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Mitochondrial respiratory rates and activities of respiratory chain complexes correlate linearly with heteroplasmy of deleted mtDNA without threshold and independently of deletion size

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Abstract

To clarify the importance of deleted protein and tRNA genes on the impairment of mitochondrial function, we performed a quantitative analysis of biochemical, genetic and morphological findings in skeletal muscles of 16 patients with single deletions and 5 patients with multiple deletions of mtDNA. Clinically, all patients showed chronic progressive external ophthalmoplegia (CPEO). The size of deletions varied between 2.5 and 9 kb, and heteroplasmy between 31% and 94%. In patients with single deletions, the citrate synthase (CS) activity was nearly doubled. Decreased ratios of pyruvate- and succinate-dependent respiration were detected in fibers of all patients in comparison to controls. Inverse and linear correlations without thresholds were established between heteroplasmy and (i) CS referenced activities of the complexes of respiratory chain, (ii) CS referenced maximal respiratory rates, (iii) and cytochrome-*c*-oxidase (COX) negative fibers. In patients with single and multiple deletions, all respiratory chain complexes as well as the respiratory rates were decreased to a similar extent. All changes detected in patients with single deletions were independent of deletion size. In one patient, only genes of ND5, ND4L as well as $tRNA_{Leu(CUN)}$, $tRNA_{Ser(AGY)}$, and $tRNA_{His}$ were deleted. The pronounced decrease in COX activity in this patient points to the high pathological impact of these missing tRNA genes. The activity of nuclear encoded SDH was also significantly decreased in patients, but to a lesser extent. This is an indication of secondary disturbances of mitochondria at CPEO.

In conclusion, we have shown that different deletions cause mitochondrial impairments of the same phenotype correlating with heteroplasmy. The missing threshold at the level of mitochondrial function seems to be characteristic for large-scale deletions were tRNA and protein genes are deleted.

Keywords: Chronic progressive external ophthalmoplegia; Respiratory chain; Mitochondrial respiration; Heteroplasmy; Deletion; mtDNA

1. Introduction

Since the first reports linking human diseases to genetic defects of the mitochondrial genome, fast and exciting progress in understanding of the pathophysiology of mitochondrial diseases has been possible [1]. However, in mtDNA-linked diseases, strong relations between genotype and phenotype are missing [2,62–64]. Chronic progressive external ophthalmoplegia (CPEO), for instance, is caused by single deletions of mtDNA occurring sporadically [3], by autosomally inherited multiple deletions of mtDNA [4], duplicated mtDNA [5], A3243G mutation [6,7], or by other point mutations [8,9]. Autosomal inheritance is a hint at mutations of the nuclear genome. Mutations in the AdN-translocator [10], twinkle [11], and polymerase γ genes [12] have been identified.

Deletions of mtDNA have at least two different consequences at different levels of mitochondrial protein synthesis. Missing protein genes cause a defined enzyme deficiency due to a defect at the level of transcription. The resulting impair-

Abbreviations: COX, cytochrome-*c*-oxidase; CPEO, chronic progressive external ophthalmoplegia; CS, citrate synthase; HEPS, high energy preservation solution; NCP, noncollagen protein; SDH, succinate dehydrogenase; SRPR, succinate-referenced pyruvate respiration [100(state $3_{pyruvate}$)/state$3_{succinate}$)]; SRNO, succinate-referenced NADH oxidation [100(complex I+III)/(complex II+III)]

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ment at the level of mitochondrial function depends on the control exercised by the deleted protein on the function in total. Missing tRNA, however, causes dysfunction due to a defect at the level of translation. This translation defect necessarily affects *all* mitochondrial encoded structure proteins and should a priori have a higher impact on the impairment of mitochondrial functioning than deletions of protein coding genes.

It is not known for certain whether or not deletions of mtDNA at different levels of heteroplasmy correlate with altered patterns of mitochondrial enzymes [13,63] and how these altered enzyme patterns influence functional properties of mitochondria [14,15] Generally, it is assumed that only pronounced enzyme defects are detectable, since the maximum activity of most single enzymes is higher than the maximum fluxes through the metabolic systems [14,16]. This is the metabolic reason for thresholds. A threshold of 55% was detected in cybrids with common deletion [17], and in resting fibroblasts, there was no detectable effect of MELAS mutation on the rate of ATP synthesis [18]. In contrast to these results, there are patients with mt-encephalomyopathies due to mutations at low heteroplasmy but with considerable clinical symptoms [7,63]. In addition, it has been recently shown that linear changes in respiratory chain complexes without threshold were detectable in muscle biopsies of CPEO patients [13]. Due to these problems, a complex set of approaches at the level of genome, enzymes, and on the level of mitochondrial function is necessary to clarify the genotype phenotype relations for the different types of mutation.

Usually, mitochondrial function is investigated in isolated mitochondria [19–22]. Due to the existence of different mitochondrial subpopulations [23–26] and the nonhomogeneous distribution of defective mitochondria [25], it is uncertain whether or not mitochondrial preparations normally obtained with a low yield [19,20] can be representative of the total amount of mitochondria. As an alternative, the skinned fiber technique allows practically all mitochondria contained in muscle fibers to be investigated [27–29]. In combination with high-resolution respirometry [30] and multiple substrate inhibitor titration [31], this method has been introduced for the diagnosis of mitochondrial disorders [27,29,31,32]. These techniques have been successfully used for detection and characterization of mitochondrial defects in heart [33] and skeletal muscle [31,33] and for estimation of flux control coefficients as well [34].

The aim of our present study was to define quantitative relations between mt-defects on genomic, enzymatic and functional level of mitochondria in the skeletal muscle of 21 patients with single and multiple deletions. We included in our study patients with deletions of different size in order to compare their importance on the degree of mitochondrial impairment. Functional properties of mitochondria in skinned fibers were compared with activities of mitochondrial enzymes, the nature, size, and heteroplasmy of the genetic defect. If functional and enzymatic properties were normalized by CS activity, both kinds of data were found to correlate linearly with the level of heteroplasmy. Non-normalized data, however, did not correlate with heteroplasmy but showed high thresholds. Results support the view that the consequences of deletions on enzymatic and functional level correlate with the heteroplasmy and not with the deletion size. Therefore, it is concluded that patients with common and similar deletions as occurring in our patients have comparable impairments at mitochondrial level, which differ only in the level of heteroplasmy.

