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Functional characterization of mitochondrial oxidative phosphorylation in saponin-skinned human muscle fibers

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The conditions of treatment of human skeletal muscle fibers from M. vastus lateralis with saponin were optimized to achieve complete permeabilization of cell membrane at intact mitochondrial oxidative phosphorylation. After 30 min of incubation with saponin all lactate dehydrogenase, 50% of creatine kinase, 30% of adenylate kinase and less then 20% of citrate synthase was released into the permeabilization medium. These skinned fibers behave similar to isolated mitochondria from human skeletal muscle: (i) the respiration with mitochondrial substrates can be stimulated by ADP, (ii) inhibited by carboxyatractyloside and (iii) it is possible to detect fluorescence changes of mitochondrial NAD(P)H on additions of substrates, uncoupler and cyanide. From a comparison of rates of respiration per cytochrome aa_3 content of isolated human skeletal muscle mitochondria and saponin-skinned muscle fibers it was possible to calculate that almost 85% of mitochondria in those fibers are accessible for the investigation of oxidative phosphorylation. As shown by the investigation of biopsy samples of two patients with undefined myopathies these fibers are a suitable object for the replacement of isolated mitochondria in the diagnosis of mitochondrial myopathies and encephalomyopathies.

Introduction

Skinned muscle fibers are widely employed by physiologists for the investigation of muscle contraction [1,2]. Different skinning procedures as mechanical skinning [3], detergent [4,5] and saponin treatment [6] allow a permeabilization of the sarcolemma leaving the contractile apparatus almost intact. Saponin as a relatively mild skinning agent was reported by Veksler et al. [6] to be even suitable for plasma membrane permeabilization in heart muscle fibers without affecting the intactness of mitochondria and the sarcoplasmic reticulum. For this reason saponin-skinned fibers seem to be an alternative to isolated mitochondria in the diagnosis of mitochondrial defects [7,8] in studies of human heart [6] or skeletal muscle [9] where very limited amounts of tissue from biopsies are available. However, the conditions of saponin treatment of those fibers remain to be carefully elucidated in order to define the normal activities of mitochondrial oxidative enzymes.

The aim of the present report is to define the optimal conditions of saponin treatment of human skeletal muscle fibers for the quantitative determination of activities of mitochondrial oxidative phosphorylation. It is shown that saponin-skinned muscle fibers can replace isolated skeletal muscle mitochondria in most of the functional studies. Due to the small amount of muscle tissue required this method is shown to be applicable for the functional determination of defects of oxidative phosphorylation in patients suffering from undefined myopathies.

Materials and Methods

Isolation of mitochondria. Between 500 mg and 1 g of M. vastus lateralis obtained from orthopaedic pa-

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Abbreviations: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

tients were placed into the isolation medium consisting of 180 mM KCl and 10 mM Na_2EDTA (pH 7.4). Then the connective tissue and fat were carefully removed, the remaining muscle tissue chopped with scissors and incubated 30 min at 4°C in the medium containing additionally 1 mg trypsin per 10 ml. Thereafter the suspension was diluted by the addition of 0.5 volumes of the isolation medium and homogenized using a Potter homogenizer. Then the homogenate was centrifuged 5 min at $600 \times g$, the supernatant percolated through a nylon mesh and recentrifuged 10 min at $10\,000 \times g$. The mitochondrial pellet was suspended in a 180 mM potassium chloride solution containing 0.5 mg bovine serum albumin per ml (pH 7.4) and recentrifuged 10 min at $10\,000 \times g$. The final mitochondrial pellet was suspended in 200–600 μl of the isolation medium.

Isolation of saponin-skinned muscle fibers. Bundles of fibers between 10 and 15 mg wet weight were placed into 1 ml of an ice-cold incubation medium containing 10 mM EGTA-CaEGTA buffer (free Ca^{2+} concentration 0.1 μM), 9.5 mM $MgCl_2$, 3 mM KH_2PO_4, 20 mM taurine, 5 mM ATP, 15 mM phospocreatine, 49 mM K-Mes and 20 mM imidazole-HCl (pH 7.1). Thereafter, 50 μg/ml saponin was added and the suspension was gentle mixed for 20 min at 4°C. Then the fiber bundles were washed to remove saponin in the medium used for respiration measurements (see below) and stored on ice.

Patients. T.L. is a 12-year-old boy with an undefined myopathy. B.K. is a 46-year-old man, with mild myopathy, ptosis and depression with psychotical symptoms (progressive external ophthalmoplegia). In both cases no ragged red fibers were detected.

Respiration measurements. The respiration measurements were performed at 25°C using a Cyclobios-oxygraph (Anton Paar, Innsbruck, Austria) in a medium consisting of 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM Na_2EDTA, 1 mg/ml bovine serum albumin and 20 mM Tris-HCl (pH 7.4). The solubility of oxygen in the medium was considered to be equal to 230 nmol/ml.

