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Direct Localization of the 51 kDa and 24 kDa Subunits of Mitochondrial Complex I by Three-dimensional Difference Imaging

Todd Clason^{1,*}, Volker Zickermann^{2,*}, Teresa Ruiz¹, Ulrich Brandt², and Michael Radermacher¹

¹ University of Vermont, College of Medicine, Dept. Molecular Physiology and Biophysics, Burlington, VT 05405, USA

² Universität Frankfurt, Fachbereich Medizin, Molekulare Bioenergetik, Centre of Excellence Frankfurt "Macromolecular Complexes", D-60590 Frankfurt am Main, Germany

Abstract

Complex I is the largest complex in the respiratory chain, and the least understood. We have determined the three-dimensional structure of complex I from *Yarrowia lipolytica* lacking the flavoprotein part of the N-module, which consists of the 51 kDa (NUBM) and the 24 kDa (NUHM) subunits. The reconstruction was determined by three-dimensional electron microscopy of single particles. A comparison to our earlier reconstruction of the complete *Y. lipolytica* complex I clearly assigns the two flavoprotein subunits to an outer lobe of the peripheral arm of complex I. Localizing the two subunits allowed us to fit the X-ray structure of the hydrophilic fragment of complex I from *Thermus thermophilus*. The fit that is most consistent with previous immuno-electron microscopic data predicts that the ubiquinone reducing catalytic center resides in the second peripheral lobe, while the 75 kDa subunit is placed near the previously seen connection between the peripheral arm and the membrane arm protrusions.

Introduction

Complex I (proton pumping NADH:ubiquinone oxidoreductase) is the first enzyme in the mitochondrial respiratory chain. It oxidizes NADH, transferring two electrons to ubiquinone with concurrent translocation of four protons across the mitochondrial membrane (for review see e.g. Brandt 2006). Complex I deficiencies are the most frequently observed disorders in the mitochondrial oxidative phosphorylation system and have been associated with a large number of mitochondrial diseases, from lethal infantile cardiomyopathies to Alzheimer's and Parkinson's disease (for review see e.g. van den Heuvel and Smeitink 2001). Despite its recognized importance, complex I is the least understood enzyme in the respiratory chain. The nature of complex I has slowed structural inquiry: it is a membrane protein of large molecular weight – 500 kDa in prokaryotes and close to 1 MDa in eukaryotes – with pronounced complexity, having at least 38 unique subunits in the yeast *Yarrowia lipolytica* (Abdrakhmanova et al. 2004).

*These authors have contributed equally to this work; all biochemistry was carried out at the Univ. Frankfurt, electron microscopy and image processing at the Univ. Vermont.

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The nomenclature of the subunits in complex I is species dependent. In the following, we adopt the most commonly used nomenclature for bovine complex I, and provide parenthetical the nomenclature for *Y. lipolytica* or *T. thermophilus* complex I where necessary.

The proton pumping mechanism of complex I is still unknown. Two fundamentally different concepts have been proposed. One concept suggests that proton translocation is achieved by a direct coupling mechanism, where the reduction of ubiquinone is chemically coupled to the transport of protons across the membrane (Brandt 1997; Dutton et al. 1998). A second concept proposes redox driven conformational changes of complex I that drive active proton transport by transporter-like subunits (Belogrudov and Hatefi 1994; Sazanov et al. 2000; Brandt et al. 2003; Yagi and Matsuno-Yagi 2003). To better understand the function of complex I, knowledge of its subunit architecture is essential. Locating the ubiquinone binding site proximal to or within the membrane arm of complex I would favor a direct coupling mode, while a catalytic center distal to the membrane would render direct coupling unlikely. Ubiquinone is reduced near the iron-sulfur cluster N2 within the Q-module, which is composed of four subunits: 49 kDa, 30 kDa, TYKY, and PSST. Putative locations for the 49 kDa and 30 kDa subunits have been identified via immuno-labeling studies combined with image analysis in two dimensions (Guénebaut et al. 1997; Zickermann et al. 2003). However, a two-dimensional analysis cannot uniquely identify the subunit relations in three dimensions.

