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Subsarcolemmal mitochondria isolated with the proteolytic enzyme nagarse exhibit greater protein specific activities and functional coupling



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ABSTRACT

Skeletal muscle mitochondria are arranged as a reticulum. Insight into the functional characteristics of such structure is achieved by viewing the network as consisting of “subsarcolemmal” (SS) and “intermyofibrillar” (IMF) regions. During the decades, most, but not all, published studies have reported higher (sometimes over 2-fold) enzyme and enzyme-pathway protein-specific activities in IMF compared to SS mitochondria. We tested the hypothesis that non-mitochondrial protein contamination might account for much of the apparently lower specific activities of isolated SS mitochondria. Mouse gastrocnemii ($n=6$) were suspended in isolation medium, minced, and homogenized according to procedures typically used to isolate SS mitochondria. However, the supernatant fraction, collected after the first slow-speed (800g) centrifugation, was divided equally: one sample was exposed to nagarse (MITO+), while the other was not (MITO-). Nagarse treatment reduced total protein yield by 25%, while it increased protein-specific respiration rates ($\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$), by 38% under “resting” (state 4) and by 84% under maximal (state 3) conditions. Nagarse therefore increased the respiratory control ratio (state 3/state 4) by 30%. In addition, the ADP/O ratio was increased by 9% and the activity of citrate synthase (U/mg) was 49% higher. Mass spectrometry analysis indicated that the MITO+ preparation contained less contamination from non-mitochondrial proteins. We conclude that nagarse treatment of SS mitochondria removes not only non-mitochondrial proteins but also the protein of damaged mitochondria, improves indices of functional integrity, and the resulting protein-specific activities.

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1. Introduction

Mitochondrial oxidative phosphorylation fulfills two critical metabolic objectives: 1) synthesizing ATP at the rate demanded by cellular ATP-utilizing processes and 2) maintaining a robust cellular energy status (low cytosolic [ADP] and high ATP/ADP ratio). Almost 40 years ago Skulachev's laboratory provided evidence that mitochondria in mammalian striated muscle are structured as a continuous network or reticulum [1]. Recently, Glancy et al. [2] provided compelling corroborative evidence for this model. Further, their report supports one of the fundamental hypotheses advanced by Skulachev over 40 years ago [1], that the reticular arrangement provides the ability to rapidly transfer intracellular energy by propagating the protonmotive force (Δp) from one region of the network to another [1,3,4]. According to this concept,

the fraction of the network located near the plasma membrane (“subsarcolemmal” or SS mitochondria) contains protein stoichiometry particularly suited for Δp development, while the region deeper within the myocyte, the intermyofibrillar (IMF) fraction, is particularly tailored for transducing Δp into ATP synthesis and export. Reticular structure therefore facilitates meeting the demands for both rapid ATP turnover and the defense of cellular energetic status. These older and more recent findings bracket decades of research supporting the mitochondrial reticulum concept.

About the time Skulachev's paper demonstrated the mitochondrial reticulum in rat diaphragm [1], Palmer et al. described procedures for independently isolating SS and IMF mitochondrial populations from rat heart [5]. Briefly, the tissue was minced, suspended in buffer, mechanically disrupted (liberating SS mitochondria) and centrifuged at slow speed, leaving SS mitochondria in the supernatant, while the IMF mitochondria were pelleted with the myofibrils. This first supernatant was then used to isolate SS mitochondria with high-speed spins. In the parallel IMF isolation procedure, the myofibrillar pellet was resuspended and

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incubated with the proteolytic enzyme nagarse to digest myofibrillar proteins and liberate the IMF mitochondria. Next, a second slow spin would yield a supernatant containing IMF mitochondria. The IMF supernatant was then centrifuged at high speed to pellet and wash the IMF mitochondria. These careful and detailed studies by Palmer et al. [5,6] provided convincing electron microscopic evidence that SS and IMF fractions were independently isolated, and their procedures became the generally accepted methodology upon which subsequent work was based. However, a curious pattern reported by Palmer et al., and many studies that followed [5–10], was that essentially all activities of individual enzymes and/or oxidative enzyme pathways were uniformly higher in the IMF fraction compared to the SS, in some cases over 2-fold higher. This consistent finding raises obvious questions: If SS and IMF mitochondria are simply part of a continuous network, then how could all protein-specific activities be higher in IMF? Another, related, question is: where and by what mechanism would the network transition from this SS (lower) to IMF (higher) protein-specific activity? One obvious alternative explanation is that these apparent differences simply reflect experimental artifact. SS mitochondria are isolated in the absence of nagarse exposure, while IMF isolation fundamentally depends on the nagarse incubation. Because nagarse treatment is the glaring difference between the two procedures, we tested the simple hypothesis that non-mitochondrial protein contamination accounts for the apparently lower specific activities of isolated SS mitochondria. Our data support this hypothesis and moreover advance the concept that nagarse treatment may also remove the protein within and perhaps also attached to damaged mitochondria that, left undigested, would otherwise diminish indices of mitochondrial structural and functional integrity.

