

# Multiple deficiencies of mitochondrial DNA- and nuclear-encoded subunits of respiratory NADH dehydrogenase detected with peptide- and subunit-specific antibodies in mitochondrial myopathies

Herman A.C.M. Bentlage <sup>a,\*</sup>, Antoon J.M. Janssen <sup>a</sup>, Anne Chomyn <sup>b</sup>, Giuseppe Attardi <sup>b</sup>, John E. Walker <sup>c</sup>, Hermann Schagger <sup>d</sup>, Rob C.A. Sengers <sup>a</sup>, Frans J.M. Trijbels <sup>a</sup>

<sup>a</sup> Department of Pediatrics, Academic Hospital Nijmegen St. Radboud, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

<sup>b</sup> Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125, USA

<sup>c</sup> Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, UK

<sup>d</sup> Department of Biological Chemistry, University of Frankfurt, Theodor-Stern-Kai 7, Haus 25B, 60590 Frankfurt am Main, Germany

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## Abstract

Antibodies have been raised against synthetic peptides corresponding to several computer-predicted epitopes of three mtDNA-encoded subunits, ND4, ND5 and ND6, of the human respiratory chain NADH dehydrogenase (Complex I). Antibodies were characterized by a sensitive immunoblotting assay using proteins from human skeletal muscle mitochondria and by immunoprecipitation of radio-labeled HeLa cell mitochondrial translation products. Only antibodies against two of six selected peptides of the ND4 subunit, i.e., the C-terminal peptide and an internal peptide close to the C-terminus, reacted in both assays with the subunit. Antibodies raised against an internal peptide close to the N-terminus of the ND5 subunit and antibodies raised against an internal epitope of the ND6 subunit also reacted in both the immunoblotting and immunoprecipitation assays. The antibodies described above and other Complex I subunit- or holoenzyme-specific antibodies were used to investigate the subunit deficiencies of the respiratory NADH dehydrogenase in the skeletal muscle of patients affected by mitochondrial myopathies associated with Complex I defects. The reduction in enzyme activity correlated in an immunoblot assay with a decrease of four mtDNA-encoded subunits of the enzyme, as well as with a decrease of other subunits of Complex I encoded in the nDNA. The present work provides the first evidence of a decrease in NADH dehydrogenase subunits encoded in the mitochondrial genome in myopathy patients.

**Keywords:** NADH dehydrogenase; Complex I deficiency; ND subunit; Peptide-specific antibody; Mitochondrial myopathy

## 1. Introduction

Mitochondrial (encephalo)myopathies are a heterogeneous group of disorders affecting predominantly skeletal muscle and the central nervous system. A defect in the mitochondrial energy metabolism is thought to be the primary cause of disease. One of the respiratory chain enzymes that is frequently found deficient in various tissues of patients is NADH-ubiquinone oxidoreductase (NADH dehydrogenase or Complex I, EC 1.6.99.3) [1,2].

It spans the mitochondrial inner membrane and oxidizes NADH generated by substrate oxidations in the matrix. This large multimeric protein consists of at least 41 subunits (review [3]), including seven (called ND) subunits that are encoded in the mitochondrial genome [4,5].

Antibodies against Complex I subunits are very useful for studying the molecular basis of mitochondrial encephalomyopathies. Polyclonal antibodies against purified beef heart Complex I have been previously shown to react with only approximately 15 subunits of the enzyme in human mitochondria. The use of these antibodies with skeletal mitochondrial proteins from myopathy patients showed a generalized reduction of all cross-reacting polypeptides, in some cases with a disproportionate and severe deficiency of a few subunits [1,6]. The ND subunits were not detected by these antibodies.

Abbreviations: ECL, enhanced chemiluminescence; FAB, fast atom bombardment; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes.

\* Corresponding author. Fax: +31 80 616428.

Table 1  
Amino acid sequences of selected peptides, as shown in Fig. 1

Peptide	(Number)	Amino acid sequence
ND4-I <sub>1</sub>	( 81– 94)	SATA-Nle-Gln-Arg-His-Leu-Ser-Ser-Glu-Pro-Leu-Ser-Arg-Lys-Lys-Leu
ND4-I <sub>2</sub>	(135–145)	SATA-Nle-Arg-Trp-Gly-Asn-Gln-Pro-Glu-Arg-Leu-Asn-Ala
ND4-I <sub>3</sub>	(277–285)	SATA-Nle-Leu-Arg-Gln-Thr-Asp-Leu-Lys-Ser-Leu
ND4-I <sub>4</sub>	(329–339)	SATA-Nle-Leu-Ala-Asn-Ser-Asn-Tyr-Glu-Arg-Thr-His-Ser
ND4-I <sub>5</sub>	(420–435)	SATA-Nle-Thr-His-His-Ile-Asn-Asn-Met-Lys-Pro-Ser-Phe-Thr-Arg-Glu-Asn-Thr
ND4-C	(451–459)	SATA-Nle-Pro-Asp-Ile-Ile-Thr-Gly-Phe-Ser-Ser
ND5-I	( 21– 35)	SATA-Nle-Thr-Thr-Leu-Val-Asn-Pro-Asn-Lys-Lys-Asn-Ser-Tyr-Pro-His-Tyr
ND6-I <sub>3</sub>	(128–144)	SATA-Nle-Glu-Gly-Glu-Gly-Ser-Gly-Phe-Ile-Arg-Glu-Asp-Pro-Ile-Gly-Ala-Gly-Ala

In brackets are given the amino acid numbers corresponding to Anderson et al. [12]. The peptides were extended with L-norleucine (Nle) and S-acetylthioacetyl (SATA) at the amino-terminus for determination of the peptide-carrier ratio and coupling-procedures, respectively [14].

Antibodies against synthetic peptides corresponding to the C- or N-terminal regions of the human mtDNA-encoded subunits have been previously used to identify the corresponding polypeptides by immunoprecipitation assays [4,5,7,8] and, occasionally, by immunoblotting [9]. Antibodies against peptides of predicted antigenic sequences can recognize epitopes on the whole protein [10,11]. Since the hydrophobic ND subunits are very difficult to purify

and have a low immunogenicity, we have raised antibodies against a few chemically synthesized peptides corresponding to predicted epitopes. In this paper, we describe the preparation and characterization of antibodies against several peptides of human mitochondrial NADH dehydrogenase subunits ND4, ND5 and ND6, and the use of these antibodies, as well as of antibodies specific for other subunits of the enzyme, for the molecular analysis of

