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Mammalian mitochondrial proteome and its functions: current investigative techniques and future perspectives on ageing and diabetes

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ABSTRACT

Mitochondria play important roles in cellular physiology and in various pathologies. Over the last decade, great achievements have been made in study of the whole mitochondrial proteome, subproteome, mitochondrial complexes and their molecular organization, and mitochondrial post-translational modifications in both physiological and pathological conditions. Mitochondrial proteomic technologies will be discussed with regard to their applications to the detection of proteins with a net impact on the capacity of the mitochondria to perform various tasks during ageing and in diabetes.

Keywords: Mitochondrial proteome; 2-DE • BN-PAGE; OXPHOS supercomplexes; **A**geing; **D**iabetes.

Introduction

Mitochondria are essential organelles, representing, in most eukaryotic cells, the primary site for the production of ATP via oxidative phosphorylation [1]. Besides the production of energy by cellular respiration, mitochondria are involved in intermediary metabolism and in anabolic processes such as the biosynthesis of amino acids, lipids, heme and iron-sulphur clusters, the cellular homeostasis of calcium [2], and in triggering apoptosis [3].

Subserving all these functions is the unique protein composition of mitochondria, with the vast majority of the proteins being encoded within the nucleus (synthesized upon cytosolic ribosomes and imported to submitochondrial destinations) and the remaining minority being encoded by the mitochondrion's own genome, which actually encodes only a few components of the respiratory chain complexes (thirteen in humans). Thus, the mitochondrial proteome should be viewed as a dynamic program generated by fine cross-talk between the two genomes and able to adapt to the needs of the tissue or disease state. Alterations in the mitochondrial proteome exert influences over mitochondrial homeostasis, leading to several pathologies in addition to disturbing such natural processes as development and ageing [4-6]. Mitochondrial dysfunction plays roles in the pathogenesis of a wide range of diseases that involve disordered cellular fuel metabolism and survival/death pathways, including neurodegenerative diseases, cancer, and diabetes. In recent years, it has emerged that the mitochondrial proteome differs greatly among tissues, depending on their functional requirements. Moreover, it is also clear that posttranslational processes modify both the localization and the function of mitochondrial proteins in tissue- and disease-specific ways.

For a better understanding of the complex mitochondrial functions, various systematic approaches to mitochondrial-

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protein identification and quantification have been made within the last decade. Already, proteomic strategies, predomonantly structural ones, have increased our knowledge of the mitochondrial compartment as well as of mitochondrial function.

Indeed, the proteomes of mitochondria from yeast [7-10], mouse [11-13], human cells [14,15], Neurospora crassa [16], rat [17-19], and various plants [20] have been systematically analyzed.

Proteomic analysis is being applied to mammalian cells and tissues both to gain insights into mitochondrial function and to identify disease-associated mitochondrial proteins [21,22]. Notably, such proteomic studies have proved capable of an almost complete analysis of the metabolic-flux potential of a given tissue in a single experiment, thus providing important and novel information concerning tissue- and disease- state modifications.

This article will provide an overview (a) of the current state of our knowledge about the mammalian mitochondrial proteome, with an emphasis on mitochondrial complexes and the mitochondrial PTMs that occur in ageing and diabetes, as well as (b) of the classical and ongoing approaches used to date in mitoproteomics.

1. Main tools used to analyze mitochondrial proteins: classical and ongoing approaches.

Even though the classical proteomic techniques were not initially developed specifically for the study of mitochondria, nowadays they, together with a few others, offer great, indeed unique, opportunities for the investigation of mitochondrial function within the cell. By so doing, they provide allowing to obtain structural, comparative/quantitative, and functional information.

Thanks to the early structural proteomic studies, it was possible to enumerate the mitochondrial proteins and it is now thought that, at least in mammals, there are approximately 1000 to 2000 of them. Knowledge of the abundance and the identities of these proteins has shed light on mitochondrial complexity. However, in view of the potential impact that any local mitochondrial dysfunction can have, directly or indirectly, on intra/extra mitochondrial metabolic pathways, large amounts of new data are still being sought concerning the mitoproteomic modulations that might occur in response to a given signal, a subject of interest both in physiology (i.e., ageing) and pathology (i.e., diabetes, neurodegeneration) [23,24]. The research in this field has been aided by the development of comparative proteomic techniques. These actually share the same principles as those used in structural proteomics, with the basic and widely used approaches being two-dimensional electrophoresis and shotgun proteomics, which differ only in the means used to separate and identify proteins [25,26].

