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Human NADH:Ubiquinone Oxidoreductase

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NADH:ubiquinone oxidoreductase consists of at least 43 proteins; seven are encoded by the mitochondrial genome, while the remainder are encoded by the nuclear genome. A deficient activity of this enzyme complex is frequently observed in the clinical heterogeneous group of mitochondrial disorders, with Leigh (-like) disease as the main contributor. Enzyme complex activity measurement in skeletal muscle is the mainstay of the diagnostic process. Fibroblast studies are a prerequisite whenever prenatal enzyme diagnosis is considered. Mitochondrial DNA mutations are found in approximately 5–10% of all complex I deficiencies. Recently, all structural nuclear complex I genes have been determined at the cDNA level and several at the gDNA level. A comprehensive mutational analysis study of all complex I nuclear genes in a group of 20 patients exhibiting this deficiency revealed mutations in about 40%. Here, we describe the enzymic methods we use and the recent progress made in genomics and cell biology of human complex I.

KEY WORDS: Mitochondria; oxphos; complex I; human; review.

INTRODUCTION

Human NADH:ubiquinone oxidoreductase (complex I), the first and largest multiprotein complex of the oxidative phosphorylation (OXPHOS) system, is an assembly product of at least 43 proteins encoded by two genomes: mitochondrial and nuclear DNA. A deficiency in the activity of this complex, first reported in 1979 (Morgan-Hughes *et al.*, 1979), is the most frequently encountered defect of the OXPHOS system and an important cause of inborn errors of metabolism in the pediatric age group (Loeffen *et al.*, 2000; von Kleist-Retzow *et al.*, 1999). The observed clinical heterogeneity of complex I deficiency means that selecting patients for mitochondrial evaluation is a significant conundrum (Chinnery and Turnbull, 1997; Thorburn, 2000). The onset of symptoms can occur from immediately after birth with severe tachypnoea to adulthood with mild exercise intolerance and virtually everything in between (Loeffen *et al.*, 2000; Pitkanen *et al.*, 1996; Robinson, 1993; Smeitink and van den Heuvel, 1999). The disease course is also variable, ranging from neonatal death to mild myopathy (Loeffen *et al.*, 2000). Determination of the enzyme complex activity in organs or tissues, preferably skeletal muscle, is the mainstay of the diagnostic process. In recent years, numerous mitochondrial as well as nuclear gene mutations have been found in conjunction with isolated complex I deficiency (Loeffen *et al.*, 2000). In this report, we summarize our current knowledge regarding clinical, biochemical, and molecular aspects related to human complex I deficiency, with special emphasis on applied enzymic methods and the recent progress made in human complex I nuclear gene and cell biology research.

CLINICAL PRESENTATION AND DISEASE COURSE

The age of the onset of the first symptoms, the course of the disease, as well as the inheritance patterns of human complex I deficiency show, like other mitochondrial disorders, great variability (Kirby *et al.*, 1999; Loeffen *et al.*, 2000; Pitkanen *et al.*, 1996; Robinson, 1993).

Recently, we re-examined the clinical and biochemical data of a cohort of isolated complex I-deficient patients (the deficiency being established at least in cultured skin fibroblasts) in whom common pathogenic