2. Materials and methods

2.1. Patients

Twenty-one patients suffering from CPEO (mean age=45±14 years; 10 male, 11 female) due to multiple (n=5) or single deletions (n=16) were included in this study. Clinical diagnosis was supported by a broad spectrum of specific investigations: characteristic histological and ultrastructural changes, increased blood lactate levels after mild bicycle exercise [35], increased muscle fatigue [36], and monitoring of muscle oxygenation during exercise by NIRS [36,37]. Control muscle specimens were obtained from 46 patients (23 male and 23 female, mean age 50±16 years) who had muscle biopsies for diagnosis of muscular symptoms. Patients were deemed to be normal controls if they were ultimately found to have no muscle disease by combined clinical, electromyographic, and histological criteria.

2.2. Biopsies

Open biopsies were taken from m. biceps brachii (14), m. quadriceps (2), m. vastus lateralis (3), m. deltoideus (1) or from m. pectoralis (1). Biopsies (about 300 mg) were divided into three parts. Two parts were immediately frozen in liquid nitrogen for enzymatic and histochemical investigations. One part was stored on ice at 4 °C in high energy buffer solution (high energy preservation solution (HEPS) [31], consisting of 8.1 mM K-EGTA, 1.9 mM CaEGTA buffer (free Ca^{2+} concentration 0.1 μM), 9.5 mM $MgCl_2 \cdot 6H_2O$, 3 mM KH_2PO_4, 20 mM taurine, 5.2 mM ATP, 15 mM PCr, 49 mM KMES, 20 mM imidazole, pH 7.1) after rough dissection. It has been shown that mitochondrial function remains intact up to 30 h under these conditions [28].

2.3. Molecular genetic investigations

DNA was extracted from muscle and digested with *Bam*HI or *Pvu*II prior to Southern blotting and hybridization to a mtDNA probe of 16252 nucleotides (Expand long PCR system, Boehringer Mannheim, forward primer 15149–

15174, reverse primer 14831–14811). DNA was labeled with the ECL kit (Amersham Life Science), before an autoradiogram was performed (Biomax-film, Kodak [7]). The deletions were mapped using restriction analysis. The proportions of mutated mtDNA were determined using ImageQuant software (Molecular Dynamics) using the 'area of rectangle method' according to the manufacturer's instructions after correction by the molecular weight. In three patients with familial CPEO and multiple deletions, the whole coding region of the ANT-1 gene was analyzed by direct sequencing according to Kaukonen et al. [10].

2.4. Isolation and permeabilization of muscle fibers

Immediately before oxygraphic measurements, the fibers were permeabilized for 30 min with 50 μg saponin/ml of HEPS as described previously [31]. After permeabilization, the fibers were washed three times (10 min) in incubation medium (see below) to remove saponin and adenine nucleotides. All procedures were performed in a cooling room on ice at 4 °C.

2.5. Respirometric measurements

We used the OROBOROS® oxygraph [30], a two-chamber respirometer with a Peltier thermostat and integrated electromagnetic stirrers. Bundles of fibers (5–10 mg) were transferred into the oxygraph chambers. The measurements were performed at 30 °C in 1.42-ml incubation medium consisting of 75 mM mannitol, 25 mM sucrose, 100 mM KCl, 10 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, 20 mM Tris–HCl and 1 mg/ml BSA, (pH 7.4) using different substrates: 10 mM pyruvate+2 mM malate and 10 mM succinate+5 μM rotenone. The oxygen concentration in the air-saturated medium was considered to be 200 nmol O_2/ml at 95 kPa barometric pressure. The weight-specific oxygen consumption was calculated as the time derivative of the oxygen concentration (DATGRAPH Analysis software, OROBOROS®). The rate of state 3 respiration was determined following the addition of 5 mM ADP. State 4 respiration was measured after the addition of 1.8 mM atractyloside.

2.6. Preparation of muscle homogenates

Small pieces of frozen tissue were homogenized (1/30 weight per volume) in a solution containing 50 mM TRIS buffer (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, and 1 mM EDTA using a glass/glass homogenizer (Kontes Glass Co. Vineland, NJ, 2 ml, 0.025 mm clearance) as described previously [38].

2.7. Enzyme determinations

Enzymatic measurements were performed in muscle homogenates. Enzyme activities were assayed at 30 °C spectrophotometrically using a DU 640 photometer (Beckman Instruments, Palo Alto, USA). Assays were run in duplicates with two different quantities of sample. Enzyme activities were referenced to noncollagen protein (NCP) or to the activity of mitochondrial marker enzyme citrate synthase (CS) as measured in the same sample.

The activity of rotenone-sensitive NADH:CoQ_1 oxidoreductase (complex I) was measured in 100 mM phosphate buffer (pH 7.4) containing 1 mM KCN, 60 μM CoQ_1 and 100 μM NADH at 340 nm according to Estornell et al. [39].

NADH:cytochrome-*c*-oxidoreductase (complex I+III), succinate:cytochrome-*c*-oxidoreductase (complex II+III), and ubiquinone:cytochrome-*c*-oxidoreductase (complex III) were measured following the reduction of cytochrome *c* at 550 nm using an ε of 19.1 mM^{-1} cm^{-1}. Activity of complex I+III was determined as the antimycin A- and rotenone-sensitive fraction of total NADH-cytochrome *c* oxidoreductase slightly modified according the method described by Hatefi and Rieske [40] in the presence of antimycin A [41]. Complex II and complex II+III were measured as described by King [42]. Complex III was measured as described by Krähenbühl et al [43]. Activity of cytochrome-*c*-oxidase (COX) was estimated by oxidation of reduced beef heart cytochrome *c* as described by Wharton and Tzagoloff [44]. The use of diluted homogenates allows linear enzyme rates to be obtained. CS was determined as described by Shepert and Garland [45].

