Congenital Deficiency of Two Polypeptide Subunits of the Iron-Protein Fragment of Mitochondrial Complex I

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Abstract

Recently, we described a patient with severe lactic acidosis due to congenital complex I (NADH-ubiquinone oxidoreductase) deficiency. We now report further enzymatic and immunological characterizations. Both NADH and ferricyanide titrations of complex I activity (measured as NADH-ferricyanide reductase) were distinctly altered in the mitochondria from the patient's tissues. In addition, antisera against complex I immunoprecipitated NADH-ferricyanide reductase from the control but not the patient's mitochondria. However, immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of complex I polypeptides demonstrated that the majority of the 25 polypeptides comprising complex I were present in the affected mitochondria. A more detailed analysis using subunit selective antisera against the main polypeptides of the iron-protein fragments of complex I revealed a selective absence of the 75- and 13-kD polypeptides. These findings suggest that the underlying basis for this patient's disease was a congenital deficiency of at least two polypeptides comprising the iron-protein fragment of complex I, which resulted in the inability to correctly assemble a functional enzyme complex.

Introduction

The terms "mitochondrial myopathy" have been used to describe a heterogeneous group of metabolic diseases and are often characterized by morphologic alterations of mitochondria in skeletal muscle. However, morphologic criteria alone are unsatisfactory, since not all mitochondrial abnormalities manifest themselves in altered morphology; moreover, it is unclear in many cases whether morphologic changes represent the primary defect or are secondary to some other metabolic consequence. Since the original description of a mitochondrial disease by Luft et al. (1) in 1962, several lesions involving distinct segments of the mitochondrial electron transport chain have been described. Specific defects in complex I (NADH-ubiquinone oxidoreductase) (2–7), complex II (succinate-ubiquinone oxidoreductase) (8), complex III (ubiquinol-cytochrome *c* oxidoreductase) (9, 10), and complex IV (cytochrome *c* oxidase) (11–15), as well as in complex V (F_0F_1ATPase) (16), have been reported. In these investigations, enzyme activities, spectrophotometric analysis of electron carriers, and/or electron paramagnetic resonance (EPR)[1] measurements were used to define the nature of the lesions. Recently, Darley-Usmar et al. (10) used specific antisera to demonstrate the absence of certain of the polypeptide subunits of complex III, thus providing a new approach to the investigation of these mitochondrial diseases.

In an earlier paper (2) we reported clinical and biochemical studies on an infant with severe congenital lactic acidosis. Investigations of mitochondria isolated from muscle biopsy and tissues obtained at atuopsy revealed defective NAD-linked electron transport due to a specific deficiency in complex I activity. This was shown to result from the near total absence of the EPR-detectable iron-sulfur centers characteristic of complex I; the other iron-sulfur centers of the mitochondria, particularly those of complexes II and III, were normal. We postulated that this may result from an absence or genetic alteration in one or more of the 25 polypeptides comprising complex I, some of which are involved in the molecular organization and function of the iron-sulfur centers (17). This study demonstrates further enzymatic and immunologic characterization of the patient's mitochondria. Our results reveal distinct abnormalities in the kinetics of the NADH-ferricyanide oxido-reductase portion of complex I, as well as complete absence of the 75- and 13-kD polypeptides normally present in the iron-sulfur protein (IP) fraction of complex I. The data suggest the lack of these polypeptides results in incomplete or incorrect assembly of a functional complex I.

Methods

Preparation of mitochondria. Liver and heart mitochondria, as well as submitochondrial particles (SMPs) from the affected patient and from normal human tissues used as controls, were isolated and stored at −70°C as described previously (2, 18). Samples for study were thawed under warm tap water, placed on ice, and used within 2 h.

Kinetic analysis of complex I. NADH and ferricyanide titrations of complex I activity were determined as follows: frozen liver SMPs and heart mitochondria were thawed and diluted to 1 mg/ml in ice-cold 1% Triton X-100, 0.25 M sucrose, and 50 mM Tris-HCl at pH 8.0. Kinetic studies were conducted using variable concentrations of ferricyanide (0.125–2.5 mM) at a fixed concentration of 0.15 mM NADH, and variable NADH concentrations (3.5–100 M) at a fixed concentration of 0.10 mM ferricyanide. Reactions were initiated by addition of solubilized mitochondria to the complete incubation mixture after thermal equilibration. The nonenzymatic rate of ferricyanide reduction was subtracted from the initial enzymatic rate. The rate of ferricyanide reduction was

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Received for publication 31 July 1986.

