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Mitochondrial ATP synthasome: Expression and structural interaction of its components

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ABSTRACT

Mitochondrial ATP synthase, ADP/ATP translocase (ANT), and inorganic phosphate carrier (PiC) are supposed to form a supercomplex called ATP synthasome. Our protein and transcript analysis of rat tissues indicates that the expression of ANT and PiC is transcriptionally controlled in accordance with the biogenesis of ATP synthase. In contrast, the content of ANT and PiC is increased in ATP synthase deficient patients' fibroblasts, likely due to a post-transcriptional adaptive mechanism. A structural analysis of rat heart mitochondria by immunoprecipitation, blue native/SDS electrophoresis, immunodetection and MS analysis revealed the presence of ATP synthasome. However, the majority of PiC and especially ANT did not associate with ATP synthase, suggesting that most of PiC, ANT and ATP synthase exist as separate entities.

1. Introduction

The complexes of respiratory chain in the inner mitochondrial membrane form higher structural entities – supercomplexes [1]. Similarly, the key component of the oxidative phosphorylation (OXPHOS) system, F_1F_o-ATP synthase, can associate into more complex structures, such as dimers and higher oligomers [2].

ATP synthase was reported to participate in a range of other supramolecular structures, most importantly ATP synthasome, which is suggested to be composed of ATP synthase, ADP/ATP translocase (ANT), and inorganic phosphate carrier (PiC). Together, they would form a single catalytic unit responsible for ATP production [3]. Since the original reports [4,5], it was also found in bovine heart mitochondria [2,6,7] as well as in the protozoan *Leishmania* [8], suggesting that ATP synthasome is an evolutionary conserved structure, albeit with questionable functional significance [5].

ANT and PiC belong to the mitochondrial carrier family of hydrophobic proteins encoded by the *SLC25* genes [9]. In rodents, three genes coding for tissue-specific ANT isoforms have been described whereas four genes have been identified in humans – *SLC25A4* (ANT1, a heart-type isoform), *SLC25A5* (ANT2, a liver-type isoform), *SLC25A6* (ANT3, expressed in highly proliferative cells, present only as a pseudogene in rodents), and *SLC25A31* (ANT4, testes-specific isoform) [11]. In the case of PiC, two isoforms PiC-A (a heart-type isoform) and PiC-B (a liver-type isoform) originate from alternative splicing of a single transcript (*SLC25A3*) [10].

While the existence of ATP synthasome is mostly accepted, little is known about its relative abundance in comparison with free forms of its constituents, stoichiometry or regulation of its biogenesis. Therefore, we set to clarify two major objectives: to examine whether the total content of the carriers (ANT and PiC) is affected by the content and function of ATP synthase and to better describe the structural associations between ATP synthasome components.

2. Materials and methods

2.1. Animals

Adult (3–4 months old) and newborn (3–5 days old) Wistar rats were killed in CO_2 narcosis. All procedures were performed in accordance with EU Directive 2010/63/EU for animal experiments. Mitochondria from rat tissues (heart, liver, kidney, brain, skeletal

Abbreviations: ANT, ADP/ATP translocase; PiC, inorganic phosphate carrier; BAT, brown adipose tissue; DDM, n-dodecyl β-D-maltoside.

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muscle, brown adipose tissue) were isolated by differential centrifugation [12,13].

2.2. Cell cultures

All human samples were obtained on the basis of written informed consent and handled in accordance with the Code of Ethics of the World Medical Association. Primary fibroblast cultures derived from skin biopsies of 3 healthy individuals, 7 patients with an isolated ATP synthase defect due to the homozygous nucleotide substitution 317-2A>G in the gene *TMEM70* [14], a patient with a mutation in the gene *ATP5E* coding for the subunit F_1-ε [15], and a patient harboring a mutation in the mitochondrial gene *MT-ATP6* coding for the subunit F_o-a [16,17] were cultivated under standard conditions [15]. Further patients' characteristics have been described previously [14–19]. Fibroblast mitochondria were isolated by hypotonic shock cell disruption [15].

2.3. RNA extraction and gene expression analysis

The total RNA was isolated using Trizol (Life Technologies) and reverse-transcribed with the SCRIPT cDNA Synthesis Kit (Jena Bioscience). cDNA was used as a template for the quantitative PCR performed on the ViiA 7 instrument (Life Technologies) with the 5x HOT FIREPol Probe qPCR Mix (Solis Biodyne) and predesigned TaqMan gene expression assays (Life Technologies; Supplementary Table S1).

