Volume 322, number 1, 51–55 FEBS 12390 May 1993

Phosphorylation of mitochondrial proteins in bovine heart

Characterization of kinases and substrates

Zuzana Technikova-Dobrova[a,b], Anna Maria Sardanelli[a] and Sergio Papa[a]

[a]*Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy* and [b]*Institute of Microbiology, Czech Academy of Sciences, Praha, Czech Republic*

Received 5 March 1993

Protein phosphorylation by [γ-^{32}P]ATP in total extract and subfractions of bovine heart mitochondria has been studied. The results show that, in addition to pyruvate dehydrogenase, three mitochondrial proteins, with molecular weights of 44,000, 39,000 and 31,000 Da, are phosphorylated by a cAMP-independent mitochondrial protein kinase. Three other proteins associated with mitochondria, with molecular weights of 125,000, 19,000 and 6,500 Da, are phosphorylated by the cytoplasmic cAMP-dependent protein kinase (kinase A).

Mitochondrion; Protein phosphorylation; Protein kinase; Cyclic AMP; Cyclic AMP-dependent protein kinase

1. INTRODUCTION

Protein phosphorylation represents a general regulatory mechanism for signal transduction and control of gene expression, metabolism, cell growth, differentiation and oncogenesis [1]. Many protein kinases (and phosphatases) have been identified in cellular compartments, and their number, substrates and roles are still increasing [1,2].

Definite progress has been made in the elucidation of the role of protein phosphorylation at the plasma membrane, in cytosol, nucleus, endoplasmic reticulum and other cellular structures [1,2]. As far as mammalian mitochondria are concerned, fragmentary and contradictory reports have appeared on cAMP-dependent [3] and cAMP-independent [4,5] phosphorylation of unidentified proteins, apparently distinct from pyruvate dehydrogenase kinase [6] and branched-chain ketoacid dehydrogenase kinase [7]. In yeast mitochondria, phosphorylation of various, as yet unidentified, proteins has been observed and evidence of the existence of mitochondrial cAMP-independent and cAMP-dependent kinases presented [8].

This paper reports on phosphorylation of mitochondrial proteins from bovine heart. Results on the cAMP dependence of protein phosphorylation, involvement of the cytoplasmic cAMP-dependent protein kinase (kinase A) [9] and existence of cAMP-independent mitochondrial protein kinase are presented.

Correspondence address: Z. Technikova-Dobrova, Institute of Medical Biochemistry and Chemistry, University of Bari, Bari, Italy.

Abbreviations: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; bc_1, ubiquinol-cytochrome *c* reductase; SDS, sodium dodecyl sulphate.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP, 3,000 Ci/nmol, and Hyperfilm-MP, were from Amersham International; DNase and RNase were from Boehringer-Mannheim; and dephosphorylated casein from bovine milk and protein kinase inhibitor (rabbit sequence) [10] were from Sigma. All other reagents were of the highest purity grade commercially available.

2.2. *Preparation of mitochondria and their fractionation*

Heavy beef heart mitochondria were isolated as in [11]. Mitochondrial fractions were prepared in two ways. (a) 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. (b) Mitochondria, 30 mg protein per ml, were suspended in the mixture described in a, and exposed to ultrasonic energy. 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, and another was centrifuged at 100,000 × *g* to obtain the pellet and supernatant fraction (S1). The presence of cytosol in the fractions was estimated from measurement of lactate dehydrogenase, matrix from measurement of aconitase, and inner membrane from spectrophotometric determination of cytochromes $a + a_3$ [12]. The resulting pellet consisted of inner membrane plus residual matrix. The orientation of the inner membrane was determined as in [12].

2.3. *Protein kinase assay*

Protein phosphorylation was assayed by incubating, for 20 min at 30°C under stirring, 600 μg protein of each mitochondrial fraction in 450 μl of 10 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 20 mM NaF, 3 μg oligomycin and 70 μM [γ-^{32}P]ATP (500 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*

Boiled samples were subjected to gel electrophoresis on slabs of 12% polyacrylamide plus 0.1% SDS. Gels were fixed in 50% methanol plus 10% acetic acid, stained with 0.1% Coomassie blue, destained in the same mixture, photographed and dried for autoradiography. Radioactive bands were visualized by exposure to Hyperfilm (Amersham). For molecular weight assignment, Bio-Rad standards, bc_1 complex [13] and cytochrome *c* oxidase [12] purified from bovine heart were used.

2.5. *Immunodetection*

Immunodetection of pyruvate dehydrogenase was carried out using a polyclonal antibody against the purified enzyme provided by Dr. M.S. Patel (Case University, Cleveland).

