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MINI-REVIEW

Molecular Defects of NADH-Ubiquinone Oxidoreductase (Complex I) in Mitochondrial Diseases

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Abstract

Defects in Complex I of the mitochondrial respiratory chain have been identified in 38 patients. The clinical and laboratory features are reviewed and the results of recently devised strategies aimed at characterizing the primary molecular and genetic abnormalities are presented. Although not exhaustive, these studies have provided a molecular basis for the contention that defects in Complex I may have their origin in nuclear or in mitochondrial genes.

Key Words: Molecular; Complex I; mitochondrial diseases.

Introduction

NADH-ubiquinone oxidoreductase (Complex I) catalyzes the transfer of reducing equivalents from NADH to coenzyme Q (Hatefi *et al.*, 1962; Hatefi and Stempel, 1969; Ragan and Hinkle, 1975). The reaction is coupled to vectorial transmembrane proton translocation and is inhibited by rotenone. Complex I, the largest of the respiratory chain assemblies, contains at least 25 different polypeptides as well as the nonprotein components FMN,

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nonheme iron, acid-labile sulfide, ubiquinone, and phospholipid (Heron *et al.*, 1979; Hatefi *et al.*, 1985). With chaotropic agents and ammonium sulfate precipitation the enzyme can be resolved into three separate fractions as shown in Table I (Hatefi and Stempel, 1969; Ragan *et al.*, 1982a,b; Hatefi, 1985). The water-insoluble fraction (HP) consists of 16 extremely hydrophobic polypeptides and carries one binuclear (2Fe–2S) and one tetranuclear (4Fe–4S) iron-sulfur cluster (Heron *et al.*, 1979; Ohnishi *et al.*, 1985; Hatefi *et al.*, 1985). The latter has been designated as cluster N2 and is thought to donate electrons directly to ubiquinone (Ohnishi *et al.*, 1974). The water-soluble iron-sulfur fraction (IP) has six major polypeptides with apparent molecular masses of 75, 49, 30, 18, 15, and 13 kDa (Cleeter *et al.*, 1985a; Hatefi *et al.*, 1985). The 75-kDa subunit has two binuclear iron-sulfur clusters and the fragment consisting of the 49-, 30-, and 13-kDa polypeptides carries two clusters, one binuclear and the other tetranuclear (Ragan, 1985; Oshnishi *et al.*, 1985). The soluble flavoprotein fraction (FP) has three subunits with apparent molecular masses of 51, 24, and 9 kDa. The 51-kDa subunit, which contains the NADH binding site (Chen and Guillory, 1981), is thought to carry a tetranuclear iron-sulfur cluster (Ragan *et al.*, 1982b), and a binuclear cluster with similar features to cluster N1b has been assigned to the 24-kDa subunit (Ragan *et al.*, 1982b; Ohnishi *et al.*, 1985). Little is known of the structural organization of Complex I and the order of the oxidoreduction steps. Studies using various chemical probes, however, suggest that the catalytic subunits of the FP and IP fractions are enclosed within a shell of hydrophobic proteins which interact directly with the lipid bilayer of the inner membrane (Earley and Ragan, 1980, 1981; Smith and Ragan, 1980; Ragan, 1985; Hatefi *et al.*, 1985).

Except for Complex II, the multimeric enzymes involved in electron transport and oxidative phosphorylation are biologically unique in that their component polypeptides are the products of two distinct genomes (Anderson *et al.*, 1981; Chomyn *et al.*, 1985a,b). Thirteen subunits are encoded in mitochondrial genes and are synthesized on mitochondrial ribosomes. They include seven complex I polypeptides, apocytochrome *b*, the three larger subunits of cytochrome *c* oxidase, and subunits 6 and 8 of H^+-ATPase (Chomyn *et al.*, 1985; Kroon and van den Bogert, 1987). The remainder are encoded in nuclear genes and synthesized on cytoplasmic ribosomes often as precursors with N-terminal extensions that specify their mitochondrial transport and membrane targeting (Schatz and Butow, 1983; Hay *et al.*, 1984). Cytoplasmically synthesized subunits are translocated into mitochondria via membrane contact sites before being processed, modified, and assembled into functionally integrated holoenzymes (Schleyer and Neupert, 1985; Hartl *et al.*, 1986a,b). Translocation of many of these subunits is dependent upon

the inner membrane being energized (Pfanner and Neupert, 1985). At least some of the complex I polypeptides encoded by mitochondrial DNA are thought to contribute to the hydrophobic shell that surrounds the catalytic subunits of the FP and IP fractions of the enzyme complex (Ragan, 1985; Chomyn *et al.*, 1985b). The product of the mitochondrial gene NDI, a 33-kDa polypeptide, is also thought to constitute part of the rotenone-binding site and as such is probably involved in the reduction of coenzyme Q (Earley and Ragan, 1984; Chomyn *et al.*, 1985b).

