Exploring the Ubiquinone Binding Cavity of Respiratory Complex I

Received for publication, June 1, 2007, and in revised form, July 16, 2007. Published, JBC Papers in Press, August 6, 2007, DOI 10.1074/jbc.M704519200

Maja A. Tocilescu, Uta Fendel, Klaus Zwicker, Stefan Kerscher, and Ulrich Brandt

From the Johann Wolfgang Goethe-Universität, Fachbereich Medizin, Zentrum der Biologischen Chemie, Molekulare Bioenergetik, Centre of Excellence Frankfurt “Macromolecular Complexes”, D-60590 Frankfurt am Main, Germany

Proton pumping respiratory complex I is a major player in mitochondrial energy conversion. Yet little is known about the molecular mechanism of this large membrane protein complex. Understanding the details of ubiquinone reduction will be prerequisite for elucidating this mechanism. Based on a recently published partial structure of the bacterial enzyme, we scanned the proposed ubiquinone binding cavity of complex I by site-directed mutagenesis in the strictly aerobic yeast Yarrowia lipolytica. The observed changes in catalytic activity and inhibitor sensitivity followed a consistent pattern and allowed us to define three functionally important regions near the ubiquinone-reducing iron-sulfur cluster N2. We identified a likely entry path for the substrate ubiquinone and defined a region involved in inhibitor binding within the cavity. Finally, we were able to highlight a functionally critical structural motif in the active site that consisted of Tyr-144 in the 49-kDa subunit, surrounded by three conserved hydrophobic residues.

Respiratory chain NADH:ubiquinone oxidoreductase (complex I) is a large membrane protein complex that catalyzes electron transfer from NADH to ubiquinone and thereby pumps protons across the inner mitochondrial or bacterial plasma membrane (1). Electron microscopy revealed that complex I is L-shaped (2–9) and is composed of a hydrophobic arm embedded in the membrane and a peripheral arm protruding into the mitochondrial matrix or the bacterial cytosol. The peripheral arm contains all known redox centers, one FMN and eight or nine iron-sulfur clusters. Based on sequence comparisons (10, 11), mutational analysis (12–15), and photoaffinity labeling studies (16), we have proposed previously (14, 17) that the PSST and the 49-kDa subunit that are homologous to the small and large subunit of [NiFe] hydrogenases form part of the quinone reducing catalytic core of complex I (note that the bovine nomenclature will be used for the central subunits of complex I throughout). Recently the crystal structure of the peripheral domain of complex I from Thermus thermophilus has been solved at 3.3 Å resolution (18). This structure (Fig. 1) shows a wire of iron-sulfur clusters connecting the NADH-binding site near FMN with a broad cavity formed by the PSST and the 49-kDa subunit that should comprise the active site for ubiquinone reduction and the binding region for the large number of inhibitors that have been found for complex I (19).

Because reduction of ubiquinone is likely to be a key event in the energy-coupling mechanism of complex I (1, 20), the quinone-binding site in the PSST and the 49-kDa subunit next to iron sulfur-cluster N2 is of particular interest. To identify the domains essential for catalytic activity and inhibitor binding, we introduced a set of point mutations in the PSST and the 49-kDa subunits of complex I from our model organism, the strictly aerobic yeast Yarrowia lipolytica. Positions for point mutations were chosen by analyzing the T. thermophilus structure so that they would probe all parts of the proposed quinone binding cavity and some surrounding residues.

EXPERIMENTAL PROCEDURES

Strains and Site-directed Mutagenesis—The Y. lipolytica nucmΔ and nukmΔ deletion strains described earlier (21, 22) were transformed with the replicative plasmids pUB26 containing a genomic fragment of the NUCM gene or pUB4 containing a genomic fragment of the NIUKM gene. All point mutations were generated in Escherichia coli by PCR mutagenesis. After transformation (23) into Y. lipolytica strain nucmΔ or nukmΔ, plasmids were recovered, and the entire open reading frames were sequenced to verify the introduced point mutations and exclude other sequence changes.