2. Material and methods

2.1. Animal and muscle preparation

All procedures were in accordance with the guidelines regarding the care and use of animals by the Institutional Animal Care and Use Committee at Mayo Clinic. A total of six C57BL/6J mice on standard chow diet and water ad libitum were used for all experiments. Mice ranged in age from 8 to 12 weeks. On the day of the experiment, the mice were euthanized by isoflourane inhalation and cervical dislocation. The left and right gastrocnemii were removed and immediately placed on an ice-cold petri dish, which was pre-rinsed with ice-cold modified Chappell-Perry Medium I (Solution I; mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 MgCl₂, 1 EDTA, 1 ATP, pH 7.5. After removing the blood, fat and visible connective tissues, the gastrocnemii were placed into a pre-massed beaker containing 2 ml of ice-cold Solution I, reweighed, and the wet muscle mass was calculated. The mean gastrocnemii mass was 138.7 ± 10.5 mg.

2.2. Isolation of mitochondria

Mitochondria were prepared according to the outline in Fig. 1; all procedures were carried out on ice or at 4 °C and all centrifugations were 10 min in duration. Muscles were cleaned, weighed, minced with scissors in 9 volumes of ice-cold Solution I, and gently homogenized by hand using a ground glass-to-glass Potter-Elvehjem homogenizer (5 passes). The homogenate was centrifuged at 800g to obtain the supernatant (SN) containing mechanically released (SS) mitochondria. This SN was divided equally into two 1.5 ml homogenization tubes. Nagarse (bacterial

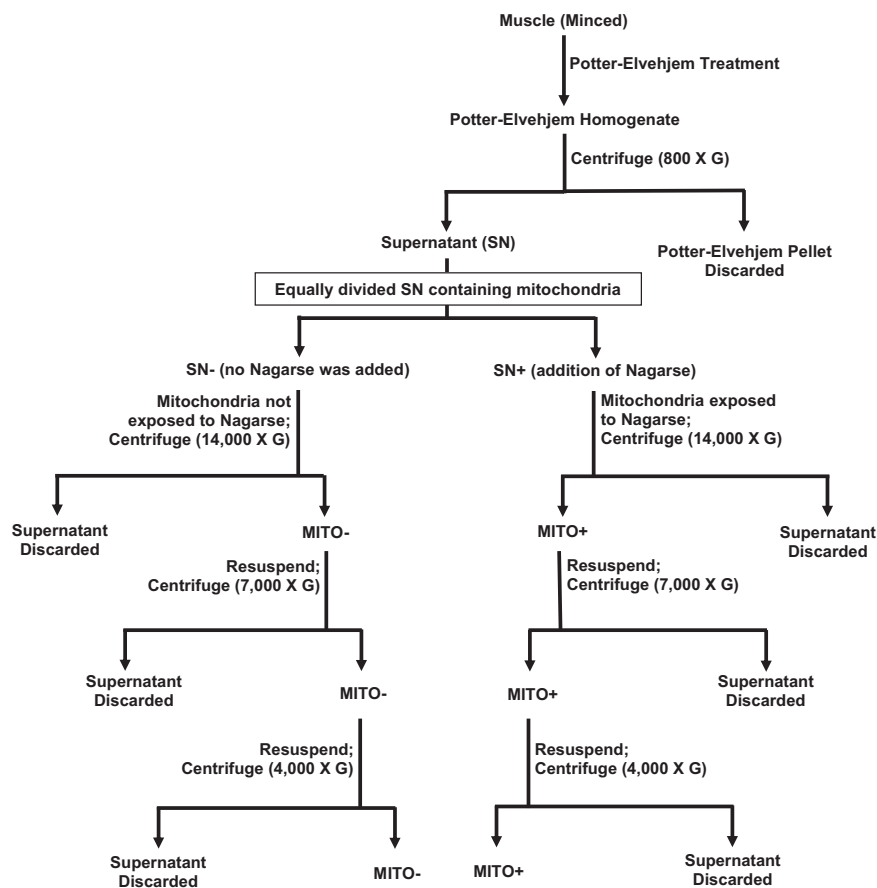


Fig. 1. Isolation and treatment of the muscle mitochondria. A flow diagram depicting the preparation of mechanically released mitochondrial sample that was subsequently either exposed or not to the enzyme nagarse. Details are given in the “Methods” (SN, supernatant).

