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Mitochondrial cytochrome *c* oxidase subunit IV is phosphorylated by an endogenous kinase

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Abstract This study was undertaken to identify novel mitochondrial membrane proteins that are potential targets for phosphorylation. Mitochondrial membranes were incubated in the presence of [γ-^{32}P]ATP and the Triton X-114 extractable protein was subjected to ion-exchange and polyacrylamide gel chromatography to purify a major phosphorylated protein of approximately 17 000 Da. The determined peptide sequence of the purified phosphoprotein corresponded to a segment of cytochrome *c* oxidase subunit IV, an inner membrane protein of 17 160 Da. The identity of the phosphoprotein was confirmed by two-dimensional electrophoresis and Western blotting. The results identify mitochondrial cytochrome *c* oxidase subunit IV as a protein which is phosphorylated by an endogenous kinase.

Key words: Mitochondria; Protein phosphorylation; Cytochrome *c* oxidase subunit IV

1. Introduction

The nuclear genome encodes the majority of proteins which reside in mitochondria [1]. In order for eukaryotic cells to function properly, mitochondria must communicate with the nucleus to regulate the synthesis and assembly of the mitochondrial proteins [2]. These proteins are translated in the cytosol and then targeted, imported and sorted into their final location within one of four compartments: the outer or inner membrane, the intermembrane space or the matrix (for reviews see [3–5]).

Communication between and within cells is often achieved through signal transduction pathways (for reviews see [6,7]). These signals can be transduced through enzymatic phosphorylation and dephosphorylation of proteins in a sequential cascade [8] which reversibly alters their structural and functional properties. Thus it is reasonable to suspect that communication between the nucleus and the mitochondria may involve one or more protein kinase, which along with some of their target proteins may reside within mitochondria.

Indeed, it has been shown that the regulation of mitochondrial pyruvate dehydrogenase [9] and branched-chain α-ketoacid dehydrogenase [10] involves phosphorylation and their distinct kinases have been cloned from rat heart [11,12]. The presence of other kinases within mitochondria has also been reported. These include cAMP-dependent protein kinase [13–17], cAMP-independent protein kinase [18–20], tyrosine kinase [21,22], protamine kinase [23], phosphorylase kinase (in brain) [24] and casein kinase II [17,23]. Phosphorylated proteins within mitochondria from mammals, yeast and plants have been examined by several investigators. To date, however, only three proteins have been routinely identified: the α-subunit of pyruvate dehydrogenase [9], the α-subunit of the branched-chain α-ketoacid dehydrogenase [10] and the autophosphorylated subunit of succinyl-CoA synthetase [25]. A limited number of other candidate phosphoproteins exists [13,22,26–34], but very few of these have been identified [35,36].

Here, we report the purification of a 17 160 Da mitochondrial membrane protein which is phosphorylated by an endogenous kinase. Amino acid sequencing and immunological analysis revealed that it is cytochrome *c* oxidase subunit IV, an inner mitochondrial membrane protein.

2. Materials and methods

2.1. General procedures

Previous articles describe the routine procedures used in this study ([37,38] and references cited therein). These include purification of mitochondria from rat heart and analysis of proteins by SDS-PAGE and fluorography. All preparative procedures and centrifugation were performed at 4°C.

