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cAMP-dependent protein phosphorylation in mitochondria of bovine heart

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Abstract

A study is presented of the cAMP-dependent phosphorylation in bovine heart mitochondria of three proteins of 42, 16 and 6.5 kDa associated to the inner membrane. These proteins are also phosphorylated by the cytosolic cAMP-dependent protein kinase and by the purified catalytic subunit of this enzyme. In the cytosol, proteins of 16 and 6.5 kDa are phosphorylated by the cAMP-dependent kinase. It is possible that cytosolic and mitochondrial cAMP-dependent kinases phosphorylate the same proteins in the two compartments.

Key words: Mitochondrion; Protein phosphorylation; Protein kinase; Cyclic AMP; Cyclic AMP-dependent protein kinase

1. Introduction

Hormones like glucacon and gonadotropins, which use cAMP as a second messenger, have been found to affect mitochondrial processes [1–3]. Glucacon also stimulates phosphorylation of mitochondrial proteins in vivo [4] or in isolated cell preparations [5]. cAMP itself seems to affect mitochondrial processes [6], and cAMP binding proteins have been detected in mitochondria [7,8]. These observations suggest that, in addition to the cytosolic enzymes [9], there are also mitochondrial cAMP-dependent protein kinases [3,10–13].

In yeast a cAMP-dependent protein kinase, loosely associated to the inner mitochondrial membrane and responsible for phosphorylation of a 40 kDa protein of this membrane, was observed [11]. In mammalian mitochondria, a cAMP-dependent protein kinase is apparently localized in the inner membrane/matrix space [12,13]. This kinase was found to phosphorylate added histones but phosphorylation of endogenous mitochondrial proteins was not reported [12,13]. In a recent study, Technikova-Dobrova et al. [14] showed that in isolated bovine heart mitochondria, in addition to pyruvate dehydrogenase [15] and branched chain α-oxoacid dehydrogenase, other mitochondrial proteins are phosphorylated by cAMP-dependent and -independent kinases. In this paper a study is presented of the cAMP-dependent phosphorylation of three proteins with apparent molecular weights of 42, 16 and 6.5 kDa in the inner membrane fraction of bovine heart mitochondria. These proteins are also phosphorylated by the cAMP-dependent protein kinase in the cytosol and by the purified catalytic subunit of this enzyme. In the cytosol, proteins of 16 and 6.5 kDa are phosphorylated by the cytosolic cAMP-dependent kinase.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; TMPD, tetramethyl-*p*-phenylene-diamine dihydrochloride; PAGE, polyacrylamide gel-electrophoresis; SDS, sodium dodecylsulphate; cAPK, cAMP-dependent protein kinase; C-cAPK, catalytic subunit of cAPK.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP 3000 Ci/nmol and Hyperfilm-MP were from Amersham International; DNase, RNase and histone H2B were from Boehringer-Mannheim; protein kinase inhibitor (rabbit sequence) and the catalytic subunit of cAMP-dependent protein kinase purified from bovine heart were from Sigma. Dynagel was from V.T. Baker Holland.

2.2. Preparation of mitochondrial fractions

Cytosol and heavy mitochondria were isolated from beef heart as in [17]. For fractionation, mitochondria (30 mg protein) were suspended in 1.5 ml of 0.25 M sucrose, 1 mM Tris-HCl, pH 7.5, 1 mM EGTA, 250 μM PMSF. 0.5 ml glass beads were added and the mitochondria ground on a Vortex at 0°C. Undestroyed mitochondria and glass beads were removed by centrifugation at 20,000 × *g*. An aliquot of the supernatant, representing the total mitochondrial extract, was directly incubated with [γ-^{32}P]ATP, another was centrifuged at 100,000 × *g* to obtain the membrane pellet and supernatant fraction. The presence of cytosol in the mitochondrial fractions was estimated from measurement of lactate dehydrogenase activity, matrix from NADP-isocitrate dehydrogenase activity, outer membrane from rotenone-insensitive NADH-cytochrome *c* oxidoreductase activity [18], and inner membrane from spectrophotometric determination of cytochromes $a + a_3$ [19]. Cytosolic contamination of the membrane and soluble mitochondrial fractions amounted to 0.4% and 5%, respectively (see also [14]). The membrane fraction exhibited a NADP-isocitrate dehydrogenase activity of 20 nmol·min^{-1}·mg prot^{-1}, an activity of the rotenone-insensitive NADH-cytochrome *c* oxidoreductase of 300 nmol·min^{-1}·mg prot^{-1} and contained 0.52 nmol hemes $a + a_3$ per mg prot. Hemes were 50% reduced directly by ascorbate; full reduction was achieved by adding TMPD together with ascorbate [19]. The soluble fraction was devoid of hemes $a + a_3$, exhibited a NADP isocitrate dehydrogenase activity of 1600 nmol·min^{-1}·mg protein and a rotenone-insensitive NADH-cytochrome *c* oxidoreductase of 1800 nmol·min^{-1}·mg prot^{-1}.