Small Scale Preparation of Mitochondrial Membranes—Mitochondrial membranes were isolated essentially according to published protocols (24). After phenylmethylsulfonyl fluoride was added to a final concentration of 2 mM, mitochondrial membranes were homogenized, shock-frozen, and stored in liquid nitrogen. Aliquots of preparations were used for activity measurements and gel electrophoresis. Protein concentration was determined colorimetrically using the DC protein assay (Bio-Rad).

Measurement of Catalytic Activity—NADH:Har2 oxidoreductase activity was measured as NADH oxidation (ε340–400 nm = 6.22 mM−1 cm−1) in the presence of the artificial electron acceptor HAR using a Molecular Devices SPECTRAMax PLUS384 plate reader spectrometer. The activity was measured at 30 °C in 20...
mm Na\(^+\)/Hepes, pH 8.0, with 250 mm sucrose, 2 mm NaN\(_3\), 0.2 mm EDTA, 0.2 mm NADH, and 2 mm HAR. The reaction was initiated by the addition of mitochondrial membranes (final concentration 25 \(\mu\)g of protein per ml). Specific NADH:HAR oxidoreductase activity was used to estimate complex I content because it is not affected by changes in the ubiquinone binding pocket.

dNADH:DBQ oxidoreductase activity of mitochondrial membranes was determined as the fraction of dNADH oxidation activity \((\varepsilon_{340-400} = 6.22 \text{ mm}^{-1} \text{ cm}^{-1})\) sensitive to the complex I inhibitor DQA in the presence of DBQ as electron acceptor. Measurements were carried out on a SPECTRAmax PLUS 384 plate reader spectrometer (Molecular Devices) at 30 °C in 20 mm Na\(^+\)/Mops, pH 7.4, with 50 mm NaCl, 2 mm KCN, 0.1 mm dNADH, and 0.07 mm DBQ. The final concentration of mitochondrial membranes was 50 \(\mu\)g/ml. The reaction was started by adding DBQ. The inhibitor-sensitive fraction of the ubiquinone reductase activity was calculated by subtracting the residual rate in the presence of 27 \(\mu\)m DQA that was usually 5–10% of the dNADH:DBQ oxidoreductase activity of the parental strain. To allow comparison between different membrane preparations, all activities were normalized for complex I content. The results are given as mean ± S.E. (\(n = 5–15\)).

**RESULTS**

**Mutations in the Ubiquinone Binding Cavity Did Not Interfere with Complex I Assembly**—To gain more insight into the function of amino acid residues that line the putative ubiquinone binding pocket (18), we generated a set of 39 point mutations covering 20 different residues of the 49-kDa and the PST subunits of complex I from *Y. lipolytica* (Tables 1 and 2). In many positions the residues were exchanged by several different amino acids and at least one conservative, and one more drastic exchange was introduced if possible. Tables 1 and 2 also list data on point mutations that we had generated and analyzed previously (14, 21, 22, 28), providing information on a total of 52 mutations at 26 positions. Mitochondrial membranes were isolated from all newly generated mutant *Y. lipolytica* strains, and complex I content was estimated as NADH:HAR oxidoreductase activity (Tables 1 and 2) and by blue-native PAGE with subsequent complex I in gel activity stain (data not shown). Membranes from most mutants contained fully assembled complex I in amounts comparable with the parental strain indicating that complex I was not destabilized in these mutants. Only very few mutations led to moderately decreased complex I contents, but even in these mutants complex I appeared to be fully assembled.
### TABLE 1  
Effects of point mutations introduced into the 49-kDa subunit