2.8. Protein determination

NCP was determined by the BCA assay [46] after digestion of the homogenate with sodium hydroxide (50 mM) and pelleting the insoluble collagen protein by centrifugation (12,000×*g* for 10 min) as described previously [38]. BSA was used as a standard.

2.9. Histochemistry

Cryostat sections of muscle biopsies were carried out on 10-μm slices. Sequential demonstration of COX and SDH activities was performed with an adapted protocol described in Ref. [47]. COX activity was demonstrated by using a medium containing 4 mM 3,3-diaminobenzidine tetrahydrochloride and 100 μM cytochrome *c*, 10 U/l catalase in 50 mM phosphate buffer, pH 7.4 at 37 °C for 60 min. After rinsing in 50 mM phosphate buffer, SDH activity was stained by using 1.5 mM nitroblue tetrazolium, 0.2 mM phenazine methosulfate and 1 mM sodium azide in 50 mM phosphate buffer, pH 7.4 at 37 °C for 40 min. All blue fibers were counted as COX-negative fibers. Other stainings were done using standard methods [48].

2.10. Statistics

The mean values and standard deviations are calculated from the number (*n*) of subjects. Significance was tested

with unpaired Students' *t*-test. Correlation for all data including controls were tested with Pearson's test using SigmaStat-software. In addition, the correlation was calculated for the patients' data only.

3. Results

3.1. Molecular defects of mtDNA

Molecular genetic investigations revealed in 16 patients single deletions with a length of 2.5–9 kb. The heteroplasmy varied between 31% and 94%. In five patients, multiple deletions were detected with a heteroplasmy between 26% and 63%. Three of these patients had an autosomal inheritance. No mutations were detectable in the AdN-translocator gene I of these patients.

To evaluate the biochemical consequences of differences in the affected genes, three patients with large differences in the size of deletion but with similar levels of heteroplasmy were selected for exact determination of the localization of the deletion. In Patient 1, with the largest deletion of 9 kb, all genes between cytochrome *b* and ATPase 8 were deleted (Table 1). In patient 3, with the smallest deletion, only ND5 and ND4L as well as tRNALeu(CUN), tRNASer(AGY) and tRNAHis were affected. Patient 2 had the common deletion. Biochemical consequences of these different deletions are presented below. Table 1 contains the structure of deleted genes of four additional patients. Two of them had the common and two had smaller deletions. In all investigated patients, the three tRNA for histidine, serine, and leucine were missing.

Table 1
Localization and heteroplasmy of deletions in skeletal muscle biopsies of three index patients and four further CPEO patients

Patient	1	2	3	4	5	6	7
Heteroplasmy [%]	69	66	62	39	61	58	62
Size of deletion [kb]	9	4.9*	2.6	4.9*	4.9*	3.5	4.0
Gene							
Cyt.b							
$tRNA_E$							
ND6							
ND5							
$tRNA_L$							
$tRNA_S$							
$tRNA_H$							
ND4							
ND4L							
tRNAR							
ND3							
$tRNA_G$							
COXIII							
ATPase6							
ATPase8							
$tRNA_K$							
COX II							

(*) common deletion.

3.2. Changes at the level of respiratory chain enzymes

As shown in Table 2, there was a significant increase in CS in the biopsies of patients with both single and multiple deletions from 63±18 U/g NCP (control) to 115±74 U/g NCP (single) and 81±19 U/g NCP (multiple). Fig. 1 shows that the specific CS activities of all patients positively correlated with heteroplasmy of deletions. This correlation was even significant for the data of the patients only (without controls). However, there are patients with high levels of heteroplasmy and normal CS activity. This large variation of CS activity (range 52–352 U CS/g NCP) is a clear indication of the necessity to scale enzymatic and functional data by activity of CS as marker for the content of mitochondria (normalization). As shown in Fig. 1B, the deletion size did not correlate with CS activity.

Normalized activities of respiratory chain enzymes for patients with multiple and single deletion and the controls are collected in Table 2. Normalized COX activity was significantly reduced (−44%) in both groups of patients. In Fig. 2A, normalized COX activities were plotted versus heteroplasmy. In both groups of patients, the normalized COX activity significantly decreased with increasing heteroplasmy. In Fig. 2B, the CS-referenced COX activity was plotted versus the size of deletions. As in the case of CS activity, there was obviously no correlation between these parameters, indicating that deletions, even with large differences in the size of deletion, have similar effects on the COX activity.

Normalized activity of complex III (Table 2) was also significantly diminished in patients with single (−41%) and multiple (−29%) deletions. Complex III activity correlated with the heteroplasmy of single and multiple deletions. Similar results were found for complex I+III (single deletions −67%; multiple deletions −33%), and for complex I (single deletions −31%; multiple deletions −27%). Only the changes in patients with single deletions were significant. The correlation with heteroplasmy was significant for complex I+III but not for complex I.

Succinate dehydrogenase is a nuclear coded mitochondrial protein and should not be influenced by deletions of mtDNA. However, the normalized activity of succinate dehydrogenase was decreased too (−27% single deletions; −19% multiple deletions) even with a significant correlation to heteroplasmy.

Furthermore, activity of complex II+III was decreased (−46% single deletions and −36% multiple deletion), significantly correlating with heteroplasmy.

