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Human Cytochrome *c* Oxidase: Structure, Function, and Deficiency

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As the terminal component of the mitochondrial respiratory chain, cytochrome *c* oxidase plays a vital role in cellular energy transformation. Human cytochrome *c* oxidase is composed of 13 subunits. The three major subunits form the catalytic core and are encoded by mitochondrial DNA (mtDNA). The remaining subunits are nuclear-encoded. The primary sequence is known for all human subunits and the crystal structure of bovine heart cytochrome *c* oxidase has recently been reported. However, despite this wealth of structural information, the role of the nuclear-encoded subunits is still poorly understood. Yeast cytochrome *c* oxidase is a close model of its human counterpart and provides a means of studying the effects of mutations on the assembly, structure, stability and function of the enzyme complex. Defects in cytochrome *c* oxidase function are found in a clinically heterogeneous group of disorders. The molecular defects that underlie these diseases may arise from mutations of either the mitochondrial or the nuclear genomes or both. A significant number of cytochrome *c* oxidase deficiencies, often associated with other respiratory chain enzyme defects, are attributed to mutations of mtDNA. Mutations of mtDNA appear, nonetheless, uncommon in early childhood. Pedigree analysis and cell fusion experiments have demonstrated a nuclear involvement in some infantile cases but a specific nuclear genomic lesion has not yet been reported. Detailed analyses of the many steps involved in the biogenesis of cytochrome *c* oxidase, often pioneered in yeast, offer several starting points for further molecular characterizations of cytochrome *c* oxidase deficiencies observed in clinical practice.

KEY WORDS: cytochrome *c* oxidase; respiratory chain; mitochondria; assembly; enzyme deficiency; Leigh's syndrome; mitochondrial myopathy (*Saccharomyces cerevisiae*, human).

INTRODUCTION

Cytochrome *c* oxidase (ferrocytochrome *c*: oxygen oxidoreductase, EC 1.9.3.1) is embedded in the mitochondrial inner membrane and forms the terminal component (complex IV) of the mitochondrial respiratory chain, the principal energy-generating system of eukaryotic cells. The enzyme is a complex metalloprotein that transfers electrons from reduced cytochrome *c* to molecular oxygen and conserves the free energy released in this exergonic reaction by maintaining a transmembrane proton gradient which is utilized to drive the synthesis of ATP or ion transport across the membrane (Hatefi, 1985). The four protons consumed in the reduction of one oxygen molecule to water are taken from the mitochondrial matrix and, coupled to this reaction, four additional protons are translocated from the matrix to the intermembrane space (Wikström, 1977, 1989). The equation of the overall reaction is

$$4 \text{ ferrocytochrome } c + 8H^+_{(\text{inside})} + O_2 = 4 \text{ ferricytochrome } c + 4H^+_{(\text{outside})} + 2H_2O$$

Over the past decade, it has become clear from amino acid sequence comparisons of subunits that the mitochondrial cytochrome *c* oxidase is a member of

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a superfamily of heme-copper containing terminal oxidases present in eukaryotes as well as prokaryotes (Calhoun *et al.*, 1994). Of the bacterial oxidases, the cytochrome *c* oxidase integrated into the cytoplasmic membrane of *Paracoccus denitrificans* is one of the most thoroughly studied. The enzyme consists of four subunits (Iwata *et al.*, 1995). Its prosthetic groups and catalytic features are almost indistinguishable from those of the mammalian enzyme (Pardhasaradhi *et al.*, 1991; Hendler *et al.*, 1991). Originally, mammalian cytochrome *c* oxidase was resolved into eight subunits by gel filtration and electrophoresis in the presence of sodium dodecyl sulfate (Steffens and Buse, 1976), but a high-resolution gel electrophoresis system developed later allowed separation into 13 subunits (Kadenbach *et al.*, 1983). The recently presented crystal structure of bovine heart cytochrome *c* oxidase assigned all 13 subunits, each in equimolar amount, in the complex (Tsukihara *et al.*, 1996), providing conclusive evidence that all are *bona fide* subunits of the enzyme. The mammalian oxidase is present as a dimer in the mitochondrial inner membrane (Capaldi, 1990) with limited contact between the monomers (Tsukihara *et al.*, 1996). The three largest subunits (I, II, and III) are encoded by mitochondrial DNA (mtDNA) and are synthesized inside the mitochondrion. The remaining subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII; nomenclature of Kadenbach *et al.*, 1983) are encoded by nuclear DNA and imported into the mitochondria. The mtDNA-encoded subunits are thought to be crucial for the catalytic functions of the enzyme because they are associated with the prosthetic groups and they are homologous to the three major subunits of cytochrome *c* oxidases found in purple bacteria such as *P. denitrificans*. There are no reports of prokaryotic subunits corresponding to any of the eukaryotic, nuclear-encoded subunits.

STRUCTURE AND FUNCTION OF SUBUNITS ENCODED BY mtDNA

More than 15 years ago, DNA sequence analysis of the human mtDNA established both the coding sequences and the primary structure of the three major subunits of human cytochrome *c* oxidase (Anderson *et al.*, 1981). The recent determination of the crystal structure of bovine heart cytochrome *c* oxidase at 2.8 Å resolution (Tsukihara *et al.*, 1996) establishes the ultimate structure of the enzyme. Subunit I spans the membrane 12 times and lacks any large extramembrane parts. Subunits II and III are associated with the transmembrane region of subunit I with no direct contact between each other. Subunit II is anchored to the membrane with an N-terminal helix-hairpin, while its large C-terminal hydrophilic domain protrudes into the intermembrane space above the surface of subunit I. The N-terminus of subunit III is on the matrix side and the subunit spans the membrane seven times with no extensive extramembrane domain.