Fluorescence microscopy. A single bundle of muscle fibers was fixed at both ends in a Heraeus Flexiperm Micro-12 chamber. Thereafter 200 μl of the medium were added. The chamber was placed on the stage of an Olympus IMT-2 inverted fluorescence microscope. The images were obtained at 340 nm excitation and above 480 nm emission from a Hamamatsu C 2400–87 intensified CCD camera and analyzed by an IBM-compatible computer using the Olympus CUE/RMS software package.

Determinations. The determination of activities of lactate dehydrogenase, citrate synthase, adenylate kinase, and creatine kinase was performed at 30°C by standard spectrophotometric procedures [10]. The content of cytochromes aa_3 was determined spectrophotometrically at 605–630 nm using $\epsilon = 12\ mM^{-1}cm^{-1}$ [11]. For the determination of cytochrome aa_3 content in muscle biopsy specimens a complete removal of hemoglobin was necessary. To reach this, the muscle tissue was homogenized using an Ultra Turrax tissue blender (Janke and Kunkel) in a buffer containing 100 mM KH_2PO_4 (pH 8.0) and centrifuged 10 min at $15\,000 \times g$. For the spectrophotometric determination of cytochrome aa_3 the pellet was solved in a medium containing 0.5% Triton X-100 and 100 mM Tris-HCl (pH 7.4).

Results

Optimization of saponin treatment

In order to optimize the permeabilization procedure of the plasma membrane of human skeletal muscle fibers the respiration rate of fibers incubated for different time intervals in a saponin-containing medium was investigated. In Fig. 1 the succinate oxidation rate after ADP stimulation (circles) and the rate after carboxyatractyloside inhibition (squares) in dependence on the incubation time with saponin is shown. It is seen that about 30 min of incubation with saponin gave the maximal rate of succinate respiration. This ADP-stimulated respiration of those fibers is sensitive to the action of the inhibitor of the adenine nucleotide translocase carboxyatractyloside indicating the intactness of mitochondrial oxidative phosphorylation. Longer incubation times with saponin led to a remarkable drop in ADP-stimulated respiration, most possibly due to cytochrome *c* release.

In Fig. 2A the release of the cytosolic marker enzyme lactate dehydrogenase (circles) and the mitochondrial marker enzyme citrate synthase (triangles) from the fibers into the permeabilization medium is shown. It can be seen that after about 30 min of incubation with saponin all of the exclusively cytosolically localized lactate dehydrogenase was liberated into the medium. The mitochondrial enzyme citrate sythase is liberated to less than 20% (triangles) indicating a rather good mitochondrial intactness after 30 min of saponin action. In Fig. 2B the release of creatine kinase (squares) and of adenylate kinase (triangles) during the incubation of the human skeletal muscle fibers with saponin is shown. The creatine kinase was liberated to approx. 50% of its total activity. Taking into account that only approx. 5% of the creatine kinase activity in human skeletal muscle is due to the mitochondrial isoenzyme [12], this is in line with reports for chicken skeletal muscle in which a substantial part of the cytosolic enzyme remains bound to the myofibrils (MM isoenzyme [13,14]) even after plasma membrane permeabilization. Interestingly, 70% of the total adenylate kinase content remained bound to the skinned fibers

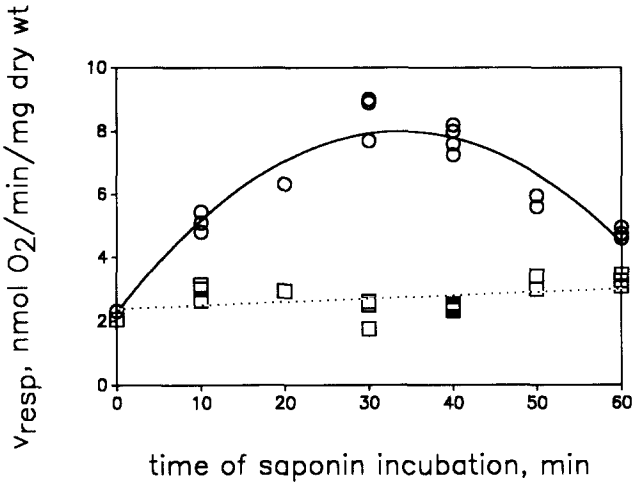


Fig. 1. Dependence of the ADP-stimulated (○) and carboxyatractyloside-inhibited (□) respiration of human skeletal muscle fibers on the time of saponin treatment. 10–20 mg of muscle fibers were incubated in the medium for the respiration measurements described in Materials and Methods after a saponin treatment of times indicated. (○), respiration rate after the addition of 10 mM succinate, 10 μM rotenone and 1 mM ADP. (□), respiration rate after the addition of 10 μM carboxyatractyloside.

after saponin treatment. This is an indication for a possible role of this enzyme in the dynamic compartmentation of adenine nucleotides [15] similar to creatine kinase.

