Changes in the mitochondrial proteome from mouse hearts deficient in creatine kinase

Florence Kerneck, Jonathan S. Minden, and Alan P. Koretsky.

Changes in the mitochondrial proteome from mouse hearts deficient in creatine kinase. Physiol Genomics 6: 117–128, 2001.—Creatine kinase (CK) is an abundant enzyme, important for maintenance of high-energy phosphate homeostasis in many tissues including heart. Double-knockout CK (DbKO-CK) mice missing both the muscle (MM) and sarcomeric mitochondrial (ScMit) isoforms of CK have recently been studied. Despite a large change in skeletal muscle function in DbKO-CK mice, there is little functional change in the heart. To investigate whether there are specific changes in cardiac mitochondrial proteins associated with the loss of MM- and ScMit-CK isoforms, we have used difference gel electrophoresis (DIGE) to compare mitochondrial proteins from wild-type and DbKO-CK mice. Mass spectrometry fingerprinting was used to identify 40 spots as known mitochondrial proteins. We have discovered that the loss of MM- and ScMit-CK isoforms did not cause large scale changes in heart mitochondrial proteins. The loss of ScMit-CK was readily detected in the DbKO-CK samples. We have also detected a large decrease in the precursor form of aconitase. Furthermore, two mitochondrial protein differences have been found in the parent mouse strains of the DbKO-CK mice.

Mitochondria; knockout mouse strain; two-dimensional electrophoresis; differential protein expression; protein map; matrix-assisted laser desorption/ionization mass spectrometry; difference gel electrophoresis

Creatine kinase (CK) catalyzes the reaction phosphocreatine (PCr) + ADP ↔ ATP + creatine (Cr). This reaction ensures that PCr can be used to supply ATP if other ATP-generating pathways such as oxidative phosphorylation or glycolysis cannot keep up with the energy requirements of the cell (4, 13). There is a large body of biochemical evidence that indicates that the specific localization of CK isoenzymes is important for muscle function (36). For example, the sarcomeric mitochondrial CK isoform (ScMit-CK) reaction coupled to ATP-ADP translocase has been shown to have a strong amplifying activation effect on oxidative phosphorylation (16). A decrease in K_m for ADP induced by ScMit-CK has also been observed in oxidative muscles and not in glycolytic ones by studying mitochondria function on permeabilized skinned fibers (35). Whether these biochemical findings on specific effects of localized isoforms of CK impact normal tissue function is not clear.

Recently, a number of transgenic mouse models have been made, and these have been used to investigate CK function in muscle and heart. Mice missing ScMit-CK show no effect on skeletal or cardiac muscle. Mice missing muscle CK (MM-CK) have decreased skeletal muscle performance (34). No effect on cardiac muscle has been described in the heart, and no contractile phenotype was detected in diaphragm muscle of MM-CK KO mice (18, 27). In addition, expressing the brain isoform of CK in the muscle of mice missing MM-CK reversed the contractile defect and metabolic alterations seen in the MM-CK knockout mice. This result calls into question the importance of specific CK isoforms in muscle function (25). Rather, it may be that having a minimum amount of CK activity is important for function regardless of the isoform. Lack of isoform-specific effects on muscle is further supported by studies in the double CK knockout (DbKO-CK) mice, missing both MM- and ScMit-CK. A defect in diaphragm muscle only occurs in DbKO-CK; no defect is found with either knockout alone (38). No contractile effects of the combined MM- and ScMit-CK knockout have been detected in heart, possibly because this tissue has some brain CK isoform remaining, which supports contractile function (26). An important issue in trying to interpret these transgenic mouse studies is the fact that the cell may have adapted to the loss of CK, causing changes that help preserve tissue function (3, 32). Indeed, there is an increase in glycogen and citrate synthase in the skeletal muscle of DbKO-CK mice (32), and a metabolic difference has been reported in hearts perfused from DbKO-CK mice missing both the MM and ScMit isoforms of CK (27).
To examine whether loss of MM- and ScMit-CK leads to changes in mitochondrial proteins in the heart, we took advantage of a new two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique called difference gel electrophoresis (DIGE) (33). This technique allows two independent samples to be run in one gel simultaneously, circumventing some of the reproducibility problems associated with 2D-PAGE. It relies on modification of two different fluorescent dyes, cyanine-3 (Cy3) and cyanine-5 (Cy5), such that they can label lysines in proteins and have the same effect on migration in 2D-PAGE. Labeling one sample with one dye and another with the other dye allows both samples to be run on the same gel but be viewed individually using the different fluorescent properties of Cy3 and Cy5. DIGE was used to look for protein changes in wild-type and DbKO-CK mice. Forty-five proteins were identified by mass spectrometry (MS) fingerprinting from wild-type mitochondria. Forty were known mitochondrial proteins, and five could not be matched to the database and may be unknown proteins. Hemoglobin was the only nonmitochondrial protein identified. Comparison of the parent strains from which the DbKO-CK mice were derived, C57BL/6 and 129/Sv, indicated three protein differences. These were a shift in position of the short-chain fatty acid dehydrogenase, probably due to an allelic variation, an isoelectric point (pI) shift in hsp70, possibly due to a phosphorylation difference between the strains, and an increase in an unidentified protein in C57BL/6. Finally, comparison of mitochondria from wild-type and MM-CK/ScMit-CK DbKO mice revealed only two protein differences. One was loss of ScMit-CK as predicted, and the other was a decrease in the precursor form of aconitase. Neither aconitase activity from mitochondria nor total aconitase content from whole heart extracts was altered, indicating that the loss of CK caused a redistribution of precursor aconitase from mitochondria to the cytosol.