In view of: the numerous functions performed by mitochondria, their high level of compartmentalization, and the existence of multipolypeptide complexes [i.e., the five components of the oxidative phosphorylation system, the Transporter Inner Membrane (TIM) and Transporter Outer Membrane (TOM) complexes, and the mitochondrial permeability transition pore] that actually contain hydrophobic proteins in close contact with the membrane lipids, peripheral proteins, and non-protein cofactors, it is likely that a deep structural/functional study of the mitoproteome requires an appropriate combination of different tools to help compensate for the limits imposed on the analysis by each individual technique. Moreover, when working on sub-cellular compartments, as in the case of mitochondria, the purity of the preparation is another critical issue. Indeed, in the process of mitochondrial preparation, several proteins can be co-isolated (i.e. from endoplasmic reticulum or other organelle membranes in close contact with mitochondria), likely impairing quantitative and qualitative analyses and, thus altering data interpretation. Several methods have been applied in efforts to obtain pure mitochondria from tissues and cells. These comprise differential centrifugation, density gradient centrifugation with Percoll™ [27], Nycodenz [28], Metrizamide [29], or sucrose [30], free-flow electrophoresis [31], and kit-based methods [32]. Mitochondria can also be highly purified by immunoisolation (by means of mitochondria-specific antibodies), although the costs are high and large amounts of sample are lost [33]. The purity of the mitochondrial preparation --a fundamental issue above all others when performing quantitative analyses of proteins with different subcellular localizations-- can be tested by measuring marker-enzyme activities and/or by Western blotting for specific markers. Thereafter, in general terms, the protein-separation strategy, critical in determining the utility of the proteomic approach, can be based either on gel electrophoresis (i.e., two-dimensional electrophoresis, DIGE, BN-PAGE) or on gel-free/MS-based techniques (i.e., shotgun proteomics). A brief description of these methods, and of their advantages and limitations, is given below.

1.1. *Two-dimensional electrophoresis (2-DE).*

2-DE allows separation of proteins first on the basis of differences in their net charge [through a technique known as isoelectric focusing (IEF); first dimension] and secondly on differences in molecular mass [through polyacrylamide gel electrophoresis (PAGE); second dimension). 2-DE is the classical, and the most popular, technique for the profiling of complex protein mixtures obtained from biological samples. It provides the highest resolution in soluble protein separation, and indeed resolves hundreds or thousands of proteins at once. In recent years, the commercial availability of preformed immobiline gradients, with various ranges of pH and sizes, has made it possible to choose the best range of pH for optimal results, depending on the origin and specific characteristics of the sample to be analyzed. The advantages of - comprise: i) fast resolution of proteins, ii) the relatively low cost of the experiments, and iii) the possibility of directly evaluating the pI and MW of the protein(s) of interest. Unfortunately, there are also several disadvantages. Among

these are: i) difficulties in the resolution of proteins with ex-extreme pI values, ii) loss of very hydrophobic proteins, iii) absence of proteins of high and low molecular weights, iv) poor resolution of low-abundance proteins, v) large amounts of sample handling, and vi) inter-gel variability. When specifically studying mitochondrial proteomes, an additional disadvantage is that, for a broad range of pH values (3–11 NL), there a slight asymmetrical distribution of the molecules, with those of molecular weights (usually) extending from 0.1 to 15 kDa, being preferentially localized in the basic parts of the gel. Proteins in 2D-gels can be detected by several staining procedures. These can be roughly divided into the following categories: (i) organic dyes; (ii) silver stains; (iii) negative stains; and (iv) fluorescent stains. For Coomassie Blue R250 (CBB), the most popular organic dye currently in use, the detection limit and linear dynamic range are from 8 to 16 ng and 125-1000 ng, respectively, depending on the proteins [34]. Silver staining is also widely used, and it is particularly suitable when the amounts of proteins are quite small [35]. Its sensitivity is relatively high (0.5-8 ng) [36], but its dynamic range is quite narrow (e.g., 8-60 ng for the alkaline/silver diamine stain) [34]. The latter obviously limits the quantitative use of silver staining when comparing samples with widely differing amounts of proteins.

Concerning negative stains, the most sensitive method is represented by the imidazole-zinc (IZ) stain. This, while having several desirable features --such as simplicity, rapidity, reversibility, and compatibility with subsequent protein analysis [37,38]-- suffers from the drawback of its poor contrast, which makes band- or spot- excision difficult [39]. Recently, a new negative detection method has been described [40]. In this method, Eosin Y is selectively precipitated in the gel background, the same precipitate being absent from those zones where proteins are located through the formation of a stable water-soluble protein-dye complex. This staining provides high sensitivity at low cost, and has a simple, fast protocol with a detection limit of 0.5 ng of a single protein band. Moreover, when compared with IZ stain, Eosin Y dye provides a broader linear dynamic range, higher sensitivity and reproducibility, and better contrast between the protein bands or spots and the background.

Among the fluorescent stains, the fluorescence-based SYPRO Ruby stain (Molecular Probes, USA) [34] has a good detection limit (0.5-5 ng) [41] and a much larger dynamic range compared to silver staining (1-1000 ng), permitting reliable quantification. Recently, Candiano et al. [42] proposed a modified Neuhoff's colloidal Coomassie Blue G-250 stain, which they dubbed "blue silver" This has high sensitivity, approaching that of conventional silver staining, with the advantages of Coomassie blue. Moreover, 2-DE electrophoresis represents the basic platform for the systematic positively identification of posttranslational modifications (PTMs) (i.e. carrying phosphate groups or groups introduced by oxidative reactions) as well as for the analysis of different splice variants. Specifically, the PTMs can be detected in gel by means of immunoblotting using antibodies against the specific added groups, or by sensitive specific probe-targeted approaches (i.e. biotin-NM).