For a visualization of the action of saponin on human skeletal muscle fibers the fluorescence of NAD(P)H was studied in microscopic investigations. To reach an almost fully reduced state of pyridine nucleotides lactate, glutamate and cyanide were present additionally in the permeabilization medium. In Fig. 3A (left picture) is shown the autofluorescence of a single bundle of human muscle fibers caused by mitochondrial and cytosolic reduced pyridine nucleotides in the absence of saponin. The action of saponin can be visualized on fluorescence micrographs after different time intervals of saponin addition. In Fig. 3A (right picture) it can be seen that after 45 min of saponin treatment the amount of the NAD(P)H autofluorescence of the muscle fibers was decreased. The total fluorescence intensities of the micrographs from different fibers plotted versus the incubation time with saponin are shown in Fig. 3B. It can be seen that after 30 min of saponin action the autofluorescence decreased to approx. 60% of its initial value and remained thereafter almost unchanged. This effect can be explained by the efflux of cytosolic pyridine nucleotides into the incubation medium. It is an independent indication for the optimal time interval of 30 min to reach a sufficient permeability of the muscle fiber sarcolemma to nucleotides.

Qualitative investigations of intactness of oxidative phosphorylation using saponin-permeabilized human muscle fibers

The functional investigation of the intactness of the processes of oxidative phosphorylation in human muscle biopsy samples can be carried out in isolated mitochondria if more than approx. 500 mg of tissue are available. This is due to the facts that the human muscle M. vastus lateralis contains between 9 and 11 mg mitochondrial protein per g wet weight (9 determinations based on the cytochrome aa_3 content) and that the fraction of mitochondria which can be isolated from a tissue does not exceed 10–15% of the total mitochondrial content [16] (the typical yield in our own isolation procedure was 10%). In Fig. 4A a typical oxygraph trace of intact human skeletal muscle mitochondria is shown in which the intactness of the different steps of substrate oxidation is investigated within

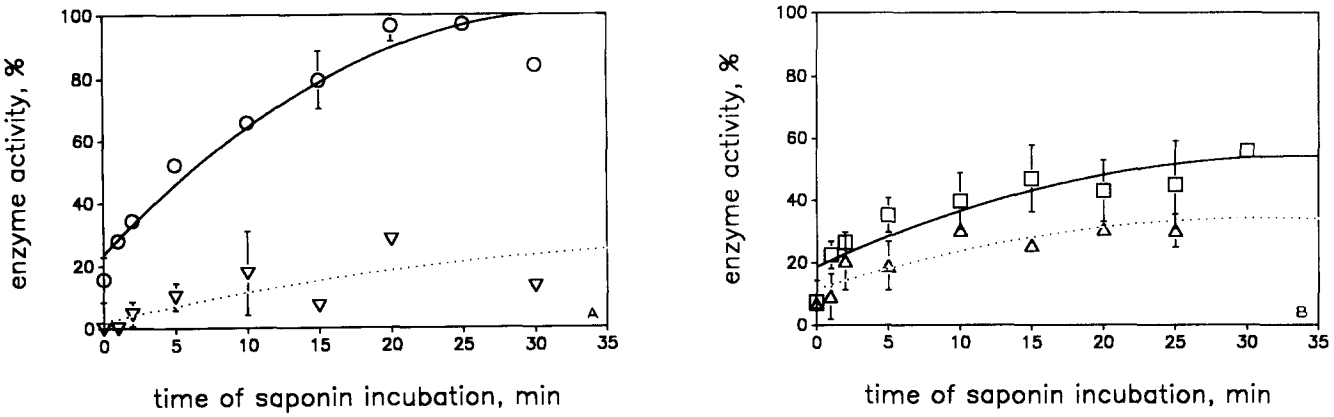
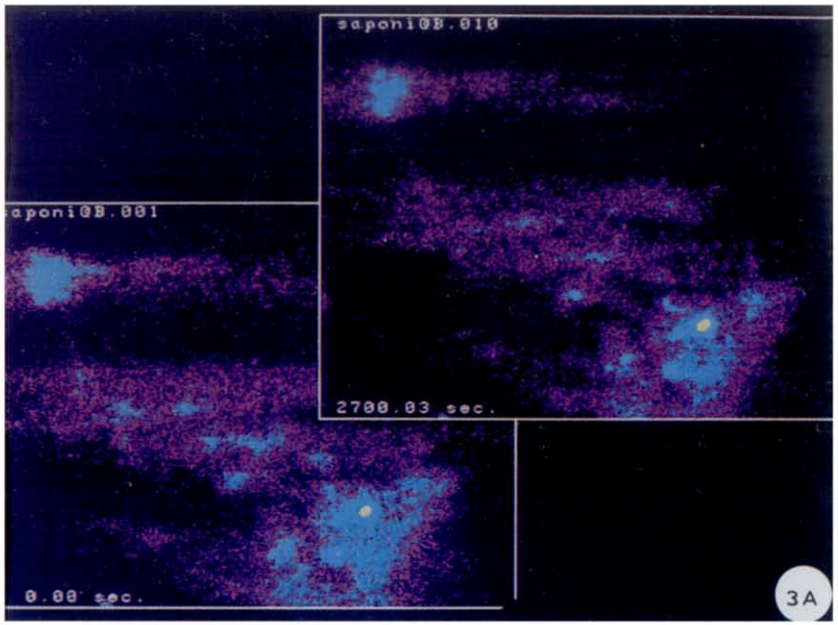
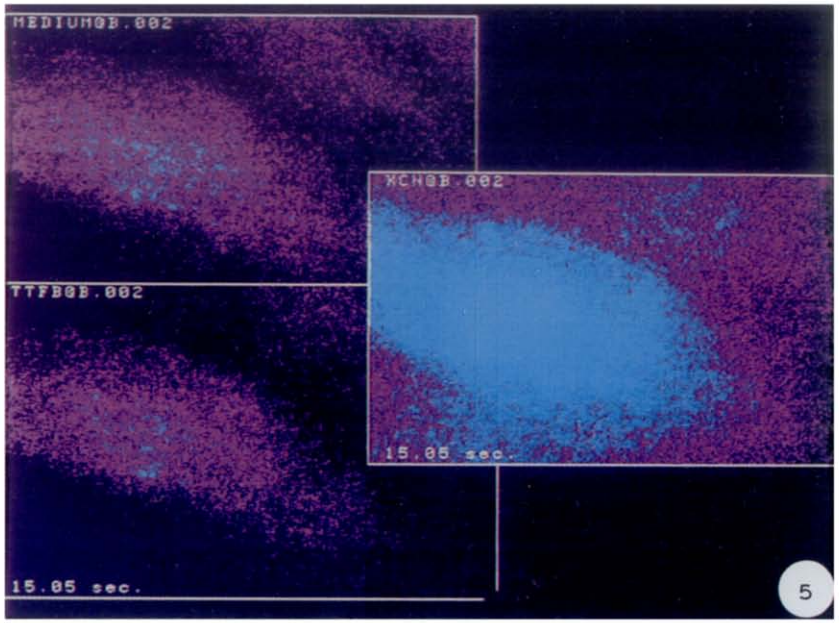


