A Central Functional Role for the 49-kDa Subunit within the Catalytic Core of Mitochondrial Complex I*

Received for publication, March 14, 2001, and in revised form, April 20, 2001
Published, JBC Papers in Press, May 7, 2001, DOI 10.1074/jbc.M102296200

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We have analyzed a series of eleven mutations in the 49-kDa protein of mitochondrial complex I (NADH:ubiquinone oxidoreductase) from Yarrowia lipolytica to identify functionally important domains in this central subunit. The mutations were selected based on sequence homology with the large subunit of [NiFe] hydrogenases. None of the mutations affected assembly of complex I, all decreased or abolished ubiquinone reductase activity. Several mutants exhibited decreased sensitivities toward ubiquinone-analogous inhibitors. Unexpectedly, seven mutations affected the properties of iron-sulfur cluster N2, a prosthetic group not located in the 49-kDa subunit. In three of these mutants cluster N2 was not detectable by electron-paramagnetic resonance spectroscopy. The fact that the small subunit of hydrogenase is homologous to the PSST subunit of complex I proposed to host cluster N2 offers a straightforward explanation for the observed, unforeseen effects on this iron-sulfur cluster. We propose that the fold around the hydrogen reactive site of [NiFe] hydrogenase is conserved in the 49-kDa subunit of complex I and has become part of the inhibitor and ubiquinone binding region. We discuss that the fourth ligand of iron-sulfur cluster N2 missing in the PSST subunit may be provided by the 49-kDa subunit.

Oxidative phosphorylation is a universal process that converts most of the energy provided by foodstuffs into the general energy source ATP (1). During this process electrons pass through a series of membrane-bound multiprotein complexes that translocate protons across the membrane. The resulting proton motive force is used by ATP synthase to make ATP (2). The subunit of [NiFe] hydrogenase is homologous to the PSST subunit of complex I, which is the first of the electron transfer complexes and accounts for up to 40% of the pumped protons. In human mitochondria, complex I is regarded as a major source of deleterious reactive oxygen species. Hereditary and acquired defects affecting this multiprotein complex have been implicated in numerous degenerative diseases and seem to promote aging (3). The physiological and medical importance of complex I contrasts sharply with our still rather limited knowledge about the molecular structure and the catalytic mechanism of this multisubunit membrane protein with a total mass close to 1000 kDa (4).

The core of complex I is formed by 14 central subunits of which in animals and most fungi seven are encoded by the mitochondrial genome (5). The mitochondrial enzyme contains up to 29 additional “supernumerary” subunits. As bacterial enzymes comprise only the 14 central subunits, these must contain all functional modules required for electron and proton transfer. Except for the electron entry point that is formed by FMN and iron-sulfur center N3 in the 51-kDa subunit, the organization of these functional modules, the location and number of prosthetic groups, and the location of the ubiquinone substrate binding site are largely unknown. In particular, the function of none of the 14 central subunits that carry none of the known prosthetic groups of complex I is obscure.

One of these proteins, the 49-kDa subunit, was found to be homologous to the large subunit of [NiFe] hydrogenases, a diverse family of enzymes found in archaebacteria and eubacteria (6). More recently, a “classical” mutation of Rhodobacter capsulatus selected for resistance to quinone analogous complex I inhibitors was found to affect this subunit (7). Prompted by these findings and guided by a more detailed analysis of the homology between the large [NiFe]-carrying subunit of hydrogenases and the 49-kDa subunit, we characterized site-directed mutations in the 49-kDa subunit using the yeast Yarrowia lipolytica as our newly developed model to study complex I (8).

We show that specific domains of the 49-kDa subunit are in fact of critical functional importance. Based on structural homology to water soluble, two-subunit [NiFe] hydrogenases, we propose that the protein fold around the [NiFe] active site of hydrogenases has been retained in the catalytic core of complex I (9).