2.4. Electrophoreses and immunoblotting

The separation of proteins with the tricine SDS-PAGE [20] was performed on 10% polyacrylamide minigels (MiniProtean III, Bio-Rad) [13]. The blue native electrophoresis (BN-PAGE) [21] was performed on 4–13 or 4–8 % gradient gels [13]. The BN gels were stained by the ATPase in-gel activity assay [22] to identify ATP synthase. The visualized bands of ATP synthase monomer and dimer were excised, incubated in 1% SDS +1% 2-mercaptoethanol and subjected to SDS-PAGE (16%) for 2D resolution under denaturing conditions.

The gels from both BN-PAGE and SDS-PAGE were blotted onto a PVDF membrane (Immobilon-FL, Merck Millipore) [13]. For the list of used primary antibodies see Supplementary Table S2. Fluorescent signals of secondary antibodies labelled with Alexa Fluor 680 (Life Technologies) or IRDye 800 (Rockland) were recorded with the infrared Odyssey Imager (Li-Cor) and quantified using the Aida 3.21 software (Raytest).

2.5. MS analysis

Protein bands from the BN gels containing ATP synthase were identified by the ATPase in-gel assay run in parallel [22], excised and processed by in-gel trypsin digestion [23]. The setup of LC-MS/MS analysis on the maXis UHR-TOF (Bruker) has been described previously [24].

2.6. Immunoprecipitation

For the co-immunoprecipitation analysis with the ATP Synthase Immunocapture Kit (Abcam), rat heart mitochondria were solubilized with 1% Triton X-100 [13] and 30,000 *g* supernatants were subjected to immunoprecipitation according to the manufacturer's protocol. Co-immunoprecipitated proteins were eluted with 0.2 M glycine (pH 2.5), neutralized with 1 M Tris-base, separated with SDS-PAGE (10%) and identified on Western blots. As a negative control, proteins captured non-specifically onto protein G-agarose beads were investigated.

3. Results

3.1. Expression of mitochondrial carriers ANT and PiC in rat

To gain insight into the physiological regulation of the biogenesis of ATP synthasome constituents, we analyzed the expression of ATP synthase subunits, ANT, and PiC at the protein and transcript level in 6 adult (heart, skeletal muscle, brain, brown adipose tissue (BAT), kidney, and liver) and 3 newborn (heart, BAT, and liver) rat tissues.

We used antibodies reacting with all PiC or ANT isoforms [25] and an antibody to subunit F_1-α as a proxy for the ATP synthase content (Fig. 1A). The ATP synthase content was highest in mitochondria from the tissues with high energetic demands – heart and skeletal muscle, lower in kidney and liver and lowest in thermogenic BAT (~30% of heart), where the down-regulated expression of subunit F_o-c limits the ATP synthase biogenesis [26]. The tissue-specific distribution of ANT and PiC was analogous to that of ATP synthase.

At the transcript level, the expression of two structural subunits of ATP synthase (*ATP5A1* and *ATP5G1* coding for F_1-α and F_o-c, respectively), all three isoforms of ANT (ANT1 – *SLC25A4*, ANT2 – *SLC25A5*, ANT4 – *SLC25A31*) and PiC (*SLC25A3*) correlated with protein profiles (Fig. 1B–D). As anticipated, the *ATP5G1* transcript level was lowest in BAT. With respect to the ANT genes, we observed the expected tissue profiles with heart and skeletal muscle expressing ANT1, liver expressing ANT2, and brain and BAT displaying a mixed profile. The *SLC25A31* transcript was hardly detectable in the analyzed tissues (not shown), in agreement with its primarily testicular expression [27]. The *SLC25A3* transcript levels paralleled the distribution of PiC across the tissues.

3.2. Expression of ANT and PiC in human fibroblasts with an ATP synthase deficiency

To find out whether PiC and ANT can be affected by an altered function of ATP synthase, we used two types of patient fibroblasts: (i) cells with a pronounced decrease in the content of ATP synthase due to a mutation in *TMEM70* or *ATP5E* and (ii) cells with a mtDNA mutation in the *MT-ATP6* gene, characterized by a normal amount of ATP synthase that lost the ability to synthesize ATP. The protein content of ANT and PiC was not only preserved, but mostly increased, regardless of the different genetic origin or clinical and biochemical manifestation of ATP synthase dysfunction (Fig. 2A). Accordingly, the ATP synthase dysfunctions were associated with an up to 7-fold increase in the ratio between the carriers and ATP synthase (Fig. 2B).