**MATERIALS AND METHODS**

**CK–/– mice genotype background.** Mice bearing a null mutation of Scmit-CK (−/−) gene were interbred with mice bearing a null mutation of the M-CK gene to generate heterozygotes for both CK isoforms (31). Sibling mating of these mice resulted in the generation of DbKO-CK mice missing both the muscle (MM) and sarcomeric mitochondrial (ScMit) isoforms of CK, as previously described (30). ScMit/MM-CK double-deficient mice had a mixed genetic (C57Bl/6 and 129/Sv) background. C57BL/6, 129/Sv, and DbKO-CK mice were used in the study. All experiments were approved by the appropriate Animal Use and Care Committee.

**Rapid isolation of mouse heart mitochondria.** Mice, 2–4 mo of age, were anesthetized with Avertin (2.5% Avertin stock solution at a dose of 0.02 ml/g mouse), and the heart was rapidly removed. Mouse heart tissue was homogenized in a tightly fitting Potter-Elvehjem glass homogenizer. A rapid isolation of mouse heart mitochondria was performed by using differential centrifugation (7,000 rpm, 2 min; 14,000 rpm, 2 min; Eppendorf model 5415D centrifuge) on a sucrose gradient (250 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 50 U/ml heparin) (28). A total heart homogenate fraction was prepared simply by homogenizing a piece of heart tissue directly in lysis buffer.

**Electron microscopic analysis of heart mitochondria preparation.** The sample was fixed as a pellet at room temperature for 30 min in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.4. Excess fixative was removed by washing in three changes of 0.1 M sodium cacodylate, pH 7.4. The sample was additionally fixed for 30 min at room temperature in 1% osmium tetroxide buffered in 0.1 M sodium cacodylate, pH 7.4. The fixative was removed by washing with changes of distilled water and was dehydrated in an ethanol series (50%, 70%, 80%, 90%, and 100%). Propylene oxide was used as a transitional solvent, and the sample was infiltrated in Epon-Araldite for 24 h. The resin containing the samples was polymerized at 60°C for 48 h.

The samples were sectioned with a diamond knife, and stained with uranyl acetate and lead citrate. The samples were viewed and photographed on a Hitachi model 7100 camera. The system is from Advanced Microscopy Techniques and is called the Advantage 10 Power Mac CCD camera system.