2.3. Protein kinase assay

Protein phosphorylation was assayed by incubating, for 20 min at

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30°C under stirring, 600 μg protein of each fraction in 450 μl of 10 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 20 mM NaF, 3 μg rotenone, 3 μg oligomycin and 70 μM [γ-^{32}P]ATP (1000 cpm/pmol). To stop the reaction, 150 μl of the suspension was mixed with 40 μl of 0.35 M Tris-HCl, pH 6.8, 10% v/v glycerol, 15% w/v SDS, 25% v/v β-mercaptoethanol, and boiled for 3 min.

2.4. Gel electrophoresis and autoradiography

Gel electrophoresis and autoradiography were performed as in [14]. Radioactive PAGE bands of given molecular weights were cut from gels immersed in Dynagel, and radioactivity was measured in a Beckman Counter.

3. Results

Fig. 1 shows the ^{32}P-labelling pattern of proteins, resolved by PAGE from freshly prepared bovine heart mitochondria and from a total extract of mitochondria, after incubation with [γ-^{32}P]ATP. Both in intact mitochondria (A) and in the total mitochondrial extract (B), in addition to the protein bands of 42 and 48 kDa, containing the E1α subunits of pyruvate dehydrogenase [15] and branched chain oxoacid dehydrogenase [16], respectively, protein bands (detected by Coomassie blue, not shown) of 44, 39, 36, 31, 16 and 6.5 kDa were labelled. In intact mitochondria, phosphorylation of all these proteins was apparently unaffected by added cAMP, except a protein band of 29 kDa which became labelled in the presence of cAMP. In the total mitochondrial extract, which was dialyzed before incubation with [γ-^{32}P]ATP, also phosphorylation of the 16 and 6.5 kDa proteins became dependent on added cAMP. Evidently freshly prepared mitochondria contain endogenous cAMP sufficient to promote protein phosphorylation by cAMP-dependent protein kinase (cAPK).

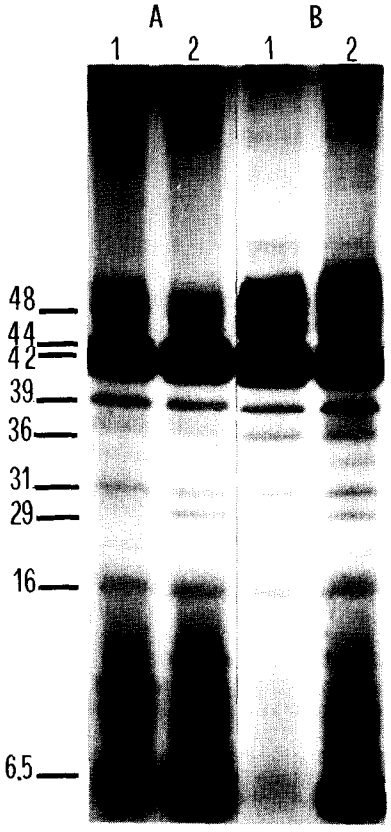

Fig. 1. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in freshly isolated bovine heart mitochondria (A) and total extract (B) obtained from glass bead-disrupted mitochondria. Mitochondria and mitochondrial extract were incubated with [γ-^{32}P]ATP as described in section 2. Lane 1, controls; lane 2, incubation in the presence of 50 μM cAMP. Molecular weights of labeled protein bands, also detected by Coomassie blue, were determined from standard proteins run on the same gel. The molecular weights in kDa, are given at the left side of the autoradiograms. For other details see section 2.