2.2. Preparation of microsomes, mitochondria and mitochondrial membranes

Dog pancreas microsomes were isolated as previously described [39] and were kindly provided by Dr. J.J.M. Bergeron's laboratory (McGill University). Liver mitochondrial membranes were prepared by a modified version of a previously described method [40]. Livers were obtained from six 200–250 g Sprague-Dawley rats and were minced and washed in ice-cold 0.25 M sucrose. The minced liver was then homogenized in a 10-fold volume of 0.25 M sucrose in a motorized Potter-Elvehjem homogenizer using eight up and down cycles at 500 rpm. The homogenate was diluted with 0.25 M sucrose to a final volume of 240 ml and centrifuged at 750×*g* for 10 min. The supernatant was recovered and centrifuged at 7000×*g* for 10 min. The resulting pellets were resuspended in 50 ml of 0.25 M sucrose and hand homogenized with five up and down passes. The homogenate was diluted with 0.25 M sucrose to 210 ml and centrifuged at 7000×*g* for 10 min. The resulting pellets (mitochondria) were washed by repeating the previous step. To obtain mitochondrial membranes, the mitochondria were resuspended in 60 ml of 10 mM Tris-PO_4, pH 7.5 and incubated on ice for 10 min, followed by the addition of 16 ml of 2 M sucrose, 2 ml of 40 mM $MgSO_4$ and 2 ml of 40 mM ATP and the incubation continued for 10 min. Aliquots (20 ml) were sonicated for 20 s using a Sonic Dismembrator (Artek Systems Corp., New York, USA) with a small probe, set at 60. Aliquots (10 ml) of the sonicate were layered onto 12 ml of 1.2 M sucrose and centrifuged at 100 000×*g* for 60 min. The interface between the two fluid layers was collected and pooled and the total volume was brought up to 200 ml with 20 ml of 1.5 M NaCl, 0.5 M Tris-HCl, pH 8.0 and distilled water. The diluted interface was centrifuged at 100 000×*g* for 60 min. The pellets were recovered and resuspended in 2 ml of 0.25 M sucrose and the protein concentration was determined (Bio-Rad Protein Assay). The resuspension was centrifuged for 35 min at 130 000×*g* and the pellets were resuspended at a concentration of 10

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Abbreviations: Tx-114, Triton X-114; COX IV, cytochrome *c* oxidase subunit IV; COX, cytochrome *c* oxidase

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mg/ml in 20 mM HEPES, pH 7.4, 50% glycerol and 1 mM dithiothreitol and stored at −70°C.

2.3. Phosphorylation of microsomes, mitochondria and mitochondrial membranes

Membranes (10–35 mg) were diluted to 4 mg/ml with 20 mM HEPES, pH 7.4 and 10% was incubated with [γ-^{32}P]ATP diluted with non-radioactive ATP to give a concentration of 28 μM and an activity of 1 μCi/μl; the remaining 90% was incubated with non-radiolabelled ATP (28 μM) using a scaled up version of the method of Wada et al. [41]. The reaction was incubated and stopped as in Rindress et al. [31]. In addition to the stop solution, 2.5 mM β-glycerophosphate was added to the terminated reaction.

2.4. Purification of pp17

The phosphorylated membranes were extracted with Triton X-114 (Tx-114) using the method of Bordier [42]. Condensed Tx-114 was a generous gift of the laboratory of Dr. J.J.M. Bergeron (McGill University). The detergent phase was subjected to anion exchange chromatography using DEAE-Sepharose Fast Flow (Pharmacia LKB Biotechnology Inc.) equilibrated with buffer A (20 mM Tris, pH 7.4 and 0.2% Tween 20), washed with 0.01 M NaCl in buffer A and eluted with a gradient of 0.01–0.5 M NaCl in buffer A containing 2.5 mM β-glycerophosphate and 5% glycerol. The collected fractions were monitored for protein (A_{280}) and radioactivity. Fractions (136–158) containing the peak radioactivity were pooled and concentrated by dialysis versus buffer A containing 2.5 mM β-glycerophosphate and 60% glycerol. The sample was precipitated with ethanol/hexanes (4:1), resuspended in 0.5 ml SDS sample buffer, incubated at 60°C for 10 min and loaded onto a Model 491 Prep Cell (Bio-Rad Laboratories) and subjected to preparative gel electrophoresis. The Prep Cell contained a 6 cm separating gel (16% acrylamide) and a 1 cm stacking gel (4% acrylamide) in the 28 mm diameter tube. The electrophoresis was performed at 40 mA for 2.5 h until the dye front started to elute and 0.5 ml fractions were collected for another 2 h. The elution buffer contained 0.02% SDS and flowed at 0.1 ml/min. The eluted fractions were monitored for radioactivity and the peak fractions were analyzed by SDS-PAGE and autoradiography. The peak fractions (16–19) were precipitated with ethanol/hexanes and resuspended in SDS sample buffer, subjected to SDS-PAGE and electroblotted to nitrocellulose membrane.