proteinase Type XXIV, Sigma, P-8038) was prepared using Solution I (5 mg g⁻¹ ww) and added to one tube (SN+). An equal volume of Solution I containing no nagarse, was added to the other tube (SN-). After 7 min incubation, 1 ml of Solution I was added to each tube (this terminated nagarse digestion in the SN+ tube). The SN+ and SN- samples were then centrifuged at 14,000g to obtain two mitochondrial pellets, one exposed to nagarse (MITO+) and the other not exposed (MITO-). After discarding the supernatant, each of the mitochondrial pellets were re-suspended in 0.5 ml of Solution II (mM): 100 KCl, 40 Tris-HCl, 10 Tris-Base, 5 MgCl₂, 1 EDTA, 0.2 ATP, and 1.5% BSA, pH 7.5. Following centrifugation at 7000g, the supernatants were discarded and the mitochondrial pellets were re-suspended in 0.5 ml of Solution III (identical to Solution II, but without BSA). After the final centrifugation at 4000g and removal of supernatant, the two final mitochondrial pellets were each re-suspended in identical volumes of mannitol-sucrose buffer containing (mM) 220 Mannitol, 70 Sucrose, 10 Tris-HCl, 1 EGTA, pH 7.40. The volume of buffer used to resuspend each mitochondrial preparation was 1 µl per mg of original wet muscle. The protein content in the final mitochondrial preparations was determined by the method of Lowry [11]. All protein concentrations reported below and in the tables relate exclusively to the final mitochondrial preparations.

2.3. Citrate synthase assay

Citrate synthase (CS) activity was determined spectrophotometrically at 37 °C by the method of Srere [12], as previously described [13]. In these assays aliquots of mitochondrial suspensions, stored at -80 °C, were assayed in buffer that included 0.05% Triton detergent to disperse the mitochondrial inner membrane and eliminate all enzyme latency.

2.4. Assays for mitochondrial O₂ consumption and ATP production

Freshly isolated mitochondria were assayed for O₂ consumption rate (J_o) and ATP production rate (J_p). O₂ consumption was measured polarographically in a respiration chamber (Hansatech Instruments, Norfolk, UK) at 37 °C following general procedures we have previously described [13]. Mitochondrial respiration was fueled with the substrate combination pyruvate (1 mM)+malate (1 mM)+glutamate (10 mM) (PMG). Aliquots, typically 20 µl in volume, of mitochondrial suspension were added to 250 µl of respiration medium adapted from Wanders et al. [14], which contained (in mM) 100 KCl, 50 MOPS, 10 K₂PO₄, 10 MgCl₂, 1 EGTA, and 0.2% BSA, pH 7.00 [13]. Next, the PMG substrate combination was added and State 2 J_o was followed (respiration due primarily to proton leak). The addition of ADP to give a final concentration of 0.67 mM stimulated state 3, (maximal) J_o. Phosphorylation of this ADP resulted in state 4 J_o [15], and the respiratory control ratio (RCR) was calculated as state 3 J_o/state 4 J_o. The ADP/O ratio was determined as previously described [15]. The State 3 (maximal) rate of ATP production was calculated as the product of state 3 J_o times the ADP/O (taking the 2:1 molecular to atomic oxygen stoichiometry into account).

2.5. Protein separation by SDS-PAGE

Final MITO+ and MITO- suspensions were diluted 1:1 in 2 × Laemmli sample buffer before running on a SDS-PAGE gel. Laemmli sample buffer containing β-mercaptoethanol was prepared according to manufacturer's instructions (Bio-Rad, Hercules, CA). Samples were heated at 95 °C for 5 minutes and then loaded onto a pre-cast 10% SDS-PAGE gel (Bio-Rad, Hercules, CA). The gel was run at 60 V for 30 minutes, 110 V for 60 minutes and then 150 V for 10 minutes. Performing gel electrophoresis under these

conditions allowed for adequate separation, visualization, and the ability to compare the MITO+ versus MITO- samples. Proteins were visualized using Coomassie blue. Gel image was captured using an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

2.6. Protein identification and quantification by mass spectrometry

To obtain an insight into the abundance of mitochondrial versus non-mitochondrial proteins contained in each of the MITO+ and MITO- preparations, aliquots from the samples analyzed on SDS-PAGE were also analyzed by mass spectrometry to identify and quantify proteins contained in each mitochondrial preparation. The mass spectrometry procedure was also used to identify and quantify the protein nagarse in the same preparations.