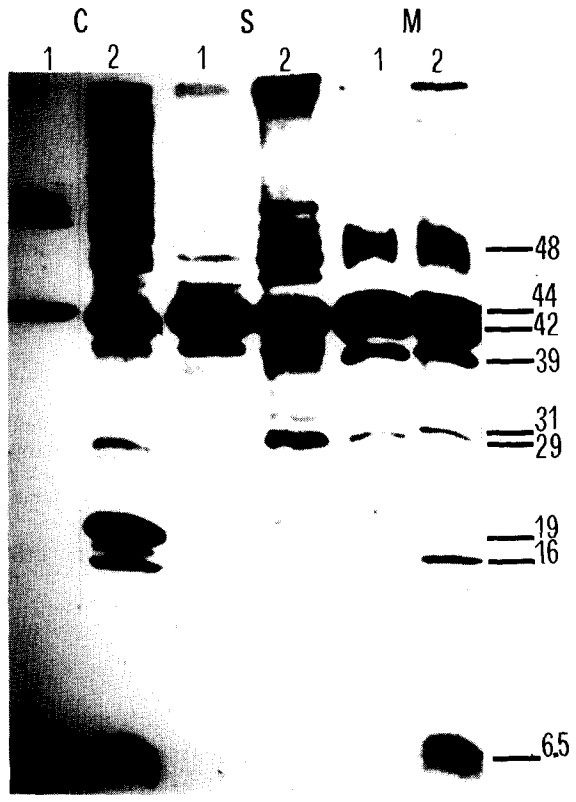

Fig. 2. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in cytosol (C), soluble (S) and membrane mitochondrial fraction (M) prepared and incubated with [γ-^{32}P]ATP as described in section 2. Lane 1, controls; lane 2, incubation in the presence of 50 μM cAMP.

Fig. 2 shows the labelling pattern by [γ-^{32}P]ATP of PAGE-resolved protein bands in the cytosol (C), the membrane (M) and soluble fraction (S) of mitochondria. cAMP-dependent phosphorylation of the protein bands of 16 and 6.5 kDa was clearly visible in the membrane fraction (M) (consisting essentially of scrambled inner membrane plus residual matrix and outer membrane; see section 2) but was undetectable in the soluble fraction (S) (consisting of matrix, outer membrane and contaminating cytosol; see section 2). In the membrane fraction, cAMP-independent labelling of protein bands of 48, 44, 42, 39 and 31 kDa was also seen. Labelling of the 42 kDa band was, however, significantly enhanced by cAMP