3.3. Detection of functional impairment of muscle mitochondria

Multiple substrate inhibitor titration and high resolution respirometry was applied to characterize the mitochondrial function in skinned fibers of muscle biopsies. Three typical oxygraphic traces (respirograms) are shown in Fig. 3. In the presence of 10 mM pyruvate, 2 mM malate, and 5 mM ADP,

Table 2
Biochemical, genetic and morphological data as detected in muscle biopsies of patients with single and multiple deletions of mtDNA

	Single	Patient 1	Patient 2	Patient 3	Multiple	Single and multiple	Controls	*r*	Slope
CS [U/g NCP]	115±74↑ (*n*=15)	55	131⇑	109⇑	83±19↑ (*n*=5)	109±67↑ (*n*=20)	63±18	−0.58	1.61
COX/CS [%]	58±24↓ (*n*=16)	51⇓	48⇓	54⇓	58±21↓* (*n*=4)	56±20↓ (*n*=20)	104±25	−0.75	−0.93
III/CS [%]	92±39↓ (*n*=16)	89⇓	53⇓	82⇓	110±33↓* (*n*=5)	94±37↓ (*n*=21)	156±48	−0.69	−0.81
II+III/CS [%]	14±9↓ (*n*=16)	12⇓	17.9⇓	24	18±5 (*n*=5)	15±8↓ (*n*=21)	28±8	−0.67	−0.80
SDH/CS [%]	36±12↓ (*n*=16)	32⇓	32.9⇓	43⇓	40±3 (*n*=5)	36±11↓ (*n*=21)	48±13	−0.45	−0.42
I+III/CS [%]	4±2↓ (*n*=16)	2⇓	3⇓	6⇓	8±3 (*n*=5)	5±3↓ (*n*=21)	12±6	−0.59	−1.07
I/CS [%]	12±6↓* (*n*=11)	n.d.	6.7⇓	n.d.	12±7 (*n*=5)	12±6↓* (*n*=16)	17±7	n.s.	n.s.
Pyruvate#	0.75±0.3 (*n*=10)	0.35⇓	0.93	0.75⇓	0.79±0.10 (*n*=3)	0.76±0.27↓ (*n*=12)	1.01±0.23	0.42	−0.47
Pyruvate/CS§	65±27↓ (*n*=10)	44⇓	56⇓	48⇓	95±42 (*n*=3)	76±23↓ (*n*=13)	125±29	−0.70	−1.15
Succinate/CS§	67±17 (*n*=10)	38⇓	38⇓	77⇓	96±46 (*n*=3)	74±28↓ (*n*=13)	112±29	−0.50	−0.74
SRPR [%]	79±21↓*(*n*=10)	n.d.	95	46⇓	81±21 (*n*=3)	85±29↓ (*n*=13)	119±36	0.43	−0.57
SRNO [%]	28±14↓ (*n*=16)	21⇓	15⇓	23⇓	43±9 (*n*=5)	32±14 (*n*=21)	43±19	0.30*	1.03
COX-negative fibers [%]	17±11↑ (*n*=14)	27⇑	48⇑	22⇑	16±7↑ (*n*=4)	16±10↑ (*n*=18)	0.18±0.17	−0.69	0.42
Type I fibers [%]	46±7 (*n*=7)	n.d.	43	53	47±4 (*n*=4)	46±6 (*n*=11)	43±6	n.s.	n.s.
Heteroplasmy [%]	50±17 (*n*=16)	69	66	62	46±18 (*n*=5)	49±17 (*n*=21)	0.0	n.def.	n.def.

Enzyme activities were detected as described in Materials and methods and given as means±S.D. of specific (U/g noncollagen protein) activities or scaled by CS activity. Rates of respiration were measured under state 3 conditions with 10 mM pyruvate/2 mM malate or 10 mM succinate/20 μM rotenone as substrates. Data as nmol O_2/min/mg permeabilized fiber (#) or as pmol O_2/min/U CS (§). SRPR, succinate-referenced pyruvate respiration; SRNO, succinate-referenced NADH oxidation. COX-negative fibers were detected by COX/SDH-double staining as described in Materials and methods. Percentage of type I fibers was determined in biopsies of m. biceps brachii by counting the dark fibers of SDH staining.

Data as means of *n* patients±S.D. Number of controls was 46 except for type I fibers and COX-negative fibers where *n* was 15. Significant changes: ↑ ,↓ $P<0.01$; ↑ *,↓* $P<0.05$. Data of three index patients 1, 2 and 3 (see Table 1) with single deletions are presented. ⇓ ⇑, outside the control range.

The last columns contain statistical results. Data of all patients with multiple plus single deletions were plotted versus heteroplasmy. *v*—correlation coefficient with $P<0.01$; or *v**—$P<0.05$ (Pearson-test) for data. Slope, slope of the regression line of normalized plots. n.s., not significant. n.def., not defined.

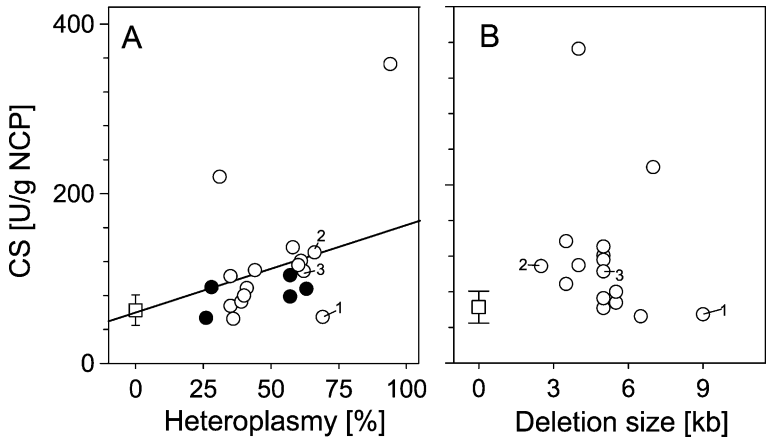

Fig. 1. Specific CS activity versus heteroplasmy and deletion size in skeletal muscle of CPEO patients. Citrate synthase activity measured in muscle biopsies of patients with single (○) and multiple (●) deletions was plotted versus the degree of heteroplasmy (A) and the deletion size (B). The control range was calculated from 46 patients without muscle diseases as means±S.D. The results of Pearson's test were (A) r=0.58, P<0.01 (n=67, with controls); r=0.49, P<0.05 (n=22, without controls). (B) n.s. (n=16, without controls).

the maximum rate of respiration (state 3) was observed. After inhibition of complex I with rotenone, the rate of respiration decreased. Since succinate metabolism does not involve complex I but complex II, it was possible to record the succinate-dependent respiration. In the control muscle (Fig. 3A), pyruvate-dependent respiration was higher (138%) than succinate-dependent respiration (100%). For all 47 controls, this succinate-referenced pyruvate respiration (SRPR) was 118±31% (Table 2). Similar results were obtained if the state 3 respiration was measured in separate incubations with single substrates. Under these conditions, pyruvate respiration (1.01±0.23 nmol O_2/min/mg s.w.; n=47) was 112% of succinate respiration (0.90±0.28 nmol O_2/min/mg s.w.; n=47). The calculated SRPR (115±32; n=47) for measurements in individual fibers was very similar to that obtained for the sequential measurement in one fiber. State 4 respiration was measured after inhibition of the AdN-translocator by 1.8 mM actractyloside.