3.3. ATP synthasome – immunoprecipitation

By co-immunoprecipitation, we found ATP synthase associated with the mitochondrial carriers ANT and PiC (Fig. 3A). While this association was specific and not caused by non-specific binding to empty beads, the enrichment of ATP synthase subunits in the immunoprecipitate contrasted with the low signal of co-immunoprecipitated carriers, especially ANT. This suggests that only a minority of ANT and PiC interact with ATP synthase, or that the interactions are weak and readily dissociate. Either way, there have to exist two forms of ATP synthase, i.e. the free ATP synthase and the ATP synthasome supercomplex.

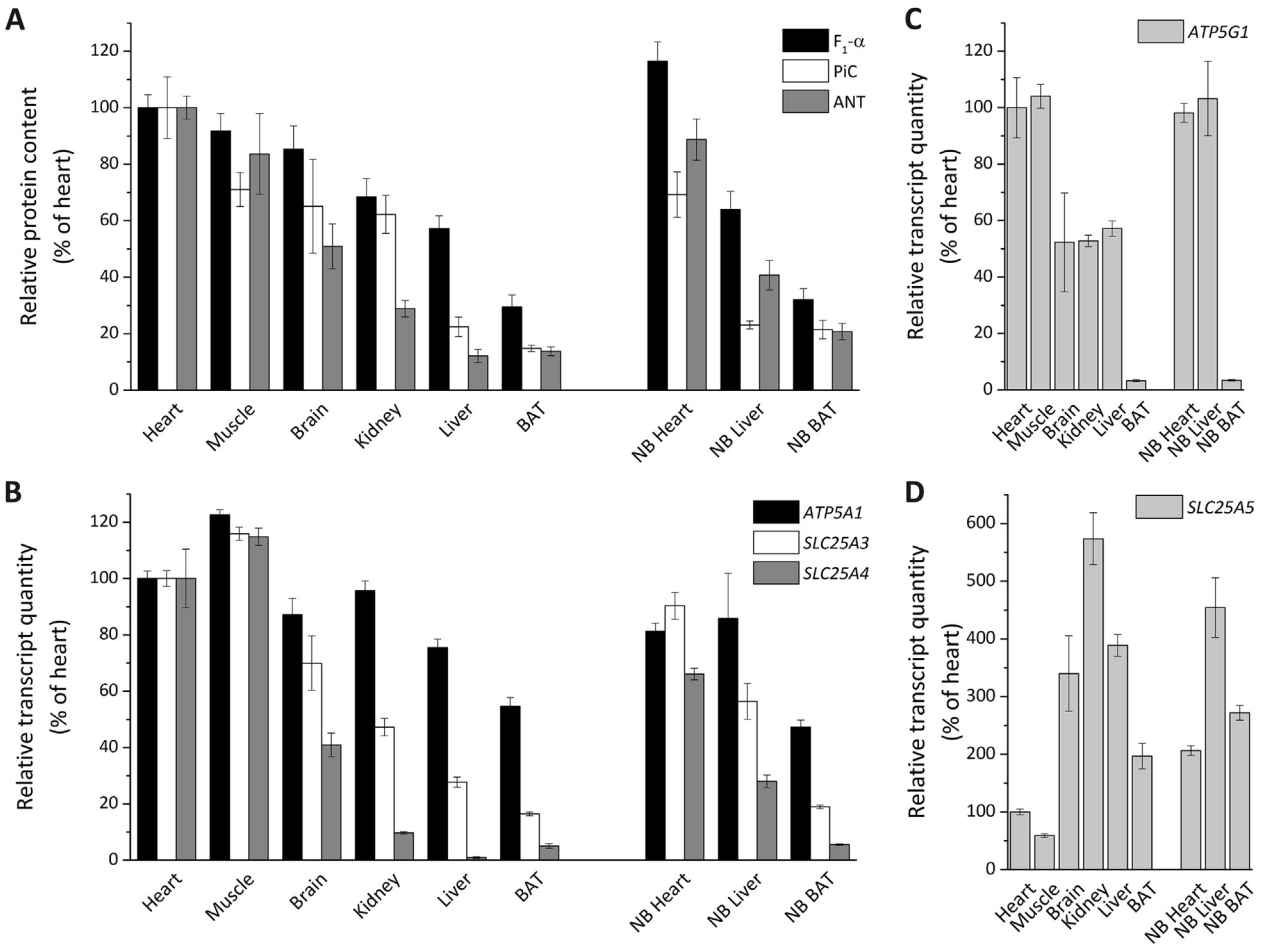

Fig. 1. The protein and transcript levels of ATP synthase and mitochondrial carriers ANT and PiC in rat tissues. (**A**) Isolated mitochondria were analyzed on Western blots (25 μg protein per lane). The signal of F_1-α, ANT, and PiC was normalized to the signal of SDHA. The values, expressed as a percentage of the heart value, are mean ± SEM (n = 