EXPERIMENTAL PROCEDURES

Sequences were analyzed using the GCG-Husar server at the Deutsches Krebsforschungszentrum in Heidelberg. Structures were analyzed using Rasmol v2.7 and SwissPdb PDB viewer v3.5 (10) software. Y. lipolytica strains E129 (MatA, lys11-23, ura3-302, leu2-270, xpr2-322) and E150 (MatB, his-1, ura3-302, leu2-270, xpr2-322) were a kind gift from Prof. C. Gaillardin, Institut National de la Recherche Agronomique, Paris, France. The diploid strain GB1 was produced by mating E129 and E150. Y. lipolytica genetic techniques were carried out according to Barth and Gaillardin (11).

Heterozygous deletion strain NK2 was generated by double homologous recombination with a 5.6-kbp 2 SpfI fragment from the NUCM locus coding for the 49-kDa subunit in which codon positions 1–448 had been replaced with a 1.6-kbp polymerase chain reaction product containing the complete URA3 gene in opposite orientation to the original NUCM open reading frame.

Random integration of a 4.7-kbp SaI fragment containing a fusion of the NUCM mitochondrial targeting sequence and a truncated NDH2 open reading frame to achieve expression of NDH2 on the matrix side of

* This work was supported by the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 472 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: kbp, kilobase pair(s); DQA, 2-decyl-4-quinazolinyl amine; HAR, hexaminerruthenium(III)-chloride; EPR, electron paramagnetic resonance; DBQ, 2-n-decylubiquinone.

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Complex I and all [NiFe] hydrogenases are marked in green cases replaced by residues that are highly conserved in all complex I 49-kDa sequences (marked in red 5 49-kDa subunit are marked in blue). The [NiFe]-ligating cysteins (marked in yellow) are in three cases replaced by residues that are highly conserved in all complex I 49-kDa sequences (marked in red). Residues found to be invariant in complex I and all [NiFe] hydrogenases are marked in green. Histidines conserved in only some subfamilies of [NiFe] hydrogenases and the 49-kDa subunit are marked in blue.

### Table I

Characterization of 49 kDa mutants

| Strain | Complex I content | Complex I activity | IC50 (nM) | N2 by EPR |
|--------|-------------------|--------------------|----------|-----------|
| Parental | 100 | 100 | 17 | 610 | normal |
| Residues corresponding to nickel ligands | | | | |
| D143E | 170 | 23 | 38 | 630 | unstable |
| D143C | 110 | 30 | 30 | 580 | normal |
| V460A | 90 | <5 | n.d. | n.d. | not detectable |
| V460M | 100 | 28 | 53 | 760 | decreased |
| E463Q | 60 | 20 | 34 | 780 | decreased |
| Surrounding conserved residues | | | | |
| R141A | 130 | 17 | 21 | 570 | not detectable |
| Y144H | 70 | <5 | n.d. | n.d. | altered |
| D458A | 90 | 28 | 520 | 5200 | normal |
| Conserved histidines | | | | |
| H120A | 93 | 39 | 24 | 580 | normal |
| H329A | 100 | 25 | 18 | 770 | not detectable |
| H384A | 70 | 47 | 12 | 550 | normal |

This allowed for a reliable alignment of the regions around the [NiFe]-ligating cysteines that are arranged in two pairs located near the N terminus and the C terminus of the sequences (Fig. 1). The four [NiFe] ligating cysteines in the hydrogenase sequence are substituted by two acidic residues, a small residue (serine, glycine, or alanine), and a valine in all known 49-kDa subunit sequences. Remarkably, the valine is the residue that was found to be mutated to methionine in an inhibitor-resistant mutant in R. capsulatus (7). An arginine two positions upstream of the N-terminal domain cysteine pair and an aspartate two positions upstream of the C-terminal cysteine pair were found to be invariant in all known [NiFe] hydrogenase and 49-kDa subunit sequences. This arginine is part of a RGXEX5 (CGX)5XXH motif reported by Albracht (17) to be present in most sequences of the large [NiFe] hydrogenase subunit. Based on this sequence analysis, we reasoned that the domains around the [NiFe] active site of hydrogenase, despite the loss of the prosthetic group, may have retained an important catalytic function in the 49-kDa subunit of complex I. To test this hypothesis we constructed and characterized a series of site-directed mutations in the homologous domains of the 49-kDa subunit of Y. lipolytica complex I.