Fig. 2. Dependence of enzyme release from human muscle fibers on the time of saponin treatment. 50 mg of muscle fibers were incubated in the medium used for saponin-skinning. After the time indicated aliquotes were taken for the assay of enzymes. The values given are expressed in % of the activity present in the total tissue extract. (A) ○, lactate dehydrogenase (three experiments), total activity in the tissue extract 100 ± 17 U/g wet wt; ▽, citrate synthase (three experiments), total activity in the tissue extract 5.4 ± 1.4 U/g wet wt. (B) △, adenylate kinase (three experiments), total activity in the tissue extract 110 ± 25 U/g wet wt; □, creatine kinase (four experiments), total activity in the tissue extract 681 ± 30 U/g wet wt.





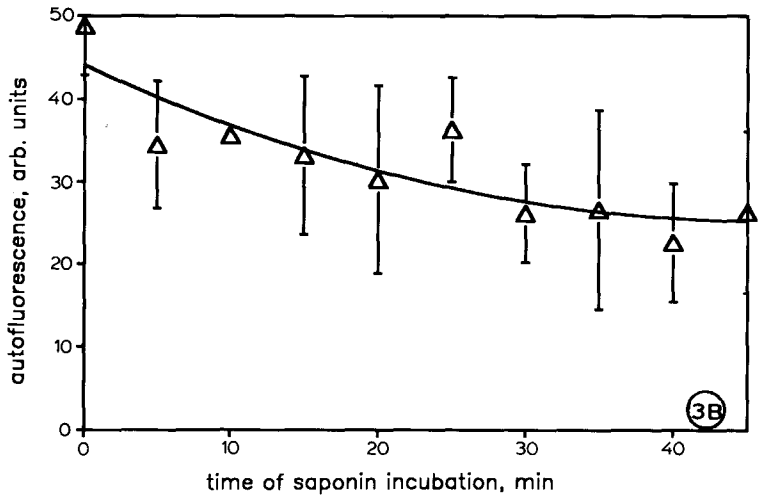


Fig. 3. (A) Fluorescence micrograph of a bundle of muscle fibers at two different times of saponin treatment. A bundle of muscle fibers (2 mg wet wt) was incubated in the skinning medium (Materials and Methods) in the additional presence of 10 mM lactate, 10 mM glutamate and 4 mM cyanide. Left picture, micrograph before saponin addition; right picture, micrograph after 45 min of saponin treatment. (B) Decay of NAD(P)H autofluorescence of human muscle fibers during saponin treatment. Muscle fibers (1.5–2.3 mg wet wt) were incubated as described above. At the time indicated fluorescence micrographs were collected. The fluorescence values (in arbitrary units) indicated are averages of the total fluorescence intensity of the micrographs from three independent experiments.

