Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5103–5106, August 1983
Medical Sciences

Deficiency in ubiquinone cytochrome *c* reductase in a patient with mitochondrial myopathy and lactic acidosis

(antibody blotting/polypeptides)

VICTOR M. DARLEY-USMAR*, NANCY G. KENNAWAY†, NEIL R. M. BUIST†, AND RODERICK A. CAPALDI*

*Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403; and †Department of Medical Genetics, Oregon Health Sciences University, 3181 S.W. Sam Jackson Park Road, Portland, Oregon 97201

Communicated by George Streisinger, May 19, 1983

ABSTRACT **The skeletal muscle of a patient with a mitochondrial myopathy was examined. A defect in the electron transport chain was identified at the position of complex III by activity measurements and the low levels of reducible cytochrome *b*. The polypeptide composition of complex III in the patient's mitochondria was determined by antibody binding experiments. The method allowed detection of individual polypeptides at a lower limit of 10–40 ng of protein. Characterization of protein composition is thus possible by using a biopsy sample of 1 g of tissue. The level of core proteins, FeS protein, and subunit VI was greatly diminished in the patient's mitochondria. Cytochrome c_1 polypeptide was found at normal levels but was sensitive to proteolysis by trypsin. These results show that complex III is not assembled in the patient's mitochondria. The possible role of cytochrome *b* as the site of the primary lesion is discussed.**

Mitochondria provide most of the energy requirements of the cell through the process of oxidative phosphorylation. The components involved are arranged in five multipolypeptide complexes located in the inner mitochondrial membrane. Four of these complexes—namely, the NADH ubiquinone reductase (complex I), succinate ubiquinone reductase (complex II), ubiquinone cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV)—comprise the electron transport chain. The energy available from electron transfer is conserved at three coupling sites (in complexes I, III, and IV) in the form of a proton gradient. This gradient is utilized by the ATP synthetase (complex V) to form ATP or can be used to drive ion transport across the mitochondrial inner membrane (reviewed in refs. 1 and 2).

Several human diseases are now known to involve deficiencies in the mitochondrial respiratory chain. Cases have been reported in which the activities of complex I (3), complex III (4, 5), and complex IV (6) are greatly diminished or, in some cases, completely absent.

In all of the studies of mitochondrial myopathies performed to date, the locus of the lesion has been identified only by activity measurements and by spectral analysis of the cytochromes (7). Such data do not indicate which of the polypeptides forming the complexes are affected and therefore are not able to identify the underlying mutation(s).

Here we describe a biochemical study of the skeletal muscle of a patient with muscle weakness and lactic acidosis. Our results establish that these symptoms are due to mitochondrial myopathy involving complex III. The clinical aspects of this case will be discussed elsewhere. The defect is characterized by a loss in activity of the cytochrome bc_1 segment of the respiratory chain and a greatly diminished amount of reducible cytochrome *b*.

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Complex III of mammalian mitochondria contains 10 different polypeptides (see ref. 1 for review). We have raised antibodies against several of these components purified from beef heart mitochondria. These antibodies were found to crossreact with human mitochondria and have been used here to examine complex III of the myopathic mitochondria.

Our analysis shows that most but not all of the individual polypeptides of complex III are missing from the mitochondria isolated from this patient's skeletal muscle. As a result, the complex does not appear to be assembled, greatly decreasing the efficiency of these mitochondria in ATP synthesis.

METHODS AND MATERIALS

Preparation of Mitochondria. Skeletal muscle was taken from the quadriceps by open biopsy under general anesthesia. Mitochondria were prepared from human control (frozen at −70°C) and the patient's skeletal muscle in the following way. Approximately 1 g of tissue was cut into small pieces and placed in 10 ml of buffer containing 0.20 M sucrose, 0.13 M NaCl, and 1 mM Tris·HCl (pH 7.4). This was stirred on ice for 30 min with 0.14 mg of collagenase (Sigma, type VII). Samples were homogenized with a loose-fitting, motor-driven, potter homogenizer and then with a tight-fitting homogenizer. The solution was spun at 500 × *g* for 10 min and the precipitate was discarded. The supernatant was spun at 8,000 × *g* for 15 min and the crude mitochondrial pellet was suspended in the isolation buffer.

Activity Measurements. Cytochrome *c* oxidase activity was measured spectrophotometrically by following the oxidation of ferrocytochrome *c* at 550 nm (8). The assay was performed in a buffer containing 0.5% Tween 80 and 50 mM potassium phosphate (pH 7.0). The use of detergent will allow disruption of the outer mitochondrial membrane, so that optimal rates can be measured. The final concentration of ferrocytochrome *c* was 10 μM and that of mitochondrial protein was 0.013 mg/ml. Rates are expressed as nmol of cytochrome *c* oxidized per min/mg of protein.