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mtDNA point mutations and major rearrangements were absent. The following distinct clinical phenotypes could be distinguished: (1) Leigh disease and Leigh-like disease, (2) macrocephaly with progressive leukodystrophy, (3) neonatal cardiomyopathy with lactic acidosis, and (4) unspecified (progressive or stable) encephalomyopathy (Loeffen *et al.*, 2000). One conclusion of this retrospective analysis is that it is extremely difficult to establish, purely based on medical history and clinical examination alone, whether or not a patient may suffer from a mitochondrial disorder and even impossible to suspect isolated complex I deficiency based solely on these items. This is mainly because of the empirical fact that the many facets with which mitochondrial disorders may present themselves are common to numerous other (pediatric) diseases. To differentiate mitochondrial disorders from other disease entities, clinicians need clues from other (laboratory, electrophysiological, and imaging) investigations, the most important of these being elevated fasting lactate to pyruvate ratio in blood and elevated lactate and alanine concentrations in body fluids (Rubio-Gozalbo *et al.*, 2000). Based on these laboratory parameters extended with, among others, the age at which the biopsy has been performed, Rubio-Gozalbo *et al.* (2000), in a retrospective study, developed a prognostic index as a diagnostic strategy in children suspected of having an OXPHOS-system disorder (Rubio-Gozalbo *et al.*, 2000). The highest prognostic index value that can be obtained shows a change of 70%, indicating that an abnormal biochemical result, among which isolated complex I deficiencies, will be found. Further studies are necessary to improve the diagnostic certainty of this index. Important in this context is, for example, that normal lactic acid concentrations in body fluids do not rule out isolated complex I deficiency (Triepels *et al.*, 1999). Whenever there is sufficient circumstantial evidence to justify further mitochondrial analysis, biochemical and pathological studies in a surgically removed fresh skeletal muscle biopsy are the golden standard. The practical implications and limitations of this statement have been recently summarized (Thorburn, 2000). In the next section we will describe the biochemical methods used in our center.

BIOCHEMICAL STUDIES

We strongly recommend that the biochemical investigations be performed immediately after removal of the muscle sample of the right vastus lateralis. This approach allows measurement of the oxidative phosphorylation capacity of the muscle mitochondria and determination of the activity of the several enzymes involved in this process. When the results of the three different approaches, i.e., assessment of mitochondrial substrate oxidation rates, ATP plus phosphocreatine production rate, and single OXPHOS enzyme complex measurements are in agreement, definite conclusions can be drawn. Here we only describe in detail the complex I rotenone-sensitive activity measurement that we use for skeletal muscle, skin fibroblasts, and native chorionic villi. Details of other methods routinely applied in mitochondrial diagnostics have been extensively described elsewhere (Fischer *et al.*, 1985; Rubio-Gozalbo *et al.*, 2000; Trijbels *et al.*, 1988, 1993).

Fresh muscle biopsies are suspended in SETH medium (0.25 *M* sucrose, 2 mM EDTA, 10 mM Tris, 5×10^4 U heparin/liter, pH 7.4). The tissue is freed from fat and connective tissue and cut into very small pieces using a Tissue Chopper. Hereafter, the skeletal muscle preparation is suspended in fresh SETH medium (10% w/v) and homogenized with a glass/Teflon Potter homogenizer at 0°C and subsequently centrifuged at $600 \times g$ at 2°C. A portion of the fresh $600 \times g$ supernatant is used for measuring oxidation rates of several ^{14}C-labeled substrates and ATP production rate from oxidation of pyruvate plus malate. The residual of the supernatant is frozen in small aliquots at −80°C until complex I and the other enzymic activities of the mitochondrial respiratory chain can be measured.

Protein content is measured according to Lowry *et al.* (1951). Citrate synthase, used as a reference enzyme, is measured according to Srere *et al.* (1969). Complex I activity is measured according to Fischer *et al.* (1985) with minor modifications: 0.08 ml $600 \times g$ supernatant is incubated in a total volume of 2.0 ml containing 0.025 mM potassium phosphate, 2.5 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 0.2 mM NADH, 2 mM KCN, 1.8 μM antimycin A, and 0.07 mM coenzyme Q_1, pH 7.4. Mitochondria are disrupted by three sonication steps of 10 s at 0°C (on a Branson B-12 sonifier with micro-tip, power output 20% of maximum value) in the incubation mixture without KCN, antimycin A, and coenzyme Q_1, with a cooling interval of 15 s between each sonication step. After sonication the incubation tube is then placed in a waterbath at 25°C for 2 min; KCN, antimycin A, and coenzyme Q_1 are added to the tube consecutively in volumes of 40, 1, and 5 μl, respectively. The rate of NADH oxidation is measured on a double-beam spectrophotometer at 340 nm at 25°C. The assay medium is equally divided between the assay and the reference cuvette. One microliter of rotenone (5 mM in ethanol absolute) is added to the reference cuvette and the decrease in absorbance measured for a few minutes. After a few minutes, 1 μl of rotenone is added to the assay cuvette; the absorbance decrease should then stop. For calculation of complex I activity, an extinction coefficient for NADH of 6.22 mM^{-1} cm^{-1} is used. We have chosen this method because, in the assay originally described by Fischer *et al.* (1985), NADH oxidation rates in $600 \times g$ supernatants from muscle tissue were often not

linear in time when measured in the absence of rotenone. This method always gives an excellent linearity of NADH oxidation rate.