4). (**B, C, D**) RT-qPCR quantification of *ATP5A1*, *ATP5G1*, *SLC25A3*, *SLC25A4*, *SLC25A5*, and *SDHA* encoding the subunits F_1-α and F_o-c of ATP synthase, PiC, ANT1, ANT2, and SDHA (used for data normalization), respectively, in adult and newborn (NB) rat tissues.

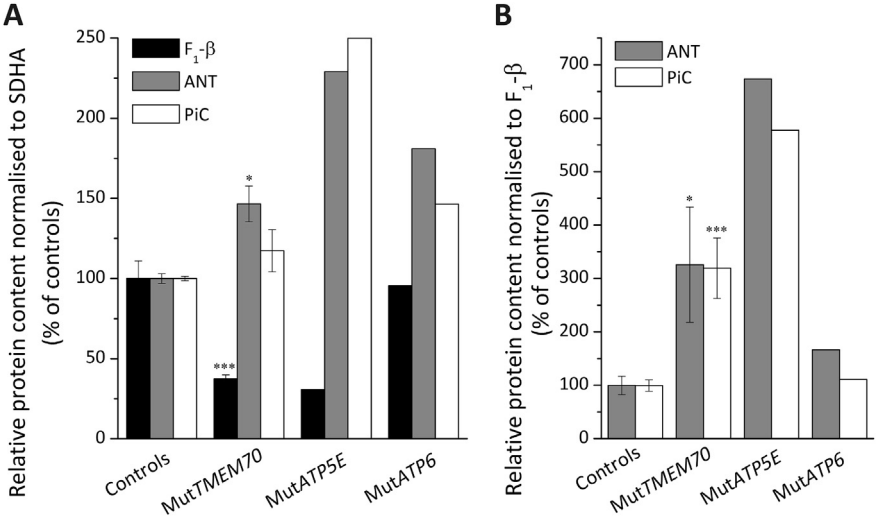

Fig. 2. The content of ATP synthase, ANT and PiC in human fibroblasts with ATP synthase deficiencies. The protein content of F_1-β subunit of ATP synthase, ANT and PiC was analyzed in mitochondria from human fibroblasts of healthy controls, patients with the mutated *TMEM70* (mut*TMEM70*), *ATP5E* (mut*ATP5E*), and *MT-ATP6* (mut*ATP6*) on Western blots (20 μg protein per lane). (**A**) The signal of specific antibodies was normalized to SDHA. (**B**) The signal for ANT and PiC was normalized to F_1-β. The values are mean from 3 experiments. The results of 3 controls and 7 patients of mut*TMEM70* are pooled for statistical analysis (***$p < 0.001$, *$p < 0.05$), mut*ATP5E* and mut*ATP6* samples represent multiple cultures of one patient each.

