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Topology of the mitochondrial cAMP-dependent protein kinase and its substrates

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Abstract In intact bovine heart mitochondria, cAMP-dependent phosphorylation of 42, 29, 18 and 6.5 kDa proteins was inhibited by carboxyatractyloside. This shows that both mitochondrial cAMP-dependent protein kinase (mtPKA) and its protein substrates are localized at the matrix side of the inner mitochondrial membrane. Proteins of 42, 29, 18, and 6.5 kDa were also bound at the outer surface of mitochondria where they were phosphorylated by the added purified catalytic subunit of PKA. In the cytosol from bovine heart proteins of the above molecular weights were phosphorylated by the cytosolic PKA.

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

1. Introduction

Recent work from our group has shown that in bovine heart mitochondria, proteins of 42, 18 and 6.5 kDa, associated to the inner mitochondrial membrane, are phosphorylated by a cAMP-dependent protein kinase also present in the inner mitochondrial membrane fraction (mtPKA) ([1,2]; see also [3,4]). The 42 kDa phosphoprotein was found to be loosely associated with NADH-ubiquinone oxidoreductase (complex I), ubiquinone–cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV) and F_0F_1 ATP synthase (complex V) of the inner mitochondrial membrane, from which it was removed upon their purification [5]. The 18 kDa phosphoprotein copurified with complex I and resulted by sequence analysis to correspond to the nuclear-encoded 18 kDa (IP)AQDQ subunit of this complex [6]. Protein bands of 42, 18 and 6.5 kDa were, however, found to be phosphorylated also by the cAMP-dependent protein kinase of the cytosol (cPKA) [2].

In this paper it is shown that, in intact bovine heart mitochondria, cAMP-dependent phosphorylation of 42, 29, 18 and 6.5 kDa proteins is strongly inhibited by carboxyatractyloside. Thus, both mtPKA and its protein substrates are localized at the matrix side of the inner mitochondrial membrane. Proteins of 42, 29, 18 and 6.5 kDa are also bound at the outer surface of mitochondria where they are phosphorylated by the purified catalytic subunit of PKA.

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Abbreviations: PMSF, phenylmethylsulfonil fluoride; PAGE, polyacrylamide gel-electrophoresis; SDS, sodium dodecylsulphate; mtPKA, mitochondrial cAMP-dependent protein kinase; C-cPKA, catalitic subunit of purified PKA

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP 3000 Ci/mmol and Hyperfilm-MP were from Amersham International; catalytic subunit of cAMP-dependent protein kinase purified from bovine heart, carboxyatractyloside and dibutyryl-cAMP were from Sigma. Dynagel was from V.T. Baker Holland. All other reagents were of the highest purity grade commercially available.

2.2. Methods

Cytosol and heavy intact mitochondria were isolated from beef heart as in [7]. Respiratory rate control of mitochondria measured as in [8] amounted to around eight with pyruvate+malate as respiratory substrates. ADP-induced stimulation of respiration was 70–80% suppressed by 5 μM carboxyatractyloside.

Protein phosphorylation was assayed by incubating, for 5 min at 30°C under stirring, 600 μg protein of mitochondria or cytosol in 450 μl of 0.25 M sucrose adjusted to pH 7.4 with Tris, 8 mM $MgCl_2$, 0.25 μM PMSF, 20 mM NaF, 3 μg rotenone, 3 μg oligomycin and 70 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol). To stop the reaction, 150 μl (200 μg of proteins) were mixed with 40 μl of 0.35 M Tris-HCl, pH 6.8, 10% glycerol, 15% w/v SDS, 25% v/v β-mercaptoethanol and boiled for 3 min.

Gel electrophoresis and autoradiography were performed as in [1]. Radioactive PAGE bands were cut from the gels, immersed in Dynagel, and radioactivity was measured in a Liquid Scintillation Counter as in [2].

3. Results

Fig. 1 (Expt. 1) shows the autoradiograms of a typical experiment in which freshly isolated bovine heart mitochondria were incubated with [γ-^{32}P]ATP and the ^{32}P-labeling pattern of mitochondrial proteins, resolved by SDS-PAGE, was analyzed. In the absence of added effectors (lane 1.A) a heavily labeled protein band of 42 kDa was observed. This phosphoprotein corresponds to the E1α subunit of pyruvate dehydrogenase [1], which is phosphorylated by its specific cAMP-independent protein kinase [9]. The addition of cAMP (lane 2.A) promoted phosphorylation of the protein bands of 29, 18 and 6.5 kDa. Phosphorylation of these bands was even more markedly stimulated by butyryl-cAMP which also enhanced the phosphorylation of the 42 kDa protein band (lane 3.A, see also Table 1). Phosphorylation of protein bands of 29, 18 and 6.5 kDa was also catalyzed by the addition to the mitochondrial suspension of the purified catalytic subunit of bovine heart cAMP-dependent protein kinase (C-cPKA) (see lane 4.A and Table 1). The cAMP- and butyryl-cAMP-dependent phosphorylation of the protein bands of 29, 18 and 6.5 kDa was strongly inhibited by treatment of mitochondria with 5 μM carboxyatractyloside which also depressed the phosphorylation of the 42 kDa protein band (see lanes 1–3 of panel B