**Protein labeling and 2-D PAGE.** Two fluorescent dyes (Cy3 and Cy5) were used to label proteins from two different samples as previously described (33). The labeling procedure affects the electrophoretic mobility of proteins from the two samples to the same extent and thus can be used to compare two samples on a single 2-D PAGE. Isoelectric focusing (IEF) was carried out on 18 or 13 cm, pH 3–10, linear precast Immobiline gels according to the manufacturer’s instructions (Pharmacia, Piscataway, NJ), except that the reswelling buffer was 2% CHAPS, 8 M urea, 1% Pharmalyte (pH 3–10), 2 mM acetic acid, and 10 mM dithiothreitol. Typical run conditions were initially 1,000–1,500 V for 1–2 h, followed by 10–16 h at 3,500 V, for a total of 30–50 kV·h per run. Gradient gels of 10–15% (16 cm × 24 cm) with a 2-cm long 3% stacking gel were used for SDS-PAGE. Gels were run at 4°C. Analytical gels were typically loaded with 50 μg of protein per sample for 100 μg of total load. Once difference proteins were identified using analytical electrophoresis, 500 μg of mitochondrial protein from one relevant sample was used without any dye labeling to run a preparative 2D gel. Proteins were visualized using Coomasie staining, and spots of interest were excised for in-gel tryptic digestion.

**Gel imaging and processing.** A home-built imager and accompanying software were used for fluorescent imaging and processing as described previously (29). Briefly, the gel was imaged with a double-wavelength band-pass emission filter and a CCD camera (587 ± 17.5 and 695 ± 30 nm). Excitation was from a quartz-tungsten-halogen lamp with band-pass filters for Cy3 (545 ± 10 nm) and Cy5 (635 ± 15 nm). Two separate images for Cy3- and Cy5-labeled proteins, respectively, were acquired and placed into a two-frame movie file. Protein differences between the two samples were detected visually by viewing this two-frame movie played in a continuous loop. Protein spots were enumerated and assigned molecular weights and pl values using the PDQuest software package. Only those differences that were independent of whether the sample was labeled with Cy5 or Cy3 and that were consistently detected from four to five gel pairs were analyzed further.

**Protein identification.** In-gel tryptic digestion of proteins was carried out using a previously described method (12) with the modifications described at the UCSF Mass Spectrometry web site (http://donatello.ucsf.edu/ingel.html). Mass fingerprinting of tryptic peptides was carried out using a Voyager model DE-STR matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF) instrument (P. E. Sciex). The Protein Prospector data-mining package was used to analyze the tryptic mass fingerprint.
MnCl<sub>2</sub>, 0.2 mM NADP, 30 mM citrate, and 1 U isocitrate dehydrogenase. Mitochondrial protein, 5–10 μg, was used in a final volume of 1 ml. Care was taken to be sure that the rate obtained changed linearly with the volume of mitochondrial protein added.

RESULTS

Mitochondria were isolated from mouse heart following a fast isolation procedure to limit artificial protein modifications (28). The yield of mouse heart mitochondrial proteins in the isolation was ~2 mg/g heart tissue. An electron micrograph obtained from the preparation shows predominantly mitochondria and little sign of other contaminants (data not shown). Figure 1A shows a typical 2D-PAGE gel obtained from the isolated mitochondria and stained with Coomassie blue. This is compared with a 2D-PAGE gel obtained from a mitochondrial sample prelabeled with Cy3 (Fig. 1B). Both labeling strategies gave a similar number and pattern of spots. Incubating the mitochondria for various lengths of time up to 2 h prior to electrophoresis did not lead to any significant changes in the number or pattern of spots (data not shown), indicating that the preparation was stable over time.

We concentrated on spots of relatively high abundance by limiting the exposure time of the cyanine-labeled gels. Peptide mass fingerprinting was performed with MALDI-TOF on in-gel trypsin-digested proteins. Examples of mass spectra obtained and peptide matches are shown in Fig. 2. The top spectrum is from a spot identified to be ATP synthase β-chain based on mouse DNA sequence. The bottom spectrum is from a spot identified to be a 24-kDa subunit of NADH-ubiquinone reductase based on Homo sapiens DNA sequence. A description of the assignment of protein identification is given in the legend of Fig. 2. Table 1 lists all of the spots on which MS identification was performed, and Fig. 3 shows a 2D-PAGE gel with all of the analyzed spots numbered to correspond with entries in Table 1.