In mitochondria-enriched fractions from cultured fibroblasts and homogenates from chorionic villi, complex I activity is measured according to Fischer *et al.* (1985). Human skin fibroblasts are grown. Sampling is done in parallel with the muscle biopsy in M199 (GIBCO) medium supplemented with 1% fetal calf serum. Complex I and the reference enzyme cytochrome *c* oxidase (the activity of citrate synthase can be artificially decreased during the mitochondria enrichment procedure due to leakage of the mitochondrial matrix) are measured in mitochondria-enriched fractions from frozen cell pellets. These are prepared with slight modifications as described (Attardi *et al.*, 1969). Approximately 10–15 $\times$ 10^6 cells are gently broken in a motor-driven glass/Teflon potter homogenizer in hypotonic medium (10 mM Tris-HCl, pH = 7.6; eight strikes at 1800 rpm at 0°C) subsequently made isotonic with 1.5 M sucrose. The homogenate is then centrifuged for 10 min at 600 $\times$ *g* at 0°C. Hereafter, the supernatant is centrifuged for 10 min at 14,000 $\times$ *g* at 0°C. Pellets are resuspended in 10 mM Tris-HCl (pH 7.6) and are frozen in aliquots of 70 μl. A part of the homogenate is saved to measure marker enzymes in order to calculate the mitochondrial recovery in the mitochondrial fraction.

Chorionic villi, after checking for maternal contamination according to established procedures, are suspended at 5–10% w/v in 100 mM potassium phosphate buffer (pH 7.4) and homogenized by hand with a glass/glass potter homogenizer at 0°C. Enzymic measurements (citrate synthase and complex I) are then performed with the crude homogenate.

Reference values for skeletal muscle were obtained from children and adults operated for nonmuscular disorders with informed consent. Reference values for cultured skin fibroblasts are obtained from healthy controls and those of chorionic villi from pregnancies without any risk of OXPHOS disease after informed consent. The products of all of these pregnancies, as based on clinical grounds, were not affected with an OXPHOS disorder. Table I summarizes the normal values for complex I and the applied marker enzymes for the various tissues.

Our center is considered a major reference center for mitochondrial diagnostics in the western part of Europe. On an annual basis, approximately 500 fresh and 300 frozen muscle samples as well as 200 fibroblasts cell lines are investigated. Complex I is, with 23% of all patients in which a deficiency is found, by far the most frequently encountered deficiency (Loeffen *et al.*, 2000). Most of the diseased complex I families are, based on the relatively high percentage of consanguineous marriage and exclusion of the most common mtDNA mutations associated with complex I deficiency, supposed to be affected by a nuclear gene mutation. Complex I deficiency shows a devastating disease course. No successful treatment is currently available. Therefore, the quest for prenatal enzyme diagnostics is constantly expanding. The prerequisites to which OXPHOS enzyme prenatal diagnostics has to fulfill will be reported separately (Niers *et al.*, in preparation). Thus far, we have performed complex I prenatal diagnosis in 23 pregnancies in 15 families. Without going into detail, we briefly mention that, in contrast to the results reported by the French group (Faivre *et al.*, 2000), complex I prenatal diagnosis on the enzyme level can be reliably performed. In only two of the families was there an unexplained discrepancy between the results in native and cultured chorionic villi, which seriously complicated the interpretation of the results (Schuelke *et al.*, submitted; Niers *et al.*, submitted). It goes without saying that the diagnostic reliability of prenatal diagnosis is improved by applying a molecular biological approach (Schuelke *et al.*, submitted). This is one of the main reasons why we started five years ago to characterize the human nuclear