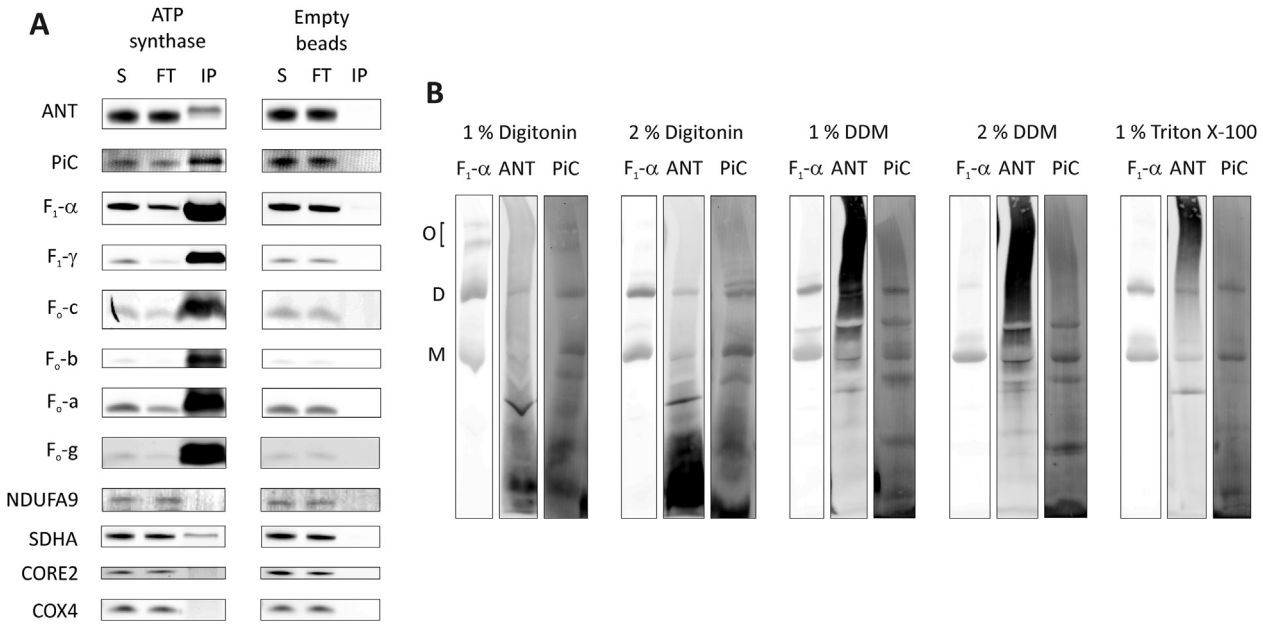

Fig. 3. Characterization of ATP synthasome by means of immunoprecipitation and BNE. (**A**) Rat heart mitochondria solubilized with 1% Triton X-100 (1 g detergent per 1 g protein) were used to perform immunoprecipitation using the ATP synthase capture kit (MS501, Abcam). The immunoprecipitates (IP) as well as input solubilizates (S), and flow-through material (FT) are shown on Western blots. As a negative control, empty protein G-coupled agarose beads were examined. (**B**) Rat heart mitochondria solubilized as indicated were analyzed by BN-PAGE (30 μg protein per lane; 4–13 % gradient). On Western blots, antibodies specific to ATP synthase (F_1-α), ANT, and PiC were used. The ATP synthase monomer (M), dimer (D) and higher oligomers (O) are indicated. The corresponding detergent to protein ratios are 1:1 and 2:1 for 1% and 2% detergents, respectively.

3.4. ATP synthasome – analysis of BN gel regions with the ATPase activity

To examine the supramolecular organization of mitochondrial ATP-synthesizing apparatus, rat heart mitochondria were solubilized with mild detergents (digitonin, DDM, and Triton X-100) and separated by BN-PAGE (Fig. 3B). Under all the different conditions that resolved ATP synthase in varying oligomerization states, we observed co-localization with PiC and ANT. However, the vast majority of PiC and ANT was present in other regions of the gel, indicating that the majority of PiC and ANT do not associate with ATP synthase. To better evaluate their interactions, we excised regions of BN gels that were stained for the ATPase activity and separated them with SDS-PAGE (Fig. 4A**).** Similar proportions among PiC, ANT, and ATP synthase were found in the monomers, irrespective of the detergent used. The detection of ANT in the dimers varied and was very weak upon digitonin solubilization. MS analysis confirmed all the components of ATP synthasome associated into a supercomplex – several ATP synthase subunits and different isoforms of ANT and PiC were detected in the regions of ATP synthase monomer and dimer, even in the digitonin solubilizates (Table 1).

In all experiments, we observed a significant difference between the total signal of PiC and especially ANT and its portion co-localizing with ATP synthase. To estimate the fraction of ANT and PiC present in ATP synthasome, we quantitatively compared solubilized mitochondria (1% Triton X-100) with excised monomers of ATP synthase (Fig. 4B and C). In the gel region containing the ATP synthase monomer and representing ≥85% of all ATP synthase present, we found only 3% of the signal for ANT and 16% for PiC, compared to the original mitochondria and normalized to the ATP synthase subunit F_1-α. Normalization to other ATP synthase subunits (F_1-γ, F_o-a) revealed similar or even smaller portions of both carriers associated with ATP synthase, confirming that most of PiC and especially of ANT was present as separate entities, not associated with ATP synthase.