Human Complex I Deficiency—Clinical and Laboratory Features

Defects localized to Complex I have been identified biochemically in 38 patients. They can be divided into four clinical subgroups as shown in Table II. The fatal infantile form presents with a progressive lactic acidosis in the neonatal period and leads to death from cardiorespiratory failure in early infancy (Moreadith *et al.*, 1984, 1987; Robinson *et al.*, 1986; Hoppel *et al.*, 1987). The defect appears to be systemic and has been identified biochemically in liver, heart, kidney, and cultured fibroblasts as well as in skeletal muscle. Autopsy studies in two cases have also demonstrated the generalized nature of the disease process (Robinson *et al.*, 1986; Hoppel *et al.*, 1987).

Fourteen patients have presented with a myopathy characterized by weakness and severe exercise intolerance (Table II). Symptoms usually begin in childhood, but one patient was weak at birth (Roodhooft *et al.*, 1986) and another remained asymptomatic until the fifth decade (Morgan-Hughes

Table I. Composition of Beef-Heart Complex I[a]

Fraction	Number of subunits	Subunit mass (kDa)	FeS clusters	Nomenclature
Hydrophobic (HP)	16	42, 39, 35, 26, 26, 25, 23.5, 22, 22, 21, 20, 18, 16, 13.5, 8.5	2Fe–2S 4Fe–4S	— N2
Iron sulfur (IP)	6	75	2Fe–2S	—
			2Fe–2S	—
		49 + 30 + 13	2Fe–2S	—
			4Fe–4S	N4
		18, 15	—	—
Flavoprotein (FP)	3	51	4Fe–4S	N3
		24 + 9	2Fe–2S	N1b

[a]Compiled from Heron *et al.*, 1979; Capaldi, 1982; Ragan *et al.*, 1982a,b; Ohnishi *et al.*, 1985; and Hatefi *et al.*, 1985.

Table II. Complex I Deficiency: Clinical Presentations in 38 Cases

Syndrome	Number of cases	References
The fatal infantile form with progressive lactic acidosis, generalized hypotonic weakness, cardiorespiratory failure	4	Moreadith *et al.*, 1984, 1987 Robinson *et al.*, 1986 Hoppel *et al.*, 1987
Myopathy with limb weakness and exercise intolerance; pigmentary retinopathy (2 cases); cardiomyopathy (1 case); adyamia (2 cases)	14	Morgan-Hughes *et al.*, 1979, 1984, 1985, 1987 Land *et al.*, 1981 Scholte *et al.*, 1983 Arts *et al.*, 1983 Clark *et al.*, 1984 Roodhooft *et al.*, 1986
Oculoskeletal myopathy with pigmentary retinopathy; sensorineural deafness (2 cases); cardiomyopathy (2 cases)	5	Morgan-Hughes *et al.*, 1984, 1987 Sherratt *et al.*, 1984
Progressive encephalopathy; dementia (11 cases); seizures (9 cases); ataxia (8 cases); deafness (7 cases); strokes (7 cases); cardiomyopathy (5 cases); pigmentary retinopathy (3 cases); involutionary movements (4 cases); sensory neuropathy (2 cases)	15	Senior and Jungas, 1974 Prick *et al.*, 1981 Morgan-Hughes *et al.*, 1982, 1984, 1985, 1987 Hayes *et al.*, 1985 Nishizawa *et al.*, 1987 Kobayashi *et al.*, 1987 van Erwen *et al.*, 1987

et al., 1985). Two patients had occasional attacks of profound generalized paralysis and a severe metabolic acidosis which were precipitated by unaccustomed exercise, fasting, or the intake of alcohol (Morgan-Hughes *et al.*, 1979, 1987). Two other patients, identical twin brothers, had asymptomatic pigmentary retinal degeneration (Petty *et al.*, 1986; Morgan-Hughes *et al.*, 1987), and a 21-year-old girl developed unexplained congestive heart failure at the age of 11 years which resolved spontaneously after several months (Morgan-Hughes *et al.*, 1985; Wiles *et al.*, 1986).

Ptosis and extraocular weakness were major features in five cases (Sherratt *et al.*, 1984; Morgan-Hughes *et al.*, 1985, 1987). All five patients had limb weakness and pigmentary retinal degeneration which was largely asymptomatic. Additional features in some but not all cases included sensorineural deafness, cardiac conduction defects, and stunted growth.

In the remaining 15 cases, the defect predominantly affected the central nervous system (Table II). Symptoms usually appeared in childhood or early adult life, but one patient presented in infancy and died from seizures and hepatorenal insufficiency during the third year of life (Prick *et al.*, 1981).