Several three-dimensional reconstructions at medium resolution have been determined from electron microscopical data (Guénebaut et al. 1997; Grigorieff 1998; Guénebaut et al. 1998; Böttcher et al. 2002). They all show a basic L-shaped structure except for one (Böttcher et al. 2002); unfortunately, none of them have the necessary substructural detail for subunit analysis. Recently, by random conical tilting and extensive classification, we have calculated a complex I reconstruction with reproducible domain structures (Radermacher et al. 2006). The peripheral arm of our reconstruction is subdivided into at least six domains (see figure 3 below): 1 and 2 are located at the distal end of the peripheral arm, while 3–6 are located at the center of the peripheral arm, closer to the membrane region. Two major protrusions facing the matrix side and a channel like feature facing the cytoplasmic side are visible in the membrane arm. The definition of these domains in our structure forms a basis for identifying the subunits that comprise each domain.

Here we report on the three-dimensional structure of *Y. lipolytica* complex I lacking the two central subunits (51 kDa and 24 kDa) that form the flavoprotein part of the so-called N-module, where electrons are delivered to the enzyme from NADH. The 51 kDa subunit contains the NADH binding site with FMN (Fecke et al. 1994) and the iron sulfur cluster N3. The 24 kDa subunit accommodates iron-sulfur cluster N1a (Ohnishi et al. 1981; Maly et al. 2006). Through a comparison of the three-dimensional structure of the subcomplex with the previous reconstruction of the holoenzyme (Radermacher et al. 2006), we have localized these two subunits in the peripheral arm of complex I.

The work presented here is the first direct localization of subunits in the 3D structure of the complete complex I. Furthermore, this subunit localization has allowed us to fit the recently published X-ray model of the hydrophilic domain from the bacterial complex of *T. thermophilus* (Sazanov and Hinchliffe 2006) into our three dimensional structure of the holoenzyme, and to predict the possible locations of seven subunits within the complete enzyme.

Materials and methods

Protein Purification

His-tagged complex I from a *Y. lipolytica* sulfur-transferase deletion strain (Abdrakhmanova et al. 2005) was purified essentially as described in (Kashani-Poor et al. 2001). Briefly, mitochondrial membranes were solubilized using n-dodecyl- β -D- maltoside and the supernatant after ultracentrifugation was applied to a Ni-NTA sepharose column. Complex I was eluted by increasing the imidazole concentration in the column buffer (400 mM sodium chloride, 20 mM sodium phosphate pH 7.2) from 55 to 140 mM. The fractions containing complex I were combined and concentrated using spin devices. Shock freezing and thawing under the specified buffer conditions resulted in specific dissociation of the 51 kDa and 24 kDa subunits from complex I. Peak fractions after subsequent size exclusion chromatography on a TSK 4000SW column were used for structural analysis. The resulting subcomplex preparation was analyzed by one and two-dimensional gel electrophoresis to confirm that only the 51 kDa and 24 kDa subunits were removed. For details and further characterization see (Zickermann et al. 2007).

Electron Microscopy

Purified subcomplex I was diluted to 0.015 mg/ml in buffer containing 25 mM Na⁺/Mops pH 7.2, 100 mM NaCl, 2 mM EDTA and 0.025% laurylmaltoside; 0.025 mg/ml Tobacco Mosaic virus (TMV) was added for magnification calibration. 8 μ l droplets of this preparation were placed on 400 mesh, glow-discharged, carbon-coated grids (EMS Electron Microscopy Sciences, Hatfield, PA, USA), blotted with #41 Whatman filter paper (Whatman Inc. Florham Park, NJ, USA), and stain embedded with 8 μ l droplets of ammonium molybdate pH 7 using a protocol based on (Stoops et al. 1992; Radermacher et al. 2001; Ruiz and Radermacher 2006). For obtaining good stain embedding after blotting, the grids were dried under a stream of dry nitrogen.

Micrographs were recorded on a Tecnai T12 transmission electron microscope (FEI, Eindhoven, NL) at 100 KV. For optimal resolution and coherence, the LaB₆ filament was run in point mode (Ruiz et al. 2003). Low-dose conditions were maintained throughout the data collection, with doses less than 10 e/ $\text{\AA}^2$ per image. Micrographs were taken as tilt pairs at $\sim 55^\circ$ and 0° for random conical analysis (Radermacher et al. 1986; Radermacher 1988). Pairs of micrographs were graded with an optical diffractometer; images without drift and resolutions better than 1 nm were selected for analysis.