| Strain      | Complex I content | Complex I activity | Apparent $K_m$ (DBQ) | $I_{50}$ | Rotenone |
|-------------|-------------------|--------------------|----------------------|---------|----------|
|             | %                 | %                  | $\mu$M             | mg      |          |
| Parental    | 100 ± 3           | 100 ± 5            | 15                  | 16      | 530      |
| A94D        | 85 ± 3            | 14 ± 2             | ND                  | ND      | ND       |
| H95A        | 130 <5            | ND                  | ND                  | ND      | ND       |
| H95M        | 120 <5            | ND                  | ND                  | ND      | ND       |
| H95R        | 100 <5            | ND                  | ND                  | ND      | ND       |
| V977W       | 78 ± 3            | 10 ± 2             | ND                  | ND      | ND       |
| L98F        | 101 ± 3           | 22 ± 2             | ND                  | ND      | ND       |
| L98K        | 71 ± 2            | 15 ± 2             | ND                  | ND      | ND       |
| R99D        | 58 ± 2            | 14 ± 2             | ND                  | ND      | ND       |
| R99T        | 71 ± 1            | 15 ± 2             | ND                  | ND      | ND       |
| R141K       | 140 ± 5           | 13 ± 15            | ND                  | ND      | ND       |
| R141A       | 130 17d           | 10 21d             | 570d                |         |          |
| Y144W       | 129 ± 2           | 8 ± 4              | ND                  | ND      | ND       |
| Y144H       | 121 4             | 12 21              | 850                 |         |          |
| V145F       | 127 ± 1           | 7 ± 3              | ND                  | ND      | ND       |
| V145T       | 122 ± 4           | 92 ± 8             | 12                  | 17      | 550      |
| S146C       | 100 ± 1           | 100 ± 2            | 12                  | 80      | 1500     |
| M188Y       | 100 ± 2           | 12 ± 2             | ND                  | ND      | ND       |
| S192E       | 110 ± 3           | 16 ± 2             | ND                  | ND      | ND       |
| S192R       | 102 ± 5           | 8 ± 2              | ND                  | ND      | ND       |
| S192Y       | 111 ± 2           | 24 ± 2             | ND                  | ND      | ND       |
| F207W       | 100 ± 2           | 63 ± 2             | 14                  | 16      | 500      |
| R210I       | 100 ± 2           | 63 ± 2             | 14                  | 16      | 500      |
| E211Q       | 124 ± 4           | 83 ± 6             | 16                  | 21      | 530      |
| E218Q       | 97 ± 2            | 97 ± 4             | 12                  | 21      | 550      |
| R224D       | 97 ± 2            | 107 ± 4            | 13                  | 12      | 550      |
| R224H       | 93 ± 3            | 102 ± 5            | 14                  | 12      | 650      |
| R224K       | 86 ± 2            | 103 ± 3            | 10                  | 11      | 550      |
| R224N       | 124 ± 2           | 99 ± 7             | 14                  | 15      | 700      |
| L225A       | 102 ± 3           | 103 ± 6            | 13                  | 13      | 550      |
| L225F       | 95 ± 3            | 110 ± 13           | 13                  | 11      | 460      |
| L225H       | 84 ± 3            | 107 ± 5            | 12                  | 14      | 700      |
| L225V       | 95 ± 2            | 91 ± 3             | 12                  | 12      | 700      |
| K407H       | 80 ± 2            | 78 ± 2             | 13                  | 13      | 500      |
| K407L       | 93 ± 3            | 105 ± 4            | 12                  | 75      | 550      |
| K407W       | 66 ± 1            | 12 ± 2             | ND                  | ND      | ND       |
| G455I       | 71 ± 3            | 16 ± 2             | ND                  | ND      | ND       |
| G455S       | 98 ± 1            | 72 ± 4             | 11                  | 22      | 700      |
| D458A       | 93 17d           | 28 ± 6             | 12                  | 12      | 650      |
| L459I       | 92 ± 7            | 92 ± 10            | 16                  | 13      | 620      |
| L459K       | 50 ± 2            | 24 ± 2             | ND                  | ND      | ND       |
| V460A       | 90 <5             | ND                  | ND                  | ND      | ND       |
| V460L       | 83 ± 3            | 21 ± 2             | ND                  | ND      | ND       |
| V460M       | 100 ± 2           | 16 ± 2             | 9°                   | 53°     | 76°      |
| V460S       | 82 ± 3            | 21 ± 1             | ND                  | ND      | ND       |
| F461W       | 97 ± 3            | 23 ± 3             | ND                  | ND      | ND       |

a 100% of complex I content corresponds to 1.25 μmol/min/mg NADH-oxidoreductase activity.
b Complex I activity of the parental strain was 0.58 μmol/min/mg-1.
c Data are from Ref. 21.
d Data are from Ref. 14.
e Data are from N. Kashani-Poor, unpublished data.
f Data are from L. Grigic, unpublished data.