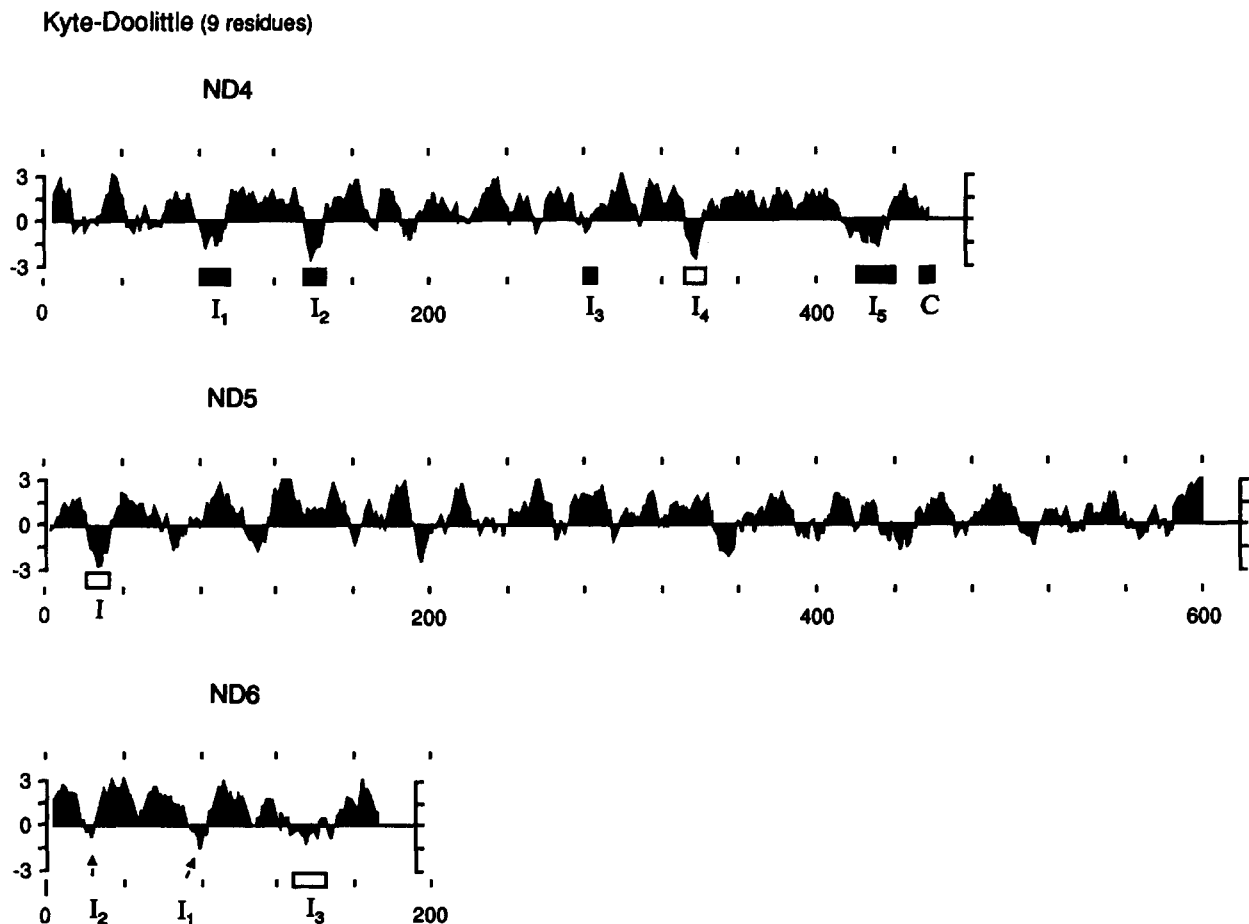


Fig. 1. The hydrophobicity profiles for the human ND4, ND5 and ND6 subunits. They are shown, as calculated with a sequence window of 9 residues. The selected peptides (Table 1) are marked by boxes below the amino acid axis. Open or closed boxes indicate coupling of the peptides to either BSA with MHS or OA with sulfo-MBS, respectively. I<sub>1</sub> and I<sub>2</sub> below ND6 indicate peptides (amino acid numbers 74–84 and 18–27, respectively) used by Chomyn et al. [5].

Complex I defects in human mitochondrial myopathies. Multiple deficiencies of mtDNA- and nuclear-encoded subunits of this enzyme complex have been detected in myopathy patients, and shown to correlate with the severity of the enzyme defect.

## 2. Materials and methods

### 2.1. Selection and synthesis of peptides

The reported sequence of the human mitochondrial genome [12] was computer analyzed using the program PEPPLOT [13]. Criteria for selection of linear epitopes were hydrophilicity, surface probability, flexibility, high  $\beta$ -turn and low  $\alpha$ -helix or  $\beta$ -sheet propensity [11]. The amino acid sequences of the selected peptides for ND4, ND5 and ND6 are shown in Table 1; their position in each subunit is shown in Fig. 1. Several candidate epitopes of ND4 were selected. The C-terminal peptide previously used by Mariottini et al. [8], even though it was not a predicted epitope by the criteria mentioned above, was synthesized for purpose of comparison. Only one internal epitope of ND5 and ND6 was chosen. For ND6, this was the only candidate epitope not used previously by Chomyn et al. [5]. The peptides were custom synthesized by Dr. G.I. Tesser (Laboratory of Organic Chemistry, University of Nijmegen, The Netherlands) by solid phase peptide chemistry. The peptides were extended with L-norleucine (Nle) and S-acetylthioacetyl (SATA) at the N-terminus for determination of the peptide-carrier ratio and coupling-procedures, respectively [14].

### 2.2. FPLC, FAB mass spectra and amino acid analysis

Peptides were analyzed by reversed-phase chromatography using a PepRPC HR 5/5 column (Pharmacia/LKB) and a continuous gradient of acetonitrile (0–40%, v/v) in 0.1% (v/v) trifluoroacetic acid created by an FPLC system (Pharmacia/LKB). FAB mass spectra of collected peak fractions were analyzed by Dr. G. van de Werken (National Institute of Health, Bilthoven, The Netherlands). The samples were hydrolyzed with 6 M HCl for 1 h at 150°C or 18 h at 120°C in vacuum hydrolysis tubes (Pierce). The amino acid content of the hydrolysates was determined with an Alpha-Plus amino acid analyzer (Pharmacia/LKB).

### 2.3. Coupling of peptides to carrier proteins

Peptides were coupled to the carrier proteins bovine serum albumin (BSA), myoglobin (MYO) or ovalbumin (OA, all from Sigma) with 6-maleimido-hexanoic acid *N*-hydroxysuccinimide ester (MHS, Pierce) according to Schielen et al. [14], except that 6-maleimidobenzoic acid *N*-hydroxysulfosuccinimide ester (Sulfo-MBS, Pierce) was used as a linker in some cases.