Patients in this group presented with a variable combination of clinical features which included headaches, vomiting, mental regression, cerebellar ataxia, myoclonic or generalized seizures, visual failure, or recurrent strokes (DiMauro *et al.*, 1985, 1987; Morgan-Hughes, 1982, 1986a,b; Morgan-Hughes *et al.*, 1987). Dystonic or choreoathetoid movements occurred in some cases (Petty *et al.*, 1986) and parkinsonian features were particularly prominent in one (van Erwen *et al.*, 1987). Half the cases in this subgroup had sensorineural deafness, pigmentary retinal degeneration, or both. Cardiomyopathy occurred in five cases (Morgan-Hughes *et al.*, 1987; Nishizawa *et al.*, 1987; Kobayashi *et al.*, 1987). Most patients showed clinical or electromyographic evidence of a myopathy, but the weakness was often slight in extent and was sometimes undetectable on clinical testing.

No consistent patterns of inheritance have so far emerged (Harding *et al.*, 1988). The 38 patients investigated biochemically include two affected sib pairs (Senior and Jungas, 1974; Morgan-Hughes *et al.*, 1987), an affected mother and son (Scholte *et al.*, 1983), and an affected mother and daughter (Clark *et al.*, 1984). In the remaining 30 patients, the family history was negative in 22 and positive in 8. Five of these had similarly affected siblings (Robinson *et al.*, 1986; Hoppel *et al.*, 1987; Morgan-Hughes *et al.*, 1987), two had an affected maternal uncle (Roodhoft *et al.*, 1986; Nishizawa *et al.*, 1987), and an affected female had an affected son (Morgan-Hughes *et al.*, 1987).

An abnormal lactic acidaemia with an elevated blood lactate-to-pyruvate ratio has occurred at rest or after modest exercise in all cases. A muscle biopsy examination carried out in 34 patients showed normal histology and histochemistry in one (van Erwen *et al.*, 1987) and an excess of neutral lipid droplets in another (Prick *et al.*, 1981). In the remaining 32 cases, the muscle biopsy showed a variable proportion of ragged red muscle fibres due to mitochondrial proliferation (Morgan-Hughes and Landon, 1983). Carnitine deficiency with or without a lipid storage myopathy occurred in one-third of all cases (Morgan-Hughes *et al.*, 1985, 1987; Scholte *et al.*, 1983; Roodhooft *et al.*, 1986). Cases with major CNS disease have shown various abnormalities on CT brain scan including cerebral and cerebellar atrophy, low-density lesions in the hemispheres, and basal ganglia calcification (Petty *et al.*, 1986; Nishizawa *et al.*, 1987). In such cases, evidence for expression of the defect in the brain has also been obtained using the noninvasive techniques of positron emission tomography (Morgan-Hughes *et al.*, 1985; Frackowiak *et al.*, 1988) and ^{31}P NMR spectroscopy (Hayes *et al.*, 1985). The defect has been identified polarographically or by direct enzyme assay usually in freshly isolated muscle mitochondria. In a few cases, mitochondria from other organs have also been studied (Moreadith *et al.*, 1984, 1987; Hoppel *et al.*, 1987; Nishizawa *et al.*, 1987). As far as is possible with the material available from human cases, defects of the citric acid cycle and

other oxidative pathways have been excluded. Although in all 38 cases the main defect has been localized to Complex I, the concentration of cytochrome aa_3 or the activity of cytochrome *c* oxidase has fallen below the control range in some cases, especially those with severe loss of Complex I activity (Sherratt *et al.*, 1984; Hayes *et al.*, 1985; Roodhooft *et al.*, 1986).

Molecular Characterization of Complex I Deficiency

ESR spectroscopy and/or antibody binding techniques have been used by other workers to further characterize the defect in two cases (Moreadith *et al.*, 1984, 1987; Cleeter *et al.*, 1985b; Tanaka *et al.*, 1986). In the infant described by Moreadith *et al.* (1984) ESR spectroscopy of liver submitochondrial particles showed virtual absence of the iron-sulfur clusters characteristic of Complex I, but normal signals were obtained from other mitochondrial clusters. Kinetic studies and immunoprecipitation experiments using antibody to the beef-heart Complex I holoenzyme indicated that the NADH-ferricyanide reductase activity associated with Complex I was virtually absent in the patient's liver mitochondria. Immunoblotting of liver and heart mitochondrial proteins using subunit specific antisera to four polypeptides of the IP fragment of beef-heart Complex I showed virtual absence of the 75- and 13-kDa subunits with reduced intensity of staining of the 49- and 30-kDa polypeptides (Moreadith *et al.*, 1987). The authors postulated that the absence of the 75- and 13-kDa iron-sulfur proteins may have prevented the assembly of a functionally intact Complex I and concluded that the genetic defect may have been at the level of nuclear transcription or may have involved translocation of these subunits into the mitochondria or to the site of complex assembly. In the second patient, immunoblotting of heart mitochondrial proteins using antibody to the bovine holoenzyme showed that most Complex I subunits were reduced but that two cross-reacting bands designated subunits 1 and 10 were more severely deficient (Tanaka *et al.*, 1986). The immunoblot of Complex III was normal is this case, but Complex IV subunits were generally decreased when compared with controls. The authors concluded that the deficiency of Complex I in their case may have been due to faulty assembly of the enzyme possibly as a result of impaired biosynthesis or incorporation of mitochondrially encoded subunits.