1.2. *DIGE.*

Mainly to avoid inter-gel variability, 2D DIGE has been developed as a multiplexing technology to enable visualization of multiple protein samples on a single 2D gel [43]. Multiplexing is ensured by pre-electrophoretic labeling of protein samples with distinct and spectrally resolvable fluorescent dyes known as CyDyes (N-hydroxysuccinimide derivatives, propyl-Cy3, and methyl-Cy5). Labeling protein samples with a CyDye is very sensitive, with a detection limit of the order of ~1 ng and a dynamic range of ~105. While two independent protein samples are labeled with Cy3 and Cy5, a third fluorescent dye (Cy2) can be used to label a pooled sample containing equal amounts of the two independent samples that are to be compared [44]. This pooled internal standard is multiplexed with the Cy3- and Cy5-labeled samples within each experimental gel-replicate, and is then used for the normalization of all spots across the gel-replicates, yielding a minimized inter-gel variation and an accurate quantification of the protein-expression differences, supported by statistical analysis. Following electrophoretic separation of multiplexed labeled samples, proteins are visualized by imaging the gel at wavelengths specific for each dye allowing the identification of protein spots and quantification within each gel-replicate. Including the internal standard on each gel used in the experiment, along with the individual biological samples, means that the abundance of each protein spot on a gel can be measured relative (i.e., as a ratio) to its corresponding spot in the internal standard present on the same gel. Ettan DIGE is a system of technologies that has been optimized to benefit fully from the advantages provided by 2D DIGE [45].

1.3. *Blue native PAGE.*

The investigation of membrane-bound protein complexes (i.e., mitochondrial respiratory chain complexes, OXPHOS) requires special tools, above all because of the high hydrophobicity of the proteins involved. These have poor focalization and cannot be efficiently separated by classical 2-DE, as stated above. Consequently, a technique called Blue Native electrophoresis (BN-PAGE) was specifically developed [46-48]. It was named BN-PAGE because of the color of the crucial compound, Coomassie Blue, which gives a charge to the protein complexes without dissociating them, thereby allowing their resolution in the native form according to their molecular mass. In the years since its development, BN-PAGE has become a very important tool, particularly in mitochondrial research. Moreover, by applying 1st dimensional BN-PAGE (under native conditions) to 2nd dimensional SDS-PAGE (where electrophoresis is performed under denaturing conditions), the individual subunit proteins of the complex can be disclosed and individually investigated. The method allows analysis of both the concentration and the

composition of protein complexes. The detergent dodecyl-b-D-maltoside, which does not dissociate protein complexes, is used to solubilize the membranes, and extraction of the protein complexes is aided by the inclusion of the zwitterionic salt aminocaprionic acid. Since this has a net charge of zero at pH 7, it does not affect electrophoresis. Moreover, Cruciat et al. [49] and Schägger and Pfeiffer [50] isolated stoichiometrically assembled respiratory chain supercomplexes from yeast and mammalian mitochondria with high yields. Functionally, the assembly of OXPHOS into respirasomes has been suggested to have a number of potential advantages versus individual complexes (e.g., substrate channeling of quinones and/or cytochrome c, sequestration of reactive intermediates such as ubisemiquinone, and stabilization of individual complexes by supramolecular assembly) [50]. Supercomplexes can be obtained by substituting, during the membrane solubilization step, dodecyl-b-D-maltoside with the mild detergent digitonin [49,50]. Among the disadvantages associated with BN-PAGE, it has to be admitted that some proteins (a) cannot be separated by this electrophoretic technique because they cannot bind Coomassie blue and (b) having a neutral or basic pI, they migrate towards the cathodes and will be lost in the running buffer. Moreover, Coomassie blue can generate micelles in combination with neutral detergents, and these may interfere with fluorimetric and catalytic assays [48]. Indeed, a new technique called high resolution clear-native electrophoresis (hrCN-PAGE) was developed to try to overcome some of the disadvantages of BN-PAGE [48,51,52]. This approach, with a separation resolution as high as that of BN-PAGE, uses non-colored mixed micelles to induce a net negative charge on the proteins. This allows the performance of fluorimetric and catalytic assays. Actually, a possible disadvantage versus BN-PAGE is that hrCN-PAGE favors the dissociation of labile proteins from protein complexes.