2.6.1. In-solution digest

A volume of isolated mitochondrial preparation, either treated or not treated with the enzyme nagarse, was added to a 9 times volume of dilution buffer [DB - 10% acetonitrile (ACN) and 25 mM Tris-HCl pH 8.5], 8 µl trypsin (Sigma; St. Louis, MO) at 0.200 µg/ml was added to the protein sample and was allowed to incubate for 16 h at 37 °C with gentle shaking, followed by addition of 50 µl 5% formic acid (FA) to halt the digestion. The resulting peptides were prepared for sample analysis similar to a previously published protocol [16]. In brief, a stop-and-go extraction tip (StageTip) [17], was fitted with two C18 disk plugs using a customized tipping syringe [17]. The Stage Tip was activated with methanol, washed in 100 µl buffer B (0.1% FA, 80% ACN), and equilibrated in 100 µl buffer A (0.1% FA) twice. The peptides were then loaded onto the activated Stage Tip, washed twice in 100 µl buffer A, followed by elution in 50 µl Buffer B. The eluate was dried by vacuum centrifugation and stored at -80 °C prior to use. 6 µl of 0.1% FA (v/v) was added to re-suspend the dried samples, followed by sonication for 2 min. The sonicated samples were briefly centrifuged and 1 µl of sample was subsequently analyzed by mass spectrometry as described below.

2.6.2. Mass spectrometry

HPLC-ESI-MS/MSn was performed on a Thermo Electron Orbitrap Elite Velos Pro fitted with an EASY source (Thermo Electron, San Jose, CA). NanoLC was performed using a DIONEX/Thermo NCS-3500RS UltiMate 3000 with an EASY Spray column (Thermo Electron, 50 cm × 75-µm inner diameter, packed with PepMap RSLC C18 material, 2 µm); loading phase for 15 min; mobile phase, linear gradient of 1–37% ACN in 0.1% FA in 150 min, followed by a step to 95% ACN in 0.1% FA over 5 min, hold 10 min, and then a step to 1% ACN in 0.1% FA over 1 min and a final hold for 19 min (total run 200 min); Buffer A=0.1% FA in 100% H₂O; Buffer B=0.1% FA in 100% ACN; flow rate, 300 nl/min. All solvents were mass spectrometry grade. A "top 15" data-dependent MS/MS analysis was performed (acquisition of a full scan spectrum followed by collision-induced dissociation mass spectra of the 15 most abundant ions in the survey scan).

2.6.3. Database search

Tandem mass spectra were extracted by ProteoWizard msConvert, version 3 [18] using the default settings. Charge state deconvolution and deisotoping were not performed. All MS/MS spectra were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search the SwissProt_02_2015 database (16,706 entries, *mus musculus*) assuming the digestion enzyme trypsin and a maximum of 2 missed cleavages permitted. Mascot searched with a fragment ion mass tolerance of 0.50 Da and a parent ion tolerance of 10.0 PPM. Phosphorylation of serine, threonine, and tyrosine as well as oxidation of methionine was specified in Mascot as a variable modification while no fixed

Table 1
Total protein yield, enzyme specific activities, and indices of functional integrity of mitochondria isolated without and with nagarse.

	MITO–	MITO+	MITO+/ MITO– (Fold Δ)	P Value
Total protein yield mg	0.386 ± 0.05	0.290 ± 0.03	0.75	0.02
Citrate synthase activity nmol min ⁻¹ mg ⁻¹	827.4 ± 65.4	1232.9 ± 83.5	1.49	0.0001
State 3 <i>J_o</i> nmol min ⁻¹ mg ⁻¹	77.6 ± 8.6	142.6 ± 16.8	1.84	0.003
State 4 <i>J_o</i> nmol min ⁻¹ mg ⁻¹	22.5 ± 2.4	31.0 ± 3.5	1.38	0.01
State 3 <i>J_p</i> nmol min ⁻¹ mg ⁻¹	384.9 ± 36.9	771.4 ± 83.4	2.00	0.002
RCR	3.6 ± 0.4	4.6 ± 0.4	1.30	0.002
ADP/O	2.5 ± 0.1	2.7 ± 0.1	1.09	0.02