2.6. *Thin layer chromatography*

Thin layer chromatography of phospholipids was carried out as in [14].

3. RESULTS

Protein phosphorylation was first examined in total extracts of bovine heart mitochondria disrupted with glass beads. A typical labeling pattern of PAGE protein bands from the extract incubated with [γ-^{32}P]ATP is presented in Fig. 1. In the absence of added cAMP protein bands (detected by Coomassie blue, not shown), with molecular weights of 105,000 (not constantly observed), 55,000, 48,000, 44,000, 42,000, 39,000, 31,000 and 29,000 Da were primarily labeled. Immunoblotting showed that the protein band of 42,000 Da reacted with an antibody against pyruvate dehydrogenase. The addition of 50 μM cAMP produced clear labeling of protein bands with molecular weights of 125,000, 29,000, 19,000 and 6,500 Da. Thin layer chromatography showed that phospholipids and the radioactive material from the mitochondrial extract migrated in two distinct spots. Furthermore, incubation of the mitochondrial extract with purified DNase and RNase did not affect the labeling pattern.

The glass bead extract of mitochondria was then fractionated into 100,000 × *g* pellet and supernatant (S1). The Coomassie blue electrophoretic patterns of these fractions (Fig. 2A) showed, in particular, that protein bands with molecular weights of 14,000 Da and lower, present in the pellet, were undetectable in the supernatant.

In the pellet fraction (scrambled inner membranes with a minor amount of residual matrix) incubation with [γ-^{32}P]ATP produced, in the absence of cAMP, labeling of the protein bands with molecular weights of 44,000, 42,000 and 39,000 Da (Fig. 2B). Addition of cAMP resulted in labeling of protein bands with molecular weights of 125,000, 19,000 and 6,500 Da. Phosphorylation of these protein bands was, however, no longer detectable when mitochondria were swollen in a phosphate buffer and the soluble material (eventual contaminating cytosol, outer membrane and inter-membrane fluid) removed before glass bead disruption and fractionation (see Fig. $2B_{iv}$). Under these conditions, cAMP-independent phosphorylation of protein bands

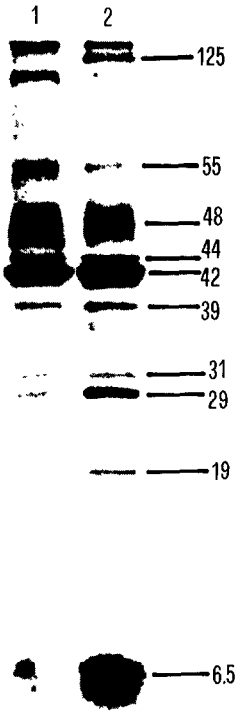

Fig. 1. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in total extract obtained by glass bead disruption of beef heart mitochondria. Mitochondrial extract was incubated with [γ-^{32}P]ATP as described in section 2. Lane 1, control; lane 2, incubation in the presence of 50 μM cAMP. Molecular 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 autoradiograms. For other details see section 2.

with molecular weights of 48,000 and 31,000 Da could also be seen.

Added casein was phosphorylated in the pellet fraction (band of 31,000 Da), both in the absence and presence of cAMP. Casein addition also caused striking enhancement of the cAMP-independent phosphorylation of the protein bands of 44,000, 42,000, and 39,000 Da, as well as of the cAMP-dependent phosphorylation of the protein bands of 125,000, 48,000, 19,000, and 6,500 Da. Addition of purified bc_1 complex or of bovine serum albumin (not shown) promoted cAMP-independent phosphorylation of the protein bands of 44,000, 42,000, 39,000 and 31,000 Da.

Incubation of the supernatant (S1) (containing matrix and outer membrane) with [γ-^{32}P]ATP gave, in the absence of cAMP, only labeling of protein bands with molecular weights of 58,000 (not constantly observed), 55,000, 44,000 and 42,000 Da. In S1, cAMP promoted phosphorylation of protein bands with molecular weights of 48,000 and 29,000 Da.