A total of 45 spots were analyzed. All but two proteins identified were of mitochondrial origin. The two contaminants were from the α- and β-chain of hemoglobin. Most of the proteins identified are involved in mitochondrial energy metabolism. Thirteen are proteins of respiratory chain complexes, 10 are of the tricarboxylic acid (TCA) pathway, and 9 are of β-oxidation of fatty acids metabolism. ScMit-CK as well as voltage-dependent anion channel (VDAC chain 1) have also been identified among these major heart mitochondrial proteins. The rest of the identified mitochondrial proteins have a variety of functions, including heat shock proteins (hsp70 and hsp60), amino acid biosynthesis pathway (aspartate aminotransferase chain 1, isovaleryl-CoA dehydrogenase), heme biosynthesis pathway (coproporphyrinogen oxidase), protein biosynthesis (p43 mitochondrial elongation factor Tu), and free radical metabolism (manganese superoxide dismutase, Mn-SOD). The identified proteins come from all compartments of the mitochondria, including the outer membrane, the intermembrane space, the inner membrane, and the mitochondrial matrix. Five of the spots analyzed could not be identified by comparison to the database, despite having high-quality mass spectra indicating these may be unknown mitochondrial proteins.

Many knockout mice are produced on a mixed genetic strain. The DbKO-CK were produced on a mouse strain mixture of C57BL/6 and 129/Sv. Therefore, mitochondrial protein differences were assessed between these two strains using DIGE. Figure 4 shows gels imaged with Cy3- and Cy5-labeled samples, illustrating the three differences detected between the strains. One difference was located in the range of 70–90 kDa and 5.5–6.5 pI (Fig. 4A). The change in this spot was a shift to the right in the pI direction of the gel. MS fingerprinting identified this spot as hsp70 (spot 5 on Table 1 and Fig. 3). Hsp70 is known to be phosphorylated, and the shift detected between the strains may be due to phosphorylation differences (19). Another...
difference was located in the range of 40–50 kDa and 7–7.5 pI (Fig. 4B). A spot present on 129/Sv 2D-PAGE clearly decreases in C57BL/6 2D-PAGE, and two new spots arise in the C57BL/6 mitochondria. The spot that decreases in C57BL/6 corresponds to spot 24 (Table 1, Fig. 3), which is identified as butyryl-CoA dehydrogenase, an enzyme important in metabolizing short-chain fatty acids. The two new spots that arise in C57BL/6 mitochondria correspond to spot 23 and the spot marked with an asterisk (*) (Table 1, Fig. 3). MS fingerprinting identified spot 23 also as butyryl-CoA dehydrogenase. The shift in butyryl-CoA dehydrogenase from C57BL/6 to 129/Sv probably represents allelic variation between the strains. The spot marked with an asterisk (*) has not yet been identified.

Proteins from heart mitochondria from mice missing both MM- and ScMit-CK isoforms were isolated for comparison with both 129/Sv and C57BL/6 heart mitochondria. Interestingly, DbKO-CK mitochondria showed the short-chain acyl-CoA from 129/Sv and the hsp70
pattern from C57BL/6, demonstrating the mixed background upon which the knockouts were made (data not shown). In addition to these strain differences, two other changes in mitochondrial proteins were detected (Fig. 6). A spot corresponding to ScMit-CK (spot 14; Table 1, Fig. 3) disappears from DbKO-CK mitochondria preparation (Fig. 5A). This indicates that DIGE was able to detect the loss of ScMit-CK caused by the knockout. In addition, there was a large drop in signal intensity of a series of spots in the range of 80–100 kDa and 7–9 pI. Peptide mass fingerprinting analysis revealed this spot to be an abundant enzyme of the TCA pathway, aconitase. Interestingly, this spot corresponds to the precursor form of aconitase that exists prior to protease processing during import of aconitase into mitochondria. No consistent or significant changes were detected in any of the other protein spots examined on the gel.

The large change in aconitase precursor was unexpected. A number of spots have been identified in the upper region of the mouse heart mitochondria 2D-PAGE as aconitase (spots 1–4; Table 1, Fig. 3). It is well known that aconitase gives a complex pattern on 2D-PAGE, probably due to the fact it is easily oxidized (21). Only the set of spots with the higher molecular mass, identified as the precursor aconitase, decreases in gels from heart mitochondria from DbKO-CK mice. No differences were detected in mature, active aconitase from the gels. Figure 6 shows mass spectra from the precursor and mature form of aconitase. The peptide peaks at 884.59 and 1,519.73 due to the signal prescence are present in the precursor but absent in the mature form.