1.4. *Shotgun proteomics and stable isotopic labeling experiments (SILE).*

In the area of mitoproteomics, the issues concerning the limitations of the above in the detection and identification of membrane (hydrophobic) proteins, as well as of low-abundance proteins, have also had to be faced during the development of large scale, so-called "shotgun proteomics", specifically with the introduction of multidimensional protein-identification technologies [53-55]. Schematically, in these procedures a complex peptide mixture is loaded on a micro capillary column packed with two stationary phases [usually, strong cation exchange (SCX) and reversed-phase (RP)] to increase protein-separation power, and this is in-line coupled between an HPLC and a tandem mass spectrometry system to enable automated protein identification and quantification [56,57]. In recent years, to add to the early adopted techniques, further approaches in shotgun proteomics have been developed, and these are being increasingly used [recently reviewed in 57]. This field is also being enriched by new, parallel developments in mass spectrometry, HPLC systems [i.e., Ultra Performance Liquid Chromatography (UPLC) technologies] as well as in peptide fragmentation techniques (i.e., ETD) which allow implementation in protein identification. Liquid chromatography (LC), which in shotgun proteomics usually supplants gel electrophoresis, has several advantages, among which are the following: it is easily automated (high throughput) and offers the possibility of studying low-abundance and highly hydrophobic proteins, which are usually masked or absent in classical 2D gels.

In quantitative shotgun proteomics the mass spectrometric (MS) data provide not only information for protein identification, but also for the determination of relative amounts, and for characterization in terms of PTM. The steps involved in protein identification by MS (viz. ionization of the peptides, separation of the obtained ions, and detection of ion masses) vary depending on the type of instrument used. Actually, various combinations of ion sources and analyzers have been developed, and each has its own advantages and inconveniences.

Paralleling the occurrence of multiplexing technology in 2-DE, quantitative LC-MS-based approaches were developed that made use of the advantages of the differential labeling of samples, which allows (provided the peptides to be compared are present in the same mass spectrum) quantitative and reproducible comparisons to be carried out. The differential labeling methods developed so far, also known as stable-isotope-labeling experiments (SILE), comprise: isotope-coded affinity tag (ICAT); isobaric tags for relative and absolute quantification (iTRAQ), and metal-coded affinity tags (MeCATs) [for technical details, see 58-60]. In 2002, Ong et al. [61] developed an additional SILE technique, now called Stable Isotopic Labeling by Amino-acids in Culture (SILAC). In this, the protein labeling is not carried out after protein extraction, but instead is "metabolically" performed during the cell culture by in vivo incorporation of either a heavy or light amino-acid during the protein translation. In general terms, SILEs offer accuracy and reproducibility in quantification, but inevitably suffer because of their intrinsic limitations (including the limited linear dynamic range, the increased time required for and increased complexity of sample preparation, and the high cost of the labeling reagents). Furthermore, to date, apart from iTRAQ, which allows comparison of up to eight samples at one and the same time, the other methods can only compare protein changes between two or three different samples.

Further, higher dynamic ranges of quantification characterize certain other proteomic approaches, namely mass spectrometry-based label-free quantitative techniques. These, although the least accurate among the MS-based quantification strategies, have proved to be successful in a variety of studies, specifically in those on the tissue-specific mitochondrial proteome changes that occur in various animal models, including normal- and diabetic- mouse models [62]. These techniques, regardless of the type of comparison --based either on measurement of the mass

spectrometric signal intensity of the peptide precursor ions of a particular protein, or on the counting of the number of fragment spectra identifying peptides of a given protein-- do not limit the number of experiments that can be compared, and so appear to be particularly advantageous for investigations of large and global protein changes between experiments.

Arguably, leaving aside the intrinsic limitations of any individual technique, the main generic advantage of 2-DE-based, as well as of non-gel based, quantitative proteomics techniques is that in one way or another, the PTMs of the proteins can be detected. In view of the central and dynamic role that mitochondria play in cellular signal transduction and in redox and nitrosative signaling, data on PTMs (such as phosphorylation, oxidation, and nitrosation) appear challenging in mitoproteomics, and indeed have novel and important information concerning the mitochondrial changes that occur during ageing and in diabetes (see below).

1.5. *Mitochondrial protein arrays.*

As happened in genomics, in proteomics, too, a variety of protein and peptide arrays have been developed (analytical as well as functional protein microarrays). In general terms, these technologies make use of protein biochips which allow the selection of specific proteins according to some of their biochemical properties (e.g., selectivity for the surface chemistry of the chip). This has the advantage of significantly reducing the complexity of the samples to be analyzed. These approaches are now widely used in biomedical studies to perform, above all, biomarker analysis, and they have begun to be applied in the field of mitoproteomics [63]. Among the various arrays, functional protein arrays have the great advantage of providing information about biochemical activities and PTMs, as well as about protein-protein, DNA-protein, RNA-protein, and drug-protein interactions.

1.6. *Mitochondrial protein databases.*

In recent years, the large-scale profiling of mitochondrial proteomes from various tissues and cells has led to the creation of more and more new databases containing mitochondrial proteome data-sets. These include Mitoproteome [64], MitoP2 [65,66], and Human Mitochondrial Protein Database and MitoMiner [67]. MitoP2, in particular, provides a comprehensive list of mitochondrial proteins from yeast, mouse, human, Arabidopsis thaliana, and Neurospora crassa, providing information about the functional annotation of proteins, as well as their subcellular locations, and their homologs, along with literature references. The recently created MitoMiner integrates 33 sets of proteomic data from six species (human, rat, mouse, Drosophila melanogaster, Caenorhabditis elegans, and Saccharomyces cerevisiae), and thereby represents the most complete mitochondrial database to date. Importantly, these data-sets have revealed that the number of mitochondrial proteins is much greater (> 3000) than the early studies estimated to be present in the mammalian mitochondrial proteome. This raises the question as to whether more mitochondrial proteins, especially membrane proteins, may be discovered in subsequent mitochondrial proteome research with the development of ad hoc proteomic technology.