The redox centers involved in electron transfer from cytochrome *c* to the active site are two heme A moieties (*a* and a_3) and two copper centers (Cu_A and Cu_B). Subunit I contains heme *a* and the heme a_3-Cu_B binuclear center where molecular oxygen is reduced to water (Tsukihara *et al.*, 1995). The C-terminal hydrophilic domain of subunit II contains the mixed-valence binuclear Cu_A center (Tsukihara *et al.*, 1995), the primary acceptor of electrons from cytochrome *c*. In addition to the five redox metal ions, the electron density distribution of the bovine cytochrome *c* oxidase crystals indicated a magnesium site situated between heme a_3 and Cu_A, at the interface of subunits I and II, with ligands from both subunits (Tsukihara *et al.*, 1995). The Mg^{2+} ion is generally not considered to be involved in the redox reaction but its central location suggests a mediating role in electron transfer, or alternatively, this metal ion may stabilize the interaction between subunit I and II.

The electron flow is in general assumed to proceed from cytochrome *c* to Cu_A, then to heme *a*, and finally to the heme a_3-Cu_B center (Hill, 1993) where oxygen binds and is reduced to water. The molecular mechanism by which the electron transfer reaction catalyzed by cytochrome *c* oxidase is coupled to proton pumping remains to be established but is likely to involve conformational changes near the redox centers. Although the resolved crystals provide structural information at atomic level, possible conformational changes will only become apparent when crystal structures of other oxidation states of the enzyme are available.

Activity and direct binding assays have indicated that cytochrome *c* donates electrons at a high-affinity binding site. In addition, at least one low-affinity binding site for cytochrome *c* is present per monomeric complex (Capaldi, 1990). The function of the low affinity site(s) is not clear. Binding of cytochrome *c* at the high-affinity site involves electrostatic interactions of a ring of conserved lysine residues around the heme crevice of cytochrome *c* (Capaldi, 1990). A photoaffinitive derivative of cytochrome *c*, modified

at Lys-13 near the edge of the heme crevice, binds covalently to subunit II of the bovine enzyme and inhibits electron transfer (Bisson *et al.*, 1980). Furthermore, cross-linking studies have demonstrated that a yeast cytochrome *c* derivative activated at Cys-107 on the opposite side of the molecule with respect to the heme crevice, cross-links to subunit III and inhibits electron transfer by blocking the entry site in the dimeric bovine enzyme but not in the monomer (Darley-Usmar *et al.*, 1984). Hence, the cross-linking experiments suggest that the substrate binds in a cleft of subunits II of one monomer and subunit III of the other monomer. Based on the crystal structure, Tsukihara and colleagues (1996) propose a cytochrome *c* binding site which involves subunits I, II, III, VIa, and VIb of one monomer. However, a Cu_A-containing water-soluble fragment of subunit II from *P. denitrificans* binds horse cytochrome *c* with a dissociation constant matching the intact bovine enzyme (Lappalainen *et al.*, 1995), suggesting that the high-affinity site is located mostly, if not completely, within the hydrophilic C-terminal domain of subunit II.

Subunit III does not contain any prosthetic groups. The polypeptide can be removed from the enzyme complex by a number of techniques (Capaldi, 1990) with only limited effects on the electron transfer activity, but some authors have reported a marked decrease in the proton translocation efficiency (reviewed in Prochaska and Fink, 1987). Dicyclohexyl carbodiimide (DCCD) covalently modifies a conserved glutamic acid residue in subunit III and abolishes proton pumping activity (Prochaska and Fink, 1987). However, loss of proton pumping upon removal of subunit III or binding of the bulky DCCD must be ascribed to nonspecific secondary perturbations of subunits I and II since cytochrome *c* oxidase from *P. denitrificans* has been isolated in a fully functional form that contains only subunits I and II (Hendler *et al.*, 1991), and site-directed mutants with substitutions for the conserved glutamic acid residue in *P. denitrificans* retain wild-type proton translocation activity (Haltia *et al.*, 1991). Deletion of the gene for subunit III in *P. denitrificans* leads to accumulation of assembly intermediates of cytochrome *c* oxidase associated with the cytoplasmic membrane, including a complex of subunits I and II, as well as heme-containing free subunit I and free subunit II (Haltia *et al.*, 1989). This *null* mutant has only residual cytochrome *c* oxidase activity, but the activity is coupled to proton pumping (Haltia *et al.*, 1991). These observations suggest that subunit III facilitates a late step in the assembly of the complex and may, in addition, stabilize the mature oxidase.

STRUCTURE AND FUNCTION OF SUBUNITS ENCODED BY NUCLEAR DNA

The nuclear-encoded subunits are synthesized on cytosolic ribosomes, mostly as precursors carrying N-terminal basic presequences for mitochondrial targeting and import. They are associated with the surface of the three core subunits but leave many areas uncovered (Tsukihara *et al.*, 1996). Seven of the ten nuclear-encoded subunits span the membrane with an extended α-helix (Fig. 1).

Genomic studies have disclosed the chromosomal localization for some of the human nuclear-encoded subunit genes (Table I). The gene for subunit Vb has been isolated and characterized (Lomax *et al.*, 1991; Bachman *et al.*, 1996) as well as the last exon of the gene for subunit VIb (Taanman *et al.*, 1991b). All ten nuclear-encoded subunits of the human enzyme have been sequenced at the cDNA level but despite this wealth of primary sequence data (Fig. 1) and the recent resolution of the quaternary structure of bovine cytochrome *c* oxidase (Tsukihara *et al.*, 1996), remarkably little is known about their function. Because there are no major functional differences between eukaryotic cytochrome *c* oxidase and the much simpler enzyme from prokaryotes, a direct role for the nuclear-encoded subunits in the electron transfer or proton pumping is unlikely. Kadenbach (1986) was the first to speculate that some of the nuclear-encoded subunits are involved in allosteric modification of the cytochrome *c* oxidase activity. The presence of tissue specific isoforms of some of the nuclear-encoded subunits, which may optimize the enzymatic activity for the metabolic demands of the different tissues, is often regarded to support this hypothesis. However, the pattern of isoform expression is not the same in different mammalian species (see, e.g., Linder *et al.*, 1995), raising doubts about the functional necessity of isoforms.