In another set of experiments mitochondria were disrupted by ultrasound and fractionated into 100,000 × *g* pellet and supernatant (S2). The Coomassie blue electrophoretic patterns showed that some of the proteins,

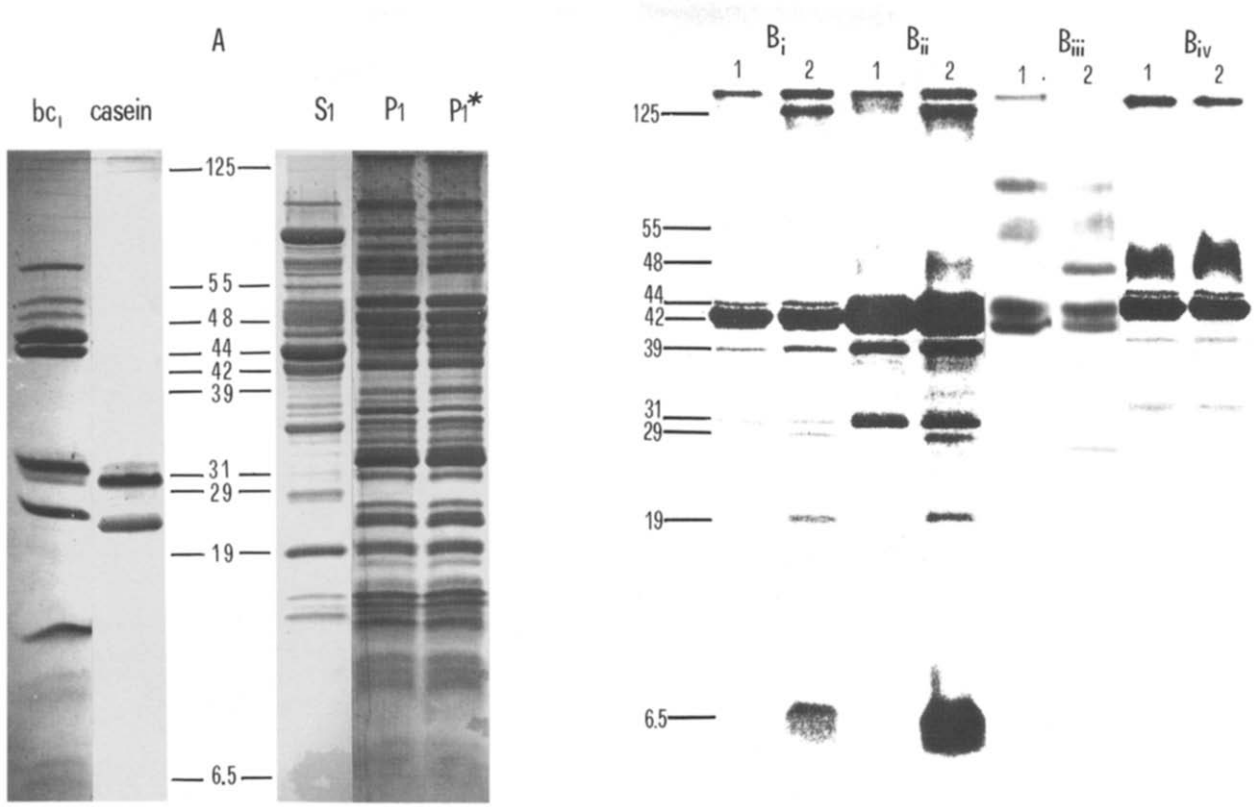

Fig. 2. Coomassie blue staining patterns and autoradiograms of protein bands labeled by [γ-^{32}P]ATP in pellet (P1) and supernatant (S1) obtained after glass bead disruption of beef heart mitochondria. Mitochondrial subfractions were incubated with [γ-^{32}P]ATP as described in section 2. (A) Coomassie blue staining pattern of PAGE polypeptide bands. (B) Autoradiograms of PAGE slabs. Intact beef heart mitochondria were disrupted by glass beads and fractionated into pellet (B_i and B_{ii}) and supernatant (B_{iii}), as described in section 2. The pellet exhibited an aconitase activity of 2 nmol · min^{-1} · mg protein^{-1}, was practically devoid of lactate dehydrogenase activity and contained 0.52 nmol hemes $a + a_3$ per mg protein. Hemes were 50% reduced directly by ascorbate; full reduction was achieved by adding phenazine methasulphate together with ascorbate [12]. The S1 fraction was devoid of hemes a and a_3; it exhibited an aconitase activity of 40 nmol · min^{-1} · mg protein^{-1} and lactate dehydrogenase activity of 30 nmol · min^{-1} · mg protein^{-1}. B_i, control; B_{ii}, 0.25 mg casein added per mg pellet protein; B_{iv}, pellet from mitochondria swollen by stirring for 15 min at 0°C in 0.1 M sorbitol, 20 mM potassium phosphate, 1 mM EGTA, 50 μM PMSF, pH 7.5, 1 mg mitochondrial protein per ml, sedimented by centrifugation at 20,000 × g and fractionated. The Coomassie blue pattern of the P1 fraction obtained from swollen mitochondria is indicated in A by an asterisk. Lanes 1, controls; lanes 2, incubation in the presence of 50 μM cAMP. For other details see section 2.

