability of phosphoproteins in the presence of inner mitochondrial membranes. Partly dephosphorylated casein (100 µg) was phosphorylated with [γ-^{32}P]ATP in the presence of 100 µg of intermembrane space proteins and 100 µM-cAMP for 60 min. Unincorporated label was removed by Sephadex G50 fine gel filtration. After 40 min of incubation with increasing amounts of inner membranes the label retained on filters after precipitation with trichloroacetic acid was determined. The radioactivity retained after 40 min in the absence of inner membranes served as the 100% value. It is only insignificantly less than that of the starting material.

Sephadex G50 fine column, the labeled casein was incubated with increasing amounts of inner membranes. As shown in Figure 5 the phosphate label is rapidly lost. This suggests that *in situ* the membrane-bound 40 kDa target protein of the cAMP-dependent protein kinase is closely associated with a protein phosphatase which is also bound to the inner membrane and which is likely to be responsible for the failure to detect significant cAMP-dependent phosphorylation of the 40 kDa polypeptide either *in vivo* (Figure 1A) or in isolated mitochondria (Figure 1B).

Similar situations, where phosphorylated proteins are associated with specific phosphatases, are, for instance, found with phosphorylase *a* (Krebs and Beavo, 1979), fructose 1,6-bisphosphatase (Rittenhouse *et al.*, 1983) and pyruvate dehydrogenase complex (Linn *et al.*, 1969). Unlike the phosphatase of phosphorylase *a* (Krebs and Beavo, 1979), we found that this phosphatase from mitochondrial membranes is not stimulated by Ca^{2+} and not inhibited by EDTA or EGTA (data not shown).

Topology of the cAMP-dependent casein-phosphorylating activity

In order to confirm the localization of the cAMP-dependent protein kinase in the intermembrane space and to test if the cAMP-binding protein recently found in inner mitochondrial membranes (Rödel *et al.*, 1985) could constitute the regulatory subunit of this kinase, we examined whether the two proteins belong to the same submitochondrial compartment. We assayed the accessibility to protease of the two activities in intact mitochondria, in mitochondria in which all accessibility barriers had been removed by lysis with detergent and in mitochondria having disrupted outer but intact inner membranes (shocked mitochondria). The resistance to trypsin with incubation time of both activities was compared to that of mitochondrial markers of known topology (Figure 6).

It can be seen from the figure that both cAMP-dependent phosphorylation and cAMP binding are resistant to protease in untreated mitochondria but are rapidly inactivated in shocked and in lysed mitochondria. In this behavior both activities resemble proteins localized outside the permeability barrier provided to trypsin by the inner membrane, e.g. cytochrome b_2 (and cytochromes *c* and c_1, data not shown), but contrasts with that of enzymes of the inner compartment, e.g. subunits α (and β, data not shown) of mitochondrial F_1-ATPase. It is concluded that both the cAMP-dependent protein kinase and the cAMP receptor are localized in the mitochondrial outer compartment: the cAMP-dependent kinase has as yet been observed exclusively in the intermembrane space as deduced from reconstitution experiments (Tables 3 and 4), whereas a cAMP receptor has recently been attributed to the outer surface of the inner membrane (Rödel *et al.*, 1985). But the cAMP-dependence of casein phosphorylation and a significant cAMP binding in the isolated intermembrane space fraction (Table 4 and Figure 3, panel 1) suggest the presence of a second soluble cAMP receptor in the intermembrane space.

DISCUSSION

The identification of a cAMP receptor protein in mitochondria is interesting both in evolutionary and in functional respect. A possible involvement of this protein in transcriptional control would lend support to the hypothesis of a prokaryotic origin of mitochondria (Ashwell and Work, 1970) and would suggest that this protein might facilitate initiation of transcription in a fashion analogous to the bacterial cAMP-binding protein. For mitochondria such a mechanism is, however, unlikely mainly for two reasons: (i) the results of this and previous work (Rödel *et al.*, 1985) have shown that the mitochondrial cAMP receptor protein and RNA polymerase