Table I. Reference Values of NADH:ubiquinone Oxidoreductase in Different Human Tissues[a]

Tissues	Complex I (mU/mg protein)	Complex I (mU/U CS)	Complex I (mU/U COX)
Frozen skeletal muscle			
Range	5.3–19.6	84–273	78–330
Mean	12.5	154	130
SD	4.6	61	67
N	13	13	13
Fresh skeletal muscle			
Range	3.6–25.0	70–251	65–159
Mean	12.1	160	108
SD	4.8	37	29
N	14	14	14
Cultured skin fibroblasts			
Range	18.9–44.2	100–310	110–260
Mean	33.1	180	190
SD	7.05	70	50
N	14	14	14
Native chorionic villi			
Range	5.3–17.9	84–263	nm[b]
Mean	11.3	170	nm[b]
SD	3.2	47	nm[b]
N	21	21	nm[b]

[a] Complex I activity values in frozen muscle are from a homogenate. Fresh skeletal muscle complex I is measured in 600 $\times$ *g* supernatant. Cultured skin fibroblasts complex I activity is measured in a mitochondrial enriched fraction. Native chorionic villi measurements are performed in crude homogenate.

[b] nm, not routinely measured.

complex I cDNA genes and perform mutational analysis in isolated complex I deficiency.

GENOMICS

Human complex I consists of at least 43 proteins, seven encoded by mitochondrial genes (ND1–6, ND4L); the remaining proteins are encoded by nuclear genes. For details concerning the mitochondrial genome and its mutations associated with isolated complex I deficiency we refer to the many excellent reviews and recent publications written on this topic (Kirby *et al.*, 1999; Loeffen *et al.*, 2000). We only want to mention that mtDNA mutations have been found in only 5–10% of our cohort of isolated complex I deficiencies (Loeffen *et al.*, 2000; Smeitink *et al.*, 1999). Here, we focus on the recent progress made with respect to the characterization of the nuclear complex I genes and complex I genomics related to human pathology. All human nuclear cDNAs and some of the gene structures have been characterized in the last few years (Table II). This characterization process has greatly benefited from the outstanding work

Table II. Human Complex I Nuclear Genes: Present Knowledge

	cDNA sequence	nDNA sequence	Chromosomal localization	Leader sequence	Functional properties
Flavoprotein group					
NDUFV1 (*NuoF*)	+	+	11q13	+	NADH-binding,
NDUFV2 (*NuoE*)	+	+	18p11.31-p11.21	+	electron transfer
NDUFV3	+	+	21q22.3	+	Electron transfer
Iron–sulfur group					
NDUFA5	+	−	7q32		
NDUFS1 (*NuoG*)	+	−	2q33-q34	+	Electron transfer
NDUFS2 (*NuoD*)	+	−	1q23	+	
NDUFS3 (*NuoC*)	+	+	11p11.11	+	
NDUFS4	+	−	5q11.1	+	Phosphorylation
NDUFS5	+	−	1p34.2-p33		
NDUFS6	+	−	5pter-p15.33	+	
Hydrophobic group					
NDUFA1	+	+	Xq24		
NDUFA2	+	−	5q31.2		
NDUFA3	+	−	—		
NDUFA4	+	−	—		
NDUFA6	+	−	22q13.1		
NDUFA7	+	−	19p13.2		Q-binding?
NDUFA8	+	−	9q33.2-q34.11		
NDUFA9	+	−	—	+	
NDUFA10	+	−	12p	+	
NDUFAB1	+	−	16p12.3-p12.1		Acyl carrier protein motif
NDUFB1	+	−	14q31.3		
NDUFB2	+	−	7q34-35	+	
NDUFB3	+	−	—		
NDUFB4	+	−	—		
NDUFB5	+	−	—	+	
NDUFB6	+	−	9p13.2		
NDUFB7	+	−	19p13.12-q13.11		
NDUFB8	+	−	10q23.2-p23.33	+	
NDUFB9	+	+	8q13.3		
NDUFB10	+	−	16p13.3		
NDUFS7 (*NuoB*)	+	−	19p13	+	Electron transfer
NDUFS8 (*NuoI*)	+	+	11q13.1-q13.3	+	Electron transfer
NDUFC1	+	−	4q28.2-q31.1		
NDUFC2	+	−	—		
Unknown					
17.2 kDa	+		—		

