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The role of a conserved tyrosine in the 49-kDa subunit of complex I for ubiquinone binding and reduction

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ABSTRACT

Iron–sulfur cluster N2 of complex I (proton pumping NADH:quinone oxidoreductase) is the immediate electron donor to ubiquinone. At a distance of only ~7 Å in the 49-kDa subunit, a highly conserved tyrosine is found at the bottom of the previously characterized quinone binding pocket. To get insight into the function of this residue, we have exchanged it for six different amino acids in complex I from *Yarrowia lipolytica*. Mitochondrial membranes from all six mutants contained fully assembled complex I that exhibited very low dNADH:ubiquinone oxidoreductase activities with *n*-decylubiquinone. With the most conservative exchange Y144F, no alteration in the electron paramagnetic resonance spectra of complex I was detectable. Remarkably, high dNADH:ubiquinone oxidoreductase activities were observed with ubiquinones Q_1 and Q_2 that were coupled to proton pumping. *Apparent* K_m values for Q_1 and Q_2 were markedly increased and we found pronounced resistance to the complex I inhibitors decyl-quinazoline-amine (DQA) and rotenone. We conclude that Y144 directly binds the head group of ubiquinone, most likely via a hydrogen bond between the aromatic hydroxyl and the ubiquinone carbonyl. This places the substrate in an ideal distance to its electron donor iron–sulfur cluster N2 for efficient electron transfer during the catalytic cycle of complex I.

1. Introduction

Mitochondrial complex I (NADH:ubiquinone oxidoreductase) is a large membrane protein and the least understood component of the respiratory chain [1–3]. It is composed of at least 40 subunits with a total mass of ~1 MDa [4,5]. In contrast to other respiratory chain complexes, a complete crystal structure of complex I has not been solved yet. However, single particle electron microscopy revealed that complex I has an L-shaped overall structure in eukaryotes as well as in prokaryotes [6–10]. The hydrophobic membrane domain of the enzyme is embedded in the inner mitochondrial membrane, whereas the hydrophilic peripheral domain protrudes into the mitochondrial matrix. Interestingly, the peripheral domain contains all known redox centers: one non-covalently bound FMN and 8–9 iron–sulfur clusters [11–14]. In contrast, no redox centers were found in the membrane domain of complex I [15]. However, this part of the enzyme must harbor the proton pumping machinery.

The mechanism by which complex I couples the redox reaction of NADH oxidation and quinone reduction to the translocation of four protons across the inner mitochondrial membrane [16,17] is still unknown. However, from recent results it seems that the reduction of quinone in the peripheral domain induces long range conformational changes in the membrane domain, which result in proton uptake at the matrix side of the inner mitochondrial membrane and proton release into the intermembrane space [3,18,19].

Previously, we suggested that the quinone and inhibitor binding pocket is located at the interface of the PSST and the 49-kDa subunit [20,21] (the bovine nomenclature for homologous complex I subunits will be used throughout). This suggestion was based on several observations. Firstly, the active site of water-soluble [NiFe] hydrogenases is located at the interface of the small and large subunits which are evolutionary related to the PSST and the 49-kDa subunit as indicated by sequence comparison [22,23]. Secondly, mutagenesis studies showed that many functionally critical residues are located in the PSST and the 49-kDa subunit and that mutations which target the former [NiFe] site conferred resistance towards complex I inhibitors which act at the quinone binding site [20,24–29]. Thirdly, photoaffinity labeling studies suggested that the PSST subunit forms part of

Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; BN-PAGE, blue-native polyacrylamide gel electrophoresis; $C_{12}E_8$, n-alkyl-polyoxyethylene-ether; DBQ, *n*-decylubiquinone; dNADH, deamino-nicotinamide-adenine-dinucleotide (reduced form); DQA, 2-decyl-4-quinazolinyl amine; EPR, electron paramagnetic resonance; FCCP, carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone; HAR, hexa-ammine-ruthenium(III)-chloride; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; log*P*, decadic logarithm of the partition coefficient; Mops, 3-(*N*-morpholino) propanesulphonic acid; PMSF, phenylmethylsulfonyl fluoride; Q_1, ubiquinone-1 (2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4-benzoquinone); Q_2, ubiquinone-2 (2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinone); Q_9, ubiquinone-9; Tris, Tris (hydroxymethyl)aminomethane

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the quinone and inhibitor binding pocket of complex I [30]. Further support came from subsequent mutagenesis studies, which identified additional functionally important residues in the PSST and 49-kDa subunit [31–33] and recent photoaffinity labeling studies which showed that the 49-kDa subunit forms part of the inhibitor binding site of complex I [34]. Finally, the crystal structure of the hydrophilic domain of complex I from *Thermus thermophilus* at 3.3 Ångström resolution [14] confirmed our proposal. The structure revealed the spatial arrangement of seven central hydrophilic subunits as well as the locations of all known redox centers of complex I. Seven iron–sulfur clusters (N1b, N2, N3, N4, N5, N6a und N6b) form an approximately 95 Å long chain of redox centers. This chain starts with iron–sulfur cluster N3 next to the FMN molecule in the 51-kDa subunit, continues via the redox centers of the 75-kDa and the TYKY subunits and ends at iron–sulfur cluster N2 in the PSST subunit adjacent to a broad cavity at the interface of the PSST and the 49-kDa subunit, which corresponds to the suggested quinone reduction domain.