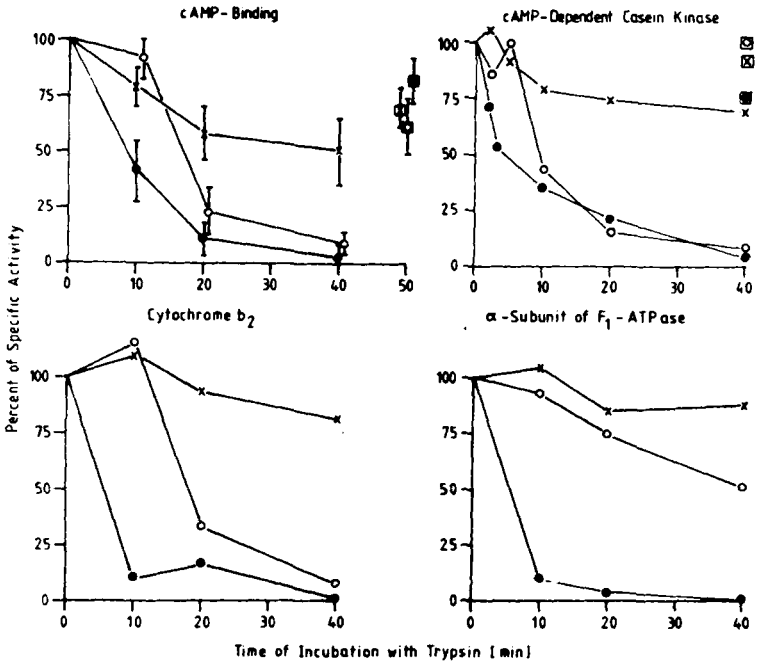

Figure 6. Topology of the mitochondrial cAMP-dependent casein kinase. Intact (×), shocked (○), and lysed (●) mitochondria were treated with 40 μg trypsin at 0°C for variable periods of time. After termination of the reaction by a 4-fold molar excess of soy bean trypsin inhibitor and 1 mM-tosyl lysyl chloromethyl ketone, cAMP binding (A), cAMP-dependent casein kinase (B), and, as markers, cytochrome b_2 (C) and the α-subunit of F_1-ATPase (D) were assayed. Cytochrome b_2 and the α-subunit of F_1-ATPase were determined by quantitative evaluation (Suissa, 1983) of immune decorations (Towbin *et al.*, 1979). Controls: presence of inhibitor during incubation of ⊠ intact, ⊡ shocked, and ▣ lysed mitochondria with trypsin.

plus its target, the DNA, belong to two different submitochondrial compartments; (ii) even if the cAMP receptor is a transmembrane protein which transmits signals from the outside to the matrix compartment, it does not control RNA polymerase since cAMP does not significantly stimulate mitochondrial RNA synthesis either *in vivo* or in isolated mitochondria (Bandlow *et al.*, 1985). The absence of cAMP-dependent protein kinases from bacteria and their presence in mitochondria could rather be taken as an indication of a eukaryotic mode of regulation.

Several groups have reported the presence of protein kinases in mitochondria of a variety of organisms (e.g. Kitagawa and Racker, 1982; Linn *et al.*, 1969; Rigobello *et al.*, 1980; Dimino *et al.*, 1981; Henriksson and Jergil, 1979; Vardanis, 1977). Their total number, specific function and submitochondrial localization is, however, not clear. The figure of four to five protein kinases we have found active in derepressed yeast mitochondria (one of them cAMP-dependent) as well as the number of phosphorylatable proteins present rather appears to be a minimum estimate. Other kinases may be active only in repressed mitochondria (like PDH kinase). The existence of still others may be masked by a rapid turnover of the respective phosphoprotein. The discrepancy in the number of phosphoproteins found after labeling *in vivo* and in isolated mitochondria may be primarily attributed to the action of cytoplasmic protein kinases which phosphorylate mitochondrial protein precursors. These are imported and possibly dephosphorylated in subsequent steps.

Recently it was reported (Steinberg, 1984) that the β-subunit of mitochondrial F_1-ATPase (molecular mass 52 kDa) becomes phosphorylated in a cAMP-dependent manner. It could not be shown that phosphorylation is a prerequisite for import into mitochondria, but it was found that it occurs during or immediately after the synthesis of this protein in its precursor form by a cytoplasmic

cAMP-dependent protein kinase. The phosphate is removed after processing and assembly of the precursor. Since the mitochondria used in the present study have been purified by gradient centrifugation from adhering cytoplasmic contaminants such as precursors prior to the subfractionation, an identity of the β-subunit with the phosphoprotein of 52·5 kDa phosphorylated in a cAMP-independent manner by the protein kinase of the inner membrane—although to be tested—appears to be less likely.

In mammalian mitochondria cAMP-dependent protein kinases of both type I and type II have been found (Dimino *et al.*, 1981; Henriksson and Jergil, 1979). We describe here that cAMP-dependent phosphorylation of casein occurs in the intermembrane space, which indicates that both the catalytic and the cAMP-binding regulatory subunit of a protein kinase must be present simultaneously in this compartment. This conclusion is further substantiated by the presence of significant cAMP-binding activity in the intermembrane space. Since cAMP-dependent phosphorylation of mitochondrial proteins is observed only when inner membranes are also present, the target of this cAMP-dependent kinase (molecular mass 40 kDa) must be associated with the inner membrane.