The ease of homologous gene replacement in haploid strains of the yeast *Saccharomyces cerevisiae* combined with the fact the organism can ferment the pyruvate formed during glycolysis (and hence can grow without functional mitochondria) have established *S. cerevisiae* as a powerful experimental organism for functional studies of cytochrome *c* oxidase. Yeast cytochrome *c* oxidase closely resembles the mammalian enzyme (Taanman and Capaldi, 1992) and

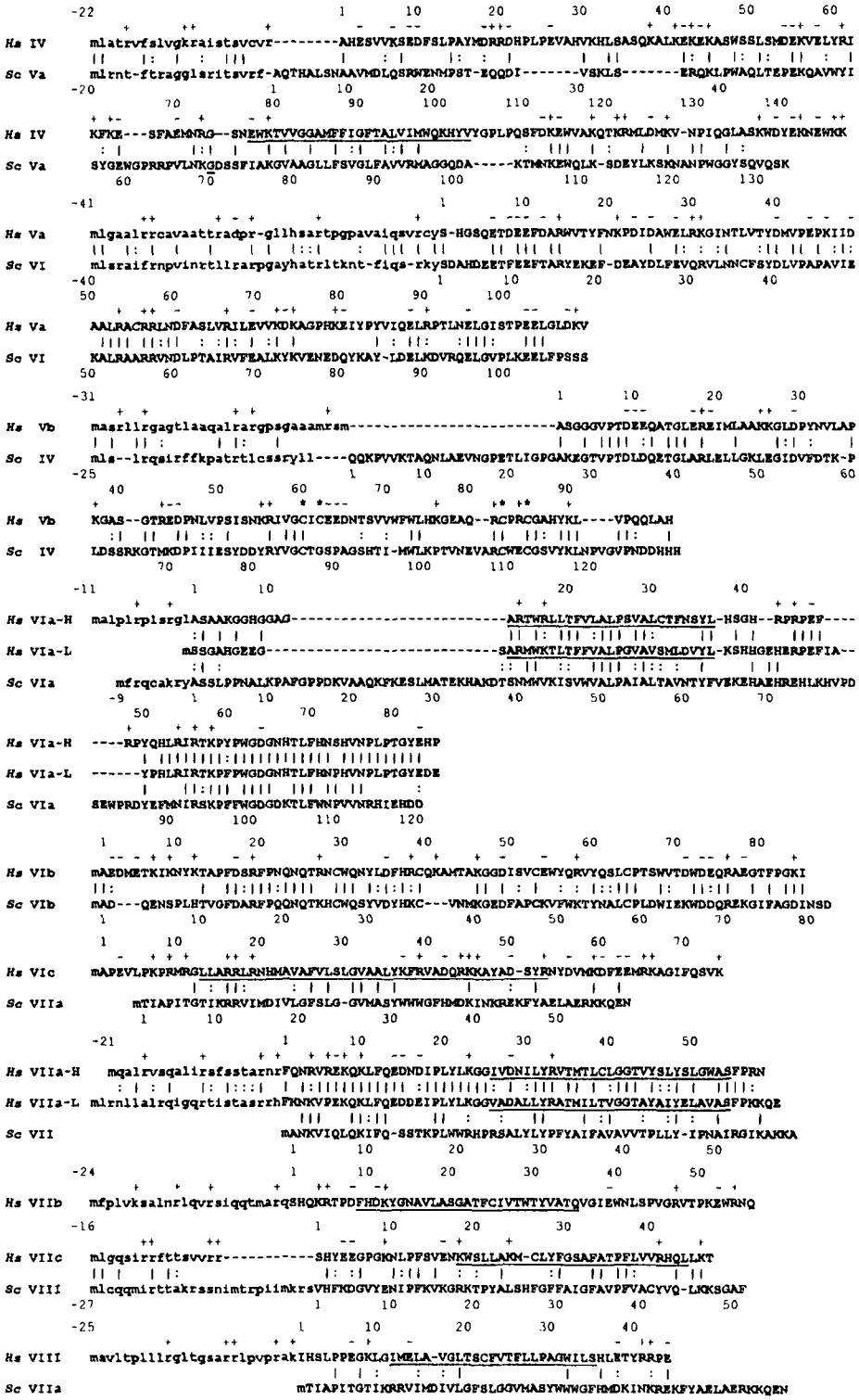

Fig. 1. Amino acid sequence alignments of nuclear-encoded cytochrome *c* oxidase subunits from humans (*Hs*) and *S. cerevisiae* (*Sc*). Presequences are in lower case, and mature polypeptides are in upper case. Hyphens in the sequence denote insertions which have been made to improve alignment. Identical amino acid residues are shown by vertical lines, and conserved replacements by colons (E:D; R:K, N:Q, A:S:T, F:Y, V:I:L). In the human polypeptides, putative membrane-spanning α-helices, based on the bovine crystal structure, are underlined, charged residues are shown, and the ligands of Zn^{2+} in subunit Vb are indicated by asterisks. Sequence data are taken from references given in Table I.

Table I. Molecular Characteristics of Nuclear-Encoded Cytochrome *c* Oxidase Subunits

Human subunit	M_r of mature polypeptide	Chromosomal localization	Yeast counterpart	Reference
IV	17,190	16q22-qter	Va, Vb	[a]
Va	12,513	—	VI	[b]
Vb	10,613	2cen-q13	IV	[c]
VIa-H	9,496	—	VIa	[d]
VIa-L	9,620	—	VIa	[d]
VIb	10,088	19q13.1	VIb	[e]
VIc	8,590	—	VIIa	[f]
VIIa-H	6,742	19	VII	[g]
VIIa-L	6,722	4q31-qter/14q21-qter	VII	[g]
VIIb	6,362	—	—	[h]
VIIc	5,356	—	VIII	[i]
VIII	4,894	11q12-q13	(VIIa)	[j]

[a] Zeviani *et al.*, 1987; Lomax *et al.*, 1990; Cumsky *et al.*, 1987.
[b] Rizzuto *et al.*, 1988; Wright *et al.*, 1984.
[c] Zeviani *et al.*, 1988; Lomax *et al.*, 1991; Maarse *et al.*, 1984.
[d] Fabrizi *et al.*, 1992; Taanman and Capaldi, 1993.
[e] Taanman *et al.*, 1990, 1991a; LaMarche *et al.*, 1992.
[f] Otsuka *et al.*, 1988; Wright *et al.*, 1986.
[g] Arnaudo *et al.*, 1992; Aggler and Capaldi, 1990.
[h] Sadlock *et al.*, 1993.
[i] Koga *et al.*, 1990; Patterson and Poyton, 1986.
[j] Rizzuto *et al.*, 1989.

will be used as comparison in the following discussion of the human nuclear-encoded subunits.