A lack of change in the mature aconitase predicts that there should be no change in aconitase activity despite the large drop in precursor form. To test whether there was a change in aconitase activity, freshly isolated mitochondria were assayed for aconitase activity. The heart mitochondrial aconitase activity measured on control mice C57BL/6 (0.27 ± 0.09 IU/mg mitochondrial protein, n = 6) and 129/Sv (0.24 ± 0.11 IU/mg mitochondrial protein, n = 5) were not significantly different from DbKO-CK mice (0.32 ± 0.08 IU/mg mitochondrial protein, n = 5), consistent with the gel results.

The decrease in precursor aconitase detected from the mitochondria could be due to a redistribution of aconitase from mitochondria to the cytoplasm. Precursor forms of mitochondrial proteins are synthesized in the cytosol and then interact with mitochondrial import machinery at the contact sites of mitochondria for import. Upon translocation into the matrix, precursor forms are processed to the mature form of proteins. It could be that in the DbKO-CK mice the binding of precursor aconitase to the contact site was affected. To test this, levels of precursor aconitase were compared in whole heart extracts by DIGE. No significant differences were detected (Fig. 7) from whole heart extract from 129/Sv, C57BL/6, and DbKO-CK mice, consistent with the idea that the decrease in precursor aconitase detected in mitochondria was due to a redistribution from mitochondria to cytosol.

DISCUSSION

With the completion of the sequence of a number of organisms’ genomes there is rapidly growing interest in efficiently characterizing changes in expressed transcripts and proteins. It is still not possible to use sequence information alone to predict the localization of a protein to mitochondria (6). Therefore, it is important to develop methods that enable efficient screening of changes in the mitochondrial proteome (10). One way to do this is to combine 2D-PAGE gel analysis with MS fingerprinting on isolated mitochondria. Close to 1,500 spots have been detected on a silver-stained human placental mitochondria 2D gel (24). However, no more than 340 genes are identified as mitochondrial in one of the most complete mitochondrial human proteins database, SCMITOP [Mitochondria Project Collaboration of several...
Table 1. Mouse heart mitochondrial proteins for 2D-PAGE gel identified by MALDI-TOF analysis and Protein Prospector data-mining package

<table>
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<th>Functional Category</th>
<th>Spot Number</th>
<th>Protein Name (SwissProt accession number)</th>
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<th>Molecular Mass, kDa</th>
<th>Isoelectric Point</th>
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</table>

Assignments were made by combining MALDI-TOF analysis and Protein Prospector dataminning package (proprietary product of The Regents of the University of California). The list was subdivided into 5 functional categories (Energy, Metabolism, Transport facilitation, Protein synthesis, Protein import, and Mitochondrial biogenesis). Numbers refer to spots on 2D gel in Fig. 3. Certainty hit is defined as follows: high, greater than 80% of major mass spectra peaks match database analysis; medium, most major peaks match, and many of the rest can be matched with conservative amino acid change or chemical modification; low, many peaks do not match, probably due to a protein mixture. MALDI-TOF, matrix-assisted laser desorption/ionization time of flight mass spectrometry; VDAC1, voltage-dependent anion channel, chain 1.
German institutions, including Munich Information Center for Protein Sequences (MIPS); [http://www.mips.biochem.mpg.de/proj/medgen/mitop/](http://www.mips.biochem.mpg.de/proj/medgen/mitop/). Consistent with this notion that many mitochondrial proteins have not been identified, 5 of the ~45 spots that were fingerprinted in this study did not match any proteins in the database. This was true even though only abundant spots were analyzed. Rigorous identification of these proteins as novel mitochondrial proteins awaits sequence information. The other 40 spots were assigned as common mitochondrial proteins involved in almost all known functions of mitochondrial biochemistry. The only nonmitochondrial protein identified was hemoglobin, indicating that the mitochondrial preparation used was not significantly contaminated.