Fig. 3B shows the respirogram of a patient with a single deletion at a heteroplasmy of 35% and a deletion size of 5 kb. Both pyruvate- and succinate-dependent state 3 respiration rates were reduced in comparison to the controls. Moreover, in this patient, the SRPR was also remarkably reduced (58%), which is in line with the strongly reduced activity of complex I+III (−80%) in this patient. Atractylate-insensitive respiration was slightly increased in comparison to the controls. The respirogram (Fig. 3C) of a second patient with 4 kb deletion (44% heteroplasmy) shows nearly normal rates of state 3 respiration. However, the CS-referenced state 3 respiration (83 pmol O_2/min/U

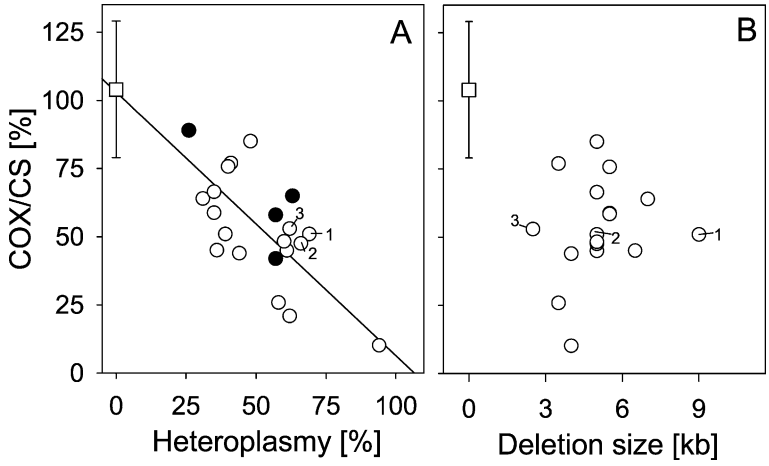

Fig. 2. Activity of COX versus heteroplasmy of deletions and the deletion size. (A) COX activity was normalized by scaling of CS activity and plotted versus heteroplasmy of mitochondrial defect in CPEO patients with single (○) and multiple (●) deletions. The correlation coefficients were r=−0.75, P<0.01 (n=67, with controls) and r=−0.58, P<0.01 (n=22, without controls). (B) Normalized COX activity versus the size of deletion. There was no significant correlation. Numbers indicate the data points of patients 1, 2 and 3.

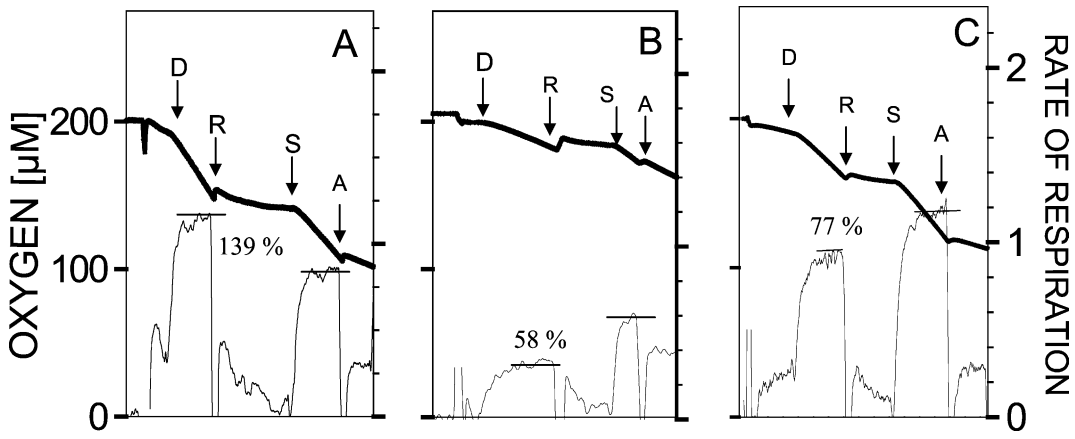

Fig. 3. Typical oxygraphic traces of mitochondrial respiration in saponin-skinned human muscle fibers of two CPEO patients with single deletions and of a control patient. Investigation of mitochondrial function by a sequential multiple substrate-inhibitor titrations. Incubation of skinned fibers as described in Materials and methods. The thick lines indicate the oxygen concentration in the oxygraph (left *Y*-axis). The thin lines represent the first derivative of these signals directly indicating the respiratory rate (right *Y*-axis). Rates of respiration were measured in the presence of 10 mM pyruvate plus 2 mM malate. Additions: D, 5 mM ADP; R, 20 µM rotenone (for inhibition of complex I); S, 10 mM succinate; A, 1.8 mM atractylate (for inhibition of the AdN-translocator). (A) Normal respiratory pattern of a control patient without muscle disease. The pyruvate-dependent respiration is higher than the succinate-dependent respiration (CS=63.0 U/g NCP, I+III/CS=12.2%). (B) Reduced rates of pyruvate and succinate respiration and of SRPR in a patient with single deletions. Deletion size=5.5 kb; 34% heteroplasmy; I+III/CS=2.4%. (C) Reduced SRPR but normal rates of pyruvate and succinate respiration in a patient with single deletions. Deletion size=4 kb, 44% heteroplasmy, CS 110 U/g NCP; I+III/CS=6.3%.