The mammalian subunit IV corresponds to yeast subunit V (Table I) although the degree of sequence conservation is low and a number of gaps have to be introduced for optimal alignment (Fig. 1). *S. cerevisiae* contains two nonidentical but similar genes for subunit V. The interchangeable isoforms, Va and Vb, have 66% primary sequence identity and disruption of the genes abolishes cytochrome *c* oxidase activity (Poyton *et al.*, 1988). Subunit Va is expressed in aerobically grown cells, whereas subunit Vb is only expressed in cells grown at low oxygen tension (Poyton and Burke, 1992). Infrared spectroscopy of carbon monoxide bound to heme a_3 has demonstrated a single conformer for the binuclear center in the apoprotein containing subunit Vb, while two discrete conformers were present in the apoprotein with subunit Va (Allen *et al.*, 1995). Isoform switching does not change the K_m for cytochrome *c*, but the enzyme with subunit Vb has an increased electron transfer rate compared to the enzyme with subunit Va (Allen *et al.*, 1995). Thus, the yeast subunit V appears to modulate the electron transfer rate by affecting the heme a_3-Cu_B environment.

Mammalian subunit Va is an extrinsic polypeptide located on the matrix side (Tsukihara *et al.*, 1996) and is homologous to yeast subunit VI (Fig. 1). Disruption of the fungal gene leads to a complete loss of cellular respiration (Poyton *et al.*, 1988), indicating an indispensable role for the subunit in the biosynthesis of the complex. On the other hand, this result precludes further analysis, and mutations leading to a milder phenotype will be required to investigate the function of this subunit in detail.

Like mammalian subunit Va, mammalian subunit Vb is also an extramembrane protein present on the matrix side (Tsukihara *et al.*, 1996). Subunit Vb is homologous to yeast subunit IV (Fig. 1). Disruption of the *S. cerevisiae* gene has been shown to prevent assembly of cytochrome *c* oxidase (Dowhan *et al.*, 1985). A yeast strain expressing a missense copy of the subunit containing three amino acid substitutions had decreased electron transfer function and decreased stability of the complex but, unlike the *null* strain, allowed normal incorporation of the hemes (Lightowlers *et al.*, 1991). Mammalian subunit Vb has a zinc site in its C-terminal domain (Tsukihara *et al.*, 1995, 1996). Three of the four cysteines that coordinate the metal ion are conserved in eukaryotes (Fig. 1), and

site-specific substitutions of Cys-109 and Cys-112 in *S. cerevisiae* impair assembly of the oxidase complex (Lightowlers *et al.*, 1991). The (mammalian) polypeptide fragment from Cys-60 to Cys-82 shows a zinc-finger motif, but the assembled subunit is unlikely to interact with double-stranded DNA, like typical zinc-finger proteins, since the domain does not protrude from the globular core of subunit Vb in the complex (Tsukihara *et al.*, 1996).

The transmembrane helix of mammalian subunit VIa interacts with subunit III, while its N-terminus is in contact with subunit I of the other monomer, an arrangement likely to stabilize the dimeric structure (Tsukihara *et al.*, 1996). There is convincing evidence for the presence of two isoforms of subunit VIa in all mammalian species investigated (Linder *et al.*, 1995), with a "liver" isoform (VIa-L) expressed ubiquitously and a "heart" isoform (VIa-H) expressed exclusively in cardiac and skeletal muscle. The degree of amino acid identity between the mature human isoforms is 62% (Fig. 1). In human skeletal muscle, subunit VIa-H appears to be the only isoform present but, in human heart, both isoforms are present in roughly equal proportions (Taanman *et al.*, 1993). Transcript analysis has indicated subunit switching from liver to heart isoform, during *in vitro* differentiation of human myoblasts and fetal development of heart and skeletal muscle (Taanman *et al.*, 1991c; Bonne *et al.*, 1993).

Conserved amino acid residues between the mammalian subunit VIa isoforms and the homologous *S. cerevisiae* subunit VIa are predominantly found in the C-terminal intermembrane space-oriented domain of the polypeptides (Fig. 1). Analysis of a *S. cerevisiae null* mutant for subunit VIa has indicated that the subunit is not necessary for assembly of the remaining complex (Taanman and Capaldi, 1993). Cytochrome *c* oxidase lacking subunit VIa shows a marked increase in electron transfer activity relative to wild-type enzyme, establishing an inhibitory function for subunit VIa (Taanman *et al.*, 1994). Covalent photolabeling of subunit VIa in yeast, and bovine heart and liver cytochrome *c* oxidases with 2-azido ATP has indicated a common nucleotide binding domain in the conserved C-terminal region (Taanman *et al.*, 1994). Binding of ATP to (yeast) subunit VIa prevents the stimulation of electron transfer activity observed in the subunit VIa-less enzyme upon binding of ATP to an unidentified second site on the complex and indicates a further inhibitory role for subunit VIa at physiologically high concentrations of ATP (Taanman *et al.*, 1994). In addition, subunit VIa from bovine heart, but not from bovine liver, was shown to bind nucleotides at its nonconserved N-terminal matrix-oriented domain (Anthony *et al.*, 1993). The presence of this nucleotide-binding site has further been suggested by the crystal structure of bovine heart cytochrome *c* oxidase (Tsukihara *et al.*, 1996). Subsequent functional studies have suggested that ATP bound to the N-terminal site of the heart isoform decouples proton pumping from electron transfer; however, minimal concentrations of ADP abolish the ATP effect (Frank and Kadenbach, 1996). Therefore, this decoupling mechanism is likely to operate only at rest and could have a function in thermogenesis of warm-blooded animals during sleep (Frank and Kadenbach, 1996).