### TABLE 2  
Effects of point mutations introduced into the PSST subunit

| Strain      | Complex I content | Complex I activity | Apparent $K_m$ (DBQ) | $I_{50}$ | Rotenone |
|-------------|-------------------|--------------------|----------------------|---------|----------|
|             | %                 | %                  | $\mu$M             | mg      |          |
| Parental    | 100 ± 2           | 100 ± 3            | 14                  | 13      | 450      |
| V88F        | 96 ± 2            | 13 ± 1             | ND                  | ND      | ND       |
| V88L        | 94 ± 4            | 92 ± 5             | 15                  | 25      | 500      |
| V88M        | 95 ± 2            | 56 ± 3             | 13                  | 32      | 330      |
| E89A        | 145 ± 7           | 14 ± 2             | ND                  | ND      | ND       |
| E89C        | 145 ± 7           | 14 ± 2             | ND                  | ND      | ND       |
| E89Q        | 100 ± 8           | 9 ± 1              | ND                  | ND      | ND       |
| E89Q        | 110 ± 9           | 50 ± 20            | ND                  | ND      | ND       |

a 100% of complex I content corresponds to 1.25 μmol/min/mg NADH-oxidoreductase activity.
b Complex I activity of the parental strain was 0.6 μmol/min/mg-1.
c Data are from Ref. 22.
d Data are from Ref. 21.
ity were observed along a path leading from the first strand of the N-terminal β-sheet of the 49-kDa subunit toward a region adjacent to iron–sulfur cluster N2.

Effects of Point Mutations on Apparent $K_m$ Value for DBQ—To test whether the mutations had altered the ubiquinone-binding site of complex I, we determined their apparent $K_m$ value for the ubiquinone oxidoreductase activity observed in a given position in complex I from Y. lipolytica. Red, several exchanges all resulted in very low activity (<25% of parental); yellow, at least one exchange resulted in very low activity (<25% of parental); green, reduced activity (between 25 and 75% of parental); blue, essentially normal activity (>75% of parental) for all exchanges. Residues of subunit PSST are marked with an asterisk. Iron–sulfur cluster N2 is shown as gray spheres. A, surface representation. B, schematic representation.

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All resistance data are illustrated in Fig. 3. Amino acid positions where mutants exhibited resistance or hypersensitivity to DQA or rotenone are shown in orange. For better orientation, other residues that were most critical for catalytic activity are shown again in red. The other mutated residues highlighted in Fig. 2 are now shown in the same color as the secondary structure schematic of the subunit. (A and B as in Fig. 2).

DISCUSSION

We have probed the proposed ubiquinone binding cavity within the peripheral arm of complex I by site-directed mutagenesis. Of the 39 mutations that were analyzed here, a
significant number resulted in a marked reduction of inhibitor-sensitive ubiquinone reductase activity. Several mutations changed inhibitor sensitivity. By localizing the corresponding residues in the partial structural complex I from *T. thermophilus* (18) and by combining these results with information from earlier studies (14, 21, 28), we could identify functionally important regions within this central domain of complex I (Figs. 2 and 3). The region identified as being most critical for activity included a group of residues that (except for Tyr-144, Ser-192, and Val-460) were not located immediately in the spacious cavity around cluster N2 but rather seemed to form a path of entry for ubiquinone (Fig. 2A). This path starts with Ala-94 at a distance of about 24 Å from the ubiquinone-reducing iron-sulfur cluster N2 within the first strand of the N-terminal three-stranded β-sheet of the 49-kDa subunit. The amphipathic loop connecting the first and second strand of this β-sheet reaches into the proposed ubiquinone binding pocket. All five mutations that we introduced here for the three consecutive, highly conserved residues Val-97, Leu-98, and Arg-99 drastically reduced ubiquinone reductase activity (Table 1). Already in an earlier study (21), we had found that all three exchanges we had introduced for the neighboring residue His-95 also abolished complex I activity. The same was found for His-91, which seems to reside in a region that is disordered in the isolated peripheral arm as it is not contained in the *T. thermophilus* structural model (18). This high density of functionally important residues strongly suggested that the N-terminal β-sheet represents a critical part of the ubiquinone binding pocket of complex I.