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J. Clin. Invest.

0021-9738/87/02/0463/05 $1.00
Volume 79, February 1987, 463–467

1. *Abbreviations used in this paper:* EPR, electron paramagnetic resonance; IP, iron-sulfur protein; PBS-BSA, phosphate-buffered saline containing 0.5% bovine serum albumin and 0.04% sodium azide.

measured in a final volume of 1.0 ml at 410 nm in 40 mM triethanolamine buffer at pH 7.8 and 30°C.

Immunoprecipitation of NADH–ferricyanide reductase activity from solubilized liver mitochondria. Samples of beef heart SMPs and liver (or heart) mitochondria from the patient and control tissues were prepared for immunoprecipitation by diluting the freeze-thawed stock samples into ice-cold 1% Triton X-100, 0.5% sodium deoxycholate, 0.5 M KCl, 0.25 M sucrose, and 50 mM Tris-HCl at pH 8.0 at a final protein concentration of 1 mg/ml. Aliquots were then removed and diluted to a final protein concentration of 0.4 mg/ml in the same buffer containing variable quantities of antiholocomplex I antiserum or preimmune serum (final volume, 0.5 ml). After incubation on ice for 1 h the samples were centrifuged at 25,000 rpm (50,000 *g*) for 30 min at 4°C and the supernatant fractions were carefully removed and assayed for NADH–ferricyanide reductase activity in a buffer containing 1 mM ferricyanide, 0.21 mM NADH, and 20 mM potassium phosphate at pH 8.0 and 30°C. The final volume was 1.1 ml and ferricyanide reduction was followed at 410 nm.

Immunoprecipitation and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) profiles using antiholocomplex I antisera. Samples for immunoprecipitation were thawed and prepared as described in (19), except that incubation with antiserum (0.4 ml/mg of mitochondria) was carried out for only 4 h at 0°C. The immunoprecipitate (derived from 2 mg mitochondrial protein) was dissolved in 1% SDS and 4% 2-mercaptoethanol in a final volume of 100 μl, and 20-μl aliquots were applied to a linear 12.5–16% gradient gel, electrophoresed, and stained according to Laemmli (20). The protease inhibitor, *p*-aminobenzamidine, was present at 5 mM final concentration in all stages except the final solubilization in SDS. Molecular weight standards were obtained from Sigma and British Drug Houses, Poole, U. K.

Western blot analysis using specific antisera to IP fragment polypeptides. Freeze-thawed mitochondria from the patient and beef heart were pretreated with 5 mM *p*-aminobenzamidine for 10 min at 4°C, then solubilized in 1% SDS wt/vol and 1% 2-mercaptoethanol vol/vol. Approximately 50 μg of protein was applied to a linear SDS-PAGE (polyacrylamide gradient gel, 12.5–16% wt/vol) and electrophoresed at room temperature using the discontinuous buffer system described by Laemmli (20). Proteins were electrophoretically transferred at constant 30 V to nitrocellulose sheets in a buffer containing 25 mM Tris-HCl, 0.19 M glycine, 20% methanol vol/vol at pH 8.0 for 3 h at 4°C. The nitrocellulose sheets were then saturated with standard phosphate-buffered saline containing 0.5% bovine serum albumin (SIGMA, fraction V) and 0.04% sodium azide (PBS-BSA) at room temperature with continuous shaking overnight. The sheets were subsequently incubated with subunit selective antisera against the four main subunits of the IP fragment (75-, 49-, 30- and 13-kD polypeptides) or anti–complex I antisera (1:200 dilutions at 37°C for 1.5 h) and rinsed with PBS-BSA for 1.5 h (at 37°C with continuous shaking). This buffer was decanted and an equal volume of PBS-BSA buffer containing ^{125}I-labeled protein A (0.5×10^6 counts/ml, 15 μl protein A per 10 ml of buffer) was added and shaken for an additional hour at 37°C. The nitrocellulose sheet was then removed, rinsed three times with distilled water, blotted dry, and allowed to completely air dry using a low speed blower. Autoradiography was performed using Kodak XOMAT-S film at −70°C for 24–48 h with the aid of a Cronex lightning-plus intensifying screen (DuPont Instruments, Wilmington, DE).