2.5. Identification of pp17

The blots were visualized using 0.2% Ponceau S stain and autoradiography and the band corresponding to pp17 was excised from the blot. The protein on the blot was subjected to trypsin digestion and the sequence of the resulting peptides was determined (Harvard Microchemistry Facility, Cambridge, MA, USA).

2.6. Two-dimensional (2D) gel electrophoresis

25 μg of phosphorylated rat heart mitochondrial membranes (0.5 mg/ml) was loaded onto the acidic end of an isoelectric focusing gel (non-equilibrium pH gradient electrophoresis) [43] containing 2% ampholytes (Bio-Lyte 3-10) and subjected to 700 V for 1 h. The protocols and procedures were as provided by Bio-Rad except for the changes which are noted above. Proteins were separated in the second dimension by 12% SDS-PAGE and analyzed either by Western blotting or by staining [44] and subsequent autoradiography or phosphorescent imaging. Anti-cytochrome *c* oxidase subunit IV (COX IV) antibodies were a generous gift from Prof. Dr. B. Kadenbach (Philipps-Universität, Marburg, Germany).

3. Results and discussion

Rat heart mitochondria were purified and constituent protein substrates phosphorylated by an endogenous kinase, using a ratio of 10% [γ-^{32}P]ATP and 90% non-radiolabelled ATP. The resulting phosphorylation pattern is shown in Fig. 1, which reveals the existence of a limited number of phosphoproteins in mitochondria (Mito, lane T) compared to microsomes (lane T). These proteins were partitioned into either the aqueous (lane A) or detergent (lane D) phase using Tx-114. Several phosphorylated proteins were detected in the

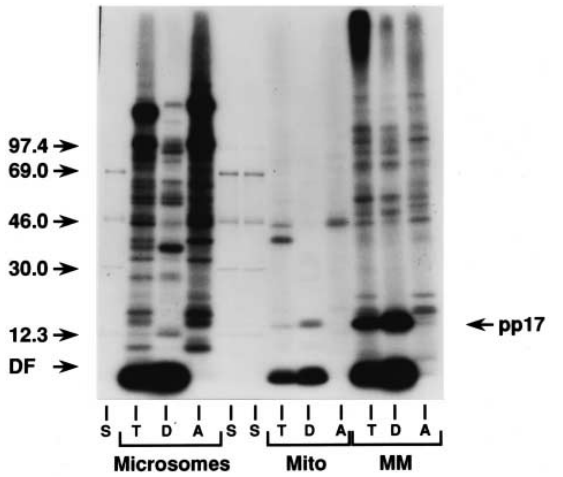

Fig. 1. Pattern of γ-^{32}P-phosphorylated proteins in microsomes, mitochondria and mitochondrial membranes. Endogenous phosphorylation with [γ-^{32}P]ATP of dog pancreas microsomes, rat heart mitochondria (Mito) or rat liver mitochondrial membranes (MM) was as described in Section 2. A 17 kDa phosphorylated protein is enriched in mitochondrial membranes and is resistant to Tx-114 extraction, its mobility is indicated by the arrow on the right (pp17). Molecular mass markers are visible in lanes S and their size (in kDa) is indicated on the left. Approximately 10 μg of microsomal protein was loaded in lane T and a total of 40 μg was loaded in D and A. For mitochondria and MM, 20 μg was loaded in lanes T and a total of 80 μg was loaded in lanes D and A. T, total fraction; D, detergent Tx-114 fraction; A, aqueous fraction; S, standards; DF, dye front.