The inhibitor-resistant mutant found in R. capsulatus (7) suggested a possible involvement of the 49-kDa subunit in forming the ubiquinone binding site of complex I. As histidine is known to be in a critical position in all ubiquinone binding sites that have so far been characterized in detail based on molecular structures (19, 20), we included the mutagenesis of three histidines that are strictly conserved in all complex I.

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*S. Kerscher, A. Eschemann, P. Ahlers, and U. Brandt, manuscript in preparation.*

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**RESULTS**

**Design of Site-directed Mutations in the 49-kDa Subunit—**

The sequence homology between the large subunit of water soluble [NiFe] hydrogenases (17) and the 49-kDa subunit of complex I is too poor to unambiguously align the sequences around the cysteines ligating the [NiFe] prosthetic group. However, the corresponding subunit from membrane-bound [NiFe] hydrogenases like the archael Ech hydrogenase (18) exhibits significantly higher homology to the 49-kDa subunit of complex I than to the large subunit of its water soluble counterpart.
sequences. The first of these histidines (His-120) is located immediately before the RGXE motif. The second histidine (His-226) is in the middle of a pattern of glycines (G\(X_3H_3G\)) found invariably in complex I and all [NiFe] hydrogenases (Fig. 1). The third histidine (His-384) lies in a 30-amino acid-long insertion of the 49-kDa subunit that is missing in the sequences of the large subunit of [NiFe] hydrogenases.

Mutagenesis of Conserved Residues in the 49-kDa Subunit Corresponding to Hydrogenase [NiFe] Ligands—

**Mutation V460M**, a reconstruction of the inhibitor-resistant mutant in \(R.\) capsulatus, resulted in a 70–80% loss of complex I activity and a 3-fold increase in the IC\(_{50}\)-value for the ubiquinone-analogous inhibitor DQA (Table I). EPR-spectra of the isolated mutant enzyme revealed that the signal of iron-sulfur cluster N2 was significantly decreased. The signals of all other EPR-detected iron-sulfur clusters (16) were unchanged (Fig. 2A). The more drastic mutation V460A led to complete loss of activity. With all other iron-sulfur clusters unaffected, cluster N2 was not detectable in the EPR spectra of isolated complex I from this mutant. Mutations D143C, D143E, and E463Q also led to a 70–80% decrease in complex I activity and a 2-fold resistance to the ubiquinone-analogous inhibitor DQA with normal or slightly increased IC\(_{50}\) values for rotenone. Mutation D143E resulted in destabilized iron-sulfur cluster N2 that was lost from the complex upon purification if high concentrations of detergent were used. If in complex I preparations from this

**Fig. 2.** EPR spectra of purified complex I from 49-kDa mutants. The EPR spectra of complex I from \(Y.\) lipolytica reduced with NADH were recorded at a temperature of 12 K, a microwave frequency of 9.48 GHz, a microwave power of 2 mW, and a modulation amplitude of 0.8 millitesla. Under these conditions the spectra show contributions from clusters N1, N2, N3, and N4 (16). The dashed line indicates the position of the \(g_x\) and the \(g_y\) signal of the iron-sulfur cluster N2. Some spectra show changes in both regions, but strong overlap by other clusters makes interpretation of changes in the \(g_x\) region of the spectra difficult. A, nickel ligands (cf. Table I); B, surrounding conserved residues and conserved histidines (cf. Table I). In complex I from mutant Y144H the spectrum of cluster N2 is shifted and decreased. The \(g_y\) signal was found at 2.054.
mutant iron-sulfur cluster N2 was detectable by EPR spectroscopy (Fig. 2), it exhibited an altered \( g_z \) region. Although this finding is difficult to interpret as several clusters contribute to this part of the EPR spectrum, it was probably due to heterogeneity of the sample. Complex I from mutant E463Q consistently exhibited a significantly decreased electron paramagnetic resonance signal of cluster N2 (Fig. 2). Again, no effects on other EPR-detectable iron-sulfur clusters were observed.