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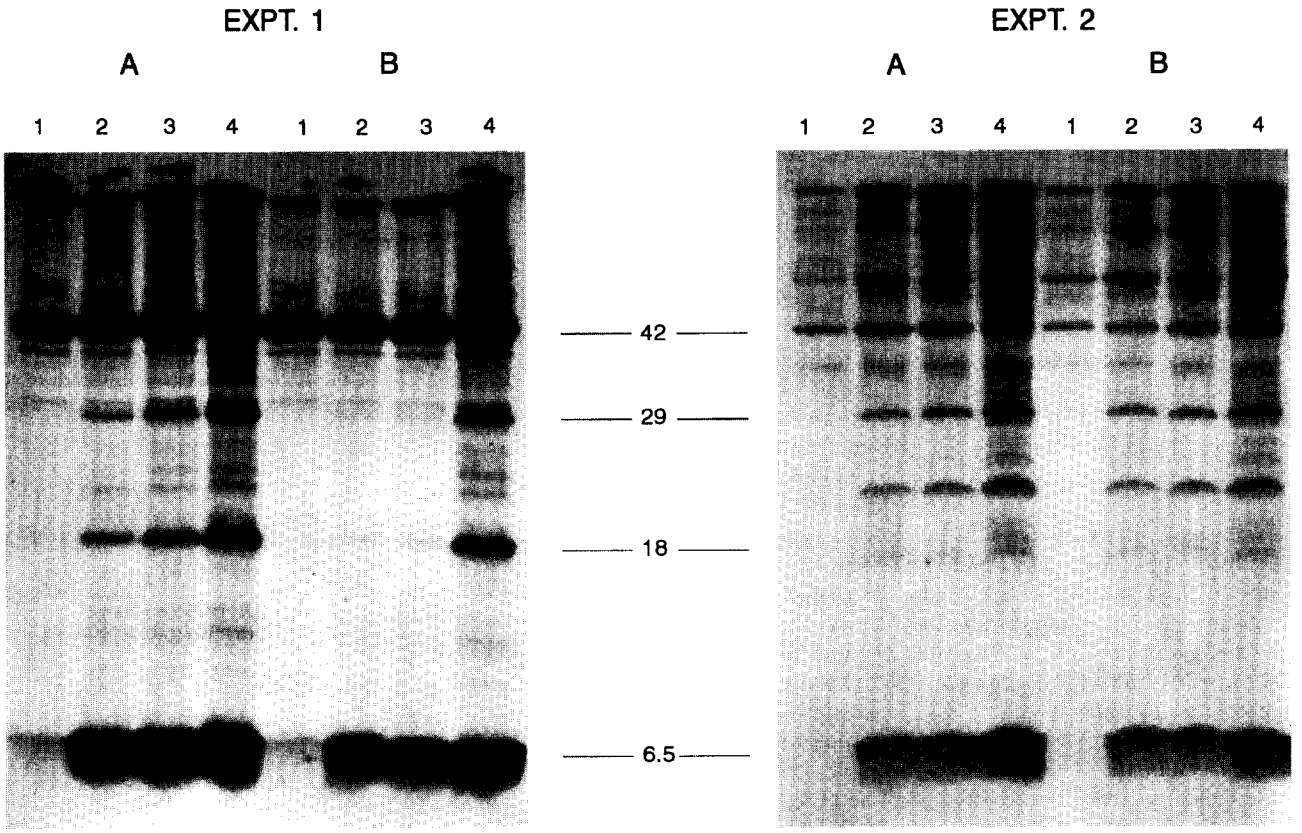

Fig. 1. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in mitochondria and cytosol from bovine heart. Freshly isolated bovine heart mitochondria (Expt. 1) and cytosol (Expt. 2), were incubated with [γ-^{32}P]ATP as described under Section 2. In Expts. 1B and 2B mitochondria and cytosol, respectively, were preincubated 5 min with 5 μM carboxyatractyloside before the addition of [γ-^{32}P]ATP. Lane 1, controls; lane 2, incubation in the presence of 50 μM cAMP; lane 3, incubation in the presence of 50 μM butyryl-cAMP; lane 4, incubation in the presence of 10 U C-cPKA. Molecular weights of labeled protein bands, also detected by Coomassie blue, were determined from standard proteins run on the same gels. The molecular weights in kDa are given between the autoradiograms. For other details see Section 2.

and Table 1). The phosphorylation of the protein bands of 42, 29, 18 and 6.5 kDa catalyzed by added C-cPKA was, on the contrary, unaffected by carboxyatractyloside (lane 4.B, see also Table 1).