Results

Kinetic characterization of NADH–ferricyanide reductase activity of complex I. In the previous investigation (2) we reported that NADH–ferricyanide reductase activity in the patient's mitochondria was normal, when compared with similar assays on normal human mitochondria. However, the overall NADH–ubiquinone oxido-reductase activity of the patient's mitochondria was essentially zero. This led us to suspect that the defect in complex I was localized exclusively in its iron–sulfur centers, which are required for reduction of ubiquinone. In these earlier tests NADH–ferricyanide activity was assayed using single fixed concentrations of NADH and ferricyanide. When the concentrations of NADH and ferricyanide were varied, a large difference in NADH–ferricyanide oxidoreductase activities of the patient's versus normal enzyme was noted, as shown in Figs. 1 and 2. Mitochondria from the patient's liver (and heart, not shown) showed on fixed ferricyanide very little dependence on NADH concentration and a low maximum velocity (V_{max}) (1.2 μmol/min per mg protein), whereas the normal human mitochondria yielded a V_{max} of 5.0 μmol/min per mg (Fig. 1). Similar results were obtained for variable ferricyanide at a fixed NADH concentration (Fig. 2). At concentrations of ferricyanide exceeding ~ 1.5 mM, a profound inhibition of activity occurred in both the patient and the control; this is caused by competition of ferricyanide with NADH. Thus, the original observation showing identical NADH–ferricyanide reductase activities at a fixed concentration of NADH and of ferricyanide must now be interpreted in light of these newer findings, which suggests: (*a*) an alteration in the enzymatic activity of complex I in the patient, manifest as a kinetic difference, or (*b*) a separate NADH–ferricyanide reductase activity in mitochondria or contaminating particles together with an absence of NADH–ferricyanide reductase activity attributable to complex I in the patient.

Immunoprecipitation of NADH–ferricyanide reductase activity. The kinetic studies raised the possibility that there may be a virtual absence of complex I altogether in the patient's mitochondria, since we could not identify a kinetically similar NADH–ferricyanide reductase activity comparable with the control. To address this possibility, we attempted to immunoprecipitate NADH–ferricyanide reductase activity using antiholocomplex I antisera known to immunoprecipitate NADH–ferricyanide reductase activity from beef heart SMPs (21). The results are shown in Fig. 3. Antiserum prepared against beef heart complex I also immunoprecipitates NADH–ferricyanide reductase activity from human liver (and heart, data not shown) mitochondria. In the experiment using control human liver mitochondria, ~ 65% of the NADH–ferricyanide reductase activity is immunoprecipitated, but no NADH–ferricyanide reductase activity was immunoprecipitated from the patient's liver mitochondria at any concentration of antisera. Multiple batches of antiholocomplex I antisera from several different preparations of beef heart complex I were used and none of the antisera immunoprecipitated NADH–ferricyanide reductase activity from the patient.

The observations in Figs. 1–3 were immediately reminiscent of a previously characterized NADH–ferricyanide reductase in beef heart SMPs that is unrelated to complex I activity (21). The nonimmunoprecipitable NADH-ferricyanide reductase activity from beef heart SMPs (which is ~ 10% of the total NADH–ferricyanide reductase activity) shows virtually no dependence

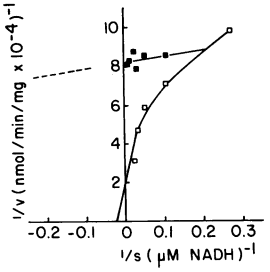

Figure 1. NADH-ferricyanide reductase activity: variable NADH concentrations at a fixed ferricyanide concentration. The assays were performed on liver mitochondria from the (*closed squares*) patient and (*open squares*) normal control as described in the Methods section. $^1/_v$, [velocity]$^{-1}$; $^1/_s$, [substrate concentration]$^{-1}$.

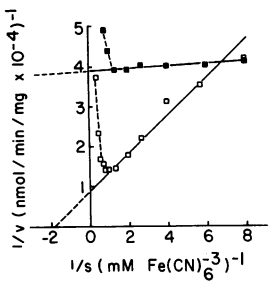

Figure 2. NADH-ferricyanide reductase activity: variable ferricyanide concentrations at a fixed NADH concentration. The assays were performed on liver mitochondria from the (*closed squares*) patient and (*open squares*) normal human control as described in the Methods section. $Fe(CN)_6^{-3}$, ferricyanide anion. $^1/_v$, $[velocity]^{-1}$; $^1/_s$, [substrate concentration]$^{-1}$.