Values are means ± SE; MITO+, mitochondria samples treated with nagarse; MITO–, mitochondria samples not treated with nagarse; total protein yield was calculated as the product of the protein concentration in the final mitochondrial suspension times the suspension volume; state 3 *J_o*, state 3 O₂ consumption rate; state 4 *J_o*, state 4 O₂ consumption rate; state 3 *J_p*, state 3 ATP production rate. RCR, respiratory control ratio, is State 3 (maximum) *J_o* divided by State 4 (resting) *J_o*; State 3 *J_p* is the product of State 3 *J_o* and the ADP/O ratio.

modifications were indicated.

2.6.4. Criteria for protein identification

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Peptide Probabilities were assigned by the Peptide Prophet algorithm [19] with Scaffold delta-mass correction as well as by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [20]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.6.5. Quantification of mitochondrial and non-mitochondrial proteins

Progenesis software (Nonlinear Dynamics; Progenesis QI; Quayside, Newcastle Upon Tyne, UK) was used to quantify abundance of peptides associated with each protein. Quantification using non-conflicting peptides was selected to generate the protein report for the peptide ion abundance associated with each identified protein. Accession numbers were imported into SwissProt_02_2015 database and used to assign the subcellular location of the identified proteins (i.e. mitochondrial, non-mitochondrial). Peptide ion abundances for all mitochondrial and all non-mitochondrial proteins in each of the MITO+ and MITO– samples were added to calculate total mitochondrial and total non-mitochondrial protein abundances, respectively.

2.6.6. Quantification of the nagarse abundance

To quantify the abundance of nagarse present in the mitochondrial preparations treated/not treated with nagarse, we used the same approach described in the “Database search,” section, but with the following exception: Mascot was set up to search the SwissProt_02_2015 database for *bacillus licheniformis*. We used the unique and stable identifier from the SwissProt_02_2015 database for nagarse, Q65LP7, to perform all queries. We also used the corresponding mnemonic identifier of the UniprotKB entry, Q65LP7_BACLD to perform all protein queries. The ion abundance for the peptides associated with the enzyme

nagarse within each of the mitochondrial preparations were compared with the total ion abundance for all the proteins within each of the mitochondrial preparations treated/not treated with nagarse from the Progenesis protein reports. The total ion abundances are in reference to alignments in Mascot search using SwissProt_02_2015 database for *mus musculus*. The ion abundances of the peptides associated with the enzyme nagarse are in reference to alignments in Mascot search using SwissProt_02_2015 database for *bacillus licheniformis*.

2.7. Statistical analyses

Differences between MITO+ and MITO– across variables of interest were compared using paired *t*-test. Data are reported as means ± SEM. Significance was set at *P* < 0.05.

3. Results

3.1. Protein content of final mitochondrial suspension

Isolating mitochondria with nagarse decreased the protein concentration of the final mitochondrial suspension by 30%. The MITO+ preparation was 2.12 ± 0.23 mg protein ml⁻¹ compared to 2.80 ± 0.37 mg ml⁻¹ in MITO–. When these protein concentrations (mg/ml) are multiplied by their respective suspension volumes (ml), the total protein yield (mg “mitochondrial” protein) of the isolation procedure is calculated. When this is done, the MITO+ protein yield was 0.290 ± 0.03 mg, compared to a MITO– value of 0.386 ± 0.05 mg (Table 1). Thus, nagarse exposure reduced, by 25%, the total protein isolated (Table 1).

3.2. Citrate synthase activity

Citrate synthase, the first enzyme of the citric acid cycle, is a sturdy matrix enzyme often used to assess muscle mitochondrial content. Expressed per mg protein in the final mitochondrial suspension, citrate synthase activity (U mg⁻¹) was 49% higher when nagarse was included in the isolation procedure (Table 1). However, the total citrate synthase activity recovered in the final mitochondrial suspension, calculated by multiplying the U mg⁻¹ by the total protein (mg) yield in the final suspension (see above), was not different in MITO+ (366.7 ± 53.0 nmol min⁻¹) compared to MITO– (329.3 ± 54.1 nmol min⁻¹) (Table 2).

3.3. Respiratory rates and respiratory control ratio

Mitochondrial O₂ consumption (*J_o*) due to the combustion of Pyruvate + Malate + Glutamate (PMG *J_o*) requires the entire

Table 2
Total yield of citrate synthase and respiratory activity of mitochondria isolated without and with nagarse.