Succinate dehydrogenase activity was measured spectrophotometrically by using 2,6-dichloroindophenol as the electron acceptor and succinate as the electron donor (9). The reaction was monitored at 600 nm. Mitochondria (1 mg/ml) were activated prior to assay by incubation with 40 mM succinate and then diluted to 0.035 mg/ml into the cuvette for assay. The assay buffer contained 1.5 mM KCN, 16 mM succinate, 0.0015% 2,6-dichloroindophenol, and 50 mM potassium phosphate (pH 7.0).

Succinate cytochrome *c* reductase activity was measured by following the reduction of ferricytochrome *c* at 550 nm. Ad-

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; nFeS protein, Rieske nonheme iron-containing protein.

ditions were identical to those described for the succinate dehydrogenase activity, except 2,6-dichloroindophenol was omitted. The concentration of cytochrome *c* in the cuvette was 13.6 μM.

ATPase activity was also monitored spectrophotometrically in the absence and presence of *N,N'*-dicyclohexylcarbodiimide (DCCD) as described in ref. 10. All assays were conducted at 37°C with a Beckman DU7 spectrophotometer.

Antibody Binding Experiments. Subunits of purified beef heart complex III were prepared by gel filtration in Bio-Gel P100 after dissociation by $NaDodSO_4$. Purity of the fractions was judged by $NaDodSO_4$/polyacrylamide gel electrophoresis. Antibodies were raised in rabbits against the purified components of complex III. One preparation of anti-complex III antibody was the generous gift of J. Hare (Oregon Health Sciences University, Portland). $NaDodSO_4$/polyacrylamide gels were made as described by Fuller *et al.* (11) and electrophoretic transfer blots were performed as described by Towbin *et al.* (12) with the addition of 0.1% $NaDodSO_4$ to the electrophoresis buffer.

RESULTS

Locus of the Mitochondrial Defect Based on Activity and Spectral Measurements. The activities of some of the inner mitochondrial enzymes involved in oxidative phosphorylation are reported in Table 1. Succinate dehydrogenase and cytochrome *c* oxidase are similar to control values, whereas succinate cytochrome *c* reductase activity is greatly diminished, being <10% of control values. These results localize the major defect to the complex III portion of the respiratory chain.

Spectral characterization of the defective mitochondria confirm that there is a lesion in complex III since the cytochrome *b* content is <20% of the control sample (Table 1).

Polypeptide Composition Detected by Antibody Binding Studies. Most of the proteins of the mitochondrial inner membrane are coded for on nuclear DNA and made in the cytoplasm. However, a small number of polypeptides, including cytochrome *b* of complex III (and the three largest subunits of cytochrome *c* oxidase), are coded for on mtDNA and synthesized inside the mitochondrion (14). Therefore, the defect leading to loss of complex III activity could be due to a mutation or mutations in either genome. To decide between these possibilities, it is necessary to identify the polypeptides altered in the defective mitochondria. This was achieved by using antibodies to determine the presence or absence of individual components of the complex.

The proteins of human mitochondria were first resolved on the basis of size by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Mitochondria from both beef heart (Fig. 1, lane 5) and human muscle (Fig. 1, lane 1) contain many different polypeptides ranging in molecular weight from 100,000 to <5,000. The polypeptide composition of the defective human mitochondria is also shown in Fig. 1. There are no major differences in composition between this and the control sample (compare lanes 1 and 2 in Fig. 1).

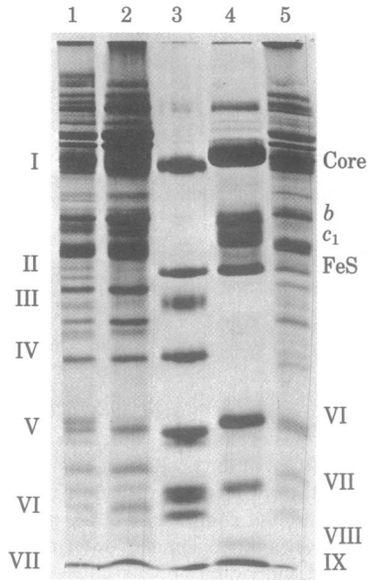

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of mitochondrial proteins. Protein samples were denatured in $NaDodSO_4$, urea, and mercaptoethanol and after electrophoresis were stained for protein with Coomassie blue. Lane 3, 40 μg of purified beef heart cytochrome *c* oxidase; subunits are numbered on the left-hand side in order of decreasing molecular weights as described by Ludwig *et al.* (15). Lane 4, 40 μg of purified beef heart complex III; subunits are labeled on the right-hand side according to their prosthetic groups and in order of decreasing molecular weight, according to Bell and Capaldi (16). Lane 1, 80 μg of mitochondrial protein isolated from control human skeletal muscle. Lane 2, 80 μg of mitochondrial protein isolated from patient with lactic acidosis. Lane 5, 60 μg of bovine mitochondrial protein.