total mitochondrial fraction (lane T). One, with an approximate molecular mass of 43 000 Da, most likely corresponds to the α subunit of the E_1 component of pyruvate dehydrogenase as previously reported [10] and it partitions into the aqueous phase as expected (lane A). A second has a molecular mass of approximately 37 000 Da and likely corresponds to succinyl-CoA synthase since it is acid labile (the band is lost with acidic gel staining, not shown) [25] and is no longer visible in either the detergent or aqueous phase after detergent extraction with Tx-114. A third phosphoprotein, with an approximate molecular mass of 17 000 Da (pp17), is partitioned into the detergent phase (lane D compared to lane T and lane A). This protein was absent in microsomes (lane D). Taken together, these data suggested that pp17 is an integral mitochondrial membrane protein. It was further characterized in order to determine its molecular identity.

As a first step, the total mitochondrial membrane fraction was obtained from rat liver and phosphorylated as described in Section 2 (Fig. 1, MM, lane T) yielding many more phosphorylated proteins compared to the situation with intact mitochondria. The major phosphorylated protein in the membranes was pp17. Again, when the membrane fraction was subjected to Tx-114 extraction, pp17 partitioned in the detergent phase (lane D compared to lane T and lane A).

3.1. Purification of pp17

In order to purify pp17, mitochondrial membranes were phosphorylated and the proteins that partitioned into the Tx-114 detergent phase were applied to a DEAE-Sepharose Fast Flow column. Most of the protein did not bind to this column and was eluted in the flow through. A small amount of bound protein eluted as a single peak (0.2–0.3 M NaCl) and the protein peak coincided with the peak of radioactivity

(Fig. 2A). The Coomassie blue stained protein profile of the mitochondrial membranes (MM) and the peak protein/radioactive fractions is shown in Fig. 2B. pp17 was greatly enriched in the peak fractions as compared with the mitochondrial membranes (MM). The autoradiographic profile of the peak fractions is shown in Fig. 2C. These fractions were pooled and applied to a preparative electrophoresis polyacrylamide gel. One major peak of radioactivity was recovered and eluted in fractions 15–19 (Fig. 3A). The Coomassie blue stained protein and radioactivity profiles of these peak fractions and of the original Prep Cell load are presented in Fig. 3B,C, respectively. The lower band of the doublet seen on the Coomassie blue protein gel corresponds with the radioactivity. The peak fractions containing the lower band were pooled and concentrated and subjected to SDS-PAGE.