### 2.4. Immunization of rabbits

Two rabbits were injected with each antigen at 2–3 week intervals. Each rabbit received a peptide-carrier protein conjugate mixed in a 1:1 (v/v) ratio with the Freund's adjuvant system (complete, FCA or incomplete, FIA, Difco). Conjugates of ND4-I<sub>3</sub> and ND4-C were also tested with the Ribi adjuvant system (RAS, Sanbio). The administration of adjuvant preparations was as follows: FCA (1 mg conjugate, total volume 2 ml) intradermally at 20 sites on the back of the animal; FIA (0.5 mg conjugate, total volume 1 ml) intramuscularly and subcutaneously; RAS (first dose 1 mg conjugate, thereafter 0.5 mg, each total volume 1 ml) at multiple places according to suggestions of the manufacturer. Blood samples were taken before the first injection (preimmune serum) and either 2 weeks after or immediately before the booster injections. The sera were prepared and stored at –20°C. Before use, they were made free of antibodies against the carrier proteins by passing them twice over a column of carrier protein conjugated to CNBr-activated Sepharose (Pharmacia/LKB).  $\gamma$ -Globulins were isolated by fractionating the sera over a Protein A Sepharose column (Pharmacia/LKB).

The rabbit antibodies against the 24 kDa subunit of bovine Complex I were raised using the purified protein over-expressed in *Escherichia coli* (Wilks, P.E., Medd, S.M. and Walker, J.E., unpublished data). The adenine nucleotide translocase (ANT) was isolated from bovine heart mitochondria by subsequent chromatography [15] and electrophoresis [16]. The ATPase (Complex V) was purified from human heart mitochondria by blue native electrophoresis [17]. Antibodies were raised in rabbits by inoculation of the purified proteins into the popliteal lymph nodes according to Sigel et al. [18]. The antibodies against the ATPase react mainly with the  $\beta$ -subunit in an immunoblot assay (results not shown). The antibodies against the bovine Complex I holo-enzyme and the ND1 subunit of *Neurospora crassa* were kind gifts from Dr. U. Harnisch (Department of Biochemistry, University of Düsseldorf, Germany) and Dr. S. Werner (Department of Physiological Chemistry, University of Munich, Germany), respectively. The former showed a reaction with approximately 10 subunits of Complex I preparations from bovine and human heart tissue (results not shown). The latter were raised against a highly conserved peptide which has 12 of 14 amino acids identical with the homologous human sequence [19].

### 2.5. Enzyme-linked immunosorbent assay (ELISA)

Antibody titers were determined by ELISA assays essentially as described by Geerlings et al. [20], using the peptides coupled with a different linker to a different carrier protein as coating antigens, in order to exclude any cross reactions. The titer is defined as the reciprocal of the serum dilution giving an ELISA-signal above background

(no serum addition), equivalent to twice the sum of the mean background and its standard deviation. The antibodies raised against the carrier and the linking part were also assayed. The specificity of each reaction was investigated by testing the serum sample after a preincubation with the corresponding peptide.

### 2.6. Labeling and immunoprecipitation of mitochondrial translation products

HeLa cells were labeled for 2 h with [<sup>35</sup>S]methionine (20–50  $\mu$ Ci/ml; 1000–1500 Ci/mmol) in the presence of emetine, an inhibitor of cytoplasmic protein synthesis, at 100  $\mu$ g/ml, and at a cell concentration of  $2 \cdot 10^6$ /ml [21], whereafter the mitochondrial fraction was isolated [7]. Immunoprecipitation was carried out using a SDS lysate of this fraction as described previously [8], except that 80  $\mu$ g of mitochondrial suspension ( $15 \cdot 10^4$  cpm) was used in each experiment. For each reaction either 8.5  $\mu$ l of anti-serum or 85  $\mu$ g of the  $\gamma$ -globulin fraction was used. Inhibition by peptides was tested by adding to the serum 5  $\mu$ l peptide solution in dimethylsulfoxide (DMSO) at 1 mg/ml. Where stated, 5  $\mu$ l DMSO was used as a control. The final immunoprecipitates were run on a 15–20% (w/v) exponential gradient SDS polyacrylamide gel [22] and prepared for fluorography [23]. The previously described antibodies against ND4-C(451-459), ND5-N(1-7), ND5-C(598-603) and ND6-I<sub>1</sub>(74-84) were used for reference purposes [4,5].

### 2.7. Patients and controls

Specimens of the quadriceps muscle were removed by biopsy from patients suspected to have a defect in oxidative phosphorylation. From patients P1 and P6 also autopsy material removed within 1 h after death was studied.

Seven patients were selected for this study based upon extensive biochemical analysis which pointed towards a Complex I deficiency. A brief overview of some clinical features and laboratory data is presented in Table 2. Control specimens were considered those in which a mitochondrial defect had been excluded by normal substrate oxidation rates and individual enzyme measurements, as previously described [24].

P5 and P7 are patients diagnosed in our hospital with the MELAS syndrome; both carry an A to G transition at position 3243 in approximately 50% of their skeletal muscle mtDNA (Van Oost, B.A., personal communication). All other patients and controls lack this as well as other known mtDNA mutations associated with mitochondrial myopathies or respiratory chain defects [25]. P5 and P6 are previously described patients [26,27].

### 2.8. Enzyme measurements

The activities of the rotenone-sensitive NADH-ubiquinone-1 oxidoreductase (Complex I), succinate-cytochrome-*c* oxidoreductase (Complex II-III), cytochrome-*c* oxidase (Complex IV) and citrate synthase were measured in  $600 \times g$  supernatant or, where stated, in mitochondria of homogenized skeletal muscle as described previously [24]. The activities in  $600 \times g$  supernatants from fresh biopsy muscle were first normalized for citrate synthase activities. All activities are expressed as a percentage of the mean of controls. Ubiquinone-1 was a gift from Hoffman-La Roche, Basel, Switzerland.

### 2.9. SDS-polyacrylamide gel electrophoresis and western blotting

Mitochondrial proteins, isolated by differential centrifugation of homogenized skeletal muscle specimens, were

Table 2  
Clinical, laboratory and morphological features of patients studied

Patient/sex	1/F	2/F	3/M	4/M	5/M	6/F	7/F
Diagnosis	encephalomyopathy	myopathy	encephalomyopathy	encephalomyopathy	MELAS	encephalomyopathy	MELAS
Age at onset <sup>a</sup>	inf	inf	inf	inf	22	4	inf
Age at biopsy <sup>a</sup>	12 d	14	81 d	16 d	25	17	20
Progression <sup>a</sup>	†27 d	–	†81 d	†30 d	slow	†17	†20
Hypotonia	+	–	+	+	–	–	–
Strokes	–	–	–	–	+	–	+
Seizures	+	–	+	–	+	–	+
Retinopathy	na	–	–	–	–	–	na
Deafness	na	–	na	na	+	–	+
Lactic acidosis	+	–	+	+	+	+	+
RRF	–	–	–	–	+	–	+
Family history	–	+	–	+	–	+	+
Complex I <sup>b</sup>	13	13	14	20	27	22¶	34
Complex II-III <sup>b</sup>	98	77	49	101	77	75¶	57
Complex IV <sup>b</sup>	88	112	102	104	57	62¶	97

<sup>a</sup> In years unless otherwise specified; inf, infantile; d, days; ¶, autopsy; na, not available.

