FEBS Letters 377 (1995) 470–474 FEBS 16478

Characterization of proteins phosphorylated by the cAMP-dependent protein kinase of bovine heart mitochondria

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Received 8 November 1995

Abstract Characterization of two mitochondrial proteins of M_r 42 and 18 kDa, respectively, phosphorylated by the cAMP-dependent protein kinase of bovine heart mitochondria (mtPKA), is presented. A 42 kDa protein is found to be loosely associated to complexes I, III and IV of the respiratory chain and complex V (ATP synthase) in the inner mitochondrial membrane. An 18 kDa protein is associated to complex I in the inner membrane and in a purified preparation of this complex where it can be phosphorylated by the isolated catalytic subunit of PKA.

Key words: cAMP-dependent protein kinase; Mitochondrial phosphoprotein; Respiratory complex; NADH-ubiquinone oxidoreductase

1. Introduction

Protein phosphorylation by the cAMP-dependent protein kinase (PKA) represents the central mechanism in the transduction of signals of hormones, like glucagon, gonadotropins, cathecolamines, etc., in eukaryotic cells [1,2]. Despite advanced knowledge on the structure [3] and the mechanism of action of PKA [2,3] its intracellular distribution is still under investigation. In addition to cytosolic PKA, there is evidence for compartmentalized pools of PKA [4–6], which could be involved in the phosphorylation of specific proteins in response to appropriate hormones [6]. Among the distinct and diverse physiological processes, that are evoked by PKA mediated hormones, it has been observed that glucagon promotes the phosphorylation of mitochondrial proteins [7,8] and affects mitochondrial functions [9]. cAMP itself seems to affect mitochondrial biogenesis in yeast [10,11] and cAMP-binding proteins have been found in mitochondria [12,13].

Recent work from our group has shown that in bovine heart mitochondria, various proteins are phosphorylated by cAMP-dependent protein kinase (mtPKA), present in these organelles [14,15]. Evidence for the existence of a mtPKA in mammalian [16–18] and yeast mitochondria [19] has also been presented by others. Among the various proteins found to be phosphorylated by the mtPKA, 42 kDa and 16–18 kDa proteins (apparent M_r depending on the electrophoretic procedure) were observed [14,15]. We have now found that an 18 kDa protein phosphorylated by mtPKA is associated with NADH-ubiquinone oxidoreductase (complex I) in the inner mitochondrial membrane and in its isolated preparation. Protein(s) of 42 kDa, phosphorylated by the mtPKA, is(are) also found to be loosely associated with complex I as well as with bc_1 complex, cytochrome c oxidase and ATP synthase (complexes III, IV and V) in the inner mitochondrial membrane.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP 3000 Ci/mmol was from Amersham International; catalytic subunit of cAMP-dependent protein kinase purified from bovine heart, from Sigma. Laurylmaltoside (dodecyl-β-D-maltoside) from Boehringer; 6-aminocaproic acid from Fluka; Coomassie blue G from Serva.

2.2. Preparation of mitochondrial membrane fraction and complex I

Heavy mitochondria were isolated from beef heart as in [20]. The inner membrane mitochondrial fraction (IM) was prepared as in [15]. Complex I was purified from bovine heart mitochondria as in [21], complexes III and IV as in [22,23], respectively].

2.3. Protein kinase assay

Protein phosphorylation was assayed by incubating, for 20 min at 30°C under stirring, 600 μg protein of membrane fraction in 450 μl of 10 mM Tris-Cl pH 7.5, 8 mM $MgCl_2$, 20 mM NaF, 0.25 μM PMSF, 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% v/v glycerol, 15% w/v SDS, 25% v/v β-mercaptoethanol and boiled for 3 min.

2.4. Electrophoresis and autoradiography

Boiled samples (200 μg proteins) of the IM fraction or of purified complex I were subject to SDS-PAGE as in [15]. For two-dimensional blue-native electrophoresis the IM fraction was solubilized with 1% laurylmaltoside, 750 mM aminocaproic acid, 50 mM Bis-Tris-HCl pH 7.00 at a concentration of 2–3 mg protein/ml for 2 h in ice. After the addition of 0.5% Coomassie blue G in 500 mM aminocaproic acid, samples were subject to electrophoresis as in [24] on exponential 5–20% (w/v) polyacrylamide gradient. Second-dimension Tricine-SDS-PAGE was performed as in [24]. Autoradiography was performed as in [15].