Mammalian subunit VIb is the only globular subunit at the intermembrane space side of the complex (Tsukihara *et al.*, 1996). This polypeptide is the most conserved nuclear-encoded subunit, sharing 41% identical amino acid residues with subunit VIb from *S. cerevisiae* (Fig. 1). Two disulfide bridges are present between the conserved Cys-29 and Cys-64, and between Cys-39 and Cys-53 (Tsukihara *et al.*, 1996). The crystal structure further revealed that the region between Cys-39 and Cys-53 contacts the corresponding domain of the other monomer (Tsukihara *et al.*, 1996). Thus, together with subunit VIa, subunit VIb may have a structural role in stabilizing the native dimer.

Both mammalian and yeast subunit VIb can be readily removed after assembly with retention of cytochrome *c* oxidase activity (cf. Power *et al.*, 1984, and Taanman and Capaldi, 1992; Weishaupt and Kadenbach, 1992). A *S. cerevisiae null* strain for subunit VIb, however, has a markedly diminished cytochrome *c* oxidase activity, though optical spectra indicate that the heme groups are assembled in the oxidase complex (LaMarche *et al.*, 1992). Therefore, the subunit appears to play a role late in the assembly of cytochrome *c* oxidase. Removal of subunit VIb results in a doubling of the enzymatic activity and alters the K_m for cytochrome *c*, while proton pumping activity of the bovine complex remains unchanged (Weishaupt and Kadenbach, 1992). Hence, in addition to a role in assembly, subunit VIb appears to function as inhibitor of the electron transfer reaction.

Mammalian subunit VIc shows homology with yeast subunit VIIa (Fig. 1). Disruption of the *S. cerevisiae* gene has indicated that the polypeptide is essential for cytochrome *c* oxidase activity (Wright *et al.*, 1986), but a more detailed functional analysis has not been performed.

Subunit VIIa exists as two isoforms in many mammalian species (Linder *et al.*, 1995). Similar to subunit VIa isoforms, the "liver" isoform of subunit VIIa (VIIa-L) is found in most, if not all, tissues, while the "heart" isoform (VIIa-H) is confined to cardiac and skeletal muscle in most species. The mature human isoforms share 63% identical amino acids (Fig. 1). N-terminal amino acid sequencing identified both isoforms in preparations of human skeletal muscle and heart cytochrome *c* oxidase (90% and 55% VIIa-H, respectively; Van Beeumen *et al.*, 1990; Van Kuilenburg *et al.*, 1992). Co-expression of both subunit VIIa isoforms in human heart and partial isoform switching, from VIIa-L to VIIa-H, during myogenesis has been further indicated by transcript analysis (Taanman *et al.*, 1991c; Bonne *et al.*, 1993).

Subunit VIIa can be removed by the detergent Triton X-100 in alkaline environment with minimal effect on electron transfer activity (Pentillä, 1983), but inhibition of subunit VIIa expression with antisense oligodeoxynucleotides has suggested that the subunit is required for biosynthesis of active cytochrome *c* oxidase (Chranowska-Lightowlers *et al.*, 1993). Furthermore, deletion of the gene for the homologous subunit in *S. cerevisiae* (subunit VII; Fig. 1) causes respiratory deficiency and a lack of spectrally detectable hemes (Aggeler and Capaldi, 1990; Calder and McEwen, 1991). Western blot and pulse-chase analyses of a yeast *null* strain have indicated a decreased rate of translation of mRNA for subunit I or a very rapid rate of degradation of nascent subunit I (relative to the accumulation of subunits II and III), while the remaining nuclear-encoded subunits were not substantially affected (Calder and McEwen, 1991). In addition, immunoprecipitation experiments have shown a markedly decreased association of subunit IV with subunits I and II (Calder and McEwen, 1991). Taken together, these results suggest a role for subunit VIIa in assembly of the holocomplex, possibly involving the proper folding and/or heme incorporation of subunit I.

No polypeptide corresponding to the mammalian subunit VIIb has been found in lower eukaryotes, and the role of this subunit in cytochrome *c* oxidase from vertebrates is completely unknown.

Mammalian subunit VIIc exhibits homology with yeast subunit VIII (Fig. 1). In a *S. cerevisiae null* strain for subunit VIII, respiration and cytochrome *c* oxidase activity was reduced by only 20% compared to the parental strain (Patterson and Poyton, 1986), indicating that the presence of this subunit in yeast is not required for assembly or homeostasis of the complex *per se* but is needed for optimal functioning of the holoenzyme.

Two tissue-specific isoforms of subunit VIII have been identified in beef, rat, and dog, but only one form appears to occur in humans, sheep, and rabbit (Linder *et al.*, 1995). Surprisingly, the amino acid sequence of the human subunit VIII is closely related to that of the bovine "liver" isoform (Rizzuto *et al.*, 1989), whereas the sequences of sheep and rabbit subunit VIII show a better match with the bovine "heart" isoform (Linder *et al.*, 1995). Mammalian subunit VIII can be aligned with subunit VIIa from *S. cerevisiae*, but the yeast subunit shares significantly more identical amino acid residues with mammalian subunit VIc (Fig. 1).

CYTOCHROME *c* OXIDASE DEFICIENCIES

Cytochrome *c* oxidase deficiency will not only affect terminal oxidation but will block the entire energy-transducing pathway in mitochondria. A complete deficiency of cytochrome *c* oxidase is, therefore, not observed clinically, but reports of cases with a partial deficiency are numerous. Disorders can be congenital or late-onset in nature, and may be restricted to a few tissues or affect all tissues (reviewed in DiMauro *et al.*, 1990).