Fig. 5. Fluorescence micrographs of human skinned muscle fibers in different functional states. 2.1 mg wet wt of a bundle of skinned fibers were incubated in the medium used for respiration measurements. Upper left picture, NAD(P)H fluorescence in the presence of 10 mM glutamate + 5 mM malate; lower left picture, NAD(P)H fluorescence after the addition of 10 μM TTFB; right picture, NAD(P)H fluorescence after the addition of 4 mM potassium cyanide.

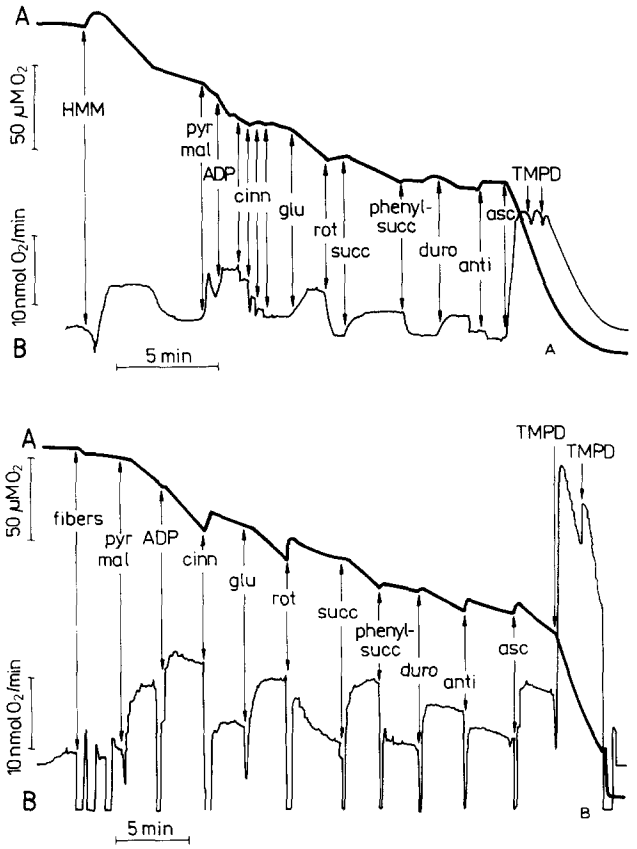


Fig. 4. (A) Oxygraph trace of respiration of isolated human skeletal muscle mitochondria. 2 mg mitochondrial protein (HMM) were incubated in 3 ml medium for respiration measurements. Additions: Pyruvate (pyr), 10 mM; malate (mal), 5 mM; ADP, 5 mM; α-cyanohydroxycinnamate (cinn), 0.65 mM (final concentration); glutamate (glu), 10 mM; rotenone (rot), 20 µM; succinate (succ), 10 mM; phenylsuccinate (phenylsucc), 20 mM; duroquinole (duro), 13 µM; antimycin (anti), 6.5 µM; ascorbate (asc), 2 mM; TMPD, 5 µM. Trace A, oxygraph trace; trace B, first derivative. (B) Oxygraph trace of respiration of saponin-skinned human muscle fibers. 2.9 mg dry weight of skinned fibers were incubated as described in the legend to Fig. 4A. All additions like in the legend to Fig. 4A.

one experiment applying specific inhibitors. Initially, mitochondrial respiration with pyruvate + malate was stimulated by ADP. Applying α-cyanohydroxycinnamate, an inhibitor of the pyruvate carrier, the respiration can be inhibited to its endogenous value. Glutamate gave under this conditions a rise in respiration which is sensitive to rotenone, an inhibitor of the NADH:CoQ oxidoreductase. To test the respiration of these mitochondria with succinate, this substrate was thereafter added and the respiration was found to be sensitive to phenylsuccinate, an inhibitor of the succinate translocator. The artificial substrate for the b-c_1 complex duroquinol caused a stimulation of respiration which is sensitive to antimycin. The addition of TMPD and ascorbate increased then the respiration of mitochondria due to the remaining uninhibited cytochrome oxidase activity. Applying this experimental protocol it should be possible to localize a defect in one of the reactions investigated: (i) pyruvate dehydrogenase; (ii) aspartate aminotransferase; (iii) NADH:CoQ oxidoreductase; (iv) succinate dehydrogenase; (v) $CoQH_2$:cytochrome c oxidoreductase and (vi) cytochrome c oxidase within one experiment. The disadvantage of this type of investigations is that a fairly high amount of mitochondria (above 1 mg of mitochondrial protein) is necessary. To overcome this limitation we tried to perform a similar experiment with saponin-skinned human muscle fibers. The result of a typical oxygraph trace is shown in Fig. 4B. It can be seen that skinned muscle fibers behave almost identical to mitochondria. No difference to isolated mitochondria was detected in respect with the oxidation of the different substrates applied and with the sensitivity to the different inhibitors. Therefore, this type of experiment can be easily performed with about 20 mg of tissue instead of isolating mitochondria from more then 500 mg of tissue.