Previous binding studies and photoaffinity labeling (Rödel *et al.*, 1985) have shown that the bulk of cAMP binding is tightly associated with the inner mitochondrial membrane and that a cAMP receptor (molecular mass 45 kDa) is accessible from the outer surface of the inner membrane. This major cAMP-binding activity either has its catalytic subunit (possibly identical with that of the cAMP-dependent kinase mentioned above) in the intermembrane space as well, or has a function different from a regulatory subunit of a protein kinase, as neither in isolated inner membranes alone nor after the addition of heterologous substrates is significant cAMP-dependent protein phosphorylation observed.

Little is known about regulatory processes in mitochondria which involve protein kinases. Among the cAMP-independent kinases only one, namely PDH kinase, has been characterized and the nature of its target protein identified. In glucose-repressed yeast cells the α-subunits of the PDH complex are inactivated upon phosphorylation by PDH kinase, which is a constituent of the complex (Linn *et al.*, 1969; Barrera *et al.*, 1972). This phosphorylated polypeptide (molecular mass 41 kDa) may be present in those lanes of Figures 1A and B showing repressed mitochondria and should be absent from derepressed ones. As the phosphorylation of PDH occurs in a cAMP-independent fashion and as PDH is localized on the matrix side of the inner membrane, the polypeptide of 40 kDa phosphorylated by the cAMP-dependent kinase is not the α-subunit of the PDH complex.

Thus, with the exception of PDH kinase, the *in situ* function of mitochondrial protein kinases remains unknown. One possible approach for revealing their physiological role without previous functional identification of their target proteins consists in the isolation of the respective genes. The *in vitro* introduction of mutations into these genes and the analysis of the resulting phenotype may then help to identify the substrates and the function of the encoded protein kinases.

ACKNOWLEDGEMENTS

We thank A. Haid and G. Schatz, The Biocenter Basel, for their generous support with antisera and G. Strobel for her skilful help with the preparation of the figures.

REFERENCES

Ashwell, M. and Work, T. S. (1970). The biogenesis of mitochondria. *Ann. Rev. Biochem.* **39,** 251–290.

Bandlow, W. (1979). Control of mitochondrial gene expression in yeast by adenosine 3':5'-cyclic monophosphate. *Hoppe-Seyler's Z. Physiol. Chem.* **360,** 228–229.

Bandlow, W., Schwarz, U., Rödel, G., Strobel, G. and Wachter, C. (1985). Isolation of cAMP receptor protein from yeast mitochondria (M_r 45 000) and comparison with mitochondrial RNA polymerase (M_r 45 000). *Biol. Chem. Hoppe-Seyler* **366,** 545–553.

Barrera, C. R., Namihira, G., Hamilton, L., Munk, P., Eley, M. H., Linn, T. C. and Reed, L. J. (1972). Studies on the subunits of the pyruvate dehydrogenase complexes from bovine heart and kidney. *Arch. Biochem. Biophys.* **148,** 343–358.

Bradford, M. M. (1976). A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analyt. Biochem.* **72,** 248–254.

Chandrasekaran, K. and Jayaraman, J. (1978). Effect of cyclic AMP on the biogenesis of cytochrome oxidase in yeast. *FEBS Lett.* **87,** 52–54.

Chock, P. B., Rhee, S. G. and Stadtman, E. R. (1980). Interconvertible enzyme cascades in cellular regulation. *Ann. Rev. Biochem.* **49,** 813–843.

Daum, G., Böhni, P. C. and Schatz, G. (1982). Import of proteins into mitochondria. Cytochrome b_2 and cytochrome *c* peroxidase are located in the intermembrane space of yeast mitochondria. *J. Biol. Chem.* **257,** 13028–13033.

De Vendittis, E., Vitelli, A., Zahn, R. and Fasano, O. (1986). Suppression of defective RAS 1 and RAS 2 functions in yeast by an adenylate cyclase activated by a single amino acid change. *EMBOJ.* **5,** 3657–3663.

Dimino, M. J., Biesczad, R. R. and Rowe, M. J. (1981). Cyclic AMP-dependent protein kinase in mitochondria and cytosol from different-sized follicles and corpora lutea from porcine ovaries. *J. Biol. Chem.* **256,** 10876–10882.

Douglas, M. and Butow R. A. (1976). Variant forms of mitochondrial translation products in yeast: evidence for location of determinants on mitochondrial DNA. *Proc. Natl. Acad. Sci. U.S.A.* **73,** 1083–1086.

Eraso, P. and Gancedo, J. M. (1984). Catabolite repression in yeast is not associated with low levels of cAMP. *Eur. J. Biochem.* **141,** 195–198.

Fang, M. and Butow, R. A. (1970). Nucleotide reversal of mitochondrial repression in *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **41,** 1579–1583.

Heil, A. and Zillig, W. (1970). Reconstitution of bacterial DNA-dependent RNA-polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. *FEBS Lett.* **11,** 165–168.

