The nuclear-encoded 18 kDa (IP) AQDQ subunit of bovine heart complex I is phosphorylated by the mitochondrial cAMP-dependent protein kinase

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Abstract In bovine heart mitochondria a protein of M_r 18 kDa, phosphorylated by mtPKA, is associated to the NADH-ubiquinone oxidoreductase in the inner membrane and is present in purified preparation of this complex. The 18 kDa phosphoprotein has now been isolated and sequenced. It is identified as the 18 kDa (IP) AQDQ subunit of complex I, a protein of 133 amino acids with a phosphorylation consensus site RVS at position 129–131.

Key words: NADH-ubiquinone oxidoreductase; cAMP-dependent protein kinase; Mitochondrial protein phosphorylation

1. Introduction

The protonmotive NADH-ubiquinone oxidoreductase (complex I) is the first, in the sequence of electron transfer, and the largest, in mass and number of subunits, of the proton-translocating enzyme-complexes by which the respiratory chain of mitochondria is made up [1,2]. Complex I of bovine heart mitochondria is composed of 41 subunits of which seven are encoded by the mitochondrial genome, the remaining by nuclear genes [1,3,4]. The cDNAs of 41 subunits of bovine heart complex I have been cloned and their primary structures are available [1]. Some of these subunits are covalently modified post-translationally. A pantothenic acid moiety is attached to subunit SDAP, which is related in sequence to acyl carrier proteins [5] cf. [6]. A number of subunits, 75 kDa (IP, iron sulphur fraction), 51 kDa (FP, flavoprotein fraction), 24 kDa (FP), 23 kDa (TYKY), 20 kDa (PSST), 19 kDa (PGIV), contribute binding sites for FMN, NADH and [4Fe-4S] and [2Fe-2S] centres (see for review refs. [1,4]). The possible function of other proteins of the FP, IP and HP (hydrophobic fraction) is unknown [1,4].

Recent work from our group has shown that in bovine heart mitochondria various proteins are phosphorylated by a cAMP-dependent protein kinase (mtPKA) present in these organelles [7–10]. An mtPKA has also been detected in yeast mitochondria [11]. It was found [12] that a protein of apparent M_r 18 kDa phosphorylated by mtPKA in the inner membrane of bovine heart mitochondria [8] is associated to complex I. In this paper it is shown that the nuclear-encoded 18 kDa (IP) AQDQ subunit of bovine heart complex I is substrate of the mitochondrial cAMP-dependent protein kinase.

2. Materials and methods

2.1. Chemicals

[γ-^{32}P]ATP was from Amersham International; catalytic subunit of cAMP-dependent protein kinase from bovine heart was from Sigma. Immobilon Poly(vinylidene difluoride) membrane was from Millipore. All reagents for protein sequencing were from Applied Biosystem.

2.2. Purification of mitochondrial membrane fraction and complex I

The inner membrane fraction (IM) from bovine heart mitochondria [13] was prepared as in [8]. Complex I was prepared from bovine mitochondria as in [14]. It exhibited the typical polypeptide pattern of complex I (cf. [1,15]) and a content of about 1 nmol FMN/mg protein with no detectable amounts of cytochromes *b*, *c* and aa_3.

2.3. Protein kinase assay

Protein phosphorylation was assayed by incubating, for 20 min at 30°C under stirring, the protein preparations (as specified in the legends to the figures) in 450 μl of 10 mM Tris-HCl 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 (25–400 μg protein) were mixed with 70 μl of 0.2 M Tris-HCl pH 6.8, 30% glycerol (w/v), 7% SDS, 15% (v/v) β-mercaptoethanol and boiled for 3 min.

2.4. Gel electrophoresis and autoradiography

Samples of the IM fraction or of purified complex I were subject to SDS polyacrylamide gel electrophoresis (SDS-PAGE) on a 12–30% linear acrylamide gradient gel [16] with addition of 1% glycerol. To isolate the 18 kDa (IP) phosphoprotein in complex I, the ^{32}P-labeled protein band of the complex I gel slab, was cut and subjected to a second SDS-PAGE [17] in the absence of glycerol. Radioactivity measurement of cut PAGE bands was performed as in [8].