A schematic representation of the overall strategies employed in mitoproteomics is shown in Figure 1.

2. Proteomic analysis pertaining to the study of mitochondria in ageing.

Several mitochondrial processes are implicated in ageing and senescence. Besides genetic determinants, increased ROS production, accumulation of oxidized proteins, and altered control of apoptosis by mitochondria have been shown to be important factors in ageing and age-related processes [68]. Proteomic studies on mammalian models have been performed in order to clarify the molecular basis of the functional deterioration that occurs in an ageing tissue [69-77]. As stated above, the precise information gained by proteomic analyses of mitochondria depends on the specific technique employed. Up to now, analytical techniques such as BN-and hrCN-PAGE combined with 2-DE have allowed the establishment of a mitochondrial protein map covering

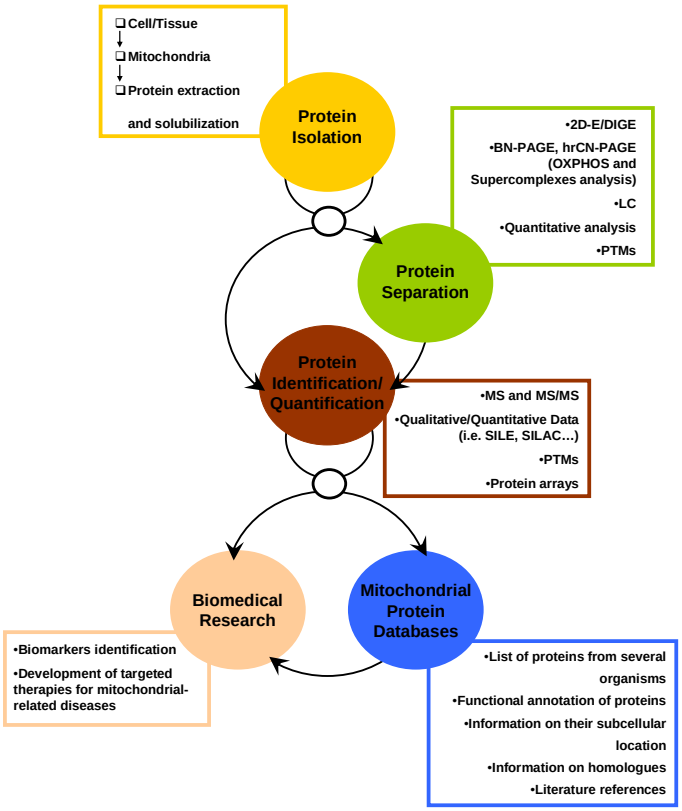

Figure 1. Main strategies and applications in mitoproteomics. The main proteomic approaches employed so far in mitochondrial studies (for mitochondrial protein separation, identification, and data analysis) are schematized, with emphasis on their mutual interrelation. For technical details and notes on both the advantages and disadvantages of the individual strategies, see text.

both the water-soluble and membrane proteins. In Table 1 reports a summary of the mitochondrial proteins so far identified through different proteomic techniques.

2.1 *Supramolecular organisation of mitochondrial respiratory chains.*

The proteomic approaches have also made possible the examination of protein redundancy, in terms of protein-protein interactions as well as PTMs. In the decade before the present one, many studies were published pointing to evidence that OXPHOS complexes do not reside as individual complexes within the inner mitochondrial membrane, but instead as large supercomplexes with a stoichiometric arrangement of complexes I, III, and IV [47,75,77-83]. Such supercomplexes are active, as shown by in-gel activity measurements within BN gels [50,84]. In mitochondria from all the tissues investigated in two studies [85,86], higher ATP synthase oligomers were separated for the first time, all of them functionally active, as displayed by in-gel ATP hydrolase activity [85].

To gain deeper insights both into ageing mechanisms and into the resulting mitoproteomic alterations, mitochondria have been studied by the BN-PAGE approach to obtain information concerning protein abundance and the supramolecular organization of OXPHOS complexes. Dencher et al. [74] reported age-modulated differences in the abundance of various proteins (see Table 1) and, age-related alterations in the oligomerization of MFoF1-ATP synthase.

The use of a combination of BN-PAGE and catalytic staining allowed detection of reduced activity in all the complexes in ageing muscle [77]. The observed reductions in the activities of respiratory complexes I, III, and V were reflections of the lower protein levels, but in contrast the reduction in complex II activity was associated with an increase in the amount of the same complex.