Polypeptide Studies in 12 Patients with Complex I Deficiency

Western blot analysis has been used to further investigate the lesion in 12 patients with Complex I deficiency who have been studied in our laboratory

(Schapira *et al.*, 1986, 1988; Morgan-Hughes *et al.*, 1987). The clinical features are summarized in Table III. The disease was clinically confined to skeletal muscle in three cases (JH, MN, and KO) but affected more than one organ system in nine and caused major CNS dysfunction in five (MT, EC, PB, PD, and CC). In each case the defect was identified in mitochondrial samples freshly isolated from a biopsy of the vastus lateralis muscle as described previously (Morgan-Hughes *et al.*, 1982) except that trypsin (Sigma type III) was used instead of protease. Samples used for polypeptide analysis were pretreated with *p*-aminobenzamidine to inhibit proteolysis (see legend to Fig. 1). Muscle mitochondria from five patients with no clinical, morphological, or other laboratory evidence of mitochondrial dysfunction served as controls.

In six cases the defect was severe, pyruvate oxidation rates being reduced to less than 25% of the mean control value (Table IV). Similar oxidation rates were obtained utilizing other NAD-linked substrates such as glutamate or 3-hydroxybutyrate. Oxygen uptake rates utilizing succinate, although substantially higher than those recorded with NAD-linked substrates, ranged from 40 to 67% of the control mean in four cases (MT, MN, PB, and IC) but were normal in JH and EC (Table IV). The concentrations of cytochrome *b* and aa_3 as determined from the low-temperature spectra were also below the control range in four cases (JH, MN, EC, and PB), and in JH the aa_3 level was reduced to 20% of the control mean. In this case, however, the low aa_3 content did not appear to be rate limiting as oxidation rates utilizing succinate or ascorbate + TMPD were normal.

In the two patients with a moderately severe defect (PD and NC) pyruvate oxidation rates were 48 and 50% of the control mean. Oxidation rates with succinate or with ascorbate + TMPD were normal in these patients, but in PD the concentrations of cytochrome *b* and aa_3 were 63 and 59% respectively of the mean control values.

In the four patients with mild Complex I deficiency, oxidation rates utilizing pyruvate were between 54 and 62% of the control mean, but rates utilizing succinate or ascorbate + TMPD were within the normal range. Cytochrome aa_3 levels were below the control range in CC and KO, being 63 and 54% respectively of the control mean. Muscle carnitine levels were low in four cases (JH, MN, PD, and KO), and in two of these (JH and KO) the plasma carnitine concentration was also reduced.

Western Blot Analysis

Muscle mitochondria were thawed at room temperature and solubilized in SDS-mercaptoethanol. Mitochondrial proteins were separated by SDS

Table III. Summary of Clinical Features in 12 Patients with Complex I Deficiency (Cases Listed in Order of Severity of the Defect

Case/age/sex	Onset	Family History	Clinical features	Reference
			Severe defect	
1 MT 47 F	Teens	−ve	Myoclonus, deafness, extraocular and limb weakness, exercise intolerance	—
2 JH 21 F	Childhood	−ve	Limb weakness, exercise intolerance, episode of adynamia	Schapira *et al.*, 1988
3 MN 49 M	47	−ve	Exercise intolerance, myalgia, progressive limb weakness	Morgan-Hughes *et al.*, 1985 (case 2)
4 EC 15 M	11	−ve	Dementia, ataxia, seizures, optic atrophy, deafness, recurrent strokes, limb weakness	Morgan-Hughes *et al.*, 1987
5 PB 58 M	20's	−ve	Dementia, ataxia, deafness, neuropathy, diabetes	—
6 IC 25 M	Childhood	Twin affected (NC)	Exercise intolerance, pigmentary retinopathy	Morgan-Hughes *et al.*, 1987
			Moderate defect	
7 PD 41 F	20's	Son affected	Dementia, ataxia, deafness, seizures, pigmentary retinopathy, recurrent strokes, limb weakness	Schapira *et al.*, 1988
8 NC 25 M	Childhood	Twin affected (IC)	Exercise intolerance, pigmentary retinopathy	Morgan-Hughes *et al.*, 1987
			Mild defect	
9 KO 24 F	Teens	Mother affected	Exercise intolerance, myalgia, limb weakness	Clark *et al.*, 1984
10 PC 42 M	Teens	−ve	Ptosis, extraocular and limb weakness, exercise intolerance, pigmentary retinopathy	—
11 CC 35 M	32	Brother died of encephalopathy	Dementia, ataxia, deafness, limb weakness, exercise intolerance	—
12 NCW 32 M	Teens	−ve	Ptosis, extraocular and limb weakness, exercise intolerance, deafness, pigmentary retinopathy	Schapira *et al.*, 1988