<sup>b</sup> Activities in  $600 \times g$  supernatant are expressed as a percentage of the mean of controls.

fractionated (2  $\mu$ g protein per lane) on 10% or 16.5% slab gels in a Mini-Protein II Apparatus (Bio-Rad) according to Schägger and Von Jagow [28]. Proteins were blotted onto 0.2  $\mu$ m nitrocellulose paper [29]. Immunodetection was performed at ambient temperature as follows. Where stated, the blot paper was cut in strips. Unoccupied sites were blocked with phosphate buffered saline solution, 0.5% (w/v) Tween 20 (PBST) and 5% (w/v) non-fat dry milk powder for 1 h. Thereafter the strips were incubated consecutively with the  $\gamma$ -globulins, a 1:5000 dilution of

biotinylated swine anti-rabbit immunoglobulins, a 1:10 000 dilution of a streptavidin/biotinylated horse radish peroxidase mixture (both Dako) in PBST with 1.5% (w/v) BSA and 1% (v/v) normal swine serum (Dako), each for 1 h. The  $\gamma$ -globulins (1 mg/ml) were diluted 100 to 2000-times in PBST with 0.5% (w/v) BSA, and occasionally, as specified below, preincubated for 1 h with 0.25 mg/ml peptide dissolved in DMSO; samples preincubated with the same amount of DMSO served as a control. Between the various steps the strips were washed three times with

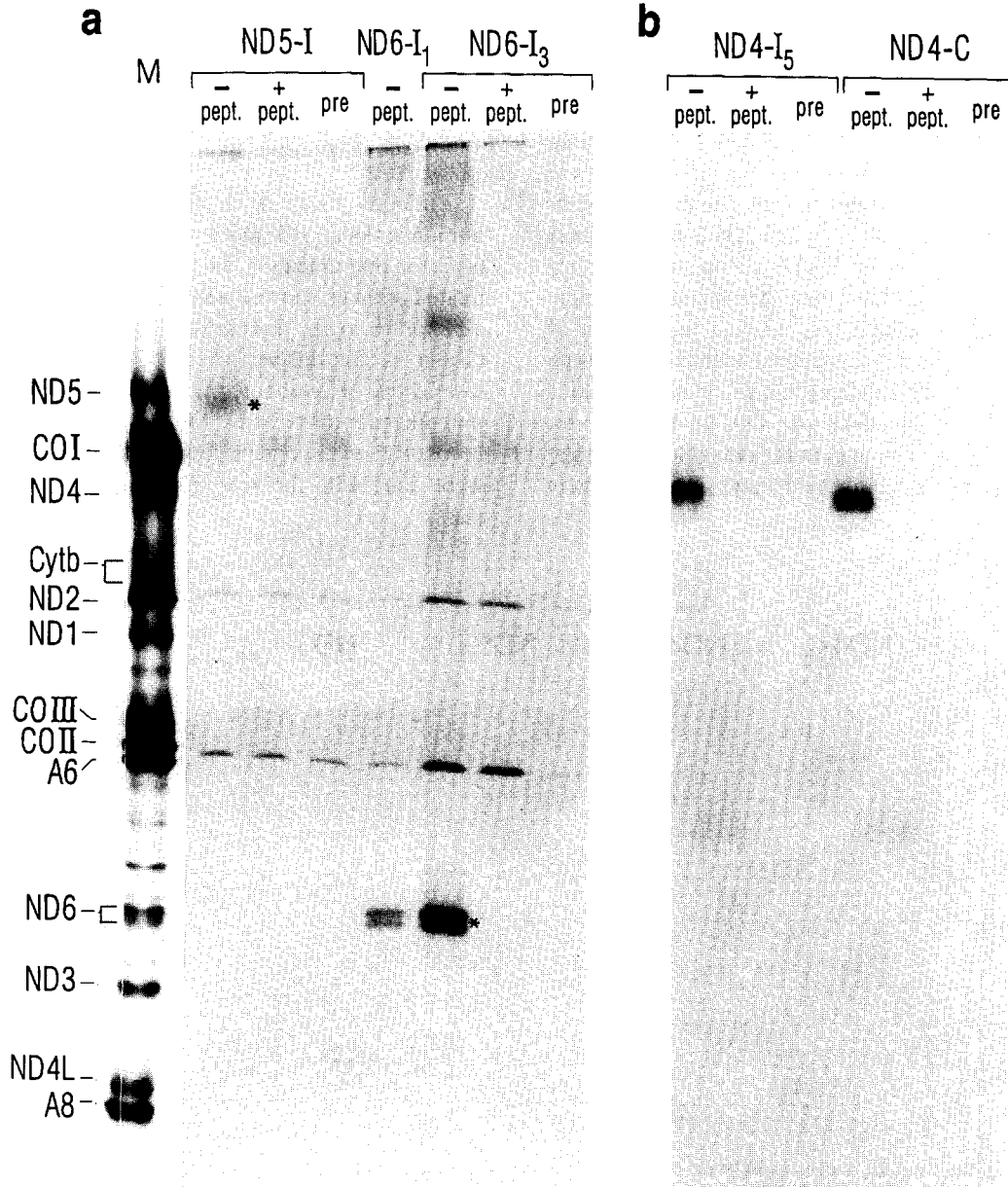


Fig. 2. Immunoprecipitation capacity of subunit-specific antibodies. In panel a, the immunoprecipitates using ND5 and ND6 peptide-specific antibodies are shown, in panel b those using ND4 peptide antibodies. Pre: pre-immune serum; – pept or + pept: with addition of 5  $\mu$ l DMSO or 5  $\mu$ l peptide (1 mg/ml DMSO), respectively. The immunoprecipitates were analyzed on a 15–20% exponential gradient SDS-polyacrylamide gel system [22], followed by fluorography [23]. The dried gels were exposed for 5 weeks (a) or 7 days (b). The asterisks in panel a show the positions of the immunoprecipitated ND5 and ND6 subunits. Lane M contains HeLa cell mitochondrial proteins labelled with [ $^{35}$ S]methionine in the presence of emetine. The 13 subunits encoded in the mtDNA are indicated on the left.

PBST. The ECL kit (Amersham) was used for detection of bound antibodies.