A similar experiment, carried out using the cytosol from bovine heart (Fig. 1, Expt. 2), showed that also in this fraction phosphorylation of protein bands of 42, 29, 18 and 6.5 kDa was promoted by the addition of cAMP, butyryl-cAMP and C-cPKA (lanes 2.A, 3.A and 4.A). In the cytosol phosphorylation of these proteins promoted by butyryl-cAMP was not inhibited by carboxyatractyloside (Fig. 1, Expt. 2 and Table 1). This shows that carboxyatractyloside has per se no direct inhibitory effect on PKA.

4. Discussion

The results presented, whilst clearly confirming the existence of a mitochondrial cAMP-dependent protein kinase (mtPKA) [1–4], show unequivocally, that the mitochondrial form of this enzyme and a number of mitochondrial proteins

Table 1
Protein phosphorylation in mitochondria and cytosol from bovine heart by endogenous cAMP-dependent protein kinase and by the catalytic subunit (C-cPKA) of purified protein kinase

Protein bands	pmol ^{32}P/mg total mitochondrial proteins				pmol ^{32}P/mg total cytosol proteins	
	Control		Carboxyatractyloside		Control	Carboxyatractyloside
(kDa)	b-cAMP	C-cPKA	b-cAMP	C-cPKA	b-cAMP	b-cAMP
42	2.05	2.45	0.15	2.10	0.69	0.67
29	0.75	1.00	0.10	1.30	0.60	0.62
18	0.68	1.44	0.18	1.00	0.22	0.49
6.5	2.5	3.20	1.35	4.50	2.85	2.50

Mitochondria and cytosol were prepared and incubated with 70 μM [γ-^{32}P]ATP in the presence and in the absence of carboxyatractyloside (5 μM) as described under Section 2 and in the legends to Fig. 1. 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 protein was calculated from the specific activity of added ATP (1000–2000 cpm/pmol). For mitochondria the values reported in the table are the means of 3–4 experiments. All the values were corrected for ^{32}P incorporation in the absence of added butyryl cAMP (b-cAMP) and C-cPKA).

substrates of this kinase are localized at the matrix side of the inner mitochondrial membrane. The inhibitory effect exerted by carboxyatractyloside on mitochondrial protein phosphorylation, promoted by cAMP and butyryl-cAMP, results from inhibition of the entry of [γ-^{32}P]ATP into the matrix mediated by the adenine translocator [10]. The inhibition is, in fact, observed also when phosphorylation of the mitochondrial proteins is stimulated by butyryl-cAMP, the lipophilic analog of cAMP which freely permeates the mitochondrial membrane. The fact that butyryl-cAMP exerts a more marked effect than cAMP in promoting phosphorylation of mitochondrial proteins, but not of cytosolic proteins (see Fig. 1), indeed provides further evidence for the location of mtPKA and its substrates at the matrix side of the mitochondrial membrane. By which mechanism cAMP is transported into mitochondria (or produced there), it remains to be clarified. The carboxyatractyloside-insensitive phosphorylation by added C-cPKA of proteins asssociated to mitochondria shows that proteins of the same molecular weights as those phosphorylated by the mtPKA inside the inner mitochondrial membrane, i.e., proteins of 42, 29, 18 and 6.5 kDa, are also associated with the outer surface of mitochondria where they can be phosphorylated by the cytosolic form of PKA (cf., [1] and [2]). In the cytosol protein bands of these molecular weights are, in fact, phosphorylated by the cytosolic PKA. Since the mitochondrial proteins phosphorylated by mtPKA do not correspond to any of the subunits of the respiratory complexes and ATP synthase coded by the mitochondrial genome [3,4], they are synthesized in the cytosol. If proteins of 42, 29, 18, and 6.5 kDa, phosphorylated in the cytosol by the cytosolic PKA and by added C-cPKA when bound at the outer surface of mitochondria, are the same as those of the same molecular weights phosphorylated at the inner side of the inner mitochondrial membrane, they could be phosphorylated 'in vivo' by the PKA in the cytosol on their way into the organelle. Reversible phosphorylation of these proteins could regulate their transport into mitochondria [1,2]. The proteins of 42 and 6.5 kDa phosphorylated by mtPKA were previously found to be loosely associated to the inner mitochondrial membrane [1,2]. They can be distributed between the matrix surface of the inner membrane and the matrix. The phosphoprotein of 29 kDa, which is detected in intact mitochondria but not in the inner membrane fraction [1,2], is apparently a matrix protein. Work is in progress in our laboratory, along the lines reported in [6], to identify the mitochondrial proteins phosphorylated by mtPKA and clarify their eventual role.

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