3. Results

In the IM fraction of bovine-heart mitochondria, consisting essentially of scrambled inner membrane, cAMP induces phosphorylation by [γ-^{32}P]ATP of proteins of apparent M_r 29, 18 and 6 kDa (Fig. 1) (see also [14,15]). cAMP promotes also additional phosphorylation of the 42 kDa electrophoretic protein band (Table I in [15]). The 42 kDa band consists essentially

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel-electrophoresis; SDS, sodium dodecylsulphate; mtPKA, mitochondrial cAMP-dependent protein kinase; CPKA, catalytic subunit of purified PKA.

SSDI 0014-5793(95)01407-1

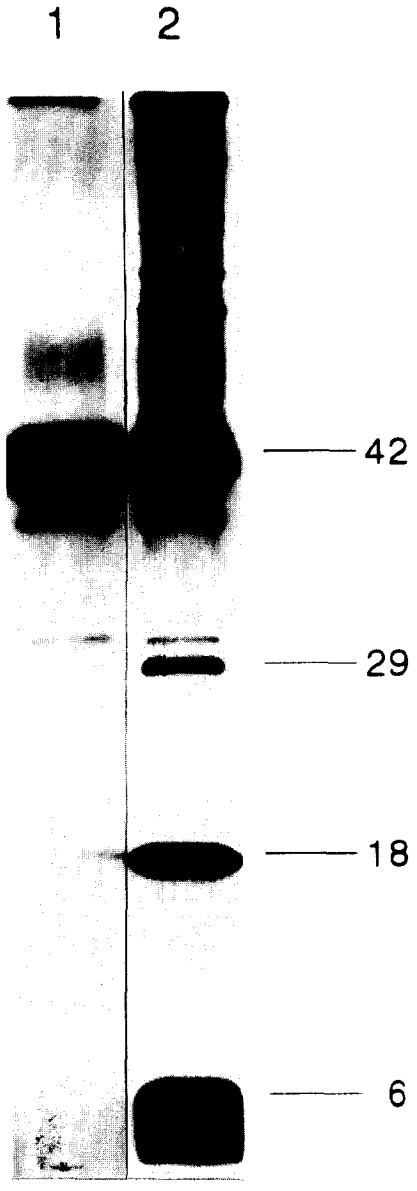

Fig. 1. Autoradiogram of SDS-PAGE protein bands labeled by [γ-^{32}P]ATP in the IM fraction. Preparation of the IM fraction and protein kinase assay were as described in section 2. Lane 1, control; lane 2, incubation in the presence of 50 μM cAMP.

of the EIα subunit of pyruvate dehydrogenase [14], which is phosphorylated by its cAMP-independent specific kinase [25]. Preliminary two-dimensional electrophoresis showed, in fact, that the 42 kDa, as well as the 18 kDa band, consist of more proteins.

In the experiment presented in Fig. 2, complexes I, V, III and IV were simultaneously separated from the IM fraction by blue-native electrophoresis and resolved in their constituent subunits by a second-dimensional SDS-PAGE [24] (Fig. 2A). Incubation of the IM fraction with [γ-^{32}P]ATP showed that cAMP induced phosphorylation of a band of M_r 18 kDa in the lane of the resolved subunits of complex I (Fig. 2Bi). This band was also phosphorylated when the IM fraction was incubated with the purified catalytic subunit of PKA (CPKA) (Fig. 2Bii). Incubation of the IM fraction with cAMP or CPKA also resulted in diffuse labeling in the region of 42 kDa, which extended from the position corresponding to the redox complexes and complex V to the right margin of the gel corresponding to low molecular masses, well below those of the complexes. In the absence of cAMP and CPKA (not shown), only this marginal labeling by [γ-^{32}P]ATP was observed, which might, in fact, correspond to the E1α subunit of pyruvate dehydrogenase [25].

In the experiment of Fig. 3, after separation by blue-native electrophoresis, the pieces of the gel corresponding to the resolved complexes were cut and subject to SDS-PAGE on one gel slab. This resulted in separation of the constituent subunits of all the four complexes (Fig. 3A). The procedure clearly showed that incubation of the IM fraction with cAMP or CPKA resulted in a marked labeling by [γ-^{32}P]ATP of a 42 kDa protein band associated, separately, with each of the four complexes. In agreement with observation in the experiment of Fig. 2, both cAMP and added CPKA also induced phosphorylation of the 18 kDa band associated with complex I.