Henriksson, T. and Jergil, B. (1979). Protein kinase activity and endogenous phosphorylation in subfractions of rat liver mitochondria. *Biochim. Biophys. Acta* **555,** 386–391.

Hixson, C. S. and Krebs, E. G. (1980). Characterization of a cyclic AMP-binding protein from baker's yeast. *J. Biol. Chem.* **255,** 2137–2145.

Kim, K. H. and Kikuchi, G. (1972). Possible participation of cyclic AMP in the regulation of δ-aminolevulinic acid synthesis in rat liver. *J. Biochem.* **71,** 923–926.

Kitagawa, Y. and Racker, E. (1982). Purification and characterization of two protein kinases from bovine heart mitochondrial membrane. *J. Biol. Chem.* **257,** 4547–4551.

Krebs, E. G. and Beavo, J. A. (1979). Phosphorylation and dephosphorylation of enzymes. *Ann. Rev. Biochem.* **48,** 923–959.

Lastick, S. M. and McConkey, E. H. (1981). HeLa ribosomal protein S6. Insulin and dibutyryl cyclic AMP affect different phosphopeptides. *J. Biol. Chem.* **256,** 583–585.

Linn, T. C., Pettit, F. H. and Reed, L. J. (1969). Purification and properties of the component enzymes of the pyruvate dehydrogenase complex from bovine heart and kidney. *Proc. Natl. Acad. Sci. U.S.A.* **62,** 234–241.

Mahler, H. R., Janes, P. K., McDonough, J. P. and Hanson, D. R. (1981). Catabolite repression in yeast: mediation by cAMP. *Curr. Top. Cell. Regulat.* **18,** 455–474.

Mahler, H. R. and Lin, C. C. (1978). Exogenous adenosine 3′:5′-monophosphate can release yeast from catabolite repression. *Biochem. Biophys. Res. Commun.* **83,** 1039–1047.

Matsumoto, K., Uno, I. and Ishikawa, T. (1983). Control of cell division in *Saccharomyces cerevisiae* mutants defective in adenylate cyclase and cAMP-dependent protein kinase. *Exptl. Cell Res.* **146,** 151–161.

Matsumoto, K., Uno, I. and Ishikawa, T. (1985). Genetic analysis of the role of cAMP in yeast. *Yeast* **1,** 15–24.

Matsumoto, K., Uno, I., Toh-E, A., Ishikawa, T. and Oshima, Y. (1982). Cyclic AMP may not be involved in catabolic repression in *Saccharomyces cerevisiae*: evidence from mutants capable of utilizing it as an adenine source. *J. Bacteriol.* **150,** 277–285.

Pall, M. L. (1981). Adenosine 3′:5′-phosphate in fungi. *Microbiol. Rev.* **45,** 462–480.

Racker, E. (1950). Spectrophotometric measurements of the enzymatic formation of fumaric and *cis*-aconitic acids. *Biochim. Biophys. Acta* **4,** 211–214.

Rigobello, M. P., Carignani, G. and Pinna, L. A. (1980). Properties and localization of a cAMP-independent protein kinase from yeast mitochondria. *FEBS Lett.* **121,** 225–229.

Rittenhouse, J., Chatterjee, T., Marcus, F., Reardon, J. and Heinrikson, R. L. (1983). Amino acid sequence of the COOH-terminal region of fructose-1,6-bisphosphatases in relation to cyclic AMP-dependent phosphorylation. *J. Biol. Chem.* **258,** 7648–7652.

Rödel, G., Müller, G. and Bandlow, W. (1985). Cyclic AMP receptor protein from yeast mitochondria: submitochondrial localization and preliminary characterization. *J. Bacteriol.* **161,** 7–12.

Steinberg, R. A. (1984). Cyclic AMP-dependent phosphorylation of the precursor to β-subunit of mitochondrial F_1-ATPase: a physiological mistake? *J. Cell. Biol.* **98,** 2174–2178.

Steinberg, R. A. and Coffino, R. (1979). Two-dimensional gel analysis of cyclic AMP effects in cultured S49 mouse lymphoma cell: protein modifications, inductions and repressions. *Cell* **18,** 719–733.

Suissa, M. (1983). Spectrophotometric quantitation of silver grains eluted from autoradiograms. *Analyt. Biochem.* **133,** 511–516.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4350–4354.

Van der Plaat, J. B. and van Solingen, P. (1979). Cyclic 3′:5′-adenosine monophosphate stimulates trehalose degradation in baker's yeast. *Biochem. Biophys. Res. Commun.* **56,** 580–587.

Vardanis, A. (1977). Protein kinase activity at the inner membrane of mammalian mitochondria. *J. Biol. Chem.* **252,** 807–813.

Wang, H. X. and Sy, J. (1985). cAMP-dependent phosphoprotein changes in the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **17,**357–360.