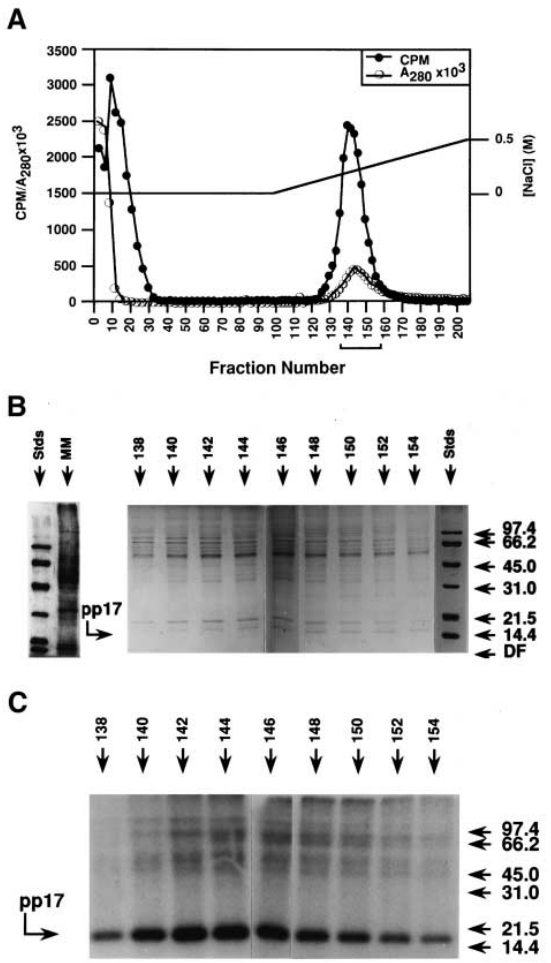

Fig. 2. Purification of pp17: DEAE-Sepharose chromatography. Mitochondrial membranes were phosphorylated with [γ-^{32}P]ATP and extracted with Tx-114. The detergent fraction after Tx-114 treatment was applied to a DEAE-Sepharose Fast Flow column as described in Section 2. A: Column fractions were monitored for their protein content (A_{280}) and radioactivity (CPM) profile. B: Coomassie blue stained protein profiles of total mitochondrial membranes (MM) and the fractions from the peak of protein and radioactivity from the column. C: Autoradiographic profile of the peak column fractions shown in B. The location of pp17 is indicated with an arrow on the left. Molecular mass markers are indicated on the right (in kDa). DF, dye front; bracket indicates the peak fractions which were pooled (136–158).

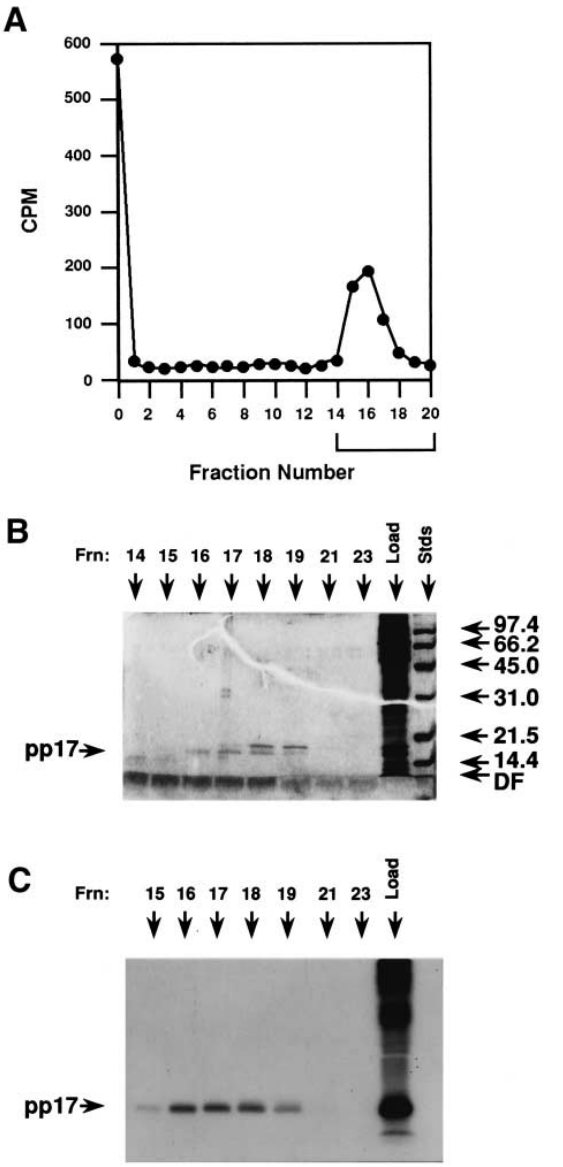

Fig. 3. Purification of pp17: preparatory gel electrophoresis. The peak fractions from the DEAE-Sepharose column were pooled, dialyzed and concentrated and applied to a preparatory polyacrylamide gel electrophoresis column as described in Section 2. A: Radioactive (CPM) profile of the eluted fractions. B: Coomassie blue stained protein profiles of the preparatory cell load (Load) and the peak radioactive fractions. C: Autoradiographic profile of the load and column fractions shown in B. pp17 is indicated with an arrow on the left, molecular mass markers are indicated on the right (in kDa). DF, dye front; bracket indicates the peak radioactive fractions.