Rabbit antibodies raised against ANT or the ATPase complex were used as an internal standard for mitochondrial protein recovery at a dilution of 1:2000. Quantitation of the blots was carried out by scanning the films with a LKB laser densitometer.

### 3. Results

#### 3.1. Analysis and coupling of peptides

Reversed phase chromatography showed that the peptides were pure to the extent of 70% or more (results not shown). Mass spectrometric analysis of the major peaks confirmed the expected mass of the peptides, indicating complete removal of the protecting groups. The crude peptide preparations were coupled to carrier proteins without further purification, since only the full-length products were expected to contain the SATA group necessary for coupling [14]. Coupling ratios were determined by amino acid analysis of the conjugates comparing the amount of L-norleucine (specific for the peptides) with that of amino acids found only in the carrier proteins. Coupling ratios were 3–10 and 7–20 peptides per OA or BSA carrier molecule, respectively. The maximum available free amino groups in both carrier proteins are 10 and 27, respectively [11].

Table 3

Antibody titers against peptides, carrier proteins or linkers

Antibody	Titer ( $\times 10^3$ ) against		
	peptide	carrier	linker
ND4-I <sub>1</sub>	197	90	8.1
ND4-I <sub>2</sub>	197	90	8.1
ND4-I <sub>3</sub>	142 (1.9)	91 (3.6)	0.3 (< 0.03)
ND4-I <sub>4</sub>	656	810	0.9 <sup>a</sup>
ND4-I <sub>5</sub>	66	270	25
ND4-C	161 (1.6)	280 (7.7)	0.9 (< 0.03)
ND5-I	656	810	1.8 <sup>a</sup>
ND6-I <sub>3</sub>	73	810	0.9 <sup>a</sup>

Sera were prepared from blood samples taken after three immunizations. The values are averages of data for two animals; between parentheses are the titers for RAS treated animals.

<sup>a</sup> Indicates protocols using BSA/MHS as carrier/linker combination; in all other cases, OA/sulfo-MBS was used.

#### 3.2. Antibody production

Antibody titers reached maximum values after three immunizations. All rabbits responded to the conjugates used for immunization. In the first experiments, the RAS-treated animals showed antibody titers that were lower, by a factor 10 to 40, than those of FCA/FIA-treated animals (Table 3). The linkers (MHS or sulfo-MBS) produced only low levels of antibodies in all animals, except for those immunized with ND4-I<sub>5</sub>. MHS has already been shown to be poorly immunogenic [30]. All subsequent experiments were done with the sera from the FCA/FIA-treated animals.

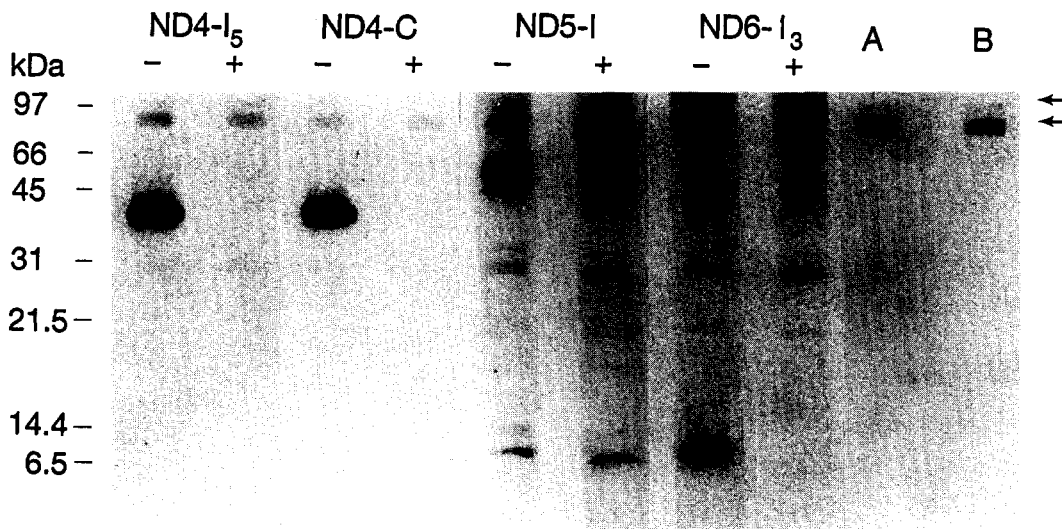


Fig. 3. Analysis of subunit-specific antibodies by Western blotting. Western blots of skeletal muscle mitochondrial proteins tested with antisera against ND4-I<sub>5</sub>, ND4-C, ND5-I and ND6-I<sub>3</sub>. Mitochondrial proteins (2  $\mu$ g) isolated from skeletal muscle of a control individual (C1 in Fig. 5) were separated on a 16.5% SDS-polyacrylamide gel according to Schägger and Von Jagow [28]. The ND4-specific antibodies were both diluted 1000-times, the ND5- and ND6-specific antibodies, 100-times. The ECL system was used for detection. The exposure time was 15 s for the ND4-I<sub>5</sub>, ND4-C and ND6-I<sub>3</sub> lanes, and 30 s for ND5-I, A, and B. + indicates a preincubation of the antiserum with the specific peptide preparation in DMSO (final concentration 0.25 mg/ml); - indicates a preincubation with the same volume of DMSO. In lanes A and B, no primary antibodies were added; in lane B, also the incubation with the secondary (biotinylated) antibodies was omitted. On the left, the migration positions of water-soluble molecular weight marker proteins (Bio-Rad) are indicated.

### 3.3. Immunoprecipitation capacity of subunit-specific antibodies

The results of the immunoprecipitation studies are shown in Fig. 2. Lane M shows the pattern of HeLa cell mitochondrial translation products labeled with [<sup>35</sup>S]methionine in the presence of emetine. Note the migration of ND6 as a doublet in the exponential gradient system used here [22] (see Discussion). It appears from Fig. 2 that only antibodies against ND4-I<sub>5</sub> and ND4-C were capable of immunoprecipitating the labeled ND4 subunit, in a reaction inhibited by the specific peptide. This immunoprecipitation was as effective as that observed with the ND4-C antiserum described by Chomyn et al. [4] (results not shown). The other ND4 peptide-specific antibodies were negative in this assay.

Also the antibodies against ND5-I and ND6-I<sub>3</sub> precipitated the ND5 and ND6 subunit, respectively, the reaction with each protein being inhibited by the corresponding peptide. The ND5 subunit could be visualized only after long exposure of the film. This resulted in some background of non-specific bands not inhibited by the specific peptide (Fig. 2). Antibodies against C- and N-terminal peptides of ND5 showed no reaction (results not shown).