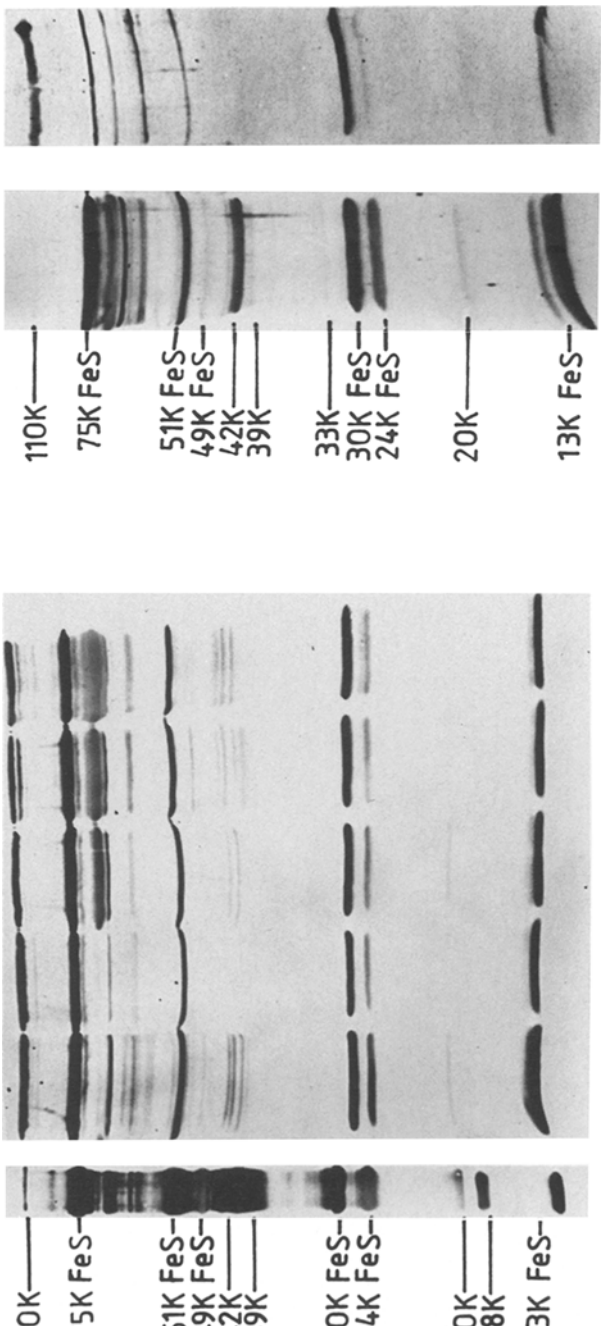

Fig. 1. Complex I immunoblots of 25 μg of purified beef-heart Complex I (track 1) and 250 μg of mitochondrial protein from control skeletal muscle (tracks 2 and 7) and patients PD (track 3), JD (track 4), PC (track 5), NCW (track 6), and JH (track 8). Mitochondria were stored in liquid nitrogen, and 5 mM aminobenzamidine was added to both samples and solubilization buffer to limit proteolysis. Samples were solubilized and run on a 12–16% linear SDS-polyacrylamide gradient using the Laemmli discontinuous buffer system (Laemmli, 1970). Proteins were then electroblotted to nitrocellulose (Towbin *et al.*, 1979) and probed with a mixture of antibody to the holoenzyme of Complex I and subunit specific antisera to the 30-, 24-, and 13-kDa FeS proteins. Bound antibody was detected with the biotin–streptavidin–horseradish peroxidase system. Equivalent protein loads of patients' mitochondrial preparations were achieved by running preliminary gels, silver staining, and integrating individual protein loads on a laser densitometer. Appropriate alterations were made to subsequent loadings to produce equal protein concentrations. Nonspecific binding to albumin, seen as a dark band at 66 kDa, occurred in those mitochondrial samples taken after use in the oxygen electrode.