Functional investigations of oxidative phosphorylation in saponin-skinned human muscle fibers can be carried out also using investigations of the autofluorescence of a single bundle of fibers (2–5 mg of muscle tissue). In Fig. 5 the fluorescence micrograph pictures of a bundle of saponin-skinned fibers are shown in different functional states of mitochondria. The fibers were incubated in a medium containing 10 mM glutamate and 5 mM malate. The obtained NAD(P)H fluorescence (left part, upper picture) could be diminished by the addition of TTFB, an uncoupler of oxidative phosphorylation (left part, lower picture). Addition of cyanide caused a large increase in fluorescence (right picture) due to NAD(P)H reduction in response to the inhibition of cytochrome-c oxidase.

Quantitative evaluation of functional characteristics of saponin-skinned human muscle fibers

As shown above, 30 min of saponin treatment resulted in the permeabilization of the cell membrane of human M. vastus lateralis fibers leaving the mitochondria almost intact. To apply this method for the quantitative determination of the different mitochondrial substrate oxidation rates it is necessary to prove how many of the mitochondria are accessible to mitochondrial substrates and to ADP. For this reason we compared the glutamate + malate oxidation rates of intact human M. vastus lateralis mitochondria with those of saponin-skinned fibers from the same muscle related to the cytochrome aa_3 content of the different objects. In order to check if the values obtained for human fast twitch skeletal muscle are affected by the rather high average age of patients (62.3 ± 6.6 years) those data were in addition compared with values obtained with the rat skeletal muscles M. quadriceps (fast twitch) and M. soleus (slow twitch) applying es-

TABLE I

Comparison of the ADP-stimulated glutamate + malate oxidation rates of skinned fibers and mitochondria from human and rat skeletal muscles

The rat skeletal muscle mitochondria were isolated according to Ref. 18 from a mixture of muscle tissue (fast twitch and slow twitch). Average values ±S.D. are presented. *n*, number of independent preparations.

Object	Required amount of tissue	Respiration in (nmol O_2/min per mg dry wt)	Respiration in (nmol O_2/min per mg protein)	Respiration in (nmol O_2/min per nmol aa_3)
Human M. vastus lateralis mitochondria	500 mg–1 g	–	168.5 ± 43.4 $n = 3$	202.3 ± 40.4 $n = 4$
Human M. vastus lateralis fibers	20–50 mg	8.65 ± 1.88 $n = 9$	–	173.6 ± 24.4 $n = 8$
Rat skeletal muscle mitochondria *	> 1 g	–	210.6 ± 28.6 $n = 4$	246.9 ± 31.5 $n = 4$
Rat M. soleus fibers	10–20 mg	18.15 ± 0.65 $n = 3$	–	223.7 ± 10.3 $n = 3$
Rat M. quadriceps fibers	20–50 mg	7.86 ± 0.07 $n = 3$	–	230.3 ± 8.2 $n = 3$

sentially the same procedures. The results of these experiments are presented in Table I. It can be seen that the ADP-stimulated respiration of isolated human skeletal muscle mitochondria expressed in nmol O_2/min per nmol aa_3 is approx. 115% of the rate obtained with saponin-skinned fibers from the same muscle. Very close values of maximal respiration rates (expressed in nmol O_2/min per mg dry wt.) with glutamate + malate were obtained for rat M. quadriceps fibers. However, if the rates are expressed in nmol O_2/min $nmol^{-1}$ aa_3 the maximal respiration activities for rat skeletal muscle mitochondria and saponin-skinned fibers are slightly higher. For fibers from rat slow-twitch muscle containing much more mitochondria per g tissue (visible from the higher rates of respiration of skinned fibers per mg dry weight) the respiration values expressed in nmol O_2/min per nmol aa_3 are very close to the value for rat fast twitch muscle fibers. Comparing the maximal respiration rates per nmol aa_3 of fibers and of mitochondria from the same object it can be stated that between 85% (human muscle) and 90% (rat muscle) of the maximal respiratory rate is adjustable by ADP in the saponin-treated skeletal muscle fibers.