CS) was clearly reduced in this patient due to increased CS activity (110 U/g NCP). Also, the SRPR (77%) was typically but modestly reduced compared to the controls, which was accompanied by slightly reduced activity of complex I+III, too. Similar respirograms were obtained from patients with multiple deletions, too (not shown). The SRPR was also decreased in these patients (Table 2).

In Fig. 4, state 3 respiratory rates of all investigated patients for the substrate pyruvate/malate were plotted versus heteroplasmy. The specific respiratory rates (scaled by the fiber weight) are shown on the right. Obviously, there was no correlation between respiration and the heteroplasmy. A complete change occurred after normalization of respiratory rates on CS, whereby a clear and significant linear correlation with heteroplasmy was detectable.

3.4. Changes on the morphological level

If COX activity inversely correlates with heteroplasmy of deletions, then it should be expected that the number of COX-negative fibers increases with the heteroplasmy of deletions too. As shown in Fig. 5, there is a clear correlation of COX-negative fibers with heteroplasmy ($r=0.69$, $P=0.0015$). The proportion of COX-negative fibers was 16.6% (single) and 16.3% (multiple) in patients with deletions (Table 2). We also detected a significant correlation

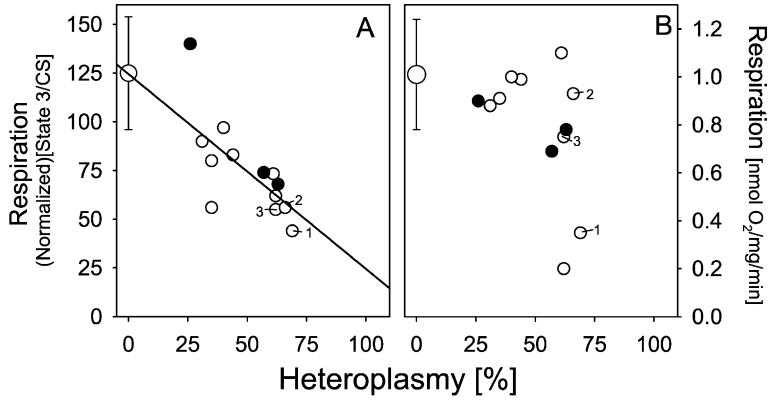

Fig. 4. Influence of different kinds of references to relation between maximum rate of pyruvate-dependent respiration (state 3) and heteroplasmy. Pyruvate-dependent rates of respiration were measured under state 3 conditions in muscle biopsies of patients with single (○) and multiple (●) deletions in comparison to controls as described in Fig. 3. Means of at least three different incubations scaled by CS (A) or by sample weight (B) were plotted versus the level of heteroplasmy. Correlation coefficients: (A) $r=-0.62$, $P<0.01$ ($n=60$, with control) and $r=-0.73$, $P<0.01$ ($n=13$, without controls). (B) Not significant. Numbers indicate the data points of patients 1, 2 and 3.

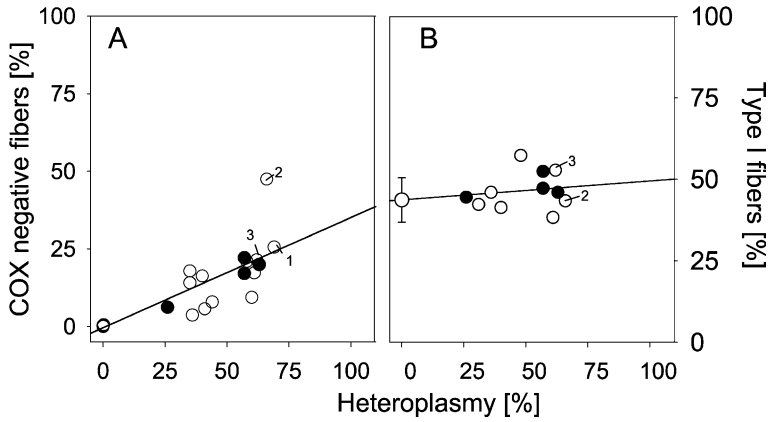

Fig. 5. Correlation of COX-negative fibers and Type I fibers with heteroplasmy in CPEO patients. Percentage of COX-negative fibers was measured by sequential staining of COX and SDH in skeletal muscle of patients with single (○) and multiple (●) deletions. Percentage of type I fibers was determined only in m. biceps brachii by succinate dehydrogenase staining. Correlation coefficients: (A) $r=0.85$, $P<0.01$ ($n=30$, with controls), $r=-0.69$, $P<0.01$ ($n=17$, without controls). (B) n.s. ($n=24$, without controls). Numbers indicate the data points of patients 1, 2 and 3.

between the number of ragged red fibers and heteroplasmy (data not shown). The percentage of type I fibers was detected by succinate dehydrogenase staining. Data obtained from m. biceps brachii in patients (46±6%, $n=11$) and controls (43±6%, $n=12$) are shown in Fig. 5B and Table 2. There was no detectable correlation between type I fibers and heteroplasmy and no change in the fiber pattern was detectable in the patients in comparison to the controls.

3.5. Comparison of changes of enzymatic and functional level

To compare the extent of deletion-dependent changes on enzymatic and functional level, we scaled both kinds of data by the respective mean values of controls and plotted them versus the level of heteroplasmy (Fig. 6). Due to this scaling, the slopes of regression line indicating the extent of heteroplasmy-dependent change are directly comparable. These slopes and the correlation coefficients obtained by the Pearson test are presented in Table 2. Slopes of regression lines are similar for the complexes of the respiratory chain (COX, III, II+III, and I+III) except for SDH. The change in SDH activity was only half of the others. Interestingly, the slopes of scaled rates of pyruvate and succinate respiration were practically the same as the slopes of the respiratory chain complexes.