Complex III has not been purified from human mitochondria and hence its polypeptide composition remains to be established. Cytochrome *c* oxidase has been isolated from human tissue (placenta) and has been found to have essentially the same subunit composition as the beef heart enzyme (17). Therefore, it seemed likely that complex III from human and beef would be similar and, on this basis, antibodies were made against beef heart complex III for the purpose of identifying their counterparts in the human tissue. Crossreactivity of beef heart complex III antibodies with the human enzyme was examined by the technique described by Towbin *et al.* (12). The mitochondrial proteins separated by $NaDodSO_4$ gel electrophoresis were transferred electrophoretically to nitrocellulose paper. Rabbit antibodies specific to individual subunits of the mitochondrial complexes were then bound to the proteins. The bound antibodies were visualized by binding fluorescein isothiocyanate-labeled goat anti-rabbit antibody to the subunit specific antibodies.

Table 1. Activities of some of the inner mitochondrial enzymes involved in oxidative phosphorylation

	Activity measurements				Cytochrome content		
Sample	ATPase	Succinate dehydrogenase	Succinate cytochrome *c* reductase	Cytochrome *c* oxidase	Cytochrome *a*	Cytochrome *b*	Cytochrome *c* + c_1
Control	1,200	94	340	945	1.0	0.63	0.66
Patient	2,000	57	8	1,222	0.6	0.1	0.66

Activity measurements are expressed as nmol of substrate utilized per min/mg of mitochondrial protein. The substrate for the ATPase assay was ADP; for succinate dehydrogenase and succinate cytochrome *c* reductase assays, succinate; and for cytochrome *c* oxidase assay, ferrocytochrome *c*. Concentrations of cytochromes (nmol/mg) were calculated from the reduced minus oxidized difference spectrum by using the extinction coefficients of Bookelman *et al.* (13).

Antibodies raised against native beef heart complex III were found to crossreact with 4 of its 10 different polypeptides, including both core proteins (which were not resolved on the gel system shown) cytochrome c_1 and subunit VI (Fig. 2*A*, lane 1). Human control mitochondria (Fig. 2*A*, lane 3) also showed the presence of the same four polypeptides as in the beef enzyme. Antibody raised against purified beef heart Rieske nonheme iron-containing protein (nFeS protein) (Fig. 2*B*) and purified core proteins (Fig. 2*C*) also crossreacted with their human counterparts. These experiments firmly establish the expected close similarity between the subunits of bovine and human complex III. In addition, they demonstrate that the Towbin blot technique can be effectively used for human mitochondria prepared from 1 g of skeletal muscle tissue. The lower limit of detection for a given antigen varies somewhat according to the affinity of the antibody but is in the range of 10–40 ng of any one polypeptide (results not shown).

Antibody binding to mitochondrial protein isolated from the patient showed that the cytochrome c_1 content was approximately the same in the two preparations, as judged by the fluorescence intensities of bound antibody (Fig. 2*A*, lanes 2 and 3). However, reaction with core protein (Fig. 2 *A* and *C*, lanes 2 and 3) and subunit VI was dramatically diminished. Similarly, the reaction of antibody against the FeS protein (Fig. 2*B*, lanes 2 and 3) was much less with the defective mitochondria than with the control.

It has not proved possible to obtain an antibody against cytochrome *b* from beef heart mitochondria, probably because of the very hydrophobic character of this polypeptide. Antibody against cytochrome *b* of yeast mitochondria (kindly provided by D. Beattie) did not react with human mitochondria. Therefore, evidence that the cytochrome *b* polypeptide is also affected is indirect and based on the low levels of *b* heme.

Antibody binding studies thus show that the absence of cytochrome *b*, as measured by heme absorbancy, is correlated with the disappearance of core protein(s), subunit VI, and nFeS protein in the defective mitochondria. Cytochrome c_1 is present, in agreement with spectral measurements of the $c + c_1$ heme content.