Table IV. Complex I Deficiency: Polypeptide Profiles in 12 Patients

Case	State 3 pyruvate oxidation rate: %mean control[a]	Complex I	Complex III	Complex IV
		Severe defect		
1 MT	1	24 K and 13 K FeS deficiencies; generalized reduction	ND	ND
2 JH	7	24 K FeS deficiency; generalized reduction	Loss of subunit VI	Subunit II deficiency; generalized reduction
3 MN	14	24 K FeS deficiency; generalized reduction	Loss of subunit VI	Subunit II deficiency
4 EC	21	24 K FeS deficiency; generalized reduction	Normal	Normal
5 PB	21	Generalized reduction	Normal	Normal
6 IC	23	Generalized reduction	ND	ND
		Moderate defect		
7 PD	48	Generalized reduction	ND	ND
8 NC	50	Generalized reduction	ND	ND
		Mild defect		
9 KO	54	Normal	ND	ND
10 PC	59	Normal	ND	ND
11 CC	62	Generalized reduction	ND	ND
12 NCW	62	Normal	ND	ND

[a]Control mean $\pm$SD = 103 $\pm$ 18 natoms O per min per mg mitochondrial protein. n = 5. ND = not determined.

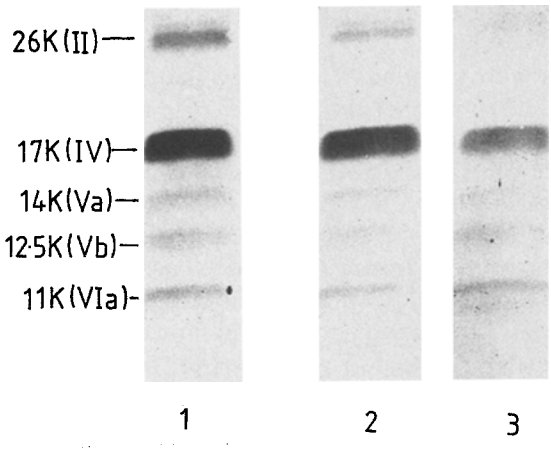

Fig. 2. Complex IV immunoblot of 150 μg of mitochondrial protein from control skeletal muscle (track 1) and patients EC (track 2) and JH (track 3). Samples were solubilized in 0.25 M Tris-HCl, pH 6.8, 2% (v/v) mercaptoethanol, 5% (w/v) SDS, and 8 M urea without boiling, and run on 12–19% linear-gradient gels as described for Fig. 1. Complex IV polypeptides were identified with antibody to the holoenzyme together with specific antiserum to subunit II.

PAGE, electroblotted onto nitrocellulose paper, and reacted with polyclonal antibody to the native beef-heart Complex I holoenzyme and also with subunit-specific antisera to six of its constituent iron-sulfur proteins (see legend to Fig. 1). The antigen–antibody reaction was identified with the biotin–streptavidin–horseradish peroxidase enzyme-linked system. Complex III and Complex IV polypeptides and muscle homogenate proteins from some of these cases were studied using similar techniques (see legend to Fig. 2).

Figure 1 (tracks 1 and 2) illustrates the significant homology that exists between the polypeptide composition of beef-heart Complex I and the human enzyme. With a mixture of holoenzyme and subunit-specific antisera, 10 cross-reacting bands were consistently identified in controls. Six of these with apparent molecular masses of 75, 51, 49, 30, 24, and 13 kDa were shown to be iron-sulfur proteins with subunit-specific antisera. The apparent molecular masses of the 75-, 51-, 49-, and 24-kDa subunits were identical in the two species. The human homologues of the 30- and 13-kDa subunits, however, show minor differences in their relative mobilities with apparent molecular masses of 29 and 13.3 kDa respectively. A seventh band of molecular mass 110 kDa probably represents the pyridine dinucleotide transhydrogenase which copurifies with Complex I. Three other cross-reacting bands with low relative staining intensities and apparent molecular masses of 42, 39, and 20 kDa have not been identified, but the 42- and 39-kDa subunits are within the molecular mass range of the mitochondrial gene products ND5 and ND4 (Chomyn *et al.*, 1985b). No significant variations in the mobilities or staining

intensities of the different cross-reacting bands were observed between the five control cases examined.