Fig. 4A shows that when a preparation of complex I from bovine heart mitochondria [21], exhibiting the typical polypeptide pattern of this enzyme [26,27], was incubated with cAMP and [γ-^{32}P]ATP, no subunit of the complex was phosphorylated. Incubation with CPKA resulted, however, in a large phosphorylation of the 18 kDa protein band in the complex. No protein phosphorylation in the isolated complex I was observed in the region of 42 kDa. Separate controls showed that isolated complexes III and IV did not show any protein phosphorylation in the region of 42 and 18 kDa (except a tiny phosphorylation in the 42 kDa band of complex IV) when incubated with CPKA and [γ-^{32}P]ATP (Fig. 4A). Fig. 4B shows that the 18 kDa protein in complex I, phosphorylated by CPKA, was also phosphorylated by the endogenous mtPKA of the IM fraction when cAMP was added and, indeed, migrated in the SDS-PAGE in the same position of the endogenous 18 kDa protein band of the IM fraction phosphorylated by mtPKA.

4. Discussion

The present work substantiates the existence in bovine heart mitochondria of cAMP-dependent mtPKA and multiple substrate proteins phosphorylated by this enzyme (see also [14,15]). The 42 kDa protein band phosphorylated by mtPKA as well as the same kinase activity were previously found to be loosely associated to the inner mitochondrial membrane from which both could be extensively solubilized by exposure to ultrasounds in a high ionic strength medium [15]. The present results show that 42 kDa protein(s), phosphorylated by [γ-^{32}P]ATP upon incubation of the inner membrane fraction (IM) with cAMP or purified CPKA, is(are) loosely associated to complexes I, III and IV. The 42 kDa phosphoprotein is, however, not a constituent subunit of these complexes as shown by the fact that no protein of this M_r was phosphorylated when the purified complexes were incubated with [γ-^{32}P]ATP and CPKA.

The nature of the 42 kDa protein(s) is unknown. One possibility is that it (they) belong(s) to the group of the anchoring proteins [5,6,28], which attach the regulatory subunit (in particular the RII type [5,6]) of PKA to specific membrane structures [4–6] and are themselves phosphorylated by the kinase [6]. It