Our ND6-I<sub>3</sub> peptide antibodies precipitated ten times more ND6 than the ND6-I<sub>1</sub> antiserum, which has been used previously to identify ND6 as a NADH dehydrogenase subunit [5]. The immunoprecipitated ND6 migrated in the gel as a doublet as in the original sample (lane M in Fig. 2). The higher molecular weight bands in the ND6-I<sub>3</sub> (–pept) lane may be a result of aggregation during the assay [5]. Purified  $\gamma$ -globulins gave an identical immunoprecipitation pattern as total serum (results not shown).

### 3.4. Analysis of subunit-specific antibodies by Western blotting

Fig. 3 shows the results of Western blotting experiments, carried out, after SDS-polyacrylamide gel electrophoresis, by using mitochondrial proteins from human skeletal muscle as antigens and  $\gamma$ -globulin-fractions as peptide-specific antibodies. With the ECL detection system 2  $\mu$ g of mitochondrial protein per lane was already sufficient for a good signal. Total serum gave a very high background signal, in part caused by the high levels of antibodies to the carrier proteins (results not shown). Therefore, purified  $\gamma$ -globulin-fractions, from which the anti-carrier-antibodies had been removed, were used.

The antibodies against ND4-I<sub>5</sub> and ND4-C reacted very strongly with a protein of apparent molecular mass of 39 kDa (as estimated from its electrophoretic mobility, relative to that of water-soluble marker proteins), the reaction being inhibited by the specific peptide (Fig. 3, first four lanes). The molecular mass of this protein is in agreement with that previously reported for bovine and human ND4, on the basis of its electrophoretic mobility in SDS-poly-

acrylamide gels [4,9,31]. The difference in size estimate relative to that predicted from the DNA sequence is due to the use, in the experiments cited above, of hydrophilic marker proteins, which bind less SDS than the hydrophobic mitochondrial translation products [8]. The antiserum against ND4-I<sub>5</sub>, which has 12 of 16 amino acids identical with the homologous bovine sequence, also reacted with a mitochondrial protein from bovine heart of approximately the same molecular mass (results not shown). The other ND4 peptide-specific antibodies were negative even in up to 100-fold lower dilutions (results not shown).

The antibodies against ND5-I and ND6-I<sub>3</sub> also showed peptide-specific reactions. The ND5-I-specific antibodies produced two bands in a reaction that could be inhibited by the specific peptide. In particular, they gave a strong reaction with a protein of apparent molecular mass of 51 kDa, and a minor reaction with a protein of a somewhat higher electrophoretic mobility (Fig. 3, fifth and sixth lane). The 51 kDa protein has the electrophoretic mobility, in an SDS-polyacrylamide gel, expected for the ND5 gene product, the difference from the mass expected for this product (66 kDa) being due to the bias in size estimates which was mentioned above. The minor reaction with the protein moving faster than the '51 kDa' protein could be explained as resulting from a cross-reaction with another mitochondrial protein with a similar or identical ND5-I epitope(s); however, a computer search did not yield a known protein containing a sequence homologous for the amino acid sequence used. The ND6-I<sub>3</sub>-specific antibodies gave a specific reaction with a protein of apparent molecular mass of  $\sim$ 8 kDa, as estimated relative to the marker proteins (Fig. 3). Here, too, the difference in molecular mass from that predicted from the DNA sequence (18.6 kDa) is due to the bias in size estimates introduced by the use of hydrophilic proteins for the standard curve [8]. Both the ND5-I- and ND6-I<sub>3</sub>-specific antibodies gave a weak reaction in the immunoblot as compared to the ND4 antibodies. Therefore, 10-times less diluted antibodies and also longer exposure times with the ECL system, in the case of ND5, had to be used. This caused the rather high background in lanes ND5-I and ND6-I<sub>3</sub> in Fig. 3. The background in the upper portion of the lanes is most likely caused by biotin-containing proteins reacting with the streptavidin/horseradish peroxidase biotin complex, as shown in lanes A and B, where the primary and, in lane B, also the secondary antibodies, were omitted during the incubations. The molecular weights of the slower-migrating proteins (arrows) are in agreement with those of biotin containing carboxylases, as reported by Robinson et al. [32].

### 3.5. Immunoblotting of skeletal mitochondrial proteins from myopathy patients with subunit- or holoenzyme-specific antibodies

The antibodies produced here and other subunit- or holoenzyme-specific antibodies were used to analyze the

quantitative behaviour of the corresponding subunits in the respiratory NADH dehydrogenase of skeletal muscle from mitochondrial myopathy patients exhibiting an enzymatical Complex I deficiency.

Fig. 4 shows that antibodies against the bovine Complex I holoenzyme (panel a) and against the ND1 peptide of *Neurospora crassa* (panel b) both gave weaker reactions with mitochondrial proteins from patients P1-P5, as compared to the reactions observed with material from control individuals; patients P6 and P7 were not investigated with these antibodies. Antibodies against the bovine Complex I holoenzyme gave a specific reaction with approximately ten proteins when tested against a Complex I preparation from bovine or human heart tissue (results not shown). Their position is indicated by arrowheads in Fig. 4, part a. These are probably all encoded in nDNA. The background in the upper portion of the lanes is, again, most likely caused by biotin-containing proteins reacting with the streptavidin/horseradish peroxidase biotin complex, as shown in Fig. 3. The bands marked with asterisks in the lower portion of the patient lanes P1, P3 and P4,

which have a stronger intensity or a different mobility, as compared to the bands from control individuals migrating at the same positions, are probably degradation products. Note that the strong reaction with a protein of apparent molecular mass of 24 kDa in the patient lanes of Fig. 4 is not the result of a reaction with antibodies against the 24 kDa subunit of Complex I (shown in Fig. 5).

The reaction with antibodies against the human ATPase  $\beta$  subunit also exhibited a normal intensity in the samples from patients P1-P5, as compared to the controls (Fig. 5, panel c), indicating the absence of significant differences in the purity of the mitochondria.