[a] *E. coli* homologous of human complex I are set between parenthesis. Data are obtained from references in Emahazion *et al.* (1998); Emahazion and Brookes (1998); Loeffen *et al.* (1998); Skehel *et al.* (1998); Smeitink *et al.* (1998); Walker (1992); and (van den Heuvel and Smeitink (in press).

of Walker (1992; Skehel *et al.*, 1998), who cloned the nuclear complex I cDNAs in *Bos taurus*, the Human Genome project, and the ever-increasing possibilities for computer-based cloning of human complex I cDNA/genes. Currently, the genomic organization has been elucidated for the following genes: *NDUFV1-V3*, *NDUFS3*, *NDUFA1*, *NDUFB9*, and *NDUFS8* (de Coo *et al.*, 1995, 1997, 1999; de Sury *et al.*, 1998; Hattori *et al.*, 1995; Lin *et al.*, 1999; Procaccio *et al.*, 2000; Schuelke *et al.*, 1998; Zhuchenko *et al.*, 1996). Potential binding sites for transcription factors have been found. Interestingly, Schuelke *et al.* (1998) found a 48-bp long complete antisense homology between the 3′ UTR of the NDUFV1-mRNA and the 5′ UTR of the mRNA for the γ-interferon-inducible protein precursor IP-30. This finding is intriguing, since both genes lie on different chromosomes. The exact function of the IP-30 is not yet known, but it may play a role in γ-interferon-mediated immune reactions. The NDUFV1-mRNA might act as an antisense suppresser, thus restraining translation of IP-30 in tissues with high energy demand. This finding could be a molecular link between complex I deficiency and inflammatory myopathy, which have been repeatedly described to occur together (Schuelke *et al.*, 1998). Simultaneously with the genetic characterization of the complex I cDNA genes, our group started with mutational analysis of complex I-deficient patients. Soon after, we performed the following strategy for patient selection: (1) the isolated complex I deficiency had to be established in at least two different tissues or organs among which cultured skin fibroblasts are a prerequisite; (2) family history could not favor maternal inheritance; and (3) mitochondrial DNA analysis had to be normal. Regarding the initial selection of genes, two criteria were choosen: (1) strong evolutionary conservation; and (2) knowledge of the function of individual complex I subunits (Smeitink *et al.*, 1998). Thus far, mutations have been found in the genes encoding the NDUFS2, 4, 7, 8, and NDUFV1 proteins. Strikingly, all these mutations are present in proteins of the protruding part of the complex (Loeffen *et al.*, 2000; Triepels *et al.*, 2000). From our initial cohort of patients, approximately 40% have been genetically elucidated. The obtained results are not only of importance for genetic counseling and prenatal diagnostics, but also have extended our knowledge about some physiological aspects of the individual subunits. This will be discussed later. Compared to human complex IV, for example, knowledge of the assembly of complex I is very limited. We assume that the disease-causing genes responsible for the complex I deficiency in our cohort of patients in which no mutations have been found belong to this class of genedisorders (Procaccio *et al.*, 1999). Similar approaches used to elucidate the majority of isolated complex-IV deficiencies, like chromosome transfer, are currently being performed in complex I-deficient cell lines. The huge number of established isolated complex I deficiencies in our center every year and the amount of genes involved, makes, from a practical point of view, routine mutational analysis impossible. In order to overcome this practical limitation, we searched for approaches which could serve as a prescreening test. In collaboration with the Capaldi group, we investigated whether or not a set of new monoclonal complex I antibodies directed against structural complex I subunits are applicable in the development of a prescreening test in selecting genes for mutational analysis (Triepels *et al.*, 2000). We examined 11 patients, four with undefined complex I defects, and the remaining ones with genetically established defects in the *NDUFV1*, *NDUFS2*, *NDUFS4*, *NDUFS7*, and *NDUFS8* genes by Western blotting with these antibodies. Our results show that different mutations in the same gene give very similar subunit profiles, which differ from mutations in other genes (Triepels *et al.*, 2000). We expect that by this approach a proper selection of the gene(s) potentially responsible for the complex I deficiency is possible. Furthermore, these immunochemical experiments show that in conjunction with sucrose gradient studies and enzymic activity measurements catalytic versus assembly defects may be distinguished (Triepels *et al.*, 2001). The latter is of great importance in the selection of patient cell lines used for chromosomal transfer experiments to establish new genes for complex I deficiency.