3.2. *Identification of pp17*

Following SDS-PAGE, proteins were electroblotted to nitrocellulose and the radioactive band corresponding to pp17 was excised. It was subjected to trypsin digestion and protein sequencing of a constituent peptide. The 10 amino acid sequence which was obtained showed a perfect match with amino acids 150–159 of rat liver COX IV.

To confirm the identity of pp17 as COX IV, 2D gel electrophoresis followed by Western blotting was performed on phosphorylated whole mitochondria from rat heart. The Coomassie blue stained protein profile of the 2D gel is shown in Fig. 5A. The 17 000 Da phosphorylated protein on the phos-

phorescent image of the 2D stained gel (Fig. 5B, arrow) has the same localization as COX IV on the Western blot of the 2D gel (Fig. 5C, arrow). Thus, COX IV is phosphorylated and corresponds to pp17 in rat heart mitochondria.

3.3. Conclusions

We have identified COX IV as the phosphorylation product of an endogenous kinase whose activity is expressed in both whole mitochondria and in isolated mitochondrial membranes. Cytochrome *c* oxidase (COX) is the terminal enzyme complex of the mitochondrial respiratory chain and catalyzes the reduction of molecular oxygen to water coupled to the translocation of protons across the inner mitochondrial membrane (for review see [45]). In mammalian mitochondria, COX is composed of 10 nuclear encoded subunits and three mitochondrial encoded subunits [46]. Subunit IV is a nuclear encoded subunit whose crystallographic structure resembles a dumbbell [47]. It contains one transmembrane domain and resides in the inner membrane with the N-terminus facing the matrix and the C-terminus in the intermembrane space. It has been proposed that the nuclear encoded subunits of COX affect the catalytic function of the mitochondrial encoded subunits by binding allosteric effectors such as substrates, cofactors, ions, nucleotides and hormones [48]. Specifically, ATP has been shown to bind to two subunits of bovine heart COX, including subunit IV [49] and to six or seven subunits of COX from bovine liver or heart, respectively, including subunit IV [50]. As well, there is evidence indicating that the yeast homologue of subunit IV, subunit V, may regulate catalysis and modulate the function of the holoenzyme [51]. Also, it has been proposed that subunit IV functions as a transmitter of signals, such as channelling protons into the proton translocating pathway [52].

There are many potential phosphorylation consensus sites for several different kinases within the mature sequence of COX IV as well as in the signal sequence (Fig. 4). Some of these consensus sites are located within evolutionary conserved regions (amino acids 26–32 and 54–84), [53], implicating them as good candidate sites for phosphorylation. The conserved region of amino acids 26–32, besides containing several phosphorylation consensus sites, is also the region identified as important in translocating protons across the membrane [52]. While speculative, it is possible that the translocation of protons could be regulated by phosphorylation.