in particular those of 14,000 Da and smaller, absent in the soluble fraction (S1) obtained after glass bead disruption of mitochondria, were released upon sonication in the soluble fraction (S2). The autoradiograms presented in Fig. 3 show that phosphorylation of the protein bands observed in the total mitochondrial extract did occur in the soluble fraction S2; in particular, both the cAMP-independent and -dependent phosphorylation of proteins observed in the particulate fraction obtained by glass bead disruption of mitochondria did occur in the soluble S2 fraction. In S2 casein (Fig. 3c), which was itself phosphorylated, enhanced, as already observed in the particulate fraction, cAMP-independent phosphorylation of polypeptide bands of 44,000, 42,000 and 39,000 Da, as well as cAMP-dependent phosphorylation of protein bands of 125,000, 58,000, 48,000, 19,000 and 6,500 Da. Added bc_1 complex (Fig. 3d) or cytochrome c oxidase (Fig. 3e) enhanced cAMP-independent phosphorylation of the polypeptide bands of 44,000, 42,000, 39,000 and 31,000 Da.

The addition of the synthetic, rabbit protein kinase A inhibitor [10] (Fig. 3b) suppressed in S2 all the cAMP-dependent protein phosphorylation (protein bands of 125,000, 48,000, 29,000, 19,000 and 6,500 Da) but did not depress, or even slightly enhanced, the cAMP-independent phosphorylation of the protein bands of 44,000, 42,000, 39,000 and 31,000 Da.

4. DISCUSSION

The present results show that 10 different proteins, at least, are phosphorylated by ATP in total extracts of bovine heart mitochondria. The protein band of 42,000 Da consists essentially of pyruvate dehydrogenase, as shown by its reaction with an antibody against the isolated enzyme and the cAMP-independence of its phosphorylation by ATP [6].

The protein bands with molecular weights of 125,000, 19,000 and 6,500 Da are loosely associated with the inner membrane where they are phosphorylated by a

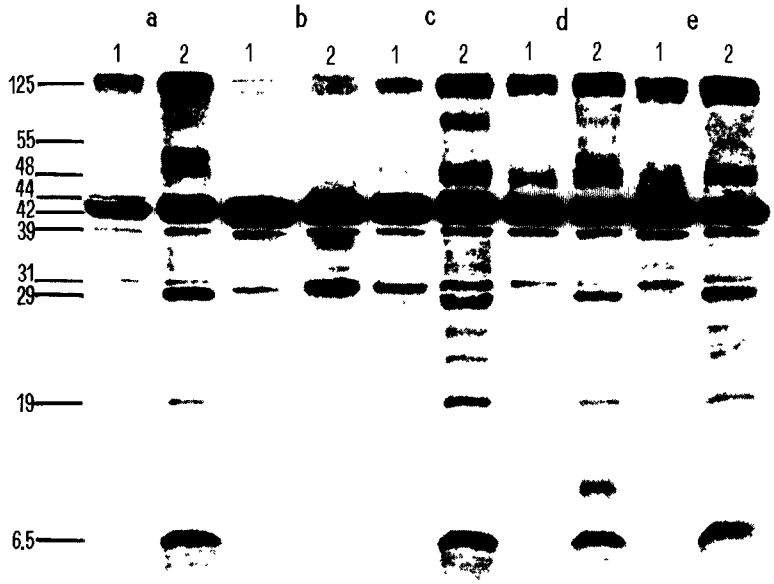

Fig. 3. Autoradiograms of protein bands labeled by [γ-^{32}P]ATP in the supernatant fraction (S2) obtained after mitochondrial disruption with ultrasound. For experimental conditions see legend to Fig. 2 and section 2. a, control; b, plus 0.25 mg kinase A inhibitor per mg protein S2; c, plus 0.25 mg casein per mg protein S2; d, plus 0.25 mg bc_1 complex per mg protein S2; e, plus 0.25 mg cytochrome oxidase per mg protein S2. Lanes 1, control; lanes 2, incubation in the presence of 50 μM cAMP. For other details see section 2.

cAMP-dependent kinase. This kinase activity is identified with the cytoplasmic cAMP-dependent protein kinase (kinase A) contaminating the mitochondrial fraction.

The protein bands with molecular weights of 44,000, 39,000 and 31,000 Da are also associated with the inner membrane where they are phosphorylated by a cAMP-independent kinase activity. This kinase and its substrate proteins are largely released upon sonication of mitochondria in the soluble fraction. Evidence that this activity is a mitochondrial protein kinase distinct from kinase A is supported by the insensitivity of labeling of the 44,000, 39,000 and 31,000 Da protein bands to the inhibitor of kinase A, and their persistent phosphorylation in the pellet obtained from swollen and washed mitochondria.