Cytochrome *c* Oxidase Deficiencies Caused by Mutations of mtDNA

The mitochondrial genome is transmitted maternally and contains genes exclusively involved in the biosynthesis of ATP synthase, cytochrome *c* oxidase, and other complexes of the respiratory chain (Attardi and Schatz, 1988). Partial cytochrome *c* oxidase deficiencies of adolescent or adult onset are often associated with specific point mutations in tRNA genes or rearrangements in a subpopulation of the mtDNA (reviewed in: Ballinger *et al.*, 1994). For instance, in patients with ocular myopathy as a key feature, as in chronic progressive external ophthalmoplegia and Kearns–Sayre syndrome, the focal defect of cytochrome *c* oxidase in individual muscle fibers has been attributed to single large-scale deletions or duplications in mtDNA. Reported deletions vary in size and location but normally include several structural genes of respiratory chain complexes as well as unique tRNA genes.

Heteroplasmy, or the co-existence of mutant and wild-type mtDNA within the same cell, allows the

otherwise lethal mutation to persist. Molecules of mtDNA with large-scale deletions or tRNA point mutations may still be transcribed, but the presence of a high proportion of mutant mtDNA molecules is expected to produce a global defect of mitochondrial protein synthesis due to the lack of sufficient tRNAs (Shoubridge, 1994). Not surprisingly, the partial cytochrome *c* oxidase deficiency observed in these patients is frequently associated with deficiencies of other enzyme complexes containing mtDNA-encoded subunits. On the other hand, reported missense point mutations and a micro-deletion in the (mitochondrial) gene for subunit III of cytochrome *c* oxidase (Johns and Neufeld, 1993; Manfredi *et al.*, 1995; Keightley *et al.*, 1996) are likely to result in isolated cytochrome *c* oxidase deficiencies due to a disruption in assembly or decreased stability of the holoenzyme.

The variable tissue involvement observed in patients with mtDNA abnormalities is generally ascribed to tissue-specific difference in dependence of mitochondrial energy and tissue-specific mutation loads (Ballinger *et al.*, 1994). Mutant mtDNA appears to accumulate with age and symptoms are progressive. Point mutations of mtDNA frequently exhibit maternal inheritance. In contrast, mtDNA rearrangements are generally sporadic, although a variant has been described with multiple deletions of mtDNA which is transmitted in an autosomal dominant mode (see, e.g., Zeviani *et al.*, 1989). An additional abnormality of mtDNA leading to partial cytochrome *c* oxidase deficiency is not qualitative but quantitative and is represented by infants suffering mtDNA depletion syndrome (see, e.g., Moraes *et al.*, 1991). The depletion is progressive but appears to be mainly limited to skeletal muscle, and liver or kidney. Family histories of patients with mtDNA depletion syndrome are compatible with an autosomal inheritance of the trait.

Diagnosis of cytochrome *c* oxidase deficiency is generally made by histochemical and biochemical analysis of the tissues involved, as well as analysis of mtDNA. More detailed diagnosis can now be performed at the protein level with subunit-specific monoclonal antibodies (Taanman *et al.*, 1993, 1996). Figure 2 compares cultured myoblasts from a patient harboring a mtDNA $tRNA^{Glu}$ point mutation (Hanna *et al.*, 1995) with control cells. Myoblasts were cultured in medium containing Mitotracker™ CM-H_2 XRos (Molecular Probes, Inc.) to label mitochondria fluorescent red. Then, cells were subsequently fixed, permeabilized, and immunostained fluorescent green with fluorescein isothiocyanate-labeled monoclonal antibodies against cytochrome *c* oxidase, while nuclei were stained fluorescent blue with bis-benzimide (Hoechst 33258). Control cultures show a uniform expression of cytochrome *c* oxidase subunits I, IV, and VIc in all cells (Fig. 2B, D, F), as demonstrated by the brightly yellow-colored mitochondrial network in the cells (red + green = yellow). On the other hand, the patient's cells show more or less reduced levels of all cytochrome *c* oxidase subunits (Fig. 2A, C, E), as indicated

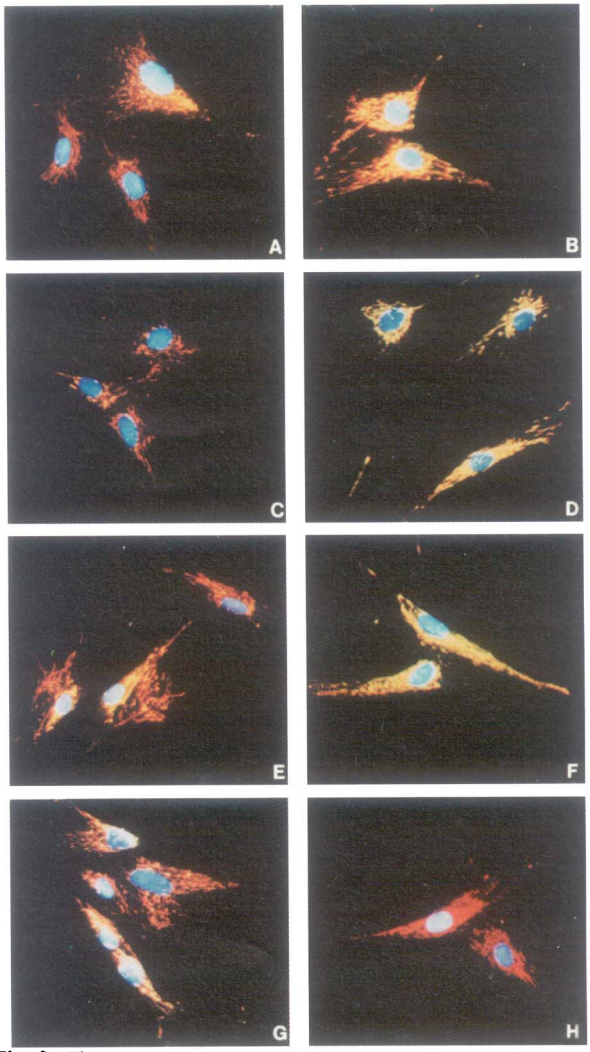

Fig. 2. Fluorescence micrographs comparing myoblast cells from a patient harboring a mtDNA $tRNA^{Glu}$ point mutation (panels, A, C, E) and a patient with depleted levels of mtDNA (panel G) with control myoblasts (panels B, D, F, H). Cells were triple-stained for mitochondria (red fluorescence), nuclear DNA (blue fluorescence), and subunits I (panels A, B, G), IV (panels C, D), or VIc (panels E, F) of cytochrome *c* oxidase (green fluorescence). Control cells in panel H were only stained for mitochondria and nuclear DNA.

by the shift from yellow to red. Similar results are obtained with myoblast cells from a patient with depleted levels of mtDNA (Fig. 2G).