3.6. Influence of the kind of deleted genes on biochemical changes

Three patients were selected with different defined deletions and nearly the same levels of heteroplasmy (Table 1). As mentioned above, these deletions included the common deletion (4.9 kb), the smallest (2.5 kb), and the largest (9 kb) deletion found in our patients. Biochemical data of these patients are shown in Table 2. In addition, in Figs. 1,2,4 and 5, the data points of these patients are marked by numbers.

As shown in Fig. 1 and Table 2 biopsies of the three patients strongly vary in specific CS activity. Whereas CS activity was normal in patient 1 (55 U/g NCP), elevated activities were detected in patients 2 (109 U/g NCP) and 3 (131 U/g NCP), indicating large differences in mitochondrial content. The specific rates of respiration are different (Table 2 and Fig. 4B) increasing with the numbers of

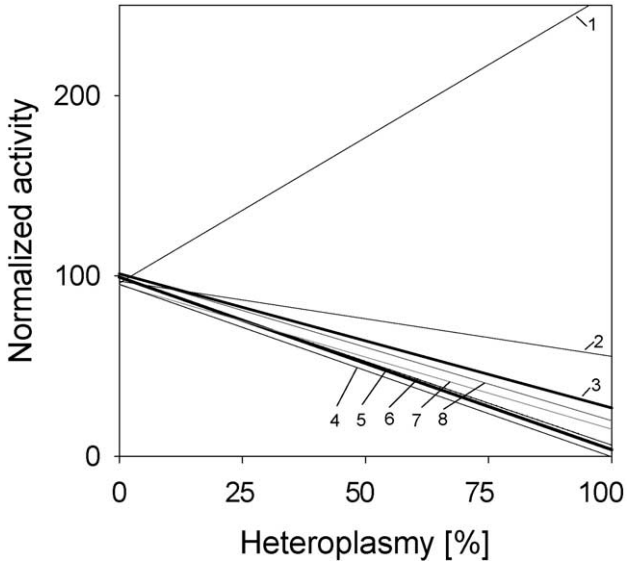

Fig. 6. Comparison of changes on enzymatic and functional level in muscle mitochondria of CPEO patients. Enzyme activities and respiratory rates were scaled by the control values (100%) and plotted versus heteroplasmy. First-order regression lines are shown. Further statistical data are given in Table 2. (1) Citrate synthase (U/g NCP); (2) SDH/CS; (3) succinate respiration (state 3/CS); (4) I+III/CS; (5) pyruvate respiration (state 3/CS); (6) COX/CS; (7) II+III/CS; (8) III/CS.

patients. After normalization, rates of respiration were essentially the same (Fig. 4A). As shown in Fig. 2B, the normalized COX-activities were also equal in the three patients, despite the large differences in the affected genes. In patient 2, besides the $tRNA_L$, $tRNA_S$, and $tRNA_H$ genes, only the genes for ND5 and ND4 were deleted. The COX activity, however, was decreased as in the two other patients where COX subunits were also affected. A similar situation was found for complex III (Table 2). The loss of COX activity in patient 2 was also accompanied by the presence of 22% COX-negative fibers.

4. Discussion

4.1. Mitochondrial function measured in skinned muscle fibers

For investigation of mitochondrial function in human muscle biopsies, we used the skinned fiber technique [27–29,31] and high-resolution respirometry [30,31]. One limiting disadvantage of the skinned fiber technique is the uncertainty of the exact amount of mitochondria present within the individual fibers under investigation, which may be varied by, e.g. pathological proliferation [49] and physical training [50]. CS activity is commonly used for quantifying the content of mitochondria due to its tight correlation with morphometric data [51] and due to its resistance to pathological changes.

In this paper, we demonstrated that in patients with single deletions at a heteroplasmy of 50±17%, the amount of mitochondria was nearly doubled. Therefore, even remarkably decreased enzyme activities can be compensated by increased amounts of mitochondria. The diagnostic relevance of this effect is that non-normalized functional and enzymatic data obtained from muscle fibers and from muscle homogenates become inconspicuous. The decreased specific functional and enzymatic data are clearly detectable only in some patients with low amounts of mitochondria. As shown in Fig. 4, the diagnostic sensitivity is increased by CS normalization. Our data therefore confirm the necessity of normalization of functional data [21,22].

It may be speculated that increasing the amount of mitochondria in diseased skeletal muscle could be a strategy to minimize functional consequences of deletions. The large variations of specific CS activity by the factor of 7, as detected in our patients, shows the possibility that the normal density of mitochondria (3–6% of cell volume) can be doubled without problems for cell functioning. However, in tissues with mitochondrial densities between 20% and 30% (e.g. heart, brain), this may be not possible without further disadvantages for the tissues. In this respect, the skinned fibers with both kinds of references are a model for understanding the consequences of mitochondrial density on genotype phenotype relations in deleted mtDNA.

A second strategy for consideration of different mitochondrial density is the sequential measurement of pyruvate- and succinate-dependent state 3 respiration in the same fiber. A decreased pyruvate respiration in one individual fiber could be the result of decreased mitochondrial content or the result of an impairment. The scaling of pyruvate respiration by the succinate respiration can easily distinguish between these possibilities since the calculated ratio of SRPR is independent of the content of mitochondria. In normal muscle, the pyruvate respiration is, in our hands, higher than the succinate respiration (112–118%). This was found in normal incubations measured with single substrates as in sequential incubations as well. Both kinds of measurements were done in the additional presence of rotenone, which is necessary to avoid inhibition of succinate dehydrogenase by oxaloactetate [52].

In patients with single deletions, SRPR was significantly decreased by 34% (Table 2) and correlated with heteroplasmy of deletions. Therefore, it seems that CPEO is associated with decreased SRPR in skeletal muscle. The reason for decreased SRPR is most probably the pronounced decrease in complex I activity in comparison to the lesser decrease in the activity of complex II. This conclusion is supported by our recent findings that diminished rates of SRPR were also detectable in the heart and skeletal muscle mitochondria of aged Fisher rats, which clearly correlated with decreased activities of complex I and I+III and the more or less unchanged activity of complex II [53].