on NADH or ferricyanide in kinetic titrations, again a result strikingly similar to the results obtained with the heart and liver mitochondria from the patient. The combined results in Figs. 1–3 illustrate several points. First, polyclonal antiserum directed against beef heart complex I also immunoprecipitates NADH–ferricyanide reductase activity from the control liver mitochondria, but not the patient's mitochondria. This implies common antigenic sites between the two sources. In addition, this is consistant with the finding that individual subunits of complex I among species as diverse as mung bean and human mitochondria are virtually identical in molecular weight (Ragan, I., unpublished observations). Second, there is an NADH–ferricyanide reductase activity associated with beef heart and human mitochondria that is not immunoprecipitated by antisera directed against holocomplex I, and this activity (left behind in the supernatant after immunoprecipitation) shows no dependence on NADH or ferricyanide in kinetic titrations. This activity is kinetically similar to the activity found in human liver mitochondria from the patient. Based on these results, we feel the NADH–ferricyanide reductase activity that shows no dependence on NADH or ferricyanide represents another enzyme activity separate from the NADH–ferricyanide reductase activity associated with complex I, and that NADH–ferricyanide reductase activity attributable to complex I is virtually absent in the patient's mitochondria.

Immunoprecipitation and SDS-PAGE profiles of human complex I. The results above suggested that NADH–ferricyanide reductase activity (attributable to complex I) was absent in the patient's liver mitochondria. This could arise from several possibilities, including a total absence of the enzyme, partial absence, or absence of essential cofactor(s). To investigate these possibilities, we used antiholocomplex I antisera to immunoprecipitate complex I and compare the SDS-PAGE profiles so obtained. The results are shown in Fig. 4. Lanes *1* and *2* represent the molecular weight markers, whereas lane *3* represents immunoprecipitated, purified beef heart complex I (this same profile is obtained when a mixture of subunit selective antisera is used for immunoprecipitation). There are 25 polypeptides comprising complex I as indicated.

Lanes *4–6* (Fig. 4) contain the immunoprecipitated human material. Lane *4* represents normal human kidney mitochondria; note the resemblance to native complex I from beef heart. Unfortunately, the presence of immunoglobulins obscures the region from 45–65 kD and prevents a detailed comparison of subunits there. However, the most remarkable results were obtained in lanes *5* and *6*. Lane *5* represents normal human liver mitochondria, and again is very similar to beef heart and virtually identical to normal kidney. Lane *6* contains the patient's mitochondria, and, surprisingly, the majority of complex I was present. Based on crude staining properties, roughly the same amount of the major subunits was present for both the normal and patient's liver mitochondria. There were some differences present, notably the near absence of a band at 75 kD in the patient's liver mitochondria, and possibly others at 39 kD, 21 kD, and 13 kD. Limited material precluded additional studies on heart or skeletal muscle mitochondria.

Although these results are highly suggestive that there may be polypeptide deficiencies in the patient's complex I, they are not conclusive. Nonetheless, several important points are apparent: (*a*) there is a remarkable similarity between the human and bovine enzyme, a result that adds to our comparative knowledge on the structure of complex I; (*b*) the identical nature of the enzyme in both normal human liver and kidney; and (*c*) the presence of a highly integrated, immunoprecipitable complex in the patient's liver mitochondria present in amounts similar to the control but devoid of both ubiquinone reductase (NADH coenzyme Q) and ferricyanide reductase activity.

Western blot analysis of the IP fragment polypeptides. The results shown in Fig. 4 documented common antigenic sites in complex I from beef heart, human, liver, and kidney mitochondria. Thus, we had reason to suspect that subunit-specific antisera might also cross-react with complex I from both sources. Furthermore, based on the EPR results reported in (2), we anticipated that the iron–sulfur clusters would be the most likely candidates for a primary alteration in subunits. Fig. 5 shows the results obtained when subunit-specific antisera generated against the purified 75-, 49-, 30-, and 13-kD polypeptides of the IP fragment of complex I were used to immunoblot SDS-separated polypeptides from the patient and control. Whole heart and liver mitochondria as well as beef heart SMPs were used in this experiment. As shown in lane *3* (Fig. 5), four major subunits are identified in beef heart SMPs. Likewise, similar polypeptides, with slightly different relative molecular masses, were identified in mitochondria from the control heart (lane *1*) and control liver (lane *4*). However, both the 75- and 13-kD polypeptides were absent in the mitochondria from both the heart and liver of the patient (lanes *2* and *5*, respectively). In addition, there is a definite indication, based on intensity of staining, of reduced amounts of the 49- and 30-kD polypeptides in the affected mitochondria. Furthermore, when only the antiserum directed against the 75-kD polypeptide was used for immunoblotting, to search for fragments of the 75-kD polypeptide that might have co-migrated with the other immuno-detected species, no degradation products of the 75-kD polypeptide were identified. Indeed, in the absence of the protease inhibitor, *p*-aminobenzamidine, the 75-kD species would characteristically undergo a single cleavage to a 37-kD