2.5. Amino acid sequence analysis

The proteins resolved by the second SDS-PAGE were electrotransferred to immobilon membrane for protein sequencing [18], performed with an Applied Biosystem sequencer (mod 473A).

3. Results

Incubation of complex I from bovine heart with the inner mitochondrial membrane fraction (IM) from the same source resulted, in the presence of cAMP and [γ-^{32}P]ATP, in the appearance of an highly radioactive ^{32}P-labeled protein band of M_r 18 kDa as estimated by the SDS-PAGE (Fig. 1). This band migrated in the same position as endogenous ^{32}P-labeled protein band of M_r 18 kDa, detected when the IM fraction alone was incubated with cAMP and [γ-^{32}P]ATP (Fig. 1) [8]. Labeling by [γ-^{32}P]ATP of other radioactive protein bands, already de-

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Abbreviations: Complex I, NADH-ubiquinone oxidoreductase; mtPKA, mitochondrial cAMP-dependent protein kinase; IM, inner membrane fraction of bovine heart mitochondria.

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tected in the IM fraction [7,8], was practically unaffected by the addition of complex I. Significant phosphorylation of the 18 kDa protein could still be detected at an IM/complex I protein ratio of 0.12. Separate controls showed that incubation of complex I alone with [γ-^{32}P]ATP did not result, in the absence or presence of cAMP, in any phosphorylation of its protein components. The specific phosphorylating activity of IM-mtPKA for the 18 kDa protein was, with added complex I, in the order of 10 pmol/min/mg.

To identify the 18 kDa protein in complex I phosphorylated by mtPKA, this complex, after incubation with the purified catalytic subunit of PKA and [γ-^{32}P]ATP was subject to SDS-PAGE. Fig. 2 shows the Coomassie-blue staining of the subunits of complex I in one PAGE lane and the autoradiogram of the gel slab, showing the ^{32}P-labeled 18 kDa band. The radioactive bands of all the lanes were cut out and applied on a new gel for a second SDS-PAGE. After electrophoresis proteins were electrotranferred on the immobilon membrane. Coomassie-blue staining of the immobilon membrane showed the radioactive 18 kDa band of the first run to be resolved in the second electrophoretic run into two protein bands of which only the lower one resulted to be labeled by ^{32}P (Fig. 3). Sequence analysis of the radioactive protein band showed this to consist of an homogenous protein with the N-terminal 15 residues corresponding to those of the 18 kDa (IP) AQDQ subunit of complex I [15]. The upper non-radioactive band was also sequenced and resulted to consist of frayed SGDH subunit of complex I [15].

4. Discussion

The results presented show that the 18 kDa (IP) AQDQ subunit of bovine heart complex I is substrate of the cAMP-