A further approach to detecting pathological changes with functional measurements is the estimation of flux control coefficients by means of inhibitor titrations [54–56]. This technique was successfully used in human [57] and animals samples [34], but the method needs relatively large amounts of material to allow sufficient replicates.

4.2. Functional investigation of AdN-translocator

It was proposed that inactive AdN-translocator due to missense point mutations could be the reason for induced multiple deletions of CPEO patients [10]. This mutation caused a lethal loss of mitochondrial function in yeast cells [10]. However, no mutations were found by sequencing the whole coding region of AdN-translocator gene 1 in the three patients with autosomal inherited multiple deletions. This result is in line with the detection of functionally sufficient AdN-translocation in mitochondria of patients with multiple deletions (Table 2). Firstly, there was a clear stimulation of respiration by ADP-addition from state 2 to state 3, indicating that ADP can be transported by AdN-translocator into the matrix space. Secondly, there was an inhibition of state 3 respiration by actractyloside in the same manner in both groups of patients. Moreover, we found that changes of enzyme activities and of mitochondrial function in patients with single and multiple deletions were quite similar (Table 2; (Figs. 1, 2 and 4, 5)). Therefore, it is concluded that at least in our patients with multiple deletions, the AdN-

translocator as well as other nuclear coded OXPHOS enzymes were not affected and probably not the cause of the autosomal inheritance of the disease.

4.3. Consequences of deletions on mitochondrial enzymes

Normalized activities of COX, complexes I, I+III, II+III and III linearly decreased with increasing heteroplasmy in biopsies of CPEO patients with both single and multiple deletions. The best fit was observed for normalized COX, which linearly decreased with heteroplasmy. These results confirm previously reported data [13] but are contradictory to others [63]. The slopes of the regression lines for all measured complexes of the respiratory chain were close together (between 1.07 and 0.83), indicating that all changes follow the same mechanism. We detected also decreased activities of complex II+III and SDH in our CPEO patients. We did not expect these changes since SDH is a nuclear coded mitochondrial enzyme. Interestingly, the decrease in SDH activity was only half of that for the other complexes of the respiratory chain. This clearly points at another mechanism of impairment. It was recently hypothesized that the Fe–S cluster-containing components of the SDH are a privileged target for superoxides [58], which might be increasingly formed at impaired respiratory chain similar to that shown in Friedreich's ataxia where frataxin is missing [59].

Interestingly, the decrease in complex II+III is more pronounced than the decrease in SDH. Activity of complex II+III mainly reflects activity of complex II [60,61], but (in a lesser extent) also the activity of complex III [60,61], which is affected like the other complexes. Therefore, the decrease in activity of complex II+III could be higher than for complex II alone.

4.4. Similar consequences of deletion caused impairment on enzymatic and functional level

Interestingly, the size of deletion had obviously no influence on the CS activity (Fig. 1B), on the normalized COX activity (Fig. 2B), and the rates of normalized respiration (not shown). Therefore, it seems that very different deletions produce the same phenotype on the level of mitochondrial function where the impairment depends only on the heteroplasmy. These results confirm reports where variation of deletion size between 1.8 to 8.8 kb did not correlate with respiratory chain enzymes [62]. Stronger support for this point of view came from the comparison of the selected patients 1, 2, and 3. In these patients, deletions differed in size and localization. Only subunits of complex I were affected at the genome level in patient 2, but the activities of complex III and COX were similarly affected as in patients 1 and 3. Obviously, the loss of the three tRNA for leucine, serine, and histidine caused the comparable disturbances in mitochondrially coded subunits of respiratory chain including those of COX and complex III.

Deletions of mtDNA have at least two different consequences at different levels of mitochondrial protein synthesis. Missing structure genes cause dysfunction at the level of transcription. The resulting impairment at the level of mitochondrial function depends on the importance of the deleted protein for the total function. Missing tRNAs, however, cause dysfunction at the level of translation. This translation defect necessarily affects *all* mitochondrial coded structure proteins and should have a higher impact on the impairment of mitochondria. Data show that, at least in our patients, mitochondrial dysfunction and COX activity linearly correlate with heteroplasmy despite differences in the pattern of affected structure genes. This very clearly points at greater importance of defects at the level of translation over those at the level of transcription. As shown earlier [63], mitochondrial proliferation was able to normalize the level of mRNA of deleted genes, but this would also further increase transcripts of nondeleted genes [63]. This might be comparable to the increased activity of CS found in affected muscles of our and other studies [13,62].

As shown in Figs. 2 and 4, the changes of COX activity and of the state 3 respiration were found to be dependent to the same extent on the level of heteroplasmy. Moreover, the slopes of the normalized activity versus heteroplasmy plots (Table 2) were nearly the same for the respiratory rates (e.g. −1.15 for pyruvate) as for the complexes of the respiratory chain (e.g. −0.93 for COX). This is only apparently not in agreement with the low flux control coefficients of COX and other single complexes of respiratory chain in human skeletal muscle. Mazat et al. [14] showed that the single COX activity is more sensitive than the rate of respiration to the inhibitory action of KCN. In those experiments, COX of normal mitochondria was specifically inhibited by KCN, and the other complexes of the respiratory chain remained unaffected. In our present measurements, however, COX is not separately decreased but is additionally accompanied by parallel declines in other respiratory chain complexes. Therefore, the changes in COX activity are an indicator of simultaneous changes in probably *all* enzymes with mtDNA coded proteins, and it is not permissible to draw conclusions on the flux control of a single affected complex. Together, these enzymes have practically entire control of oxidative phosphorylation, which explains the parallel decrease in functional and enzymatic data and the absence of thresholds in this kind of disease.

In conclusion, we have shown that in skeletal muscle of CPEO patients with deleted mtDNA, normalized mitochondrial function *and* normalized activity of respiratory chain complexes linearly correlate with heteroplasmy. Mitochondrial impairment did not correlate with the size of deletion in mtDNA, which points at a large pathogenicity of the deleted tRNAs. The missing threshold at the level of mitochondrial function due to large-scale deletions seems to be a characteristic of CPEO as a special form of mitochondrial translation disease.

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