The stimulation by casein of cAMP-dependent and -independent phosphorylation of mitochondrial proteins is of particular interest. Stimulation of cAMP-independent protein phosphorylation was also observed upon addition of isolated bc_1 complex and cytochrome *c* oxidase, or of bovine serum albumin. These three proteins did not, however, affect the phosphorylation of the protein bands of 19,000 and 6,500 Da, which thus do not appear to contain subunits of the two redox enzymes. Stimulation of protein kinases, by serum albumin and cellular protein factors, has already been reported. Non-substrate proteins can apparently activate protein kinases by conformational effects (chaperons) [15]. It is worth mentioning in this respect that core protein I of the bc_1 complex has been proposed to belong to a family of chaperon proteins, which modulate the activity of mitochondrial processing peptidase [16].

The subcellular localization of the protein of 29,000 Da, which distributed in the soluble fraction of mitochondria and was apparently phosphorylated by protein kinase A, remains to be ascertained. The cAMP-dependent fraction of the phosphoprotein band of 48,000 Da, has to be different from branched-chain ketoacid dehydrogenase of the same molecular weight, the mitochondrial specific kinase of which is cAMP-independent.

In conclusion in bovine heart three mitochondrial proteins associated with the inner membrane are phosphorylated by a cAMP-independent protein kinase present in the same fraction. Three other proteins apparently associated with this fraction are phosphorylated by the cytoplasmic cAMP-dependent protein kinase A. In vivo these proteins (125,000, 19,000 and 6,500 Da) might be phosphorylated by kinase A in the cytosol before they are imported into mitochondria.

It is conceivable that phosphorylation of mitochondrial proteins by mitochondrial and cytosolic protein kinases plays a critical role in the biogenesis and function of mitochondria. There is evidence that glucacon [17] and gonadotropins [3] might influence mitochondrial metabolism. It has also been reported that glucacon stimulates phosphorylation of mitochondrial proteins in vivo [18].

Acknowledgements: This work was supported in part by Grant 91.04203.ST75 of CNR, Italy.

REFERENCES

[1] Edelman, A.M., Blumenthal, D.K. and Krebs, E.G. (1987) Annu. Rev. Biochem. 56, 567–613.

[2] Papa, S., Azzi, A. and Tager, J.M. (1992) Adenine Nucleotides in Cellular Energy Transfer and Signal Transduction, Birkhäuser Verlag Basel, Switzerland.
[3] Dimino, H.J., Bieszczad, R.R. and Rowe, M.J. (1981) J. Biol. Chem. 256, 10876–10882.
[4] Vardanis, A. (1977) J. Biol. Chem. 252, 807–813.
[5] Kitagawa, Y. and Racker, E. (1982) J. Biol. Chem. 267, 4547–4551.
[6] Reed, L.J. and Yeaman, S.J. (1987) Enzymes 18, 77–95.
[7] Randle, P.J., Patson, P.A. and Espinal, J. (1987) Enzymes 18, 97–121.
[8] Müller, G. and Bandlow, W. (1987) Yeast 3, 161–174.
[9] Taylor, S.S., Buechler, J.A. and Yonemoto, W. (1990) Annu. Rev. Biochem. 59, 971–1005.
[10] Cheng, H.C., Kemp, B.E., Pearson, R.B., Smith, A.J., Misconi, L., Van Patten, S.M. and Walsh, D.A. (1986) J. Biol. Chem. 261, 989.
[11] Low, H. and Vallin, I. (1963) Biochim. Biophys. Acta 69, 301–302.
[12] Capitanio, N., De Nitto, E., Villani, G., Capitanio, G. and Papa, S. (1990) Biochemistry 29, 2939–2945.
[13] Lorusso, M., Cocco, T., Sardanelli, A.M., Minuto, M., Bonomi, F. and Papa, S. (1991) Eur. J. Biochem. 197, 555–561.
[14] Landriscina, C., Gnoni, G.V. and Quagliarello, E. (1972) Eur. J. Biochem. 29, 188–196.
[15] Racker, E. (1991) Methods Enzymol. 200, part A, 107–111.
[16] Weiss, H., Leonard, K. and Neupert, W. (1990) Trends Biochem. Sci. 178–180.
[17] Halestrap, A.P. (1978) Biochem. J. 172, 399–405.
[18] Zahlten, R.N., Hoehberg, A., Stratman, F.W. and Lardy, H.A. (1972) Proc. Natl. Acad. Sci. USA 69, 800–804.