2.2 *Supramolecular architecture of OXPHOS complexes.*

To elucidate whether the ageing process also alters the functional/structural organization of the respiratory chain, in terms of the assembly of supercomplexes, mitochondria have been extracted using the mild detergent digitonin, since this largely retains inner mitochondrial membrane supercomplexes [50]. The major supercomplex bands in aged mitochondria were those representing the larger supercomplexes (I1 III2 IV1-4), and the majority of complex I was assembled within supercomplexes [77]. This significant increase in the supramolecular assembly of respiratory chain complexes into respirasomes might be a compensatory mechanism that, in ageing muscle, is functionally directed towards substrate-channeling and catalytic enhancement advantaging. Indeed, mitochondrial oxidative phosphorylation is more efficient in aged than in young skeletal muscle, since in old rats there is an increased respiratory control ratio (attributed principally to a reduction in the reactions able to dissipate the proton motive force not associated with ATP synthesis) [77]. This could be interpreted as a compensation for the reduced level and activity of FoF1-ATP synthase (see Figure. 2). Frenzel et al. [87] detected a profound decrease in the proportion of the supercomplexes of the respiratory chain complexes I, III2,

Table 1. Summary of the models used and of the major findings obtained by applying proteomic technologies to the study of mitochondria in ageing

Protein identified	Species, tissues	Analytical method	References
NADH dehydrogenase, cytochrome C oxidase, serum albumin precursor, casein alpha-S1, desmin, voltage-dependent anion channel 2, and adenylate kinase 2	Bovine heart	2-DE	71
3-oxoacid CoA transferase 1, ATP synthase, F1 complex α, and creatine kinase 2	mouse heart	2-DE and LC/MS	72
3-hydroxy-3-methylglutaryl-CoA synthase, acyl-CoA oxidase, enoyl-CoA hydratase, glutamine synthetase, and ornithine aminotransferase	senescence-accelerated mouse liver	2-DE and ICAT	76
NADH dehydrogenase, mitofilin, peroxiredoxin isoform III, ATPsynthase, succinate dehydrogenase, mitochondrial fission protein Fis1, succinate-coenzyme A ligase, acyl-coenzyme A dehydrogenase, porin isoform VDAC2, ubiquinol-cytochrome c reductase core I protein, and prohibitin	Rat skeletal muscle	DIGE	73
Na,K-ATP ase, HSP60, V-type ATPase, mitochondrial aconitase-2, MFoF1-ATP synthase, and complex I-IV of OXPHOS	Rat brain	BN-PAGE	74
complex I –V of OXPHOS	Rat skeletal muscle	BN-PAGE	77

and IV, as well as of the MFoF1 ATP synthase, in aged rat cerebral cortex. Complex I was observed solely in supercomplexes and was much lower in quantity in aged cortex mitochondria. An age-associated decline was observed especially in supercomplexes I1 III2, I1 III2 IV1 and I1 III2 IV2, as well as in ATP synthase, together with a pronounced increase in unbound F1 [87]. Semi-quantitative in-gel activity analysis revealed no age-dependent decline in the specific activities of the supercomplexes, favoring a compensatory mechanism for the overall decline in the abundance of respiratory supercomplexes with ageing. Contrary to the findings of Lombardi et al. [77] and Frenzel et al. [87], Gomez et al. [83] --using BN-PAGE separation of membrane proteins and LC-MS/MS analysis-- found that cardiac mitochondria display a significant age-related decline in the assemblies of supercomplexes, especially those of the highest molecular weight. These conflicting results could be due to the different model used (viz. Wistar vs. Fisher rats) or to the different methodological approaches.

2.3 *Posttranslational protein modifications.*

The nuclear transcriptional regulation of mitochondrial activity, which provides the required level of protein to accomplish a given function, is just one side of the diamond in the identification of all the mitochondrial proteins. Indeed, the dynamics of the mitochondrial proteome are manifest at multiple levels, including such PTMs as reversible phosphorylation (4-6) carbonylation, and nitrosilation. Carbonylation can be considered to be an oxidative modification that may render a protein more prone to degradation. Feng et al. [88] identified rat mitochondrial proteins that exhibited muscle type (slow- vs. fast-twitch)-dependent and age-dependent susceptibilities to carbonylation. Carbonylated mitochondrial proteins were more abundant in fast-twitch than in slow-twitch muscle. Twenty-two proteins showed significant changes in carbonylation state with age, the majority of these exhibiting increases in their amount of carbonylation. Ingenuity pathway analysis (IPA) revealed that these proteins belong to various functional classes and pathways, including cellular function and maintenance, fatty acid metabolism, and the citrate cycle. That study provided a unique catalogue of protein targets warranting further investigation because of their potential role in the muscle decline that occurs with age.