	MITO–	MITO+	MITO+/ MITO– (Fold Δ)	P Value
Citrate synthase activity nmol min ⁻¹	329.3 ± 54.1	366.7 ± 53.0	1.11	0.11
State 3 <i>J_o</i> nmol min ⁻¹	31.1 ± 5.7	42.5 ± 7.5	1.37	0.01
State 4 <i>J_o</i> nmol min ⁻¹	9.1 ± 1.8	9.1 ± 1.3	1.00	0.98
State 3 <i>J_p</i> nmol min ⁻¹	153.5 ± 27.5	229.5 ± 38.8	1.49	0.01

Values are means ± SE; MITO+, mitochondria samples treated with nagarse; MITO–, mitochondria samples not treated with nagarse; total yield of activity was calculated as the product of specific activity times the total protein yield (see mean values in Table 1); state 3 *J_o*, state 3 O₂ consumption rate; state 4 *J_o*, state 4 O₂ consumption rate; state 3 *J_p*, state 3 ATP production rate.

oxidative pathway of fully intact mitochondria, including several soluble matrix cofactors such as Coenzyme A and NAD^+ . Saturating ADP was added to stimulate the maximum O_2 consumption rate (state 3 Jo). When mitochondria phosphorylate all of the added ADP to ATP, they transition to “resting” (state 4) respiration.

Table 1 reports that nagarse treatment increased both state 3 Jo (by 84%) and state 4 Jo (by 38%), when these values are expressed per mg protein ($\text{nmol O}_2 \text{ min}^{-1} \text{ mg}^{-1}$). Thus, nagarse treatment especially increased State 3 Jo, while it increased State 4 more modestly. As a result, nagarse increased the respiratory control ratio ($\text{RCR} = \text{State 3 Jo}/\text{State 4 Jo}$) by 30% (Table 1). The total yield of State 3 Jo activity recovered in the final mitochondrial suspension was calculated as above for citrate synthase, by multiplying the respiration rates per mg by the total protein (mg) yield in the final suspension. Unlike citrate synthase, which was not statistically different, total state 3 Jo was 37% higher in MITO+ ($42.5 \pm 7.5 \text{ nmol min}^{-1}$) compared to MITO- ($31.1 \pm 5.7 \text{ nmol min}^{-1}$) (Table 2). The total yield of State 4 Jo activity was essentially identical in MITO- and MITO+ (Table 2).

3.4. ADP/O coupling and maximum ATP production

Nagarse exposure modestly improved, by 9%, the ADP/O ratio, 2.7 ± 0.1 vs. 2.5 ± 0.1 , in MITO+ vs. MITO-, respectively (Table 1). The product of State 3 Jo times the ADP/O yields the State 3 ATP production rate (State 3 Jp). Because both factors were elevated by nagarse treatment, State 3 Jp was dramatically (100%) higher in MITO+ compared to MITO-, 771.4 ± 83.4 vs. $384.9 \pm 36.9 \text{ nmol ATP min}^{-1} \text{ mg}^{-1}$ respectively (Table 1). Again, multiplying these protein-specific State 3 Jp values by the total protein (mg) yield indicated that nagarse exposure increased the total yield of State 3 Jp by 49%: $229.5 \pm 38.8 \text{ nmol ATP/min}$ in MITO+ vs. $153.5 \pm 27.5 \text{ nmol ATP/min}$ in MITO- (Table 2).

3.5. Protein detection by SDS-PAGE and identification and quantification by mass spectrometry

Consistent with the Lowry protein determinations, nagarse treatment decreased the visually apparent protein detected using SDS-PAGE in representative MITO+ vs. MITO- preparations (Fig. 2A). Mass spectrometry analysis of all samples also showed less protein, including mitochondrial protein, content in the MITO+ suspensions (Fig. 2B). In MITO+ preparations approximately 83% of the total protein content was identified as mitochondrial proteins. In contrast, in MITO- preparations mitochondrial proteins could account for only approximately 73% of total protein. The ratio of identified MITO proteins versus non-MITO proteins was higher in MITO+ samples compared to MITO- (Fig. 2C).

Nagarse (apr; subtilisin Carlsberg; EC:3.4.21.62; UniProtKB accession number, Q65LP7) was essentially absent in the final suspensions of mitochondria subjected to nagarse treatment. In MITO+ preparations the tryptic peptide abundance of nagarse represented only 0.008% of the total peptide abundance. In MITO- this value was 0.0002% (data not shown).