Taking advantage of our model organism, the strictly aerobic yeast *Yarrowia lipolytica*, we exchanged all amino acid residues located at the surface of this large cavity. Subsequent structure/function analysis allowed the identification of functional domains [35]. A possible ubiquinone access path leading from the N-terminal β-sheet of the 49-kDa subunit into the cavity to a tyrosine residing next to iron–sulfur cluster N2 was identified. Within the cavity many amino acid residues were found to be critical for oxidoreductase activity of complex I. In a more recent study, we used the large array of mutations targeting the quinone binding pocket to identify the binding sites of different complex I inhibitors [36]. The obtained results suggested that type A, B and C inhibitors [37] indeed bind to the quinone binding pocket at the interface of the PSST and the 49-kDa subunit and that the binding sites overlap spatially, as had been deduced from earlier competition experiments [38].

According to the crystal structure of the hydrophilic domain of complex I from *T. thermophilus* [14], Y144 (*Y. lipolytica* numbering) of the 49-kDa subunit is located only ~7 Å away from iron–sulfur cluster N2, the immediate electron donor to ubiquinone (Fig. 1). This tyrosine is highly conserved between prokaryotes and eukaryotes (Fig. 2). The side chain of Y144 seems to define the border between the PSST subunit coordinating iron–sulfur cluster N2 and the 49-kDa subunit forming most of the quinone binding pocket. By systematically exchanging this tyrosine to aromatic, hydrophilic and hydrophobic amino acids, we show here that this residue is critical for ubiquinone binding and reduction in complex I.

2. Materials and methods

2.1. Materials

Asolectin (total soy bean extract with 20% lecithin) was purchased from Avanti Polar Lipids (Alabaster, AL), *n*-dodecyl-ß-D-maltoside from Glycon (Luckenwalde, Germany), and octyl-ß-D-glucopyranoside from Biomol (Hamburg, Germany). 9-amino-6-chloro-2-methoxyacridine (ACMA) was obtained from Invitrogen/Molecular Probes (Eugene, OR) and decylubiquinone (DBQ) from Alexis Biochemicals (Lausen, Switzerland). DQA (2-n-decyl-quinazolin-4-yl-amine, SAN 549) was from AgrEvo (Frankfurt, Germany). Carbonyl-cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and the ionophore valinomycin were from Sigma. ACMA, DBQ, Q_1, Q_2, DQA, roteonone, and the ionophores were dissolved in dimethylsulfoxide.

2.2. Site-directed mutagenesis

Site-directed mutagenesis, preparation of mitochondrial membranes, determination of protein concentration, and structural image preparations were performed as described in [35].

2.3. Analytical methods

Measurement of NADH:HAR and dNADH:DBQ oxidoreductase activities was performed as described [35]. Inhibitor insensitive activities were determined in the presence of 27 µM DQA. *Apparent* K_m and V_{max} values for DBQ, Q_1 and Q_2 were measured as described for dNADH:DBQ oxidoreductase activity [35] except that the concentrations of DBQ, Q_1 or Q_2 were adjusted to the respective ranges and that DQA was omitted. A DQA insensitive rate could not be determined because the concentrations needed to completely block complex I would have been too high for the mutant strains (see Results). Instead, background rates determined in parallel with mitochondrial membranes of the *Δnucm* strain were subtracted from the rates measured with the parental and mutant strains in the absence of inhibitor. Since the *Δnucm* strain does not contain complex I, these background rates that were in the same range as the inhibitor

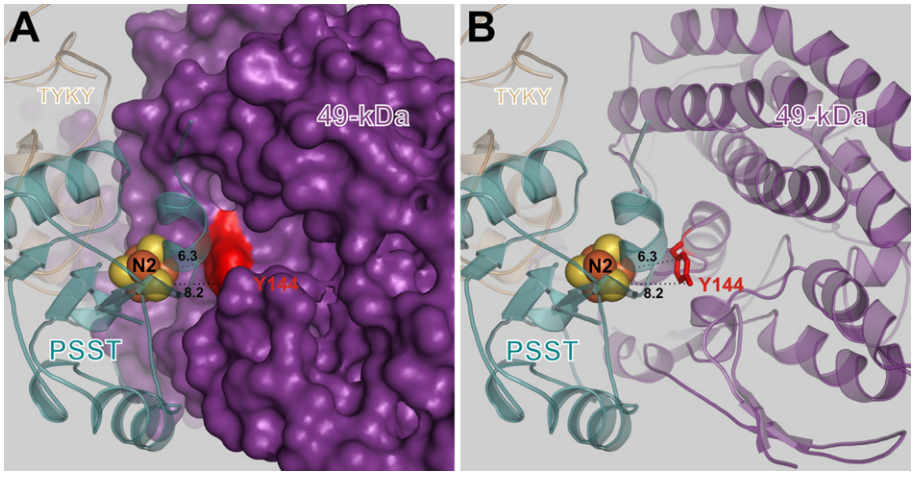

Fig. 1. Y144 from the 49-kDa subunit is located between iron–sulfur cluster N2 and the quinone binding pocket. The PSST, the 49-kDa and the TYKY subunits are colored in cyan, purple and beige, respectively. Y144 is highlighted in red. Iron–sulfur cluster N2 is depicted as spheres. Numbers give center-to-center distances between atoms in Ångström. The figure was prepared with the PyMol software package (version 0.99) using the coordinates from the crystal structure of the hydrophilic domain of complex I from *T. thermophilus* (2FUG). A, Surface representation of the 49-kDa subunit is shown in order to visualize the quinone binding pocket. B, Cartoon representation of the 49-kDa subunit showing the side chain of Y144.