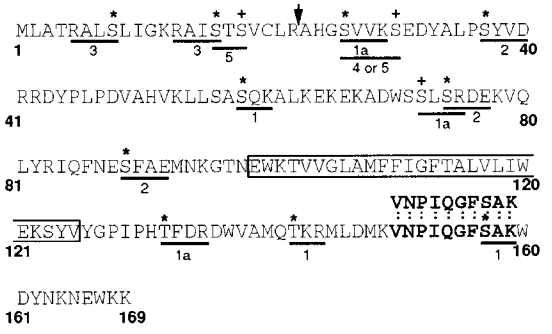

Fig. 4. Comparison of trypsin generated peptide sequence with cytochrome *c* oxidase subunit IV. Amino acid sequence of rat cytochrome *c* oxidase subunit IV [55,56], with the peptide obtained from trypsin digestion and sequencing, shown in bold. Several potential phosphorylation consensus sites are underlined, 1 and 1a are protein kinase C, 2 is casein kinase II, 3 is CAM kinase II or cAMP dependent kinase, 4 is glycogen synthase kinase-3 and 5 is casein kinase I (1 and 2 were identified by Prosite; 1a, 3, 4 and 5 were identified from Kennelly and Krebs [57]). Bold numbers indicate amino acid position relative to initiator methionine, which is 1. Arrow, the site of cleavage in the signal sequence where processing would otherwise take place in the matrix. The predicted transmembrane domain is boxed [47]. The single letter amino acid code is used. +, potential sites of phosphorylation for consensus sites 5 or 1a at position 72; *, potential sites of phosphorylation for the other consensus sites.

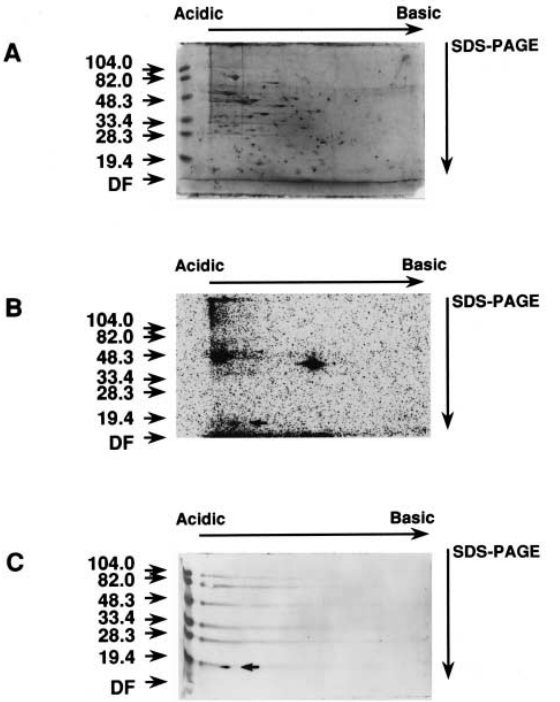

Fig. 5. Identification of pp17 as cytochrome *c* oxidase subunit IV. Rat heart mitochondria were phosphorylated as described in Section 2 and loaded onto the acidic end of an isoelectric focusing gel (non-equilibrium pH gradient electrophoresis). The proteins were then separated in the second dimension by 12% SDS-PAGE and analyzed by autoradiography, phosphorescent imaging and Western blotting. A: Coomassie blue stained profile of the protein pattern on the 2D gel. B: Phosphorescent image of the gel in A after it was analyzed using a Fuji BAS-2000 Bio-Image Analyzer. The migration of pp17 is indicated with an arrow. C: Western blot of a 2D gel electroblotted to nitrocellulose and probed with mouse anti-cytochrome *c* oxidase subunit IV antibody. The position of cytochrome *c* oxidase subunit IV is indicated with an arrow. Molecular mass markers are indicated on the left (in kDa). The directions of the first and second dimensions are indicated by the arrows on the top and side of the gels. DF, dye front.

Several candidate endogenous kinases in mitochondria have been identified, including cAMP-dependent, cAMP-independent, tyrosine, protamine and phosphorylase kinases, and casein kinase II. It is noteworthy that one of these is casein kinase II [17,23] since there are several consensus sites for casein kinase II located within the COX IV sequence, including one within the conserved region of amino acids 54–84. The presence of phosphorylation consensus sites within the presequence may be relevant to import of the protein into the mitochondria, as was shown recently for chloroplast precursor protein transit sequences [54].

An 18 kDa protein of complex I (18 kDa (IP) AQDQ) has also been shown to be phosphorylated in bovine heart mito-

chondria [36] indicating that other proteins of the respiratory complexes may be regulated by phosphorylation as well.

The site and significance of the phosphorylation of COX IV is not known at this time. It is reasonable to assume, however, that it may contribute to the signalling pathway which regulates COX IV, and may play a key role in modulating COX activity and mitochondrial function.

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