Fig. 5 shows that antibodies against the ND4-I<sub>5</sub>, ND5-I and ND6-I<sub>3</sub> peptides, as well as antibodies against the purified bovine 24 kDa FeS protein of Complex I, which is encoded in the nuclear genome, gave weaker reactions when tested with material from all seven patients studied, as compared to the reactions with samples from control individuals. This was not caused by differences in the purity of the mitochondria, because, except for patient P4, no large differences were seen among the various samples

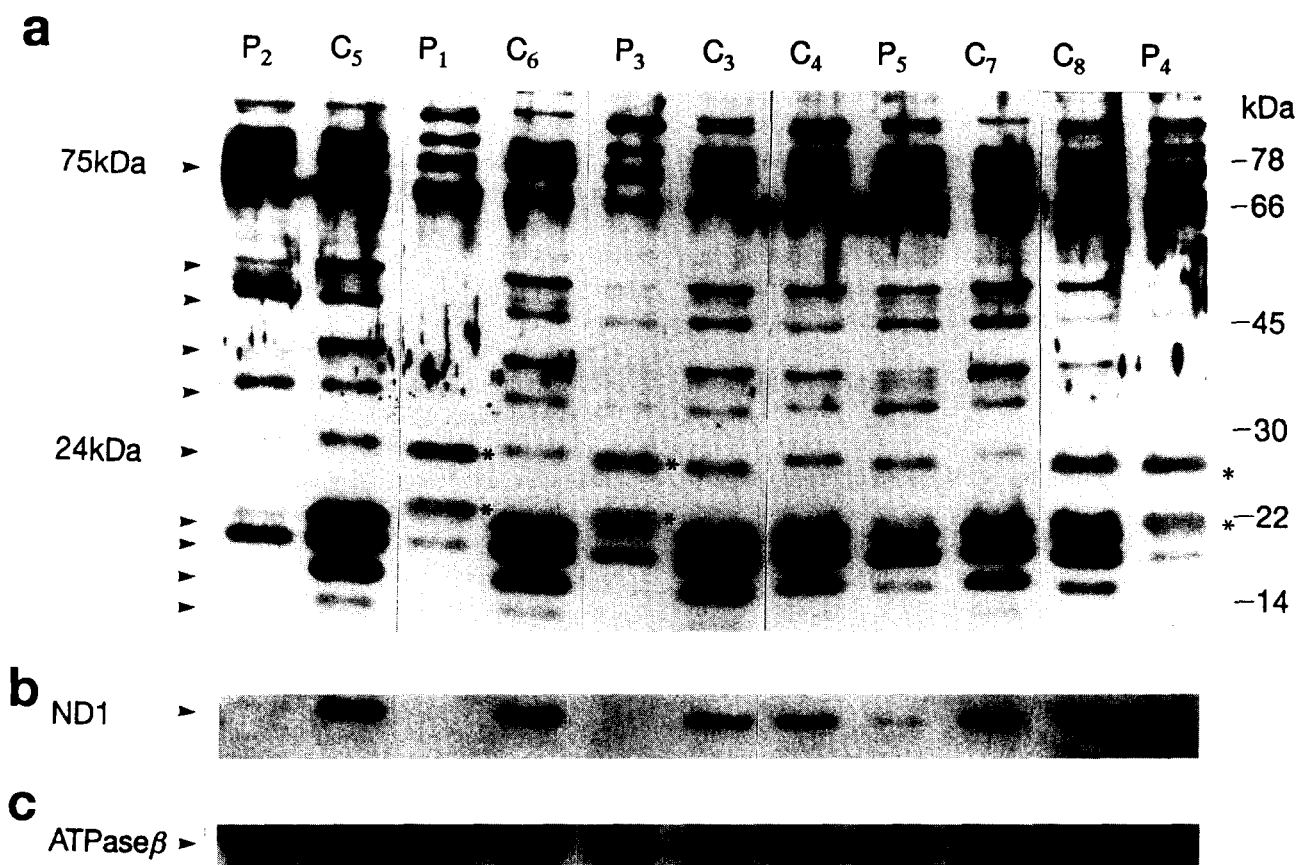


Fig. 4. Western blots using antisera against whole Complex I, its ND1 subunit and the ATPase  $\beta$  subunit. Control and patient mitochondrial proteins (2  $\mu$ g each) were separated on two identical 10% SDS-polyacrylamide gels [28]. Panel a shows a portion of one blot incubated with antibodies against the bovine Complex I holoenzyme. The positions of the 24 kDa, 75 kDa and other subunits, which show a reaction between the antibodies and blots of purified Complex I preparations, are indicated on the left. On the right, the migration positions of water-soluble molecular weight marker proteins (Pharmacia/LKB) are indicated. The other blot was cut into horizontal strips. Panel b shows the reaction with antibodies against the ND1 peptide of *Neurospora crassa*. Panel c shows the reaction with antibodies against the  $\beta$ -subunit of human ATPase. The asterisks in lanes P1, P3 and P4 indicate the positions of possible degradation products (see Discussion). The ECL system was used for detection in all blots.



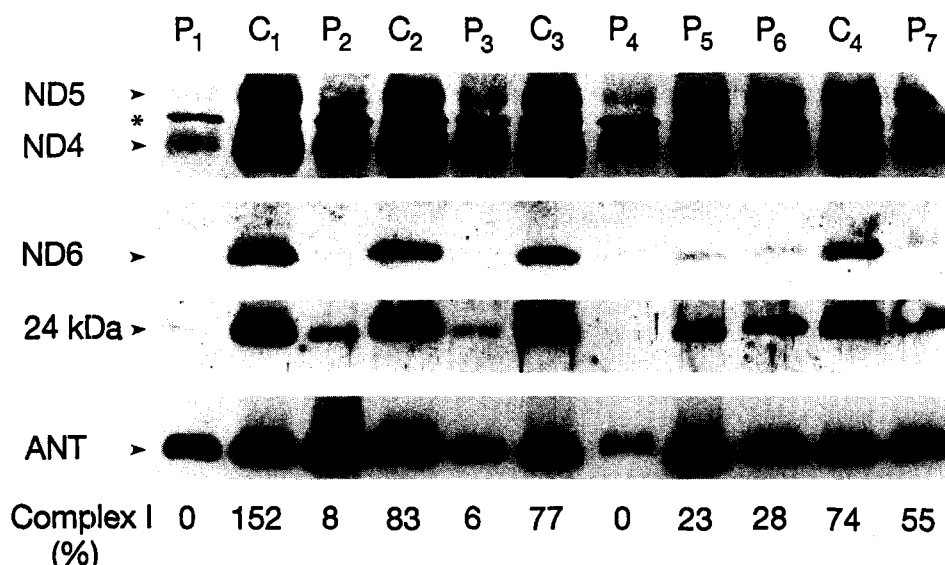


Fig. 5. Western blots using antisera against the ND4, ND5, ND6 and 24 kDa subunits of Complex I or the ANT protein. Control and patient mitochondrial proteins (2  $\mu$ g each) were separated on a 16.5% SDS-polyacrylamide gel [28]. After the transfer, the nitrocellulose paper was cut into four horizontal strips corresponding to the expected molecular weights of the subunits of interest and the parts were incubated with antibodies against ND4-I<sub>5</sub> and ND5-I, ND6-I<sub>3</sub>, 24 kDa protein or ANT. The activity of Complex I in the isolated mitochondria is given below each lane. It is expressed as a percentage of the mean of controls (mean  $\pm$  S.D.: 168  $\pm$  59 ( $n$  = 11) or 200  $\pm$  85 nmol/min per mg protein ( $n$  = 15) for mitochondria isolated from frozen or fresh muscle homogenate, respectively). \* Indicates the position of the band cross reacting with the ND5-I antibodies (see also Fig. 3).

in the reaction with antibodies against the bovine ANT protein (Fig. 4, bottom) or human ATPase  $\beta$  subunit (Fig. 4, panel c). The strong reduction of ANT in mitochondria from patient P4 may have been caused by an additional deficiency of ANT protein in this patient. Support for this interpretation comes from the observation that the protein cross-reacting with the ND5-I antibodies, which is marked by an asterisk in Fig. 5, appeared to be present in mitochondria of patient 4 in the same amount as, e.g., in the samples of P5 and P6, which show a good reaction with the ANT-specific antibodies. The possibility of an ANT deficiency in patient P4 is presently under further investigation.