4. Discussion

The major findings reported here are that nagarse exposure substantially reduced (by 25%) the protein yield in the final mitochondrial suspension, and proteomic analysis indicated that the elimination of non-mitochondrial protein accounted for much of this reduction. By removing non-mitochondrial protein, nagarse treatment increased mitochondrial enzyme and enzyme pathway protein-specific activities. Expressed per mg protein, nagarse

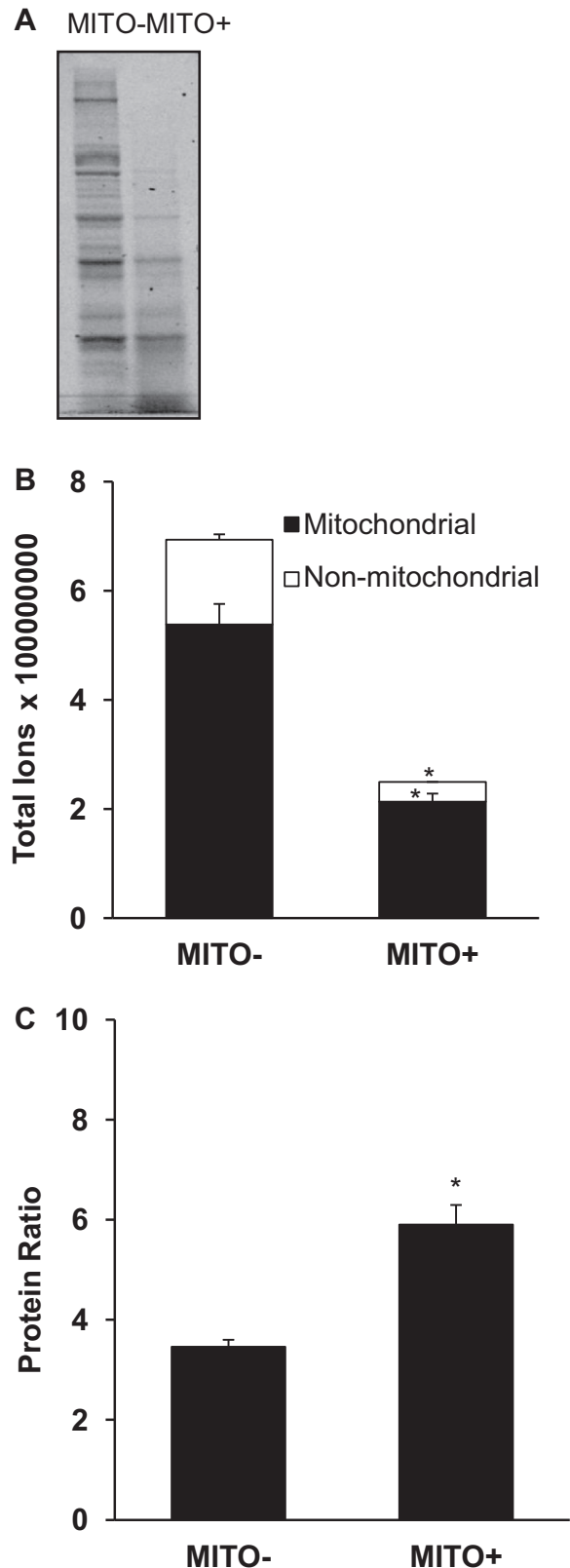


Fig. 2. Protein abundance in mitochondrial suspensions isolated without and with nagarse. Protein abundance detected by SDS-PAGE/Coomassie Blue (A), HPLC-ESI-MS/MS quantitative proteomics analysis to determine peptide ion abundance of mitochondrial and non-mitochondrial proteins in MITO+ and MITO- samples (B), and protein ratio of mitochondrial-to-non-mitochondrial protein in the same samples (C). Values are means \pm SE; MITO+, mitochondria samples treated with nagarse; MITO-, mitochondria samples not treated with nagarse; * $P < 0.05$ between MITO+ and MITO-.

treatment increased citrate synthase activity by 49% and State 3 Jo even more dramatically, by 84%. Moreover, nagarse also modestly increased the ADP/O ratio, so that, per mg protein, State 3 Jp improved by 100%. The data generally indicate that nagarse treatment effectively removes contaminating non-mitochondrial proteins and substantially improves indices of mitochondrial catalytic potential and energetic coupling.