The main goal of the present work was to screen for changes in mitochondrial proteins associated with lack of MM- and ScMit-CK in cardiac mitochondria. To quantitatively compare mitochondrial samples from wild-type and DbKO-CK mitochondria, we made use of the recently developed 2D-PAGE technique, DIGE. DIGE enables two samples to be compared on the same gel, through the development of different fluorescent dyes that can be conjugated to each respective sample, run together, and specifically detected due to the different wavelength properties of the dyes (30). Here we used DIGE, first to examine differences in the parent strains of the DbKO-CK mice, C57BL/6 and 129/Sv. There is a large literature demonstrating different phenotypes of mice from different strains (1, 11, 23). These differences are presumably due to variation in DNA sequence in the different strains. For example, it has been shown that hearts from 129/Sv mice grow much larger than do hearts from C57BL/6 mice when challenged with phenylephrine (B. B. Roman, personal communication). Comparison of mitochondria from C57BL/6 and 129/Sv using DIGE indicated three major differences. There was a shift in mobility of the short-chain acyl-CoA dehydrogenase, presumably due to a mutation that changes the amino acid composition. Whether this variation in short-chain acyl-CoA dehydrogenase causes any strain differences in fatty acid metabolism is an interesting question for future work. A change in the phosphorylation state of hsp70 was also detected with the protein from C57BL/6 being more phosphorylated than in 129/Sv. Hsp70 has been previously shown to be phosphorylated (19); however, the significance of this phosphorylation for the physiological function of Hsp70 is unknown, making it difficult to assess the consequences of this difference in mouse strains. There was a third difference between

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**Fig. 3.** A 129/Sv heart mitochondrial protein 2D-PAGE gel with all of the analyzed spots numbered. Numbers refer to protein identifications given in Table 1. VDAC, voltage-dependent anion channel; CK, creatine kinase; ScMi-CK, sarcomeric mitochondrial isoform of CK (“Sc-Mit-CK” is used in main text).
C57BL/6 and 129/Sv mitochondria; however, the protein has not yet been identified.

Interestingly, when mitochondria from the DbKO-CK mice were studied, one could see from the hsp70 and short-chain acyl-CoA dehydrogenase spots that the DbKO-CK mice were on a mixed background. This highlights the growing appreciation for strain differences among mice and the fact that knockouts that are on mixed strains may be difficult to analyze because of lack of an appropriate control. This result also highlights that proteins can be used to study allelic differences in mouse strains along with the use of DNA sequence differences.

Comparison of mitochondria from DbKO-CK mice with both parental strains revealed only two differences. First, as expected, ScMit-CK was missing from the DbKO-CK mice. This demonstrated the ability of DIGE to identify protein changes. Furthermore, it raises the interesting possibility of using DIGE to look for the protein changes associated with mutations in the mouse genome. There are a number of naturally occurring mouse mutants and growing interest in random mutagenesis of the mouse. Identifying mutation through analysis of DNA remains extremely time consuming. Utilizing DIGE to find candidate proteins that may have been altered in mutagenesis strategies may complement the analysis of DNA.

ScMit-CK, adenine nucleotide translocator, and porin have been shown to be involved in a complex of proteins called the permeability transition pore complex (PTPC) (2, 22). This complex has been hypothesized to play a crucial role in the regulation of heart mitochondrial respiration (36). In particular, ScMit-CK is believed to associate with porin and the adenylate translocase in a manner that has functional significance for the transfer of high-energy phosphates out of the mitochondria. Although hearts from DbKO-CK mice have not been reported to contain in vivo functional deficiencies, a metabolic defect has been demonstrated in perfused DbKO-CK hearts containing C57BL/6 and 129/Sv mitochondria; however, the protein has not yet been identified.

Fig. 4. 2D gels imaged with Cy3- and Cy5-labeled samples illustrating differences in mitochondrial proteins detected between mouse strains. A: difference gel pairs are side by side for 129/Sv and C57/B6 labeled samples and reverse-labeled samples. Three changes are identified by the arrows in the 40- to 50-kDa and 7- to 7.5-pI ranges. Asterisks indicate an unidentified protein. B: difference gel pairs are above and below one another with labeled and reverse labeled pairs on the right and left. A difference was detected in the 70- to 90-kDa range that represents a shift in pI as indicated by the arrows.
consistent with an important role of MM- and ScMit-CK in cardiac energetics (27). Thus one might expect adaptations in members of the PTPC in response to elimination of ScMit-CK. Using DIGE, we were not able to detect any significant changes in porin content. We have not identified a protein spot corresponding to the adenylate translocase protein (ANT1), probably because this protein has a very basic pI (~10.54) that was out of our 2D gel detection range. This illustrates a shortcoming of 2D-PAGE to search for changes in proteins. Another shortcoming in the present study is that only abundant mitochondrial proteins were studied. It will be possible to study very low levels of proteins with DIGE using more sensitive gel imaging and more extensive protein labeling strategies.