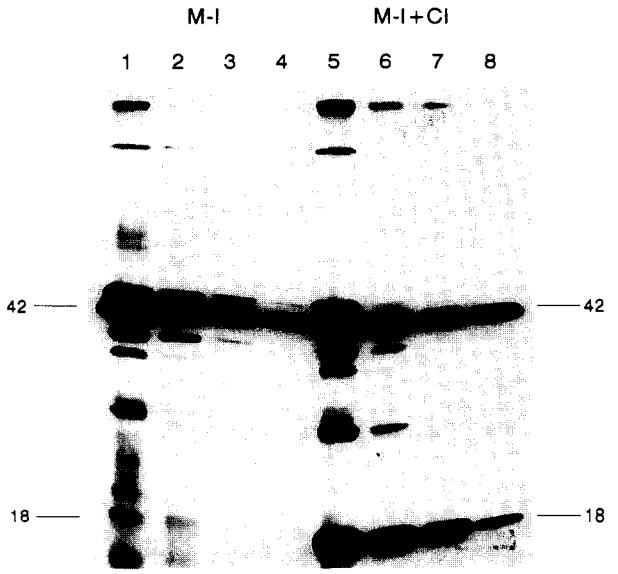

Fig. 1. Autoradiogram of protein bands labeled by [γ-^{32}P]ATP in the IM fraction of bovine heart mitochondria and in combination of (IM) and complex I (CI). Incubation of IM and IM+CI with [γ-^{32}P]ATP was as described in section 2. Lanes 1–4: incubation of different amounts of the IM fraction, 200 μg protein (1), 100 μg protein (2), 50 μg protein (3) and 25 μg protein (4). Lanes 5–8: incubation of 200 μg complex I in combination with 200 μg (5), 100 μg (6), 50 μg (7) and 25 μg (8) of the IM fraction, respectively. All incubations were carried out in the presence of 50 μM cAMP.

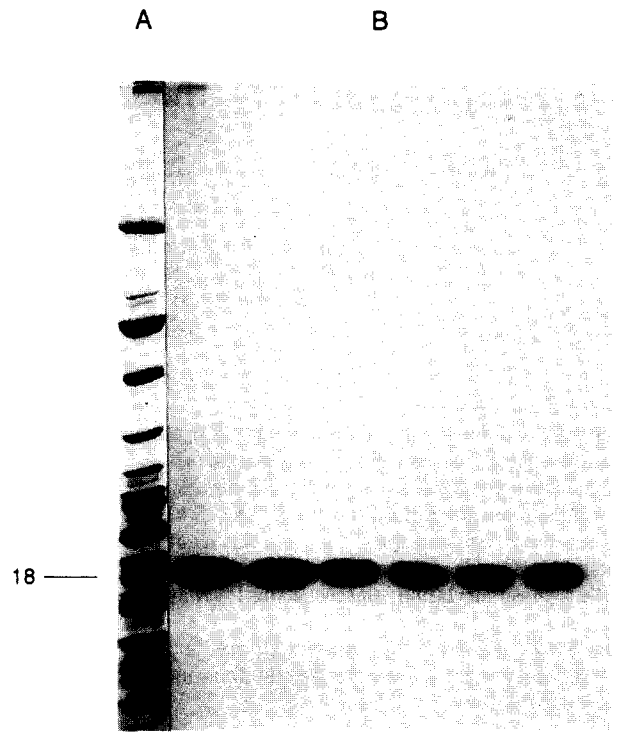

Fig. 2. Complex I Coomassie blue staining pattern and autoradiogram of protein labeling by [γ-^{32}P]ATP. Complex I (200 μg protein/150 μl incubation mixture) was incubated with [γ-^{32}P]ATP as described in section 2 in the presence of 10 U of the purified catalytic subunit of PKA (rotenone and NaF were omitted from the incubation mixture). Seven aliquots of 200 μg protein complex I were applyed on separate wells of the same gel slab. After electrophoresis, one gel lane (A) was stained with Coomassie blue. The other lanes of the gel (B) were used for autoradiography. Control experiments showed that no phosphorylation of subunits of purified complex III or IV could be detected when these complexes were incubated with PKA and [γ-^{32}P]ATP [cf. 12].