The absence of complex III subunits could be due to a block or blocks in their synthesis or could have a trivial explanation—i.e., that there is enhanced proteolysis of the inner membrane polypeptides in the defective mitochondria by endogenous proteases when the mitochondria are denatured in $NaDodSO_4$ (many proteases remain active in $NaDodSO_4$). This possibility was tested by studies with other inner membrane components, cytochrome *c* oxidase and succinate dehydrogenase. Fig. 3 shows that antibodies against subunits II, IV, V, and VI of beef heart cytochrome *c* oxidase react with the defective (Fig. 3*A*, lane 2) and control mitochondria (Fig. 3*A*, lane 3) to about the same extent. The same is true of antibodies against succinate dehydrogenase (Fig. 3*B*, lanes 2 and 3). There is a small amount of proteolytic breakdown of both cytochrome *c* oxidase and succinate dehydrogenase subunits in the defective mitochondria, not seen in control samples. This proteolysis is not significant enough to account for the dramatic reduction in the amount of complex III components.

The lability of cytochrome c_1 to proteases is prevented if this polypeptide is incorporated into complex III. This provides a test for assembly of cytochrome c_1 with polypeptides to which we do not have antibodies and which therefore could be present in the patient's mitochondria. The mitochondrial proteins were solubilized in Triton X-100 and exposed to proteolysis with trypsin. Fig. 4 shows the antibody binding to complex III polypeptides before (Fig. 4, lanes 2 and 4) and after (Fig. 4, lanes 1 and 3) digestion with trypsin. In the mutant mitochondria, cytochrome c_1 is cleaved (Fig. 4, lane 1). This does not occur in purified beef heart complex III or in the control human mitochondria (Fig. 4, lane 3) and therefore indicates that cytochrome c_1 is not in its normal association with the other polypeptides of complex III. This result is further evidence that proteolytic digestion does not occur significantly in the pa-

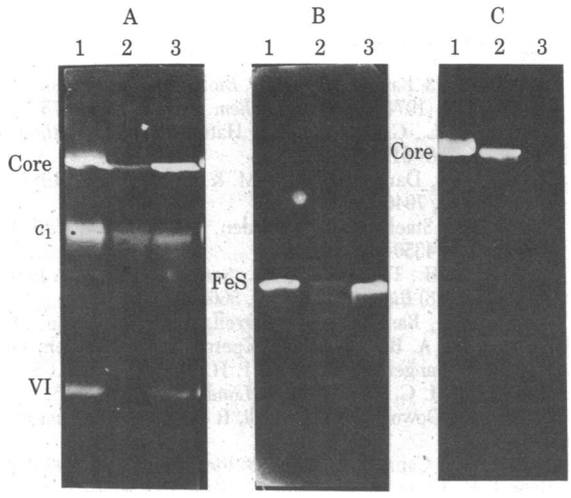

FIG. 2. Antibody binding to subunits of ubiquinone cytochrome *c* reductase (complex III). Mitochondrial proteins were bound to nitrocellulose paper and incubated with antibodies as described in the text. (*A*) Binding of anti-holocomplex III antibody. Lane 1, 2 μg of purified complex III from beef heart; lane 2, 80 μg of mitochondrial protein isolated from mutant mitochondria; lane 3, 80 μg of control human mitochondrial protein. (*B*) Binding of anti-FeS protein antibody. Lane 1, 2 μg of purified beef heart complex III; lane 2, 80 μg of mutant human mitochondrial protein; lane 3, 80 μg of control human mitochondrial protein. (*C*) Binding of anti-core protein (I and II) antibody. Lane 1, 2 μg of purified complex III antibody; lane 2, 80 μg of control human mitochondrial protein; lane 3, 80 μg of mutant mitochondrial protein.

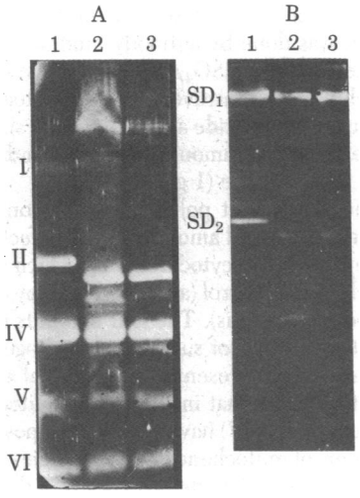

FIG. 3. Antibody binding to subunits of cytochrome *c* oxidase and succinic dehydrogenase. Mitochondrial proteins were resolved by $NaDodSO_4$/polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. Antibodies were bound to the proteins and visualized by a second fluoresceinated antibody directed against them. (*A*) Binding of anti-holo beef cytochrome *c* oxidase antibody. Lane 1, 2 μg of purified beef heart cytochrome *c* oxidase; lane 2, 80 μg of mutant mitochondrial protein; lane 3, 80 μg of control human skeletal muscle mitochondrial protein. (*B*) Binding of succinate dehydrogenase (SD) flavoprotein antibody. Lane 1, 80 μg of beef mitochondrial proteins; lane 2, 80 μg of control human mitochondrial protein; lane 3, 80 μg of mutant mitochondrial protein.