In Table II the maximal oxidation rates with different substrates of saponin-treated human skeletal muscle fibers from orthopaedic patients are compared with fibers from patients suffering of an undefined myopathy (T.L.) and a progressive external ophthalmoplegia (B.K.). Interestingly, only in the case of B.K. substantially lowered respiration rates of saponin-treated muscle fibers were observed (almost 50% of the control values). In order to investigate which of the complexes of the respiratory chain is affected different enzyme

TABLE II

Maximal respiration rates of saponin-skinned human muscle fibers from orthopaedic patients and myopathy patients

The values presented are averages ±S.D. *n*, number of independent preparations from different patients, *n**, number of different measurements; n.d., not determined.

Substrate	Rate of respiration (nmol O_2/min per mg dry wt) (orthopaedic patients)	Rate of respiration (nmol O_2/min per mg dry wt) (T.L.)	Rate of respiration (nmol O_2/min per mg dry wt) (B.K.)
10 mM Glutamate 5 mM Malate	8.65 ± 1.88 $n = 9$	7.17 ± 0.19 $n^* = 3$	3.52 ± 1.14 $n^* = 6$
5 mM Pyruvate 5 mM Malate	8.15 ± 1.74 $n = 6$	8.12 ± 0.16 $n^* = 2$	4.38 ± 1.2 $n^* = 2$
1 mM Octanoylcarnitine 5 mM Malate	7.38 ± 2.29 $n = 6$	5.52 ± 0.22 $n^* = 2$	3.52 ± 0.6 $n^* = 3$
10 mM Succinate 20 μM Rotenone	9.05 ± 3.09 $n = 6$	n.d.	6.17 ± 1.87 $n^* = 5$

TABLE III

Enzyme activities in muscle homogenates from orthopaedic patients and myopathy patients

The given activities are means (±S.D.) of triplicate determinations. n.d., not determined.

Enzyme	Orthopaedic patients (U/g wet wt)	T.L. (U/g wet wt)	B.K. (U/g wet wt)
Lactate dehydrogenase	100.1 ± 17	80.2 ± 6.1	73.2 ± 13
Citrate synthase	5.4 ± 2	6.2 ± 0.66	4.0 ± 0.5
Adenylate kinase	110.3 ± 25	119 ± 23	133.6 ± 10.9
Creatine kinase	681 ± 30	1171 ± 190	668.2 ± 127
Succinate : cytochrome-*c* reductase	0.52 ± 0.03	n.d.	0.51 ± 0.04
NADH : cytochrome-*c* reductase	0.68 ± 0.09	n.d.	0.51 ± 0.1
Cytochrome-*c* oxidase	1.57 ± 0.24	n.d.	1.06 ± 0.18 [a]
Heme aa_3 (nmol/g wet wt)	5.57 ± 1.8	4.86 ± 0.24	2.9 ± 0.1 [a]

[a] The difference to the control values was tested to be significant ($P < 0.001$).

activities in the muscle homogenates were compared to the orthopaedic controls. The results are summarized in Table III. It was observed that the reason for the diminished substrate oxidation rates of saponin-treated muscle fibers of patient B.K. is a lowered content of cytochrome-*c* oxidase as detected by the spectrophotometric determination of hems aa_3 and the measurement of the enzyme activity in the muscle homogenate.

Discussion

As already shown by Veksler et al. [6] for rat heart muscle and by Letellier et al. [9] for human skeletal muscle saponin can be used as a mild skinning agent for the investigation of mitochondria in an in-situ-like environment. Saponin perforates the sarcolemma due to a high amount of cholesterol in this membrane [17]. We observed, applying three independent techniques, a slightly longer optimal permeabilization time with saponin for human skeletal muscle fibers than the values used for skinning of rat heart [6] and human skeletal muscle fibers [9] in previous publications. However, in the reports mentioned, no detailed investigation of the optimal permeabilization time was made. Most probably due to this reason Letellier et al. [9] reported lower respiratory controls (ADP stimulated respiration divided by carboxyatractyloside inhibited respiration) – 2.64 ± 0.55 – than we observed under optimal conditions −4.0 ± 0.9. The maximal respiration activities with succinate (2.57 ± 0.73 natom O/min mg^{-1} wet wt.) given in this paper are however close to the values found by us but the glutamate or pyruvate oxidation rates (1.55 ± 0.43 natom O/min per mg wet wt) are substantially lower (60%) than our values taking into account a wet weight/dry weight quotient of 6.52 ± 0.52 (nine independent determinations).

We observed that the maximal glutamate + malate oxidation rates of human muscle mitochondria and saponin-skinned human muscle fibers are in the range of 200 nmol O_2/min per nmol aa_3. This value is close to 178 nmol O_2/min per nmol aa_3 reported for rat heart mitochondria by Veksler et al. [6] who obtained with saponin-skinned heart muscle fibers 87% of mitochondrial respiration rates. Interestingly, the values obtained by us for rat muscle tissue are even somewhat higher. The smaller maximal rates of respiration obtained for mitochondria and saponin-treated fibers can be explained by either an incomplete accessibility of fibers for substrates or ADP, or by an partial damage of mitochondria during the skinning procedure.