In patients with Complex I deficiency the immunoblot appearances correlated with the severity of the defect as determined polarographically. Three out of the four cases with a mild defect (KO, PC, and NCW) showed a normal profile of cross-reacting bands with relative staining intensities indistinguishable from controls (Fig. 1, tracks 5 and 6). In the remaining nine cases the staining intensities of all cross-reacting bands were decreased except for the pyridine nucleotide transhydrogenase which in some cases appeared to be increased (Fig. 1, track 8). In five of these cases (PB, IC, PD, NC, and CC) the bands corresponding to the six iron-sulfur proteins appeared to be proportionately reduced (Fig. 1, track 3). In the four most severely affected cases, there was a selective and disproportionate loss of one or two Complex I polypeptides which were identified as the 24- and 13-kDa iron-sulfur proteins in MT and the 24-kDa iron-sulfur protein in JH, MN, and EC (Fig. 1, track 8). These findings were confirmed using high protein loads and subunit-specific antisera. In patients with a generalized deficiency of Complex I polypeptides, the 49-, 42-, 39-, and 20-kDa subunits which cross-reacted weakly in controls were only just detectable on the blots and were not sufficiently intense to be reproduced in the photographic records. The immunoblots of muscle homogenate proteins from these cases were similar in profile to those obtained using purified mitochondria although cross-reacting bands appeared less intense. Some Complex III and Complex IV polypeptides were analyzed in four patients with severe Complex I deficiency and were normal in two cases (EC and PB), but JH and MN showed selective loss of subunit VI in Complex III and subunit II in Complex IV (Table IV and Fig. 2, track 3).

Discussion

Inherited defects of human Complex I may originate in either the nuclear or mitochondrial genomes. Nuclear mutations may cause loss of activity by altering the transcription or translation of cytoplasmically synthesized catalytic subunits or the polypeptides involved in posttranslational events that determine the entry of these subunits into the mitochondria or their modification and subsequent assembly into a functionally integrated holoenzyme. Nuclear genes also code for enzymes and factors involved in the transcription and translation of mitochondrial DNA so that nuclear mutations may have a profound effect upon mitochondrial gene expression. Such a mechanism is thought to be responsible for certain respiratory-deficient yeast mutants (Cabral and Schatz, 1978) and Chinese hamster cell lines

(Burnett and Scheffler, 1981; Malczewski and Whitfield, 1984). Mutations in mitochondrial DNA may similarly alter the transcription or translation of mitochondrially synthesized subunits and thereby affect enzyme assembly. A functionally abnormal mitochondrial genome may also alter the expression of certain nuclear genes although the nature of this type of intergenomic regulation has not been identified (Parikh *et al.*, 1987; Kroon and Van den Bogert, 1987). Coincidental mutations resulting in a mismatch between pairs of functionally related polypeptides encoded by each of the two genetic systems has been proposed as another hypothetical cause of respiratory-chain dysfunction (Kroon and Van den Bogert, 1987). As the authors point out, this would help to explain the apparent rarity of unequivocal mitochondrial inheritance in these cases (Rosing *et al.*, 1985) as well as the observed differences in clinical expression and in biochemical severity in affected members of the same pedigree.

In view of the multiplicity of subunits involved and the complexities of their biosynthesis and assembly, it is perhaps not surprising that limited experiments aimed at characterizing human Complex I defects at a molecular level have, so far, provided somewhat inconclusive results. Interpretation of the findings has been limited by the fact that less than half of the component polypeptides of human Complex I consistently cross-react with available antibodies, and of these only six iron-sulfur proteins can be identified with any degree of certainty. Despite these shortcomings, the different immunoblot appearances observed in the 14 reported cases would be difficult to explain on the basis of a single molecular or genetic alteration and would be more in keeping with multiple molecular etiologies. The wide differences in clinical phenotype and in biochemical severity encountered in such cases would also be consistent with this hypothesis.

The normal immunoblots obtained in three out of the four patients with a mild functional defect (KO, PC, and NCW) may be explained by the presence of a relatively small proportion of defective mitochondria in the sample examined or by the selective loss of one or more subunits which failed to cross-react with antibody but were not essential for the assembly of other Complex I polypeptides. The former hypothesis would imply heterogeneity of mitochondrial DNA in skeletal muscle. It is interesting, therefore, that a recent restriction enzyme analysis in cases PC and NCW has demonstrated two distinct populations of mitochondrial DNA in muscle (but not in blood), one of which was partly deleted (Holt *et al.*, 1988). This finding was confirmed by mapping the deleted regions with small probes derived from Hela cell mitochondrial DNA. In PC the deletion extended from at least 1307 to 5274 kb and resulted in complete loss of the coding regions for ND1 and ND2, the 16S rRNA and 5 tRNA's. In NCW it extended from at least 12,640 to 14,258 kb and involved large parts of coding regions for ND5 and ND6.

Deletions of mitochondrial DNA were not detected in any of the other patients in this series, but the family history in KO, the third patient with a normal immunoblot, was consistent with non-mendelian mitochondrial inheritance (Clark *et al.*, 1984).

Polypeptide studies in the remaining nine cases show two different patterns of abnormality (Table IV). In five cases the staining intensities of all cross-reacting bands, except for the 110-kDa polypeptide, appeared to be decreased to a similar extent, the degree of reduction being proportional to the severity of the metabolic block. This type of change would imply decreased synthesis and/or increased degradation of all identified subunits and may be due to an alteration in one or more component polypeptides which are essential for enzyme assembly but fail to cross-react with antibody. The putative subunits involved could be the products of either genome. The disease was sporadic in PB, but IC and NC were identical twins and CC had a brother who had died from a progressive encephalopathy in his early twenties. Patient PD had a son with mental regression seizures and ragged red fibers on muscle biopsy but normal mitochondrial biochemistry and a normal immunoblot (Fig. 1, track 4).