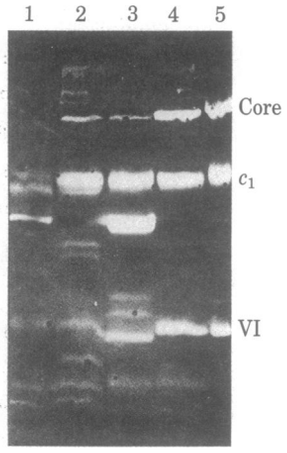

FIG. 4. Trypsin digestion of human mitochondria. Human mitochondria were dissolved in 0.4% Triton X-100/20 mM Tris·HCl, pH 7.8/200 mM NaCl at a concentration of 9.4 mg/ml. Trypsin was added (1 mg/ml) and the samples were left at room temperature for 1 hr. Control samples contained no trypsin. $NaDodSO_4$ gel electrophoresis and the Towbin blot technique were carried out as described previously. Lane 1, 160 μg of trypsin-treated mutant mitochondria; lane 2, 160 μg of mutant mitochondria (no trypsin); lane 3, 160 μg of beef heart mitochondria treated with trypsin; lane 4, 160 μg of beef heart mitochondria (no trypsin); lane 5, 1 μg of purified beef heart complex III.

tient's mitochondria and cannot account for the loss of complex III polypeptides.

DISCUSSION

Here we describe a study of the skeletal muscle mitochondria of a patient with a mitochondrial myopathy. These mitochondria have impaired respiratory function that was localized to complex III by activity measurements. The visible spectra show a greatly decreased content of cytochrome *b*.

The protein composition of the respiratory chain of the defective mitochondria has now been examined and compared with a control. This was done by antibody binding after separation of polypeptides by $NaDodSO_4$/polyacrylamide gel electrophoresis. The technique is sensitive enough to detect as little as 40 ng of a particular polypeptide and can thus be successfully employed on the very small amounts of mitochondria that can be isolated from biopsy tissue (1 g).

Most of the component polypeptides of complex III were found in greatly decreased amounts in the defective mitochondria, the exception being cytochrome c_1, which was present at the same levels as the control (as judged both by antibody binding and by spectral analysis). The four subunits of cytochrome *c* oxidase and two subunits of succinate dehydrogenase for which we had antibodies were present in near normal amounts and in an unaltered form from that in the control mitochondria.

Morgan-Hughes *et al.* (7) have recently proposed a biochemical classification of mitochondrial deficiencies. The case we describe here comes under the category of a deficiency in cytochrome *b*. However, in the present study, this classification based on spectra and activity measurements is not appropriate since the lesion extends to components of the enzyme not detected by visible spectroscopy.

Our results show that at least four components (core proteins, nFeS protein, and subunit VI) in addition to cytochrome *b* are not present in the defective mitochondria. This is consistent with complex III no longer being assembled.

Cytochrome *b* of complex III is coded for on mtDNA; the remaining components are coded for on nuclear DNA. Mutations causing deletions in the cytochrome *b* gene have been found to affect the assembly of complex III in yeast (18). As well as cytochrome *b*, the nFeS protein is missing but not cytochrome c_1, which is found in normal amount (18). When mitochondrial protein synthesis is blocked in *Neurospora* with chloramphenicol, there is no synthesis of cytochrome *b* and no assembly of complex III. Again, cytochrome c_1 is found in the mitochondria (19). These effects of altered synthesis of cytochrome *b* are remarkably similar to the alterations in complex III found in the present study.

An important feature of the patient being studied here is that the mitochondrial abnormality does not extend to all tissues. The same is true of other reported cases of similar mitochondrial disfunctions (3–7). The tissue specificity is hard to explain if the mutation is in mtDNA, implying that one (or more) of the nuclear coded proteins is altered or missing. This could be a subunit of complex III or a protein involved in the correct transcription or processing of apocytochrome *b* such as described recently by Dieckmann *et al.* (20) in yeast.

We thank H. R. Loetscher for his assistance in the ATPase assays. This study was supported by National Institutes of Health Grant HL-22050 to R.A.C.

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