PHYSIOLOGY AND CELL BIOLOGY OF HUMAN COMPLEX I

The physiology of normal human complex I functioning as well as the cell biological aspects of a complex I deficiency are only grossly understood. The characterization of the nuclear-encoded complex I genes, the detection of mutations in human complex I deficient cell lines, and the results of studies in lower species have provided new opportunities for detailed physiological and cell biological studies of human complex I in health and disease. This is illustrated by the results of further studies of the *NDUFS4* gene. The first complex I-deficient patient described with a nuclear gene mutation showed a 5-bp duplication in the *NDUFS4* gene, among others, resulting in the destruction of the consensus RVS phosphorylation site (van den Heuvel *et al.*, 1998). Papa *et al.* (1996) have found that in bovine heart mitochondria, the serine in the consensus site of this complex I protein can be posttranslationally phosphorylated by a cAMP-dependent protein kinase. In a collaborative work with both groups, we recently studied the cAMP-dependent phosphorylation of

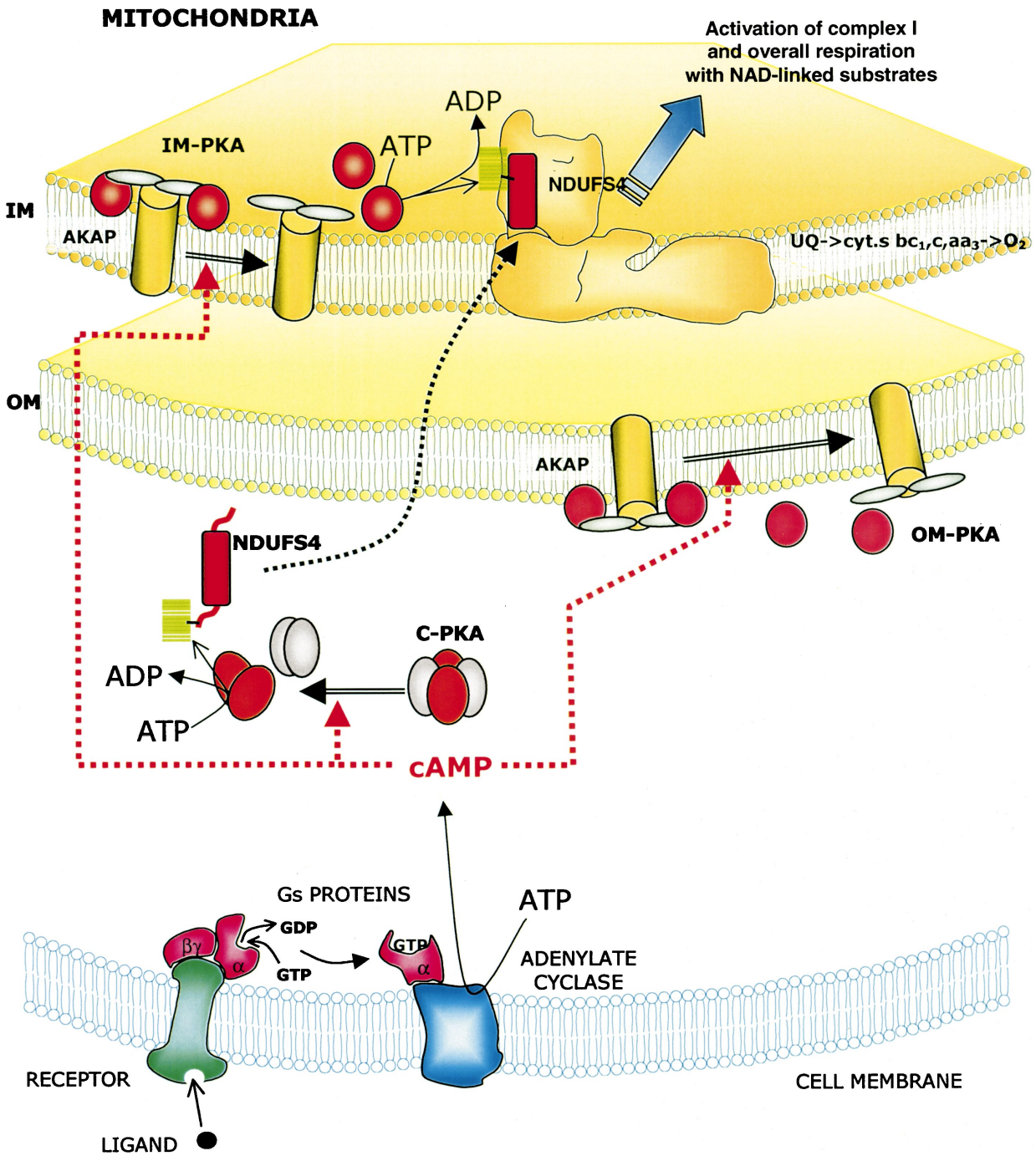

Fig. 1. Scheme of the current view of the *NDUFS4* complex I cAMP-dependent phosphorylation. A ligand, in the experimental conditions (cholera toxin), binds to a receptor of the cell membrane. Consequently, via a G-protein-mediated pathway, ATP is converted to cAMP via adenylate cyclase. cAMP, at its turn, at various subcellular locations, stimulates PKA mediated phosphorylation. Phosphorylation of the NDUFS4 (18-kDa) protein can be performed in the cytosol and in the inner mitochondrial compartment. Experimental evidence shows that following this pathway, NDUFS4 serine phosphorylation enhances activation of complex I and overall respiration with NAD-linked substrates. IM, inner mitochondrial membrane; OM, outer mitochondrial membrane; C, cytoplasmic; PKA, protein kinase A; AKAP, A-kinase anchoring protein.