4. Discussion

The presence of mitochondrial ATP synthasome has been reported in several species [2–8], but without further insight into the regulation of its biogenesis. Therefore, we focused on co-expression analysis of PiC, ANT, and ATP synthase and structural investigation of the purported ATP synthasome.

First, we investigated tissue-specific differences at the transcript and protein level of ATP synthase subunits, ANT, and PiC (Fig. 1). Our results indicated that the protein levels of ANT and PiC are controlled in accordance with the ATP synthase content. Indeed, expression of many OXPHOS proteins is transcriptionally regulated and shares common regulatory pathways [28], although the control of some genes may still be independent, such as distinct regulation of *ATP5A1* and *ATP5G1* in BAT [26] or tissue-specific up-regulation of ANT2 expression by thyroid hormones in rat heart and liver [29]. To observe possible developmental features of ANT and PiC expression, we also compared three tissues from newborn rats. While BAT did not show any major developmental changes, the neonatal heart was characterized by a decreased content of ANT and PiC. Furthermore, a reduction in the *SLC25A4* transcript was accompanied by an increase in *SLC25A5*, reflecting that the fetal and neonatal heart depends predominantly on anaerobic glycolysis whereas the mature adult heart is almost exclusively aerobic [30]. Apparently, the protein content of ATP synthase rises faster than that of ANT and PiC in heart after the birth.

A distinct type of regulation of ATP synthasome constituents appears to be associated with the pathophysiological conditions. In all of the studied fibroblasts from ATP synthase deficient patients (Fig. 2), the protein levels of ANT and PiC remained normal or even raised. In these cells, we have previously described an adaptive increase in the respiratory chain complexes III and IV that was not accompanied by any changes in transcript levels of individual ANT and PiC isoforms [19]. Thus, while the physiological regulation of ATP synthasome components likely occurs at the transcriptional level, the observed adaptive responses are regulated post-