**Mutagenesis of Conserved Residues in the 49-kDa Subunit**—The diagrams show the inhibition of the dNADH:ubiquinone oxidoreductase activity of wild type (■) and mutant D458A (○) mitochondrial membranes by increasing concentrations of n-decyl-quinazoline-amine (a) and rotenone (b). Compared with wild type membranes a 30-fold higher concentration of n-decyl-quinazoline-amine and a 10-fold higher concentration of rotenone were required for 50% inhibition of inhibitor-sensitive complex I activity in D458A membranes (see Table I).

**DISCUSSION**

The Pattern of Functional Changes Suggests Conservation of Functional Domains—Guided by sequence homology to the hydrogen reactive subunit of [NiFe] hydrogenases, we have characterized a series of 11 point mutations in the 49-kDa subunit of *Y. lipolytica* complex I. Complete loss of activity was caused by two mutations, V460A and Y144H. One mutation, D458A, increased the IC\(_{50}\) values for DQA and rotenone dramatically, while five other exchanges, D143E, D143C, V460M, E463Q, and H120A, caused moderate resistance to DQA. Although no effects on any other EPR-detectable iron-sulfur clusters were observed, isolated complex I from seven mutants exhibited changes in the EPR spectrum of iron-sulfur cluster N2; in V460M and E463Q cluster N2 was decreased, and in V460A, R141A, and H226A it was not detectable. In D143E this iron-sulfur cluster was destabilized and in Y144H a broadening of the N2 EPR spectrum and a specific shift of the \( g_z \) value was observed. The fact that some of these mutations had pronounced, but specific effects on ubiquinone reductase activity, inhibitor sensitivity, or the EPR spectrum of iron-sulfur cluster N2 demonstrated that the 49-kDa subunit plays a central functional role in complex I. Some amino acid exchanges near or at the residues corresponding to the cysteine pairs ligating the [NiFe] center in hydrogenase had rather severe consequences on complex I function, whereas two of the histidine to alanine exchanges further away (H120A, H1384A) had only very minor effects. This strongly supports the notion that domains around the hydrogen reactive [NiFe] site of hydrogenase have been retained as a functionally critical module in the 49-kDa subunit of complex I.

Structural Homology Explains Effects on Iron-Sulfur Cluster N2—The Fe\(_{5}\)S\(_{4}\) cluster N2 is assumed to be the immediate electron donor to ubiquinone in complex I. By virtue of its pH-dependent redox potential (21) it has been suggested to be an important component of the proton-translocating machinery (22, 23). There has been conflicting evidence whether this redox group resides in the TYKY (24) or in the PSST subunit (25). In either case one would not have expected mutations in the 49-kDa subunit to have pronounced and specific effects on iron-sulfur cluster N2, as was observed in this study. A straightforward explanation became obvious when we combined information from the molecular structure of the water soluble [NiFe] hydrogenase from *Desulfovibrio fructosovorans* (26) and the sequence homology to model the arrangement of the mutated residues in the 49-kDa subunit of *Y. lipolytica* complex I (Fig. 4, 9): In hydrogenase, the [NiFe] site is located at the interface between the large and small subunit adjacent to the proximal iron-sulfur cluster of the small subunit. The small subunit of hydrogenase is homologous to the PSST subunit of complex I (17). Three of the cysteines ligating the proximal iron-sulfur cluster are conserved in the PSST
subunit (Cys-86, Cys-150, and Cys-180) and are therefore expected to ligate iron-sulfur cluster N2 of complex I (if this subunit in fact binds this redox group). It is immediately obvious from Fig. 4 that the effects on iron-sulfur cluster N2 by the mutations analyzed here correlate well with the proximity of the corresponding residues in water soluble hydrogenase to the proximal iron-sulfur cluster. It is most remarkable, that H226A, the only mutation studied here with a sequence position distant from the residues corresponding to the [NiFe]-ligating cysteines, exhibited such a pronounced effect on complex I abolishing the EPR signal of iron-sulfur cluster N2. In the hydrogenase structure the corresponding residue His-228 resides at the tip of a loop flanked by three invariant glycines (cf. Fig. 1). Thus in the light of the molecular structure of [NiFe] hydrogenase, analysis of our data strongly suggests that the proximal iron-sulfur cluster has in fact become cluster N2 in the PSST subunit of complex I and resides at the interface to the 49-kDa subunit. However, as one of the cysteines ligating the proximal iron-sulfur cluster is missing in PSST, it remains an unsolved question which residue may serve as the fourth ligand of cluster N2. Glu-89 had been proposed as a possible candidate (22) but could be excluded recently by site-directed mutagenesis (8). Based on the structural homology outlined above one is tempted to speculate that the fourth ligand of N2 actually resides on the 49-kDa subunit. Possible candidates are the invariant His-226, which already in hydrogenase makes a hydrogen bond to the proximal iron-sulfur cluster (26), and fully conserved Tyr-144, which corresponds to a conserved glycine in hydrogenase (cf. Fig. 1). Observations were made that may lend support to either option; mutant H226A in the 49kDa subunit did not exhibit an EPR signal for iron-sulfur cluster N2 and Y144H led to a total loss of complex I activity along with a shift in the $g$ value of iron-sulfur cluster N2.