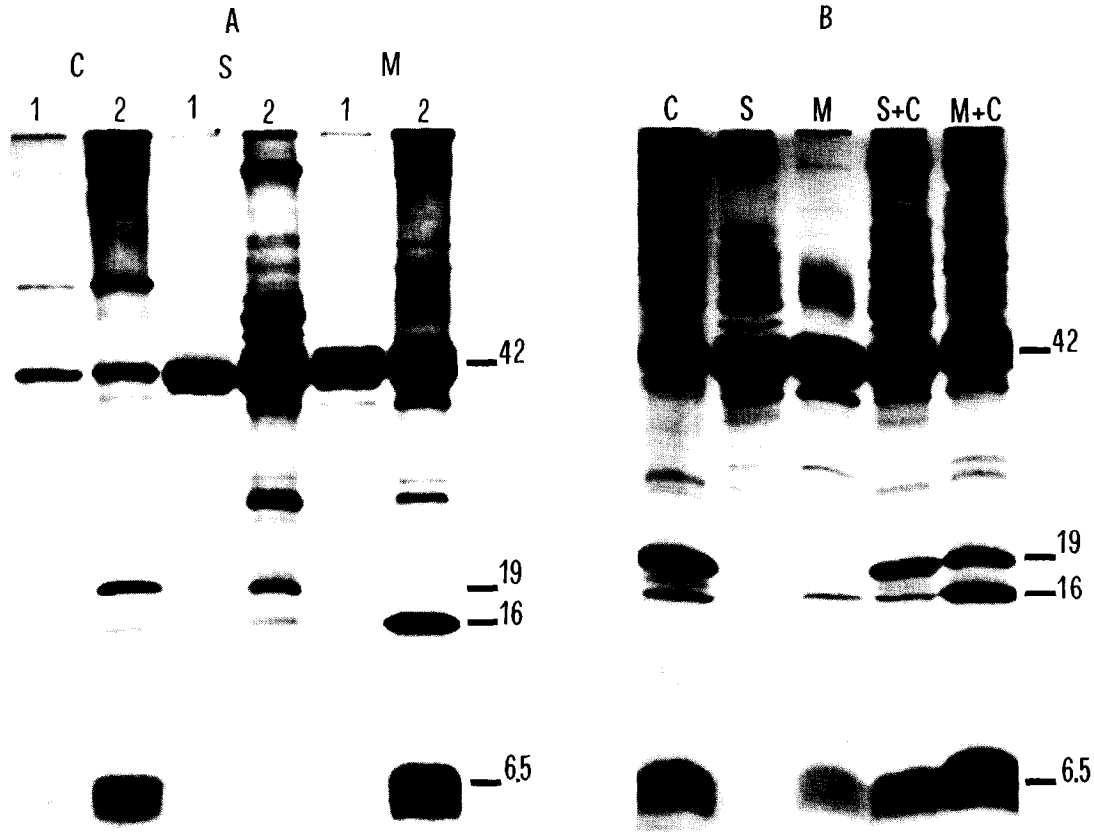

Fig. 3. (A) Phosphorylation of proteins in cytosol and mitochondrial fractions by added purified catalytic subunit of bovine heart cAPK. Cytosol (C), soluble (S) and membrane mitochondrial fraction (M). Preparation of fractions and incubation with [γ-^{32}P]ATP was as described in section 2. Lane 1, controls; lane 2, incubation in the presence of 10 U C-cAPK. (B) cAMP-dependent protein phosphorylation in cytosol (C), soluble (S), and membrane mitochondrial fraction (M), of mitochondria and combinations of (S) + (C) and (M) + (C). When fractions were combined half of the amount of each fraction (300 μg protein of each in 450 μl incubation mixture) was used. All the incubations were in the presence of 50 μM cAMP.

(see also Table 1). In the cytosol (C), cAMP-dependent phosphorylation of protein bands of 29, 19, 16 and 6.5 kDa was observed. cAMP also enhanced the labelling of the protein band of 42 kDa in the cytosol. The cAMP-dependent phosphorylation of protein bands was completely abolished in all the fractions by the synthetic rabbit cAPK inhibitor (not shown [14]).

The autoradiograms of Fig. 3A show that protein bands of 6.5 and 16 kDa in the membrane fraction (M) and, to some extent, also in the cytosol (C) were labelled by [γ-^{32}P]ATP upon addition of the purified catalytic subunit (C-cAPK) of cAPK from bovine heart. Added C-cAPK revealed little if any $^{32}P_i$-labelled 16 and 6.5 kDa proteins in the soluble mitochondrial fraction (S) (see also Table 1). C-cAPK in addition also phosphorylated the 42 kDa band in the membrane and soluble fractions of mitochondria. On the other hand, the amount of the 19 kDa protein band phosphorylated in the cytosol by added C-cAPK decreased going from this fraction to the soluble and membrane fraction of mitochondria.

Fig. 3B shows that addition of the cytosol to the membrane fraction resulted in a marked enhancement of cAMP-dependent phosphorylation of the protein bands of 42, 16 and 6.5 kDa.

In Fig. 4 the phosphorylation of mitochondrial proteins in the soluble and membrane fraction obtained by glass-bead disruption of the organelles is compared with that observed in these fractions obtained after exposure of mitochondria to ultrasound. Upon sonication the cAPK activity of the membrane fraction responsible for phosphorylation of the 42, 16 and 6.5 kDa proteins was released, together with these proteins, into the soluble fraction. A large part of the 16 and 6.5 kDa proteins, however, still remained associated to the membrane fraction where they were phosphorylated by added C-cAPK.

In Table 1, quantitative data on cAMP-dependent protein phosphorylation in the mitochondrial fraction and cytosol are presented. Phosphorylation by purified C-cAPK of the 6.5 and 16 kDa proteins in the membrane fraction, amounting to 6 and 9 pmol ^{32}P per mg total protein, was one order larger than in the soluble mitochondrial fraction. Phosphorylation by C-cAPK of the 42 kDa protein in the membrane fraction amounted to 12 pmol ^{32}P. About the same amount of this protein was phosphorylated in the soluble mitochondrial fraction by purified C-cAPK. The cAPK associated to the membrane fraction gave practically the same extent of phosphorylation of the 42 kDa protein as added C-cAPK.