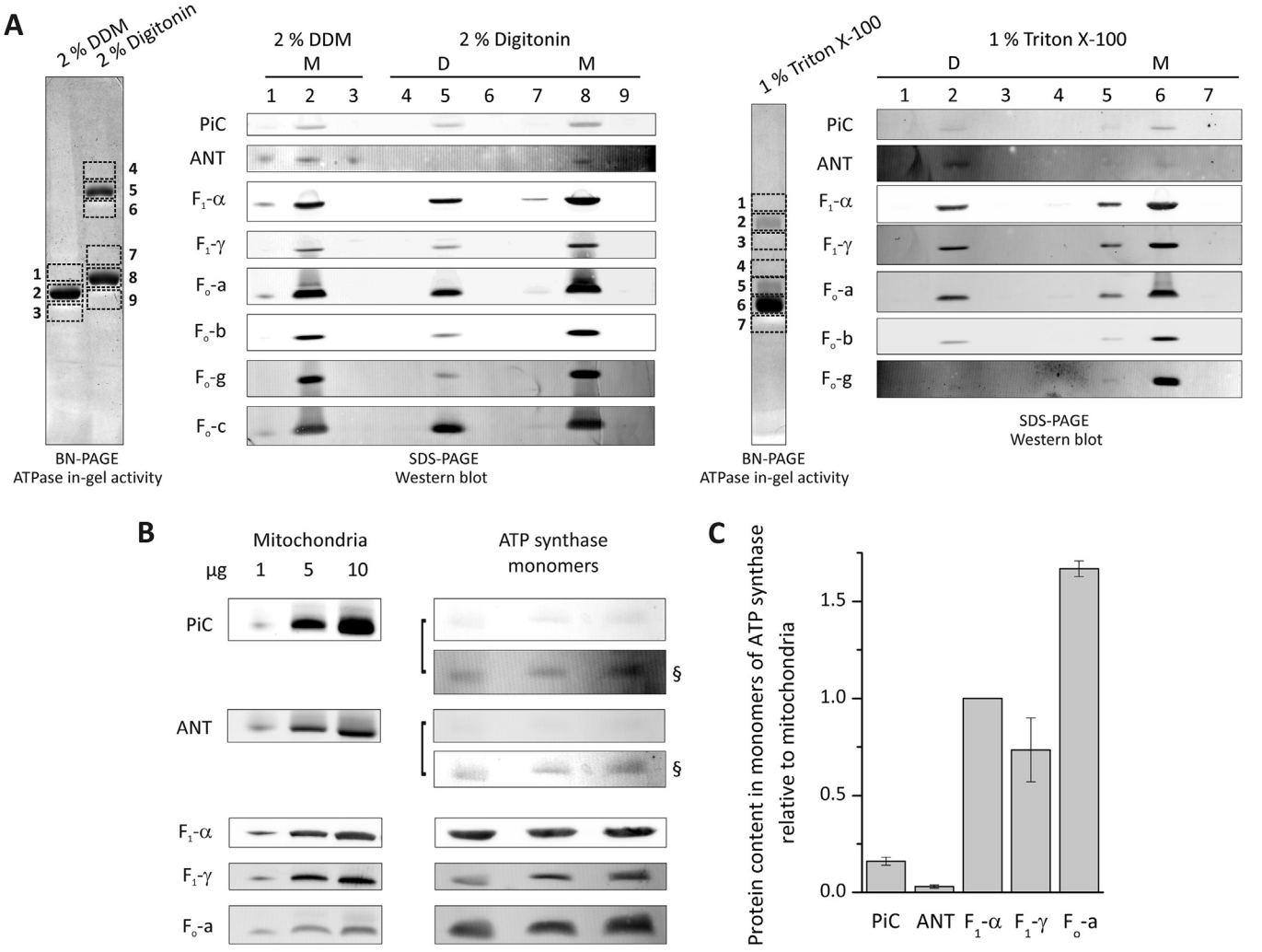

Fig. 4. Analysis of BNE excised ATPase bands in the second dimension. (**A**) Rat heart mitochondria were solubilized with mild detergents as indicated and resolved in the first dimension using BN-PAGE (30 µg protein per lane; 4–8 % gradient). The bands of ATP synthase monomers (M) and dimers (D) as well as adjoining gel pieces were excised and resolved on SDS-PAGE (16%). (**B**) The protein composition of ATP synthase monomers solubilized with 1% Triton X-100 (50 µg protein per lane; 4–8 % gradient) was compared to the input mitochondria. The antibody signals in mitochondria and excised ATP synthase monomers are shown at the same detection sensitivity. In the case of PiC and ANT, a higher sensitivity is also shown (§). (**C**) Calibration curves were constructed for dose response of each antibody, based on the signal in mitochondria. The antibody signals were normalized to the respective signal of the antibody against F_1-α and all the signals in the monomers were expressed relatively at the theoretical unit quantity. The values are mean ± SEM, n = 3.

transcriptionally, possibly at the level of protein stability. This would be similar to OXPHOS complexes whose observed compensatory up-regulation also occurred at the post-transcriptional level [19].

In the second part of our study, we examined the structural association of ATP synthase, ANT, and PiC. While it was likely observed already in the 1970's [31], the so-called ATP synthasome was first reported only decades later, when all its components co-localized in vesicles of mitochondrial cristae membrane from rat liver [4,5]. Since then, the interaction of ATP synthase and ANT has been described in bovine heart while separating digitonin-solubilized mitochondria on clear native [6] or blue native gels [2].

We found ANT and PiC in the ATP synthase immunoprecipitate from rat heart mitochondria (Fig. 3A), where we also detected a signal of SDHA that was much stronger than in empty beads, and hence represented a true interaction between succinate dehydrogenase and ATP synthase. Such an interaction has been described previously as a part of a putative mitochondrial ATP-sensitive K^+ (mitoK_{ATP}) channel [32,33].

Subsequently, we used BN-PAGE for a more detailed investigation into the stability and quantity of ATP synthasome. As detergents, we chose digitonin, DDM and Triton X-100, which effectively solubilize the intact and active ATP synthase [4] and also yield stable ANT preparations [34]. A well-documented problem with ANT is smearing on BN-PAGE (Fig. 3B), due to its high pI and hydrophobicity [6,35] an also its high abundance [36]. Therefore, we analyzed the excised gel pieces containing monomers and dimers of ATP synthase by Western blotting and LC-MS/MS and found them to contain most of the ATP synthase subunits, ANT1, ANT2, and PiC (Fig. 4A, Table 1). The subunits of ATP synthase that were not detected by LC-MS/MS represent highly hydrophobic parts of the F_o domain, and their absence rather reflects the limits of MS approach than the actual lack of these proteins. Interestingly, we detected ATP synthasome upon solubilization with 2% DDM, arguing for rather stable interactions between the ATP synthasome constituents. ATP synthasome may also be detected by the complexome profiling studies that represent another approach aimed at detection of novel mitochondrial supercomplexes. While one study on DDM-solubilized HEK293 mitochondria observed this supercomplex [28], another study on digitonin-solubilized rat heart mitochondria failed to detect any such association [37].