Cytochrome *c* Oxidase Deficiencies Caused by Mutations of Nuclear DNA

In infants, the clinical phenotypes of cytochrome *c* oxidase deficiency fall into two main categories. In one group of patients myopathy is the principal manifestation, but many of these patients have additional renal disease (De Toni–Fanconi syndrome). Another group of patients have a multi-system disorder, dominated by dysfunction of the central nervous system (DiMauro *et al.*, 1990). This group is typically represented by infants suffering Leigh's syndrome, which is a progressive neurodegenerative disorder, characterized by subacute necrotizing encephalomyelopathy. The syndrome can result from several inborn errors of energy metabolism, including cytochrome *c* oxidase deficiency (see, e.g., Rahman *et al.*, 1996).

Pedigree analysis of families with infantile cytochrome *c* oxidase deficiency generally indicate an autosomal recessive mode of inheritance. With the exception Pearson's syndrome (Rötig *et al.*, 1991) and a maternally inherited form of Leigh's syndrome (Tatuch *et al.*, 1994), qualitative defects of mtDNA are normally not found in early childhood. This suggests that the cytochrome *c* oxidase defects in this age group are predominantly due to nuclear mutations, though such mutations have not yet been identified.

In order to determine which genome is primarily involved, cell hybridization experiments are increasingly being used, among others, to demonstrate a nuclear involvement in cytochrome *c* oxidase-deficient Leigh's syndrome (Miranda *et al.*, 1989; Tiranti *et al.*, 1995). Tiranti and colleagues (1995) generated two lines of transmitochondrial cybrids by fusing enucleated cell cultures with cultures experimentally depleted of mtDNA (ρ^0 cells). In the first combination, transformed ρ^0 fibroblasts derived from a patient with cytochrome *c* oxidase-deficient Leigh's syndrome were fused with enucleated control fibroblasts, while in the second combination, enucleated fibroblasts from the patient were fused with transformed ρ^0 control cells. The cytochrome *c* oxidase deficiency was preserved in the first but not in the second combination, demonstrating a nuclear origin for the disease as a new nuclear environment is able to correct the defect in the case studied.

In the three patients with cytochrome *c* oxidase-deficient Leigh's syndrome studied by Lombes and colleagues (1991), the partial enzyme deficiency appeared to be related to a reduced amount of otherwise normal enzyme which had a K_m for cytochrome *c* similar to values found in controls. In another study, however, a higher K_m for cytochrome *c* was found in a patient with cytochrome *c* oxidase-deficient Leigh's syndrome (Zimmermann and Kadenbach, 1992). Furthermore, an infant with severe mitochondrial encephalomyopathy was recently shown to have normal levels of all cytochrome *c* oxidase subunits, but kinetic studies revealed a decreased electron transfer and an increase in K_m for cytochrome *c* (Nijtmans *et al.*, 1995). DNA sequencing of the gene for subunit II, the primary binding site for cytochrome *c*, did not reveal any sequence abnormalities, but the presence of mutations in other cytochrome *c* oxidase genes was not investigated (Nijtmans *et al.*, 1995).

In the population of northeastern Quebec, particularly in the Saguenay-Lac-Saint-Jean region, a unique form of cytochrome *c* oxidase deficiency exists (Morin *et al.*, 1993). The patients have very low cytochrome *c* oxidase activities in brain and liver, while skin fibroblasts, amniocytes, and skeletal muscle show a 50% reduction, and kidney and heart tissue give near normal values (Merante *et al.*, 1993). Western blot analyses suggested a general decrease of cytochrome *c* oxidase subunits, proportional to the decrease in cytochrome *c* oxidase activity (Merante *et al.*, 1993). The tissue profile of cytochrome *c* oxidase deficiency does not match the pattern of the isoform expression of subunits VIa or VIIa. Therefore, it is not surprising that cDNA sequence analysis failed to reveal any mutations in the coding sequences of subunits VIa-L and VIIa-L, nor that Northern blot experiments indicated normal transcription of the genes (Merante *et al.*, 1993). Segregation analysis was consistent with an autosomal recessive mode of inheritance, and genealogical reconstruction of the family trees suggested that the nuclear gene defect was introduced in the French-Canadian population by early settlers (Morin *et al.*, 1993).

Although the tissue-specific phenotype of cytochrome *c* oxidase defects are difficult to explain by a subunit isoform deficiency in children with a predominant neurological presentation, deficiency of an isoform may provide a molecular basis for the occurrence of certain cytochrome *c* oxidase-deficient myopathies of infancy. Clinically, there are two forms of cytochrome *c* oxidase-deficient infantile myopathies, a fatal and a benign form (DiMauro *et al.*, 1990). In

both forms, infants have little or no cytochrome *c* oxidase activity in skeletal muscle but often normal levels in other tissues. While the fetal form has a relentlessly downhill clinical course and leads to early death, in the benign myopathy, the low cytochrome *c* oxidase activity in skeletal muscle at birth spontaneously progresses to normal levels in 1–3 years. Immunohistochemical analysis has shown a specific deficiency of subunits VIIa and/or VIIb in both groups of patients, while patients affected by benign myopathy showed an additional lack of immunodetectable subunit II (Tritschler *et al.*, 1991). This result contradicts an earlier study in which a general decrease of cytochrome *c* oxidase subunits was observed in muscle mitochondria from a patient with the fatal form (Bresolin *et al.*, 1985), but a more recent investigation did demonstrate a specific deficiency of the subunit VIIa-H isoform in skeletal muscle from an infant with the fatal form (Possekel *et al.*, 1995). The expression of subunit VIa isoforms has not been investigated in any of these cases.