The other major change in a mitochondrial protein detected in the DbKO-CK mice, in addition to the loss of ScMit-CK, was a large drop in the precursor form of aconitase. Aconitase is a key enzyme of the TCA cycle.

Fig. 5. 2D gels imaged with Cy3- and Cy5-labeled samples illustrating the two mitochondrial protein differences detected between normal and double-knockout (DbKO)-CK mice (“DbKO” here and in Fig. 7). A: Cy3 and Cy5 pairs are side by side with the reversed labeled pairs below. Arrows indicate location of M-CK for each set. ScMit-CK disappears in DbKO-CK mice. B: Cy5 and Cy3 pairs are side by side, with the reverse-labeled pair below. The arrow indicates the region in the gel that contains the pre-cursor form of aconitase that decreases in DbKO-CK mice.

A number of protein spots in the molecular mass range of 80–100 kDa and pI range of 7–8.5 of the heart mitochondria 2D gel have been identified as aconitase. Aconitase is known to run in a complex pattern on 2D gels with numerous oxidation forms (21). Only the line of spots with the highest molecular mass decreased in CK knockouts. MS fingerprinting identified these spots as the aconitase precursor form. It is known that the precursor form of aconitase is processed upon import into mitochondria to a lower molecular mass, mature, and active form of the enzyme (40). Consistent with the change in aconitase being the precursor and not the mature form of aconitase is that no decrease in aconitase activity was measured from the mitochondria of the knockout mice. Aconitase is a complex protein; activity only measures the fully mature enzyme, whereas gel electrophoresis measures total protein, so that total protein levels could change without change in activity (Fig. 7). There is a cytosolic form of aconi-
tase, coded by a separate gene, involved in iron regulation; however, in the heart the majority of aconitase is known to be localized mainly in the mitochondria. The lack of any decrease in the high molecular mass precursor form of aconitase in whole heart extract compared with isolated mitochondria argues that there has been a redistribution of the precursor form of aconitase from the mitochondria to the cytosol in the knockout mice.

The significance of a redistribution of the mature form of aconitase from the mitochondria to the cytosol in the DbKO-CK mice is not clear. Import of mitochondrial precursor proteins is thought to occur at contact sites. Indeed, contact sites between outer and inner mitochondrial membranes are formed when there is import of mitochondrial matrix proteins (8). Contact sites between the outer and inner mitochondrial membranes arise from dynamic mechanisms (7, 8, 16). One
of these mechanisms involved ScMit-CK, which has been shown to stabilize contact sites due to its interaction to porin (outer membrane) and ANT (inner membrane) (29). It may be that the drop in precursor aconitase associated with the mitochondria occurs due to an impairment in contact site formation due to lack of ScMit-CK. However, a previous analysis by Steeghs (30) found no differences in the number of contact sites between DbKO-CK mice and wild type.

Interestingly, the most abundant aconitase form in the 2D gels is the precursor form. Furthermore, we were unable to identify precursor forms of any other mitochondrial proteins. Thus it is tempting to speculate that the presence of the precursor form of aconitase plays some role in mitochondria and that disruption of ScMit-CK influences this role. Aconitase has a very reactive sulfhydryl (15) and is known to be modified during procedures that alter cellular redox state (5). Interestingly, CK also has a reactive sulfhydryl that is known to be modified during changes in cellular redox state (20, 39). Both proteins are located near contact sites and both are in high abundance compared with what is necessary for the rate of the reactions they are known to catalyze. It is possible that ScMit-CK and aconitase are localized to the contact sites to act as redox buffers to protect other components of the contact site or to be involved in redox signaling. Based on the change in aconitase precursor in the DbKO mice, it should be possible to design experiments to test this hypothesis.

In conclusion, we have used DIGE in combination with MS fingerprinting to study mitochondrial protein changes associated with C57BL/6 and 129/Sv mouse strains and in MM- and ScMit-CK knockout mice. Forty known mitochondrial proteins were identified, of which three varied with mouse strain. Among the most abundant spots analyzed, five still remain without any identification. The lack of ScMit-CK in the double knockouts was easily detected. The only other significant change was a large decrease in the precursor form of aconitase, which is attributed to redistribution of the protein. The significance of this change and the connection to ScMit-CK is not clear, but it is intriguing in light of the overlap in localization and the similar sensitivity of these proteins to sulfhydryl reactive agents, such as nitric oxide.

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