Table 1
MS analysis of ATP synthase bands from blue native gels. Excised monomers (M) and dimers (D) of ATP synthase solubilized with 1% detergents and resolved on 4–8 % BN-PAGE were analyzed by LC-MS/MS. The detected proteins (black dots; empty dots represent detected proteins with a score too low to be reported as a valid detection; n.d. – not detected) are indicated in the overview of proteins relevant to the ATP synthasome supercomplex. For complete datasheets see Supplementary Table S3.

Gene	Protein	MW (kDa)	Digitonin		DDM		Triton X-100	
			M	D	M	D	M	D
SLC25A3	PiC	39.6	●	●	●	●	●	●
SLC25A4	ANT1	33.0	●	●	●	●	●	●
SLC25A5	ANT2	32.9	●	●	●	●	●	●
ATP5A1	F_1-α	59.8	●	●	●	●	●	●
ATP5B	F_1-β	56.4	●	●	●	●	●	●
ATP5C1	F_1-γ	30.2	●	●	●	●	●	●
ATP5D	F_1-δ	17.6	●	●	●	●	●	●
ATP5E	F_1-ε	5.8	○	○	○	○	●	○
ATP5G1	F_o-c	14.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MT-ATP6	F_o-a	25.1	n.d.	n.d.	n.d.	n.d.	●	n.d.
ATP5O	OSCP	23.4	●	●	●	●	●	●
ATP5F1	F_o-b	28.9	●	●	●	●	●	●
ATP5H	F_o-d	18.8	●	●	●	●	●	●
ATP5I	F_o-e	8.3	●	●	●	●	●	●
ATP5J2	F_o-f	10.5	●	●	●	●	●	●
ATP5L	F_o-g	11.4	●	●	●	●	●	●
ATP5J2	F_o-F6	12.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MT-ATP8	A6L	7.6	●	●	●	●	●	●
ATPIF1	IF1	12.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
USMG5	DAPIT	6.4	●	●	●	●	●	●
C14ORF2	MLQ	6.8	n.d.	n.d.	n.d.	○	n.d.	○

When we tried to quantify ANT and PiC associated with ATP synthase in comparison to their total mitochondrial content, we confirmed that a large portion of PiC and especially of ANT was not organized into ATP synthasome (Fig. 4B and C). Indeed, this is also in agreement with our immunoprecipitation data, where the observed recovery for PiC and ANT was 4.2 and 14.9 times lower, respectively, than that of ATP synthase. This may well reflect differences in the protein content of ATP synthase, ANT, and PiC: ANT is estimated to represent ~10% of all mitochondrial proteins [36] and the molar ratio between ANT and PiC is ~4:1 [38]. Indeed, we found approximately 16% of the total PiC and 3% of the total ANT to be incorporated into ATP synthasome, which is in line with the ATP synthasome components proposed stoichiometry of 1:1:1 [5]. As the carriers are likely present in molar excess to ATP synthase [38], it may not be surprising that most of the ANT and PiC proteins are detected in a free form. However, we cannot make clear-cut conclusions regarding the presence of free ATP synthase. If we take into account the ratio between ANT and cytochrome *c* oxidase in mitochondria (2.5:1 – likely being similar to the ratio between ANT and ATP synthase) [38] and our ratio between the free and ATP synthasome-bound ANT (33:1), it can be expected that also ATP synthase exists predominantly in the free form.

In conclusion, we have demonstrated that ATP synthasome is present in rat heart mitochondria and that its constituents likely share common transcriptional regulation. However, its components do not mutually depend on one another and a large portion of the carriers was found out of the association with ATP synthase. Given the relative minority of the proteins present as the ATP synthasome supercomplex, its physiological role in tighter coupling of the whole ATP production machinery remains questionable.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.07.034.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.07.034.

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