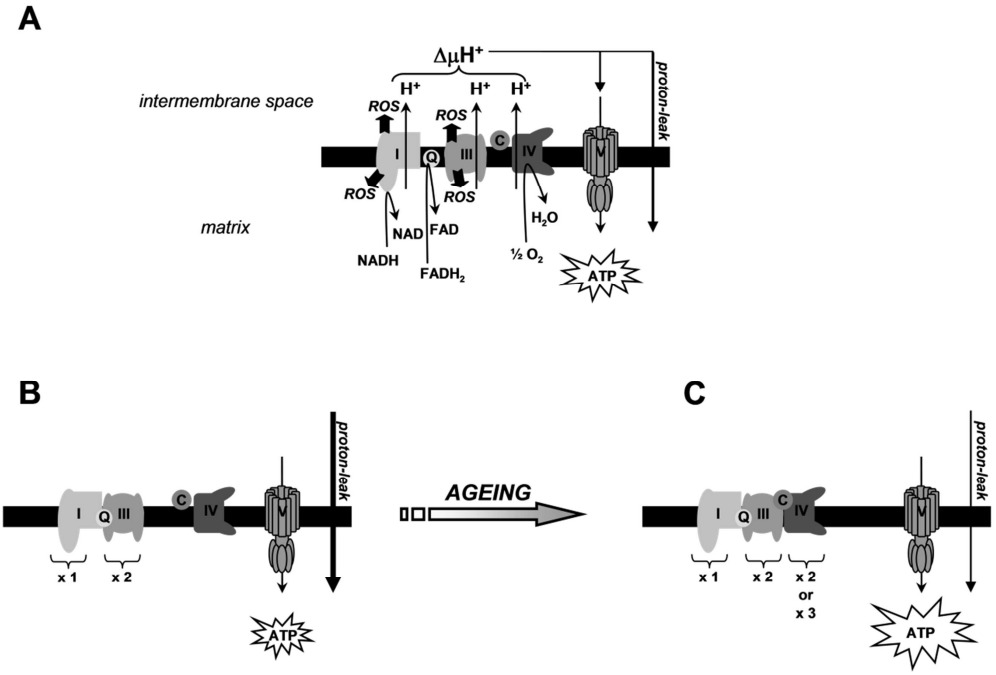

Figure 2. *Respiratory chain individual complexes (A) and their supramolecular organization in some of the main respiratory chain supercomplexes identified so far (B and C): effects of ageing in skeletal muscle and brain [according to (77, 87)].* (A) Schematic representation of mitochondrial respiratory activity. The respiratory chain (OXPHOS system) transfers electrons from reduced coenzymes (NADH and FADH2) to O2, and by pumping out H+ from the matrix into the intermembrane space, generates an electrochemical gradient, ΔμH+. This gradient provides the driving force for ATP synthesis by FoF1-ATPase (Complex V). H+ can also enter the matrix by mechanisms not coupled to ATP synthesis (generically represented as the inner membrane proton-leak). Abbreviations: I (Complex I, NADH:ubiquinone oxidoreductase), III (Complex III, ubiquinol:cytochrome c oxidoreductase), IV (complex IV, cytochrome c oxidase, COX), V (Complex V, FoF1-ATP synthase), c (cytochrome c), Q (coenzyme Q). (B and C) BN-PAGE and single particle electron microscopy has furnished evidence of specific interactions among individual protein complexes of the OXPHOS system that result in the formation of defined supramolecular structures. Various models have been suggested for these OXPHOS supercomplexes have been suggested (for review, see 78 and 108). Depending on the identity and number of the individual complexes involved, such supercomplexes may be lighter or heavier. In two different studies (77, 87), it has been demonstrated that in both skeletal muscle and brain mitochondria, the ageing process is associated with a shift towards an enrichment with heavier supercomplexes containing complex IV. This, together with a reduction in the proton-leak (at least in muscle), should represent an adaptative mechanism functionally directed towards substrate channeling and catalytic enhancement advantaging. For simplicity, dimers and supercomplexes of complex V are not represented.

Carbonylation is irreversible, however, so this modification may be of special importance in directing the affected protein along the path toward degradation.

3. Proteomic analysis pertaining to the study of mitochondria in diabetes.

Mitochondria, by virtue of their numbers or functional properties, or both, are critically involved in the pathophysiology of diabetes [89]. Comparative mitochondrial proteomics have been used to highlight the role played by mitochondria in diabetes, as well as to identify potential therapeutic markers.

Following light and heavy acrylamide labeling, 2-DE, and MS, Turko et al. [90] found that heart mitochondria from streptozotocin-induced diabetic rats displayed no alterations in the expressions of proteins from the tricarboxylic acid cycle, although an upregulation of fatty acid-oxidation proteins was detected. Other changes included down-regulations of the protein levels of creatine kinase, voltage-dependent anion channel 1, HSP60, Grp 75, and the mitochondrial-associated level of albumin but an upregulation of catalase [90]. The mitochondrial proteome has been investigated across four tissues (kidney, brain, liver and heart) from wild-type (WT) and type 1 diabetic Akita mice by means of label-free proteome expression analysis [62]. In diabetic versus WT mice, the fatty acid-oxidation proteins were less abundant in liver mitochondria, but more abundant in mitochondria from the other three tissues. In diabetes, tricarboxylic acid (TCA) cycle proteins were repressed in cardiac mitochondria, but kidney mitochondria showed a coordinated induction of TCA-cycle enzymes. Only liver mitochondria showed increased levels of OXPHOS subunits in diabetes. Mitochondrial respiration, ATP synthesis, and morphology were decreased only in cardiac mitochondria in diabetes, and these changes were accompanied by coordinated repression of OXPHOS and peroxisome proliferator–activated receptor (PPAR)-γ coactivator (PGC)-1α transcripts. These results support the idea that type 1 diabetes causes tissue-specific remodeling of the mitochondrial proteome, with a central role for mitochondrial dysfunction in diabetic cardiomyopathy [62]. Munusamy et al. [91] analyzed mitochondrial complexes by BN-PAGE, and assayed their activities in kidneys from streptozotocin-treated (diabetic) rats. They reported a reduction in Complex III, as well as a failure of this complex to assemble correctly, thus contributing to the mitochondrial oxidant production occurring in the early stages of diabetes.