The large scatter of experimental data reported in the literature for human skeletal muscle mitochondria preparations (cf., Refs. 19, 20) if expressed in nmol O_2/min per mg of mitochondrial protein is most probably caused by different amounts of contaminating proteins. Applying our isolation procedure we observed that the extent of those protein contaminations is higher in mitochondrial preparations from fast-twitch muscles if the amount of tissue is below 500 mg.

Investigating the time-dependence of the NAD(P)H fluorescence in cyanide-inhibited human skeletal muscle fibers during saponin treatment we detected an approx. 40% decrease in the total fluorescence intensity after 30 min. Therefore, the remaining 60% of the total pyridine nucleotide fluorescence can be assigned to fluorochromes located in the mitochondria. This value is much lower than the value reported for heart muscle, in which the NAD(P)H fluorescence of the tissue seems to be exclusively of mitochondrial origin [21,22]. The difference is caused most probably by the much lower content of mitochondria in fast-twitch human skeletal muscle. So, in tissues having a lower specific content of mitochondria than rat heart, like rat kidney or bull spermatozoa, 25% [23] or even as much as 50 % [25] of the total NAD(P)H fluorescence originate from the extramitochondrial compartment.

Summarizing, saponin skinning of human muscle fibers is an attractive alternative to the isolation mito-

chondria for functional investigations of defects in oxidative phosphorylation. Applying this procedure and oxygraphic measurements it was possible to detect a case of a mitochondrial myopathy caused by a deficiency of cytochrome-*c* oxidase. The rates of respiration of fibers of this patient were substantially diminished in comparison to the controls and to a yet undefined myopathy case.

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References

1 Best, P.M. (1983) Am. J. Physiol. 244, H167–H177.
2 Hoar, P.E., Mahoney, C.W. and Kerrick, W.G. (1987) Pflügers Arch. 410, 30–36.
3 Kawata, H. and Fujishiro, N. (1990) Jpn. J. Physiol. 40, 243–251.
4 Ventura-Clapier, R., Saks, V.A., Vassort, G., Lauer, C. and Elizarova, G.V. (1987) Am. J. Physiol. 253, C444–C455.
5 Clark, J.F., Khuchua, Z. and Ventura-Clapier, R. (1992) Biochim. Biophys. Acta 1100, 137–145.
6 Veksler, V.I., Kuznetsov, A.V., Sharov, V.G., Kapelko, V.I. and Saks, V.A. (1987) Biochim. Biophys. Acta 892, 191–196.
7 Morgan-Hughes, J.A., Hayes, D.J., Clark, J.B., Landon, D.N., Swash, M., Stark, R.J. and Rudge, P. (1982) Brain 105, 553–582.
8 Siemes, H. (1990) Mitochondriale Myopathien und Enzephalomyopathien, Zuckschwerdt, München.
9 Letellier, T., Malgat, M., Coquet, M., Moretto, B., Parrot-Roulaud, F. and Mazat, J.-P. (1992) Pediatric Res. 32, 17–22.
10 Bergmeier, H.U. (1970) Methoden der enzymatischen Analyse. 2. Auflage, Akademie Verlag, Berlin.
11 Van Gelder, B.F. (1966) Biochim. Biophys. Acta 118, 36–46.
12 Smeitink, J., Wevers, R., Hulshof, J., Ruitenbeck, W., Van Lith, T., Sengers, R., Trijbels, F., Korenke, C. and Wallimann, T. (1992) Ann. Clin. Biochem. 29, 196–201.
13 Wallimann, T. and Eppenberger, H.M. (1985) Cell Muscle Motil. 6, 239–285.
14 Wallimann, T., Turner, D.C. and Eppenberger, H.M. (1977) J. Cell Biol. 75, 318–325.
15 Gellerich, F.N. (1992) FEBS Lett. 297, 55–58.
16 Halperin, M.L. and Pande, S.V. (1979) Methods Enzymol. 56, 368–378.
17 Daum, G. (1985) Biochim. Biophys. Acta 822, 1–42.
18 Reichert, M., Schaller, H., Kunz, W. and Gerber, G. (1978) Acta Biol. Med. Germ. 37, 1167–1176.
19 Byrne, E. and Trounce, J (1985) J. Neurol. Sci. 69, 319–333.
20 Zheng, X.X., Shoffner, J.M., Voljavec, A.S. and Wallace, D.C. (1990) Biochim. Biophys. Acta 1019, 1–10.
21 Nuutinen, E.M. (1984) Basic Res. Cardiol. 79, 49–58.
22 Eng, J., Lynch, R.M. and Balaban, R.S. (1989) Biophys. J. 55, 621–630.
23 Krinelke, L. and Kunz, W.S. (1990) Biochim. Biomed. Acta 49, 1119–1130.
24 Halangk, W. and Kunz, W.S. (1991) Biochim. Biophys. Acta 1056, 273–278.