It should be noted that although no iron-sulfur cluster N2 was detectable in complex I from mutants R141A and H226A, complex I in these two mutants exhibited significant residual ubiquinone reductase activity of around 20–30%. This seems difficult to explain if iron-sulfur cluster N2 is in fact an essential component of the electron pathway of complex I. However, as both residues are predicted to be in close vicinity to iron-sulfur cluster N2, the mutations may have shifted the midpoint potential to very negative values. This may prevent reduction of the iron-sulfur cluster by NADH necessary for detection by EPR spectroscopy. Although this is one possible explanation, further studies will be needed.

**Inhibitor Resistance Suggests Involvement of the 49-kDa Subunit in Ubiquinone Binding**—Several of the mutations analyzed here exhibited resistance to ubiquinone-analogous inhibitors of complex I that was most pronounced for the residue most distant from the predicted position of iron-sulfur cluster N2. We conclude that this part of the 49-kDa subunit contributes to the inhibitor (and ubiquinone) binding pocket of complex I and has evolved from the [NiFe]-binding fold of hydrogenase. The very recent identification of two additional inhibitor resistant point mutations G409A and D412E (Gly-462 and Asp-465 in Y. lipolytica) in the 49-kDa subunit in R. capsulatus (27) by Dupuis and co-workers corroborates this view. Also the finding that the PSST subunit is labeled specifically by a derivative of pyridaben, another ubiquinone-analogous inhibitor of complex I (28) fits well with the structural model of Fig. 4. A recent report from the Nijmegen Center for Mitochondrial Disorders that two independent point mutations on the loop carrying the possible fourth ligand of cluster N2 cause hypertrophic cardiomyopathy and encephalomyopathy in humans (29), provides further support for this paradigm. It seems inevitable to conclude that the 49-kDa and the PSST subunit form a central part of the catalytic core of complex I and contribute to the ubiquinone binding domain.

Our results demonstrate a remarkable degree of structural conservation between a homologous subunit from two classes of quite functionally different enzymes on a molecular level. We conclude that not only the overall fold of the active site is the same in [NiFe] hydrogenase and complex I but also that both enzyme families share a number of functionally important residues that are invariant in all known sequences. This is of particular significance as a central part of the catalytic core of complex I itself seems to have evolved from a hydrogen reactive [NiFe] center to a site reducing the much larger ubiquinone that may comprise the proton pumping module as well. We suggest that the residues invariant between the large subunit of hydrogenase and the 49-kDa subunit of complex I may be involved in electron and proton pathways serving the active site as these functions have to be present in both enzymes.

**Acknowledgments**—We thank Gudrun Beyer and Franz J. Streb for excellent technical assistance.

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