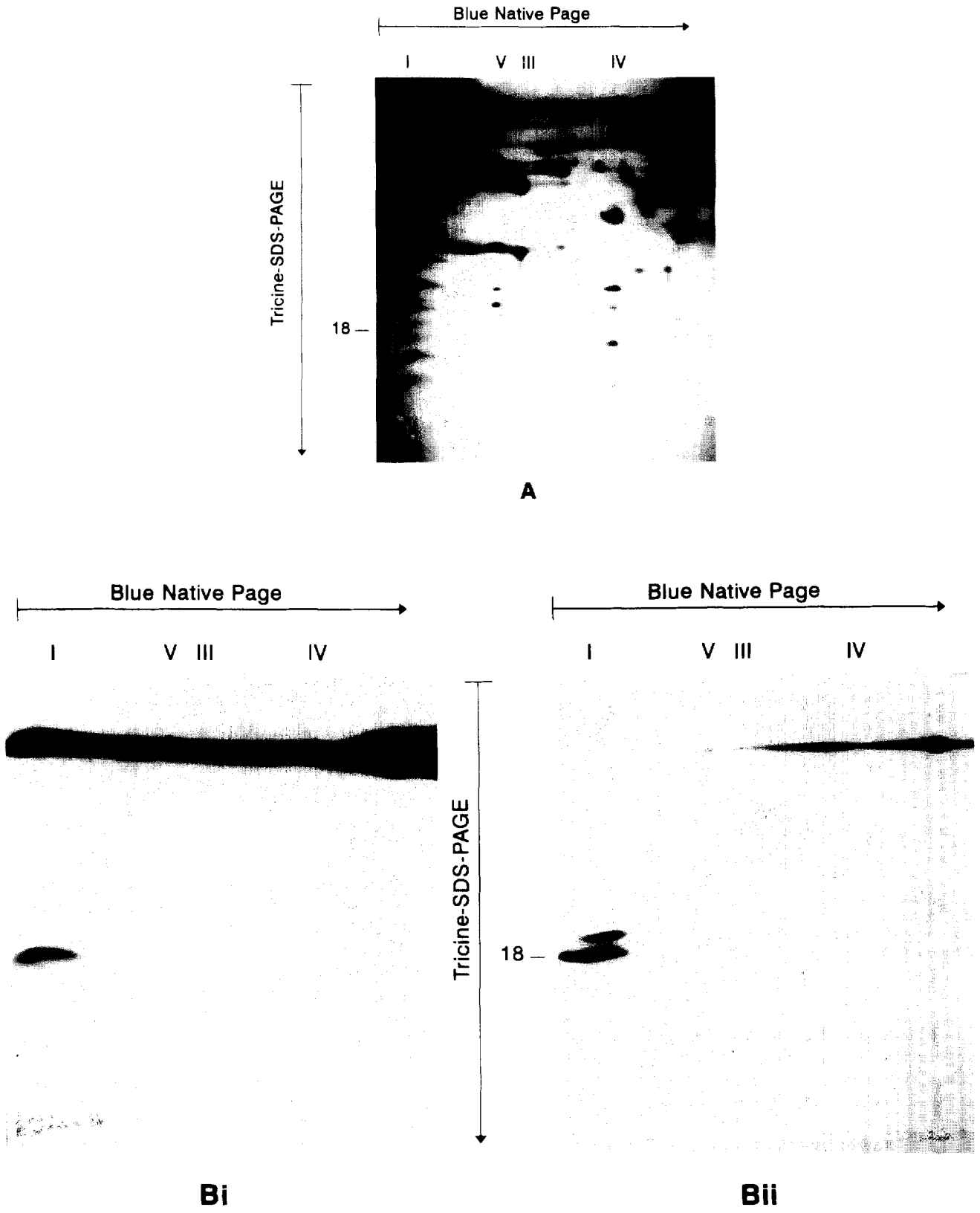

Fig. 2. Two-dimensional electrophoretic resolution of oxidative phosphorylation complexes from the IM fraction and autoradiograms of protein labeling by [γ-^{32}P]ATP. 400 μg protein of the IM fraction were incubated with [γ-^{32}P]ATP and processed for two-dimensional electrophoresis as described in section 2. (A) The complexes were identified, after the second SDS-PAGE, by Coomassie blue staining of their characteristic polypeptides (see [24]). (B) autoradiograms of the second SDS-PAGE slabs. (Bi) incubation in the presence of 50 μM cAMP; (Bii) incubation with 10 U CPKA.