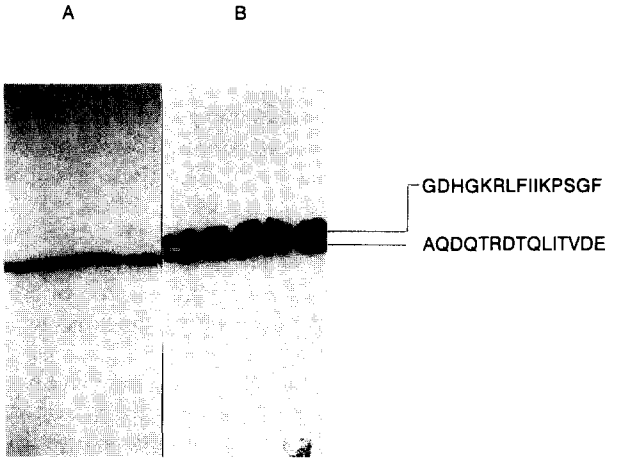

Fig. 3. Coomassie blue staining and autoradiogram of the radioactive protein band of complex I electrotransferred on the immobilon membrane. The radioactive band of complex I identified as described in the legend to Fig. 2 was cut and subject to the second SDS-PAGE as described under Materials and Methods. After electrophoresis was completed proteins were transferred on the immobilon membrane. The stained protein bands were cut out and subject to protein sequencing as described in section 2. The first 15 N-terminal residues of the two protein bands are shown on the right side of the figure. (A) autoradiogram of the immobilon membrane. (B) Coomassie blue staining of the immobilon membrane.

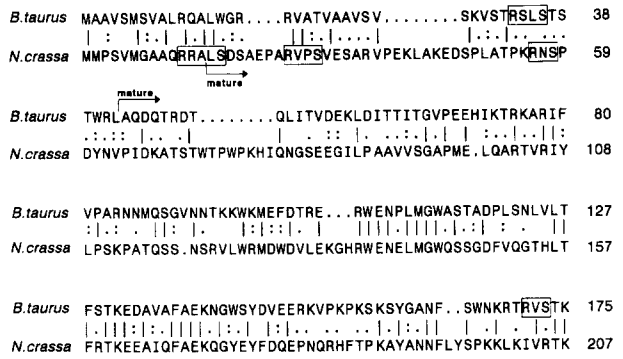

Fig. 4. Alignment, conservation analysis and localization of phosphorylation consensus sites of the 18 kDa (IP) subunit of bovine heart complex I and the 21 kDa subunit of *Neurospora crassa* complex I. The analysis was carried out by FASTA [20] and BESTFIT [22] programs using SWISSPROT and NBRF/PIR data banks. Phosphorylation consensus sites [19,20] are boxed.

dependent protein kinase associated to the inner membrane of mitochondria. The cDNA of this subunit has been cloned and its sequence is available [15]. The mature protein consists of 133 residues, at positions 129–131 there is a phosphorylation consensus site RVS [19,20] (Fig. 4). The protein has a leader sequence, removed after import in mitochondria [15], which also presents a phosphorylation consensus site RSLS at positions −10 to −7. Although the protein can be obtained associated to the IP fraction [15], it has no cystine residues which might provide ligands for iron-sulphur centres. The *Neurospora crassa* complex I has a homologous hydrophylic subunit (M_r 21 kDa), 52% similar and 37% identical with the bovine heart AQDQ subunit [21,22,cf. 23]. The mature *Neurospora crassa* protein has two phosphorylation consensus sites, RVPS and RNS at position 9–12 and 34–36, respectively. The *Neurospora crassa* M_r 21 kDa has also a leader sequence, removed after import, and presents a second phosphorylation consensus site RRALS, encompassing the C-terminal of the leader sequence and the first two N-terminal residues of the mature protein. The presence of phosphorylation consensus sites in both the leader sequences and the mature proteins would suggest phosphorylation of the precursor protein by cAMP dependent protein kinase in both the cytosolic and mitochondrial compartments [7]. This dual phosphorylation might regulate import and/or maturation of the protein. The 18 kDa (IP) AQDQ subunit of bovine heart complex and the homologous 21 kDa protein of *Neurospora crassa* complex I do not show by FASTA program [21,22] any significant homology to other proteins in the SWISSPROT, NBRF/PIR and EMBL data banks.

cAMP-dependent protein phosphorylation is a central process in signal transduction of different hormones [24,25]. cAMP-dependent phoshorylation by mtPKA of the 18 kDa (IP) AQDQ subunit of bovine heart complex I might have a role in the regulation of the function and/or biogenesis of the complex.

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