this particular cell line and observed that the complex I phosphorylation and activation was abolished (Papa *et al.*, 2001). These findings provide substantial evidence showing that cAMP-mediated intracellular signal transduction, through serine phosphorylation of the NDUFS4 subunit of complex I, regulates the activity of the complex in cAMP-responsive mammalian tissues. The present knowledge is summarized in Fig. 1.

FUTURE PERSPECTIVES

The final goal of our mitochondrial research is to obtain comprehensive insight into the causes and consequences of a disturbed function of human NADH: ubiquinone oxidoreductase, enabling the development of more rational treatment strategies than is currently available. In approximately 40–50% of our initial isolated complex I-deficient patient cohort, we now know the underlying genetic defect, either in the mitochondrial genome or nuclear genome. We expect that the genetic causes in the remaining patients will be elucidated in the next few years by a combination of laboratory tools, like the rho-zero cell and chromosome transfer technique, in combination with positional and functional cloning and the knowledge obtained from the various genome projects. The results of cell biological studies in genetically determined human tissues, like cultured skin fibroblasts and myoblasts, as well as those of complex I-deficient models, like *e.g.*, *Escherichia coli*, *Saccharomyces cerevisiae*, *Yersinia lipolytica*, and mice will hopefully determine which one(s) of the factors involved in pathology are the most crucial and suitable for new forms of therapeutic intervention. Both cDNA and protein microarray technologies, in combination with bio-informatics will, as we expect, lead to a strong advancement of this essential knowledge. The expanding progress in on-line visualization of subcellular compartments, like mitochondria, the developments in the generation of molecular probes, as well as of genetic engineering, like safe vehicles for gene and enzyme replacement therapies, will be of great help in achieving the final goal—stabilizing or even curing human complex I deficiency. This goal, however, can only be reached by a strong and open collaboration between researchers from the various disciplines involved.

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