Recent research findings have supported the notion that impaired mitochondrial function leads to insulin insensitivity in myocytes and adipocytes as a result either of an insufficient supply of energy or of defects in the insulin signaling pathway [92]. A comprehensive analysis of the mitochondrial proteome in the livers of type 2 diabetic rats has recently been performed by Deng et al. [93], who identified 1091 mitochondrial proteins, 228 phosphoproteins, and 355 hydroxyproteins. Their semiquantitative analysis revealed upregulation of proteins involved in beta oxidation, the TCA cycle, OXPHOS, and other bioenergetic processes, but down-regulation of proteins involved in apoptosis and oxidative stress [93]. Activation of oxidative phosphorylation was related to the overproduction of ROS, which caused oxidative stress, as also evidenced by the presence of heavily hydroxylated mitochondrial proteins [93].

Besides changes in mitochondrial protein expression, mitochondrial protein modifications play important roles in the pathology of type 2 diabetes. Protein tyrosine nitration is a common PTM occurring under conditions of oxidative stress in a number of diseases, including diabetes [94-97]. Protein tyrosine nitration alters the structure and function of proteins, and may prevent tyrosine phosphorylation [94,95,98].

Heart mitochondria from diabetic mice are prone to tyrosine nitration of proteins involved in major mitochondrial functions, such as energy production (succinyl-CoA: 3-oxoacid CoA transferase, and creatine kinase), antioxidant defense (peroxiredoxin 3), and apoptosis (voltage-dependent anion channel-1) [90]. Those findings support the general conclusion that nitration of mitochondrial proteins may result in dysfunctional mitochondria in diabetes. Kartha et al. [99] detected an increased mitochondrial protein tyrosine nitration in kidneys from high calorie- and fat diet-induced diabetic mice. Reversible phosphorylation is emerging as a central mechanism in the regulation of mitochondrial functions [100,101], and the mitochondrial phosphoproteome has been characterized in a series of studies on yeast, mouse and rat livers, porcine heart, and various plants [93,102-105]. To date, the largest data-set, published by Deng et al. [93], identified 228 different phosphoproteins and 447 phosphorylation sites in rat liver mitochondria. Hojlund et al. [106], who examined human skeletal muscle biopsies, identified eight potential protein markers for type 2 diabetes in the fasting state. The observed changes in protein expressions indicate increased cellular stress, as well as perturbations in ATP synthesis and mitochondrial metabolism, in the skeletal muscle of patients with type 2 diabetes. In particular, Hojlund et al. demonstrated that the catalytic β-subunit of ATP synthase is phosphorylated in vivo, and that the down-regulated level of the ATP synthase β -subunit phosphoisoform in diabetic muscle correlated inversely with the fasting plasma glucose level. Finally, Cui et al. [107] applied shotgun proteomics to the profiling of mitochondrial proteins, and identified potential phosphorylation sites in rat pancreatic insulinoma-1 (INS-1) beta cells. More than 800 mitochondrial proteins and 84 mitochondrial phosphoproteins were identified in that study.

4. Conclusions and future perspectives.

Mitochondrial proteomic research is expanding and will continue to expand to give further insights into the molecular mechanisms involved in both mitochondrial physiology and mitochondria-associated diseases. Proteomic approaches and

technologies developed over the last decade have provided novel insights into mitochondrial functions and have increased our knowledge of the relationship between mitochondrial dysfunction and the onset of disease. No single technique can be used for all studies of mitochondrial proteins. BN-PAGE has proved successful not only for the dissection of the inner membrane OXPHOS system, but also for the identification of the components of the outer membrane, such as those involved in protein import. Identification of PTMs, such as phosphorylation, acetylation, and nitration of mitochondrial membrane proteins, has been greatly improved by the use of affinity techniques. However, many challenges remain, largely due to the dynamic properties of these organelles. The striking differences in their composition within a given tissue and their rapid dynamics when metabolic challenges have to be faced, make mitochondria a fascinating subject for proteome-based studies in the future. An important approach could be the integration of more technologies in the fields of transcriptomics, proteomics, biochemistry, and bioinformatics, which should provide useful information concerning tissue/cell-specific mitochondrial functions.

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