The condition in children with the malignant form could in principle be caused by a mutation in the gene for subunit VIa-H or VIIa-H, which would profoundly affect skeletal muscle function but not necessarily impair the performance of the heart, given the large proportion of VIa-L and VIIa-L isoforms in adult heart tissue. Still, such a mutation may explain why some patients with fatal infantile myopathy have an associated cardiomyopathy, with the defect in skeletal muscle being more severe than in heart (DiMauro *et al.*, 1990). The benign form, however, is more difficult to explain by subunit isoform deficiency. Because the enzyme defect is not expressed in liver, deficiency of a liver isoform is unlikely, and because of the spontaneous remission of symptoms, deficiency of a heart isoform is also unlikely. Possibly, an as yet unidentified neonatal specific isoform plays a role in the pathogenesis or, alternatively, a defect in a (post)transcriptional regulation mechanism accounts for a deficiency of a liver-type isoform during early skeletal muscle development. This deficiency would then be corrected once the heart-isoforms are fully expressed in the developing muscles.

PROSPECTS

The biogenesis of cytochrome *c* oxidase is controlled by many factors. All are in principle subject to genetic lesions and may lead to cytochrome *c* oxidase deficiency. Mutations of mtDNA affecting either directly or indirectly the synthesis of the three core subunits have now been documented in many patients. Mutations of nuclear genes encoding the ten smaller subunits have not yet been identified but are likely to occur. In *S. cerevisiae*, nuclear gene disruptions have demonstrated that six of the eight associated polypeptides in yeast are indispensable for assembly of the remaining enzyme complex. In humans, lack of subunit VIa-H or VIIa-H isoforms may explain fatal infantile cytochrome *c* oxidase-deficient (cardio)myopathies, but deficiency of the other subunits is likely to cause a general cytochrome *c* oxidase defect and, therefore, an embryo which is not viable. More subtle mutations, however, may lead to milder phenotypes. Missense mutations in structural genes of cytochrome *c* oxidase may result in a decreased assembly or stability of the complex, or may alter the electron transfer rates or affinity of the enzyme for cytochrome *c* as observed in some patients.

Deletion analysis of nuclear genes coding for mitochondrial proteins have revealed a mosaic of *cis*-acting upstream and intron sequence elements required for maximal transcription (reviewed in Scarpulla, 1996). Consequently, not only mutations in the coding regions of the structural genes may affect cytochrome *c* oxidase function but also mutations in these transcriptional regulatory elements and mutations in the *trans*-acting factors may result in decreased subunit synthesis and eventually lead to decreased enzyme levels.

Cytochrome *c* oxidase gene expression appears to be at least in part post-transcriptionally regulated, involving glutamate dehydrogenase interacting with the 3′ untranslated region of "liver" isoform transcripts (Preiss and Lightowlers, 1993; Preiss *et al.*, 1995). This interaction appears to be tissue and developmental specific (Preiss *et al.*, 1994; Schillace *et al.*, 1995), and offers a potential mechanism for tissue-specific cytochrome *c* oxidase deficiencies.

Complementation analyses of cytochrome *c* oxidase-defective mutants of *S. cerevisiae* have identified several nuclear genes that provide an essential function at a post-translational stage of the biogenesis of cytochrome *c* oxidase (Schulze and Rödel, 1989; Nobrega *et al.*, 1990; Tzagoloff *et al.*, 1990; McEwen *et al.*, 1993; Bonnefoy *et al.*, 1994a; Glerum *et al.*, 1995, 1996a; Church *et al.*, 1996). Two of the gene products appear to be involved in heme A biosynthesis (Tzagoloff *et al.*, 1993), while two other products seem to have a role in recruitment of the copper prosthetic

groups of cytochrome *c* oxidase (Glerum *et al.*, 1996a, b). Human homologs of two of these yeast genes have been isolated by functional complementation of the yeast *null* mutant (Glerum and Tzagoloff, 1994; Bonnefoy *et al.*, 1994b). Genetic lesions in the human homologs may provide additional explanations for cytochrome *c* oxidase deficiencies.

Finally, mutations in genes controlling the many steps involved in mitochondrial protein import, protein folding, and assembly of the respiratory chain complexes may also result in cytochrome *c* oxidase deficiencies in combination with deficiencies of other mitochondrial enzymes. Again, groundbreaking work has been performed in fungal systems (Stuart *et al.*, 1994; Lithgow *et al.*, 1995), but it is already clear that defects at these stages may cause cytochrome *c* oxidase deficiency. This is illustrated by an infant with markedly decreased steady-state levels of heat shock protein 60, a mitochondrial molecular chaperone involved in re-folding of polypeptides imported into the mitochondrial matrix. Cultured skin fibroblasts of the patient showed low activities of all examined matrix and inner membrane-associated enzymes, including cytochrome *c* oxidase (Agsteribbe *et al.*, 1993).

Given the many steps involved in the biogenesis of cytochrome *c* oxidase, the clinical heterogeneity of cytochrome *c* oxidase disorders is hardly surprising. The conventional approach to diagnosis, using enzyme assays or histochemistry, measures total enzyme activity and gives little additional information about the primary genetic basis of any abnormality or its prognosis. I expect that, in future, traditional methods will increasingly be followed up by a dissection of the deficiency with subunit-specific antibodies, cDNA probes, and cell fusion experiments to determine the genetic origin. Research will take advantage of the rapid progress being made in yeast and, where appropriate, mutations found in patients will be introduced in yeast to investigate their pathogenic significance. These detailed case studies will not only help to understand the molecular mechanisms of the enzyme defect but also provides an opportunity to study aspects of the biogenesis of cytochrome *c* oxidase in humans.

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