In four patients with severe Complex I deficiency, the immunoblots indicated selective loss of one or two cross-reacting bands which were identified as the 24- and 13-kDa iron-sulfur proteins in MT and the 24-kDa iron-sulfur protein in JH, MN, and EC. These polypeptides which cross-reacted strongly in controls were virtually undetectable on the blots unless very high protein loads were used. In all four cases there was an additional but less marked reduction in the other cross-reacting bands, suggesting down regulation of the genes coding for these subunits or increased proteolytic degradation possibly as a consequence of faulty enzyme assembly.

Although selective loss of a nuclearly encoded subunit such as the 24-kDa iron-sulfur protein may represent the primary molecular abnormality in these cases, other mechanisms need to be considered. It is possible, for example, that in the presence of a defect which compromises the assembly of the holoenzyme, certain iron-sulfur proteins, by virtue of their structure or position within the complex, may be more susceptible than others to proteolytic degradation. The bovine 24-kDa subunit is known to contain no hydrophobic sequences long enough to penetrate the inner mitochondrial membrane (Bahr-Lindström *et al.*, 1983), so that it may be especially vulnerable in this respect. Although such a hypothesis cannot be excluded; it would seem unlikely, however, as changes of this type were not seen in two other cases (PB and IC) with an equally severe deficiency of Complex I polypeptides.

Defective translocation into the mitochondria also seems unlikely as the deficient polypeptides were also undetectable in muscle homogenate blots. This was in contrast to the findings in another patient studied in our laboratory

who had a combined deficiency of Complex II and Complex III on biochemical investigation (Schapira *et al.*, 1987). Immunoblots of muscle mitochondrial proteins in this case using antibodies to beef-heart Complex III revealed selective absence of the Rieske iron-sulfur protein, a finding which was confirmed using high protein loads and subunit-specific antibody. Immunoblots of muscle homogenate protein in this case, however, indicated that the Rieske protein was present in an amount similar to that seen in controls. These results pointed to a defect in the translocation of the Rieske protein into the mitochondria possibly at the level of the receptor molecule on the outer membrane.

The polypeptide changes in Complex III and Complex IV which were encountered in two patients in the present series are consistent with the observation that human Complex I deficiency is sometimes associated with low cytochrome *b* or aa_3 levels or reduced cytochrome *c* oxidase activity. Such changes occurred in seven patients in the present series and have been reported in other cases (Sherratt *et al.*, 1984; Roodhooft *et al.*, 1986; Nishizawa *et al.*, 1987). Although it is likely that these changes are secondary to the defect in Complex I, it is interesting that in the two patients presented here, the deficiency involved a nuclearly encoded subunit of Complex III (i.e., subunit VI) and a mitochondrial gene product of Complex IV (i.e., subunit II). These findings illustrate the potential complexities of intergenomic regulation in these diseases and emphasize the need to examine the polypeptide components of other respiratory enzymes in patients with Complex I deficiency.

The presence of a partially deleted population of muscle mitochondrial DNA in some cases and the loss of specific nuclearly encoded iron-sulfur proteins in others provides further support for the hypothesis that defects in human Complex I may have their origin in either genome. If the loss of specific iron-sulfur proteins such as the 24-kDa subunit represent the primary molecular abnormalities in these cases—and further studies are needed to confirm this—then the observed differences in clinical phenotypes would suggest that the nuclear genes encoding for at least some of these polypeptides are expressed in a tissue-specific manner. The genetic regulation of these proteins has not yet been studied, but duplicate genes with tissue-specific control have been identified for both the bovine and human ATP synthase proteolipid, a functional component of Complex V and for the bovine ADP/ATP translocase (Gay and Walker, 1985; Walker *et al.*, 1987). Nucleocytoplasmically regulated developmental and tissue-specific isozymes of cytochrome *c* oxidase have also been demonstrated in rat tissues (Kuhn-Nentwig and Kadenbach, 1985) and may exist in man (DiMauro *et al.*, 1987). The amino acid sequence of the 24-kDa iron-sulfur protein of beef-heart Complex I is known (Bahr-Lindström *et al.*, 1983), and a complimentary DNA clone has recently been isolated (Walker, personal communication).

The development of gene probes for the nuclearly encoded Complex I subunits should provide further information about the molecular and genetic mechanisms underlying these diseases and will facilitate accurate diagnosis and genetic counseling. Such strategies will also open the way for a more rational approach to therapy (Przyrembel, 1987).

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