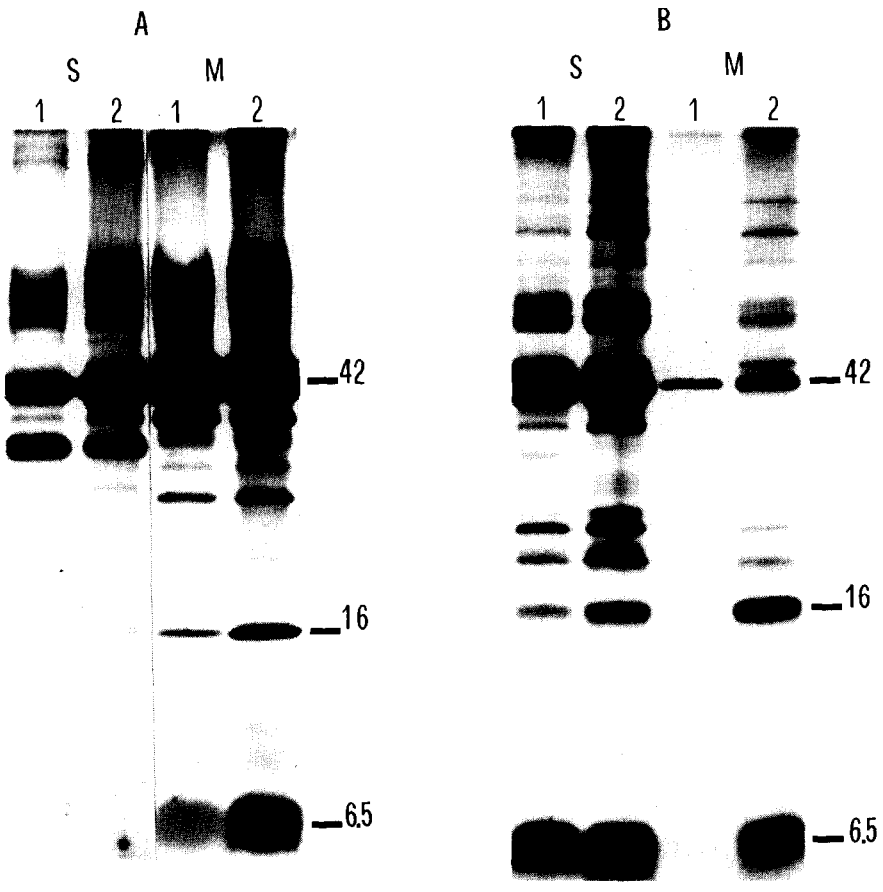

Fig. 4. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in soluble and inner membrane fractions of bovine mitochondria. (A) Fractions were obtained after glass bead disruption of mitochondria as described in section 2. (B) Mitochondria were disrupted by exposure to ultrasound (3 min at 0°C). After removal of residual mitochondria by centrifugation at 20,000 × g, the extract was incubated for 30 min at 0°C in 2 M KCl and membrane (M) and soluble fraction (S) separated by centrifugation at 100,000 × g. Fractions were incubated with [γ-^{32}P]ATP as described in section 2. Lane 1, incubation with 50 μM cAMP; lane 2, incubation with 10 U C-cAPK.

These data thus clearly show that the 42 kDa protein band, in addition to the E1α subunit of pyruvate dehydrogenase (the prominent component of this band [14]) which is phosphorylated by its cAMP-independent kinase, contains a different protein that is phosphorylated by the mitochondrial cAPK.

The 16 and 6.5 kDa proteins in the membrane fraction were, on the other band, less actively phosphorylated by the cAPK associated to this membrane than by added C-cAPK. Addition of cytosol to the membrane fraction resulted in full cAMP-dependent phosphorylation of the 16 and 6.5 kDa protein in the membrane. The amount of these proteins labelled by [γ-^{32}P]ATP was, in this case, larger than the sum of the extent of their labeling in the two fractions separately.

Added histone H2B, which was actively phosphorylated by the cytosol, was also phosphorylated in mitochondria. With this exogenous substrate cAMP-dependent phosphorylation was, however, ten times more active in the soluble mitochondrial fraction than in the membrane fraction (cf. [12,13]).