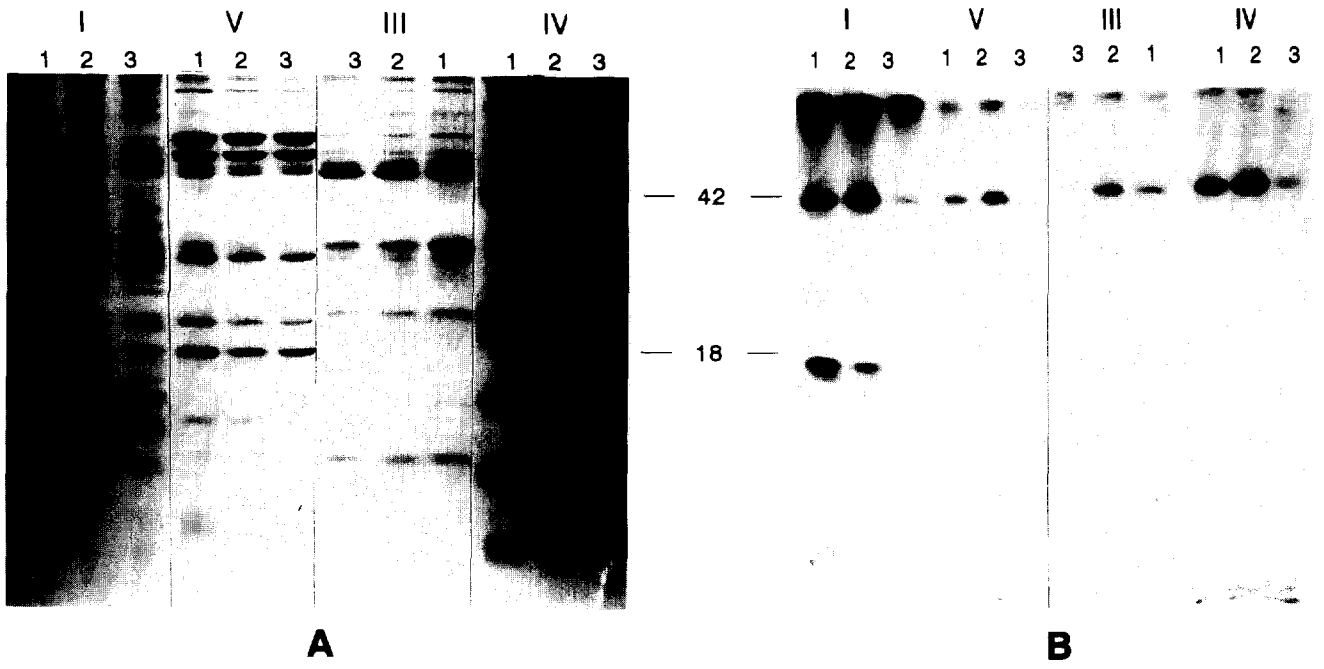

Fig. 3. Coomassie blue staining patterns and autoradiogram of protein labeling by [γ-^{32}P]ATP in oxidative phosphorylation complexes. (A) Coomassie blue staining of oxidative phosphorylation complexes. The oxidative phosphorylation complexes from the IM fraction, separated by blue-native electrophoresis (see section 2), were cut, placed on a 16.5% polyacrylamide gel and subject to Tricine-SDS-PAGE [24]. (B) Autoradiogram of oxidative phosphorylation complexes separated from the IM fraction by the procedure described in (A). Lanes 1, incubation of IM in the presence of 10 U CPKA; lanes 2, incubation in the presence of 50 μM cAMP; lanes 3, controls.

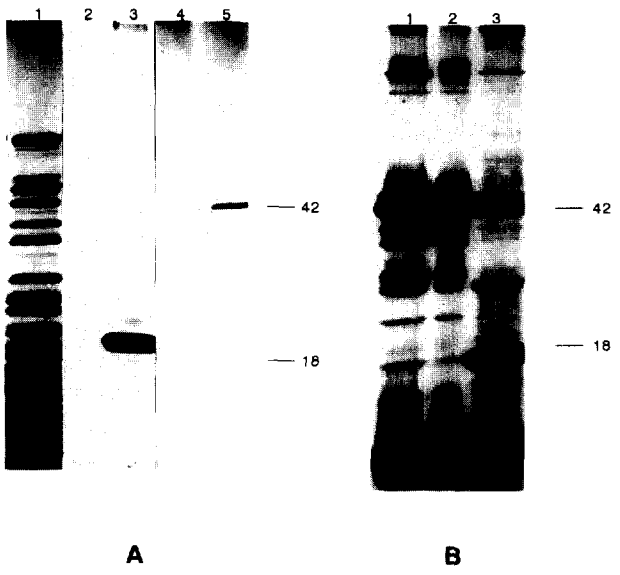

Fig. 4. Autoradiograms of protein labeling by [γ-^{32}P]ATP in the IM fraction and in purified complexes I, III and IV. (A) Lane 1, Coomassie blue staining of SDS-PAGE protein bands of the preparation of complex I (~1 nmol FMN/mg protein); lane 2, autoradiogram of complex I incubated in the presence of 50 μM cAMP; lane 3, autoradiogram of complex I incubated with 10 U CPKA; lanes 4 and 5, autoradiograms of complexes III and IV, respectively, incubated with 10 U CPKA. Phosphorylation of purified complexes was carried out in the absence of rotenone and NaF. (B) cAMP-dependent protein phosphorylation in the IM fraction and combination of the IM fraction and complex I. Lane 1, 200 μg IM fraction; lane 2, 100 μg IM fraction; lane 3, combination of 100 μg IM fraction and 200 μg complex I. In all incubations 50 μM cAMP was added.

(they) could also represent assembly factor(s) for the complexes.

An 18 kDa protein phosphorylated in the IM fraction of mitochondria by mtPKA is shown here to be associated to complex I when separated directly from the inner mitochondrial membrane by native electrophoresis. It should be recalled that preliminary two-dimensional electrophoresis of IM (not shown) revealed the 18 kDa electrophoretic band from the inner membrane to consist of three proteins, at least, which were phosphorylated by mtPKA or added CPKA (cf. [8,29]). Our results show that an 18 kDa protein in purified complex I is actively phosphorylated by purified CPKA as well as by mtPKA and exhibits the same electrophoretic migration as the 18 kDa protein of the IM fraction phosphorylated in response to cAMP. It can be noted that various subunits of complex I migrate in the region of M_r 18 kDa where the radioactive band appears [26,27].

Acknowledgements: This work was supported in part by CNR ACRO Grant 104283/39/9410582.

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