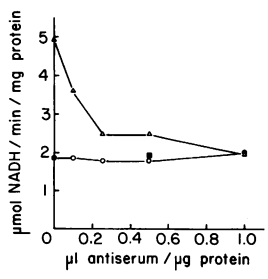

Figure 3. Immunoprecipitation of NADH-ferricyanide reductase activity from human liver mitochondria (patient and control). Liver mitochondria from the patient and control were incubated with variable amounts of antiholocomplex I antiserum and centrifuged as described in the Methods section. NADH-ferricyanide reductase activity was measured in the supernatant after centrifugation. (*Triangles*) Control human liver mitochondria; (*circles*) patient human liver mitochondria; and (*boxes*) NADH-ferricyanide reductase activity in the presence of anticomplex I antiserum in an uncentrifuged patient sample and patient sample plus preimmune serum (centrifuged and uncentrifuged).

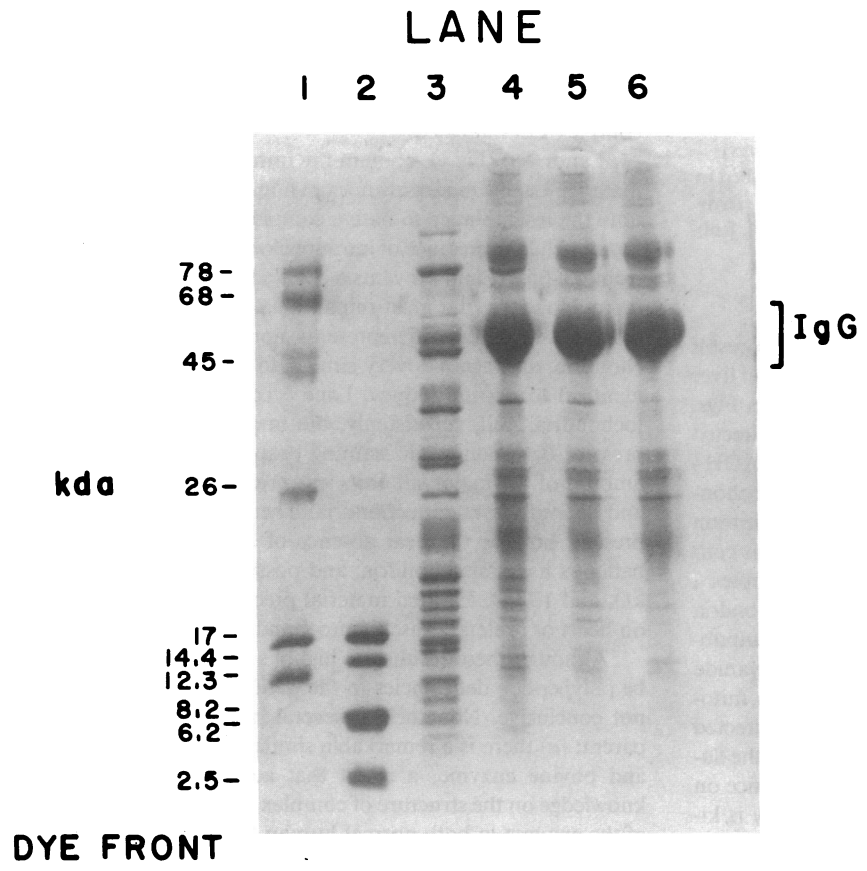

Figure 4. SDS-PAGE profiles of immunoprecipitated complex I. Samples were prepared, electrophoresed, and stained with Coomassie Blue as described in the Methods section. Lanes *1* and *2* represent molecular weight standards. Lane *3* represents purified complex I, 35 μg of protein. Lane *4* represents normal human kidney mitochondria. Lanes *5* and *6* represent normal and patient liver mitochondria, respectively. kda, kilodalton.

species in both the beef heart SMPs and human control mitochondria (data not shown). These observations demonstrate several points: (*a*) the major polypeptides of the IP fragment of human complex I cross-react with antisera raised against those of beef heart, and are similar in molecular weight; (*b*) the 75- and 13-kD polypeptides are lacking in human liver and heart mitochondria from the patient; and (*c*) there are reduced amounts of the remaining IP fragment polypeptides, 49- and 30-kD, in the affected mitochondria.