4. Discussion

The results presented identify in bovine heart three mitochondrial proteins of 42, 16 and 6.5 kDa, respectively, which are phosphorylated by mitochondrial cAMP-dependent protein kinase (cAPK).

These three phosphoproteins appear to be preferentially associated to the inner mitochondrial membrane. cAPK activity is also present in the inner membrane fraction. Most of this activity is, however, found in the soluble fraction of mitochondria (cf. [12,13]), where it is completely released upon exposure of mitochondria to ultrasound (see Fig. 4). Evidence has been presented showing that a mitochondrial cAPK is associated to the inner membrane [11–13]. This kinase is evidently responsible for the cAMP-dependent phosphorylation of the 42, 16 and 6.5 kDa proteins in the inner membrane. The possibility we previously entertained [14], that phosphorylation of these proteins could be due to contaminating cytosolic cAPK (cytosol contamination of the membrane fraction is not higher than 0.5%), is excluded by the

Table 1
Phosphorylation of mitochondrial proteins by endogenous cAMP-dependent kinase and by the catalytic subunit (C-cAPK) of purified protein kinase from bovine heart

Protein bands	pmol ^{32}P/mg total protein						
	Exp. 1: Mitochondrial inner membrane		Exp. 2: Soluble mitochondrial fraction		Exp. 3: Cytosol	Exp. 4: Inner membrane and cytosol	Exp. 4−(1 + 3)
(kDa)	cAMP	C-cAPK	cAMP	C-cAPK	cAMP	cAMP	
6.5	1.6 ± 0.20	6.4 ± 1.7	0.2 ± 0.06	0.2 ± 0.03	5.2 ± 0.3	13.0 ± 0.5	6.2
16	0.5 ± 0.05	8.7 ± 0.2	0.1 ± 0.03	0.5 ± 0.10	2.6 ± 0.1	11.5 ± 0.3	8.4
42	9.1 ± 1.10	12.5 ± 0.6	3.6 ± 0.70	10.1 ± 0.80	2.7 ± 0.0	13.1 ± 0.2	1.3
Histone (H2B)	37.5		375		3750		

The fractions were prepared and incubated with 70 μM [γ-^{32}P]ATP as described in section 2. PAGE bands were cut from gels and after measurement of radioactivity of the individual bands of interest, the amount of ^{32}P incorporated in the proteins was calculated from the specific activity of added ATP (1,000–1,500 cpm/pmol). Where indicate 150 μg of histone H2B were added in 450 μl of the incubation mixture as described in section 2. Phosphorylated histone was separated by PAGE and radioactivity measured on the isolated band. The values reported in the Table are the means of 4–5 experiments (except for histone) and were corrected for $^{32}P_i$ incorporation in the absence of added cAMP (50 μM) and C-cAPK (10 U). Histone phosphorylation in the membrane and soluble mitochondrial fractions was corrected for contribution by contaminating cytosol, amounting, respectively, to 0.4 and 5.0%.

present quantitative data on protein phosphorylation in the cytosol and mitochondrial fractions (Table 1). The 42 kDa protein, which is the protein to be more actively phosphorylated in the inner membrane fraction by the cAPK associated to this membrane, is easily solubilized together with cAPK. In addition to the E1α subunit of pyruvate dehydrogenase [14] the 42 kDa band contains other proteins, as revealed by preliminary two-dimension electrophoretic analysis.

The proteins of 16 and 6.5 kDa, which are less actively phosphorylated by the mitochondrial cAPK, appear to be more firmly associated to the inner mitochondrial membrane, from which they can not be completely released, even after prolonged exposure to ultrasound in high ionic strength medium (see Fig. 4).

The finding that the 16 and 6.5 kDa proteins in the inner membrane are fully phosphorylated by cAPK of the cytosol, when the two fractions are incubated together, is of particular interest. Proteins of 16 and 6.5 kDa in the cytosol are phosphorylated by the cAPK of this fraction. If the 16 and 6.5 kDa proteins phosphorylated in the cytosol are the same as those phosphorylated in the inner membrane fraction it would mean that the cytosolic and the mitochondrial cAPK isoenzymes phosphorylate the same proteins in the two cellular compartments. Reversible phosphorylation of these two proteins could regulate their post-translational traffic in the cell, post-translational proteolytic maturation and/or turnover [20], and their biological activity. Work aimed to isolate and characterize these phosphoproteins is in progress in our laboratories.

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