Somewhat deeper into the crevice but on its opposite side, we could identify another region, where amino acid exchanges M188Y and S192Y significantly impaired catalytic activity (Fig. 2). These residues are located within the lower half of a four α-helical bundle that could be called the backbone of the ubiquinone binding pocket. Remarkably, mutagenesis of a group of strictly conserved and polar residues in the most remote part of the cavity (Glu-211, Glu-218, and Arg-224) and ubiquinone reductase activity. Element A lines the interface between the 49-kDa subunit and the iron-sulfur subunits PSST and TYKY. Remarkably, mutagenesis of Val-88 in the PSST subunit that is located at only about 4 Å distance from Tyr-144 and Val-145 resulted in loss of activity when a bulky phenylalanine was introduced (Table 2). In contrast, mutation V88M resulted in an about 2–3-fold resistance to DQA and slight hypersensitivity to rotenone suggesting that substrate and inhibitor binding are closely linked in this region of the pocket.

Exchanging the fully conserved Arg-224 that resides within the long disordered loop of element B (Fig. 4A) had no significant effect on catalytic activity. Note however, that His-226 found at the tip of the hairpin loop of element B is the redox-Bohr group of cluster N2 (20). Lys-407 is situated on a loop forming element C that is arranged in parallel to element A (Fig. 4A). Mutation K407W caused a marked reduction in ubiquinone reductase activity, whereas mutation K407R resulted in an almost 5-fold resistance toward DQA. Note that in this position an arginine is found in *T. thermophilus*. Element D is a highly conserved mostly random coil C-terminal stretch that approaches the region around cluster N2 from the side opposite to elements A and C. It hosts several previously reported mutations (12, 13, 14) that lead to marked inhibitor resistance or, like mutations F461W and G455L studied here, to reduced catalytic activity.

A more detailed analysis of the effect of the mutations on the conserved fold around iron-sulfur cluster N2 revealed a remarkable structural feature that may well play a central role in the catalytic mechanism of complex I; a triad of three hydrophobic residues, Val-88 in the PSST subunit and Val-145 and Val-460 in the 49-kDa subunit that are spaced only a few Ångströms from each other, seem to form a hydrophobic platform around Tyr-144 (Fig. 4B). Tyr-144 has been shown previously to be critical for ubiquinone reduction (14) and is only about 7 Å away from cluster N2. Although mutating Val-460 to alanine resulted in complete loss of activity, introduction of a bulky
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It seems however hard to envision how the extremely hydrophobic substrate ubiquinone-10 and the hydrophobic inhibitors could reach a binding site that resides far above the level of the membrane. One option would be that complex I undergoes extensive conformational changes during turnover that brings the ubiquinone binding pocket down to the membrane. Another option would be that the peripheral domains of some of the membrane-bound subunits and the PSST subunit form a ramp or channel that could shuttle the substrate between the membrane domain and the ubiquinone binding pocket. Based on the orientation of the pocket predicted from our structural studies (32), it is tempting to speculate that the long ubiquinone tail acts as a tether that slides along this ramp or channel, whereas the headgroup of the substrate diffuses through the water phase. Solving a high resolution structure of the entire complex will be necessary to decide whether such an unusual substrate binding mode is operational in complex I.

Acknowledgment—We thank Gudrun Beyer for excellent technical assistance.

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