The total isolated CS activity was not significantly affected by nagarse treatment, while nagarse increased the total yield of State 3 Jo by 37%, and State 3 Jp by 49%. The spectrophotometric assay of citrate synthase activity versus the polarographic assay of mitochondrial oxidative phosphorylation fueled by PMG evaluate two vastly different parameters of mitochondrial structure and function. Citrate synthase is a high activity matrix enzyme with well-known stability [21], and is routinely measured in either whole tissue homogenates or mitochondrial suspensions in the presence of a detergent such as Triton X-100, as it was in the present study. In marked contrast, the oxidation of the substrate combination PMG, whether the mitochondria are “resting” or stimulated by saturating ADP, requires, at the least, the entire integrated oxidative pathway and, in particular, robust inner membrane structural integrity. It can be argued that the different outcomes of the CS and Jo assays suggest that nagarse treatment somehow modified the first, slow speed, supernatant in a way that protected mitochondrial units (vesicles) from damage during the subsequent high speed centrifugation and resuspension steps of the isolation procedure. This interpretation is developed below.

Whether mitochondria are partially damaged, have been briefly opened and then resealed, or are fully intact, citrate synthase activity may well remain unaffected. Thus, it can be envisioned that in the absence of nagarse treatment some mitochondria, both fully intact and partially damaged, in the first supernatant were subsequently further damaged during the course of the isolation procedure, but they nevertheless carried citrate synthase activity into the final suspension. Nagarse treatment, on the other hand, would be expected to proteolytically remove the activity of exposed citrate synthase in damaged mitochondria. The data of Table 2 indicate that these intact mitochondria in the final MITO+ suspension carry roughly similar citrate synthase as the MITO-. The net effect is that the total yield of citrate synthase activity in the final suspension is not significantly different. However, in MITO+ more of this citrate synthase activity resides in fully intact mitochondria, which are capable of coupled oxidative phosphorylation.

The total yield of State 3 Jo was 37% higher and State 3 Jp activity was 49% higher in MITO+ final suspensions. In contrast, the total yield of State 4 Jo was essentially identical in the two preparations. Nagarse in the first supernatant may have somehow protected against mitochondrial damage during subsequent centrifugation and resuspension steps, perhaps by digesting non-mitochondrial proteins in some type of linkage [22,23] to mitochondria. In the absence of nagarse treatment, during the high-speed centrifugations and pellet resuspensions these linkages somehow increased the likelihood of structural insult to mitochondrial vesicles. The damage was not severe enough to liberate citrate synthase from its matrix binding [21], but was sufficient to preclude the development of the very high driving forces required for oxidative phosphorylation. Damaged organelles in MITO- preparations are consistent with the lower RCR and ADP/O values observed in MITO-. It therefore appears that nagarse promoted the release of mitochondria from non-mitochondrial protein, which was digested and resulted in less protein yield and less damage to mitochondria during isolation.

Mass spectrometry data further support this interpretation. MITO- preparations had much greater abundance of non-mitochondrial proteins. In nagarse treated preparations, both

mitochondrial and non-mitochondrial protein yields were reduced (Fig. 2B). This suggests that sufficiently damaged mitochondria in the first supernatant were exposed to nagarse entry/attack, digested and did not make it into the final suspension.

The mitochondria within a muscle cell are interconnected as a reticulum (network) [1–3,24,25]. Mechanical homogenization, however gentle, of muscle tissue therefore must destroy this reticular structure, because electron micrographs of isolated mitochondria, for example those of Palmer et al. [5], clearly show the isolated organelles as individual vesicles. During homogenization, as these vesicles form, some membrane damage and leakage of essential matrix cofactors (NAD, CoA, adenylates, etc.) into the isolation medium would be expected [26]. Nevertheless, when these procedures are performed carefully by experienced hands, and particularly when nagarse is included in the isolation procedure, the resulting vesicles are capable of nearly matching the maximum O₂ consumption rates [27,28] and ATP free energy [29–31] measured in intact muscle using Fick O₂ mass balance and ³¹P-MRS, respectively. Moreover, the isolated mitochondria also control respiration and ATP production over the same range of energy phosphate levels observed in vivo [13,30,32,33]. Thus, mitochondria isolated using mechanical homogenization and exposure to protease can be nearly as functional as mitochondria in vivo.

5. Conclusions

Nagarse treatment of mitochondria removes non-mitochondrial proteins as well as proteins of damaged mitochondria, and improves indices of functional integrity and resulting protein-specific activities.

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Appendix A. Transparency document

Transparency document associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bbrep.2016.03.006>.

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