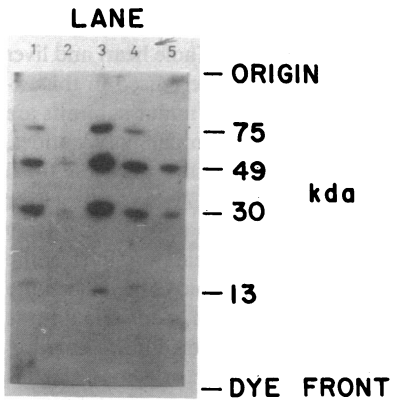

Figure 5. Western blot analysis of the IP fragment polypeptides in complex I. Beef heart SMPs, liver, and heart mitochondria from the patient and normal human tissues were separated by SDS-PAGE, transferred to nitrocellulose sheets, and immunoblotted using subunit-selective antisera against the four major polypeptides of the IP fragment. Lane *1*, 50 μg protein of normal human heart mitochondria; lane *2*, 50 μg protein of patient's heart mitochondria; lane *3*, 50 μg protein of beef heart SMPs; lane *4*, 50 μg protein of normal human liver mitochondria; and lane *5*, 50 μg protein of patient's liver mitochondria. kda, kilodalton.

Discussion

In a previous study, we identified a patient who had deficient complex I activity in four separate tissues due to an absence of the characteristic iron–sulfur clusters detected by EPR (2). In the present study, we have extended those observations to a more detailed molecular level. Using specific enzymatic and immunological techniques we have identified the absence of two of the polypeptide constituents of the IP fragment of complex I in the patient's mitochondria. We propose the underlying molecular basis for this patient's severe lactic acidosis resulted from the absence of (at least) the 75- and 13-kD polypeptides. Their absence likely results in an inability to correctly assemble either the entire polypeptide complex or, possibly, an inability to incorporate iron into a stable and functional iron–sulfur cluster, thus resulting in an inability for electron transfer to occur from NADH to ubiquinone.

Our initial observations regarding the presence of identical NADH–ferricyanide reductase activities in the patient and control, a result which led us to investigate the iron–sulfur centers by EPR, has now been shown to be incorrect but fortuitous. It is clear that a complete kinetic titration of the NADH–ferricyanide reductase activity must be used instead of a single fixed concentration of substrates. The ease with which the assay can be performed should provide a simple and reliable screening test in suspected cases of complex I deficiency.

Although our results show an absence of two of the polypeptide constituents of the IP fragment, we cannot be sure these are the only missing polypeptides in complex I. However, most of the complex I polypeptides are present in the mitochondria from the patient, and the presence of the 49- and 30-kD poly-

peptides indicates at least part of the IP fragment is present as well. Our postulate that the absence of the 75- and 13-kD polypeptide results in an inability to assemble a functional complex I cannot readily be tested, but its precedence lies in the assembly of complex III in yeast (22), where the genetic absence of cytochrome *b* (coded by mitochondrial DNA), a necessary component in complex III, results in the inability to correctly assemble functional complex III. Similar mechanisms may have been present in the case described by Darley-Usmar et al. (10).

Another intriguing aspect of our work relates to the mode of inheritance. The parents of the infant studied here showed no family history of lactic acidosis or muscle defect, and they have subsequently had a normal male child. Thus, if the lesion was transmitted via the mitochondrial genome, one would predict all children would be affected, since mitochondria are of maternal inheritance. It appears more likely that the genetic defect is autosomally recessive or was the result of a spontaneous lethal mutation. Therefore, we presume the genetic defect is one that either involves the nuclear transcription (or translation) of the 75- and 13-kD polypeptides, or involves the transport of these polypeptides (or others) into the mitochondria or to the site of assembly of complex I. The demonstration of a distinct abnormality in this case by use of subunit selective antibodies may allow prenatal diagnosis using either amniocytes or chorionic villi.

Acknowledgments

The authors wish to dedicate this work to the memory of Dr. Albert L. Lehninger.

The expert secretarial assistance of Ms. Peggy Ford is appreciated. The authors also wish to thank Dr. Perry G. McLimore and Dr. James J. Onorato for their review of the manuscript.

This work was supported by National Institutes of Health grant P01HD 10981.

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