There is a good correlation between the signal of immunodetected Complex I subunits, as determined by densitometry, and the activities of Complex I in isolated mitochondria from different individuals, as shown in the lower part of Fig. 5. The correlation coefficients for the ND4, ND5, ND6 and 24 kDa subunits, normalized for the ANT signal, were 0.82, 0.93, 0.86 and 0.92, respectively. Patients P1, P2, and P3, who exhibit a complete or near-complete absence of enzyme activity, appear to contain negligible amounts of the tested Complex I subunits in their skeletal muscle mitochondria. Patients P5, P6 and P7, who have moderately or substantially decreased Complex I activities, show a proportional reduction in reactivity of their mitochondrial proteins with all four Complex I subunit antibodies, whereas they exhibit amounts of ANT and ND5-I cross-reacting protein comparable, e.g., to those of control C4.

#### 4. Discussion

We have shown that it is possible to raise antibodies successfully against computer-predicted linear epitopes of mitochondrial membrane proteins. Furthermore, these antibodies were shown to react with the corresponding proteins not only in immunoprecipitation assays, but also in Western blots. Using the Ribi adjuvant system for immunization, consistently much lower antibody titers were observed, as compared to the immunizations carried out with the Freund's adjuvant (Table 3). This phenomenon has previously been observed in rabbits using rat microsomal epoxide hydrolase as an antigen [33].

Only two of six predicted epitopes of ND4 yielded antibodies positive in both the immunoprecipitation and the immunoblot assays. These were the C-terminal peptide and an internal peptide (I<sub>5</sub>) close to it. Fearnley and Walker [34] predicted that all five internal peptides are situated between transmembrane segments. Whether these predictions are incorrect, or whether these peptides do not form linear epitopes in the presence of SDS is not clear at present.

Antibodies against internal peptides of ND5 and ND6 reacted also in both the immunoprecipitation and the immunoblot assays. For ND5, these are the first antibodies reported with this capacity. The previously-tested ND5-N and ND5-C peptide-specific antibodies [8] were not capable of precipitating ND5. In the immunoblot analysis carried out here, the observed reaction with two bands could be inhibited by preincubation of the ND5-I antibody-

ies with its corresponding peptide. The protein corresponding to the upper, strong band is most likely ND5, because it has the expected molecular mass, as estimated relative to hydrophilic marker proteins, and because it is reduced in amount in mitochondria from Complex I-deficient patients. The lower, weak band may have been produced by a reaction with an unknown protein containing a sequence homologous to the ND5-I peptide sequence.

In the exponential gradient gel system used here, the ND6 subunit migrated as a doublet band, possibly resulting from an unknown post-translational modification of a fraction of the protein molecules. The immunoprecipitated ND6 also appeared as a doublet. In electrophoretic runs of proteins from isolated HeLa cell mitochondria and of fibroblast whole-cell extracts, ND6 has always been found to migrate as a doublet, making the possibility of proteolytic degradation as the cause of the doublet formation less likely (H. Bentlage, unpublished observations). Also in 20 cm long 16.5% acrylamide gels using the Tricine buffer system [28] ND6 appeared as a doublet band (results not shown). The strong immunoprecipitation reaction of the ND6-I<sub>3</sub> antibodies, as compared to the ND6-I<sub>1</sub> antibodies, can be explained by a difference in either the titer or affinity of the two types of antibodies. The nature of the difference was not further investigated. In the Western blotting experiments, ND6 appeared as a single band, probably because of a different size (8 cm) of the gel system used.

In the immunoblots, it was essential to use  $\gamma$ -globulin fractions depleted of antibodies to the carrier proteins in order to reduce the background. The remaining background could be partially explained by the presence of biotin-containing proteins in skeletal muscle mitochondria. A lower background appeared in the immunoprecipitation assays, since only 13 proteins were labeled that could give rise to non-specific signals.

The antibodies produced in the present work and other subunit- or holoenzyme-specific antibodies were used to investigate the presence and amount of mtDNA- and nuclear-encoded subunits of the respiratory NADH dehydrogenase in the skeletal muscle of patients affected by myopathies associated with a Complex I deficiency. The results obtained indicated lower levels of the three mtDNA-encoded subunits (ND4, ND5 and ND6) and one nuclear encoded subunit (24 kDa) in all seven patients studied. Also ND1 and several other nuclear encoded subunits were found to be less abundant in five patients studied, when ND1 peptide-specific and Complex I holoenzyme-specific antibodies, respectively, were used. The latter antibodies gave a reaction, in the samples from patients, with two bands that were either not present (20 kDa) or less strongly represented (24 kDa) in the samples from control individuals. The band that migrated with an apparent molecular weight of 24 kDa in the samples from patients (Fig. 4) was shown not to react with 24 kDa subunit-specific antibodies (Fig. 5). Both bands probably

represent degradation products. This degradation phenomenon has been previously observed by Robinson [35].

A generalized reduction of immunoreactive Complex I subunits has previously been found in mitochondrial myopathy patients with a Complex I deficiency by immunoblotting experiments utilizing polyclonal antibodies against bovine Complex I and antibodies specific for six nuclear-encoded iron-sulfur proteins [1,6]. However, the mtDNA-encoded subunits were not identified in these experiments. Therefore, the present work provides the first evidence of a decrease in the latter subunits in mitochondria of myopathy patients. It was found here that the remaining amount of reacting subunits correlated with the residual Complex I activity in the skeletal muscle mitochondria.

In the case of the two MELAS patients (P5 and P7), a reduced rate of mitochondrial protein synthesis [36,37] would account for the reduced amount of mitochondrial translation products and a resulting decreased assembly of Complex I. In the other patients, the underlying defect could again be a reduced protein synthetic capacity, or a disturbed assembly and subsequent degradation of Complex I subunits. The latter process has been shown to occur in a HeLa cell mutant lacking the ND4 subunit [38]. Whether this pathogenetic mechanism operates in any of our patients remains to be determined. The antibodies described here will be useful for identifying defects in biosynthesis or stability of subunits encoded in mtDNA.

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