Image Processing

70 tilt pair micrographs were digitized at 7 μ m pixel size on an Intergraph SCAI scanner (Z/I Imaging Corporation, Huntsville, AL); after binning by a factor of 3, the calibrated pixel size was 4.02 $\text{\AA}$ on the specimen scale. Scans included a transparent section of each micrograph for accurate optical density calculations. Complex I particle coordinates were picked interactively from the tilt pairs using WEB (Frank et al. 1996), windowed, and contrast normalized based on the average density in a ring around each particle. A total of 5548 image pairs of single molecules were selected and processed further using SPIDER 5.0 with modifications (Frank et al. 1981; Frank et al. 1996). The contrast transfer function (CTF) parameters were determined, amplitude contrast, defocus, and astigmatism, and CTF correction was applied to the windowed particles by flipping the phases with a smooth function (Radermacher et al. 2001). All alignments were carried out using Radon transform techniques, allowing for simultaneous rotational and translational alignment (Radermacher 1994, 1997). A first reference was created by reference free alignment (Marco et al. 1996). This overall average was centered to a low-pass filtered disk, and used as an alignment reference for the entire particle set. The aligned particles were analyzed with correspondence analysis (van Heel

and Frank 1981; Frank and van Heel 1982; Bretaudiere and Frank 1986), and classified using Diday's technique of moving centers (Diday 1971) followed by hierarchical ascendant classification (CL CLA command in SPIDER). Six class averages were realigned to the previous alignment reference, and used for multi-reference alignment. Two subsequent cycles of correspondence analysis were performed and five final classes were obtained and used for three-dimensional reconstruction. 3D reference-based alignment (Radermacher 1997; Ruiz et al. 2003) was performed to refine the angular and translational parameters of the tilt data of each of the five classes until the resolution in three dimensions remained stable. During refinement, care was taken to properly adjust the low-pass filter in each alignment step to avoid overfitting of high resolution components. The final reconstructions were low pass filtered to their measured resolution. Resolutions were determined using the Fourier ring/shell correlation with a cutoff criterion of 0.3 (Saxton and Baumeister 1982; Rosenthal and Henderson 2003).

Volumes of the subcomplex and the complete complex I were aligned manually using the Chimera software package (Pettersen et al. 2004) and by three-dimensional correlation alignment as described in (Ruiz et al. 2001; Radermacher and Ruiz 2006). The X-ray structure was fitted into two volumes from our earlier 3D reconstruction. One was the volume of the combined set, with resolution 24 Å, as a second volume we used the higher resolution (17 Å) structure from class 8_2 (see Radermacher et al. 2006). For each fit, the X-ray structure of the hydrophilic fragment from *T. thermophilus* was first visually placed into a probable position in the structure of the holoenzyme and then fitted using the fitting option in Chimera.

Results

Images of the subcomplex showed, at first inspection, a similar distribution and shape as had been observed for the holoenzyme (figure 1). Notably, however, was a change in the appearance of the peripheral arm.

Removal of subunits can lead to conformational changes throughout a multi-subunit macromolecular assembly. While conformational changes are present in the subcomplex (see discussion), they are minor, with a substantial number of particles retaining the conformation of the holo-enzyme. Image processing and classifications resulted in the division of the data set into five major classes (figure 2). The reconstructions from classes 1 and 2, comprising more than 45% of the data set, most closely resemble the shape of the holoenzyme, lacking just one of the major domains, yet exhibiting all other domains in the same position as seen in the holoenzyme. Classes 3 to 5 exhibit larger differences. The reconstruction of class 1, which has the highest resolution (31 Å), was used for a detailed comparative analysis with our previous 3D reconstruction of the holoenzyme (Radermacher et al. 2006). The comparison of the 3D structures clearly shows that domain 1 is missing in the subcomplex (figure 3), while all other major domains of the peripheral arm are present in the same relative positions as in the holoenzyme. The size of the missing domain is sufficiently large to accommodate a globular mass of 75 kDa, which is the combined mass of the two removed subunits (51 kDa and 24 kDa).

The subcomplex lacking the flavoprotein subunits was purified from a sulfurtransferase deletion strain of complex I (st1Δ) (Abdrakhmanova et al. 2005), while the 3D reconstruction of the complete complex I was carried out from the wild type complex. Sulfurtransferase is a subunit of 34.6 kDa with rhodanese activity, and can be found in substoichiometric amounts in preparations of complete complex I from *Y. lipolytica*, but not in preparations from other species (Abdrakhmanova et al. 2005). To ensure that this difference did not contribute to the difference that we observe in domain 1, we imaged the intact complex I purified from the st1Δ strain and compared the images to those obtained from the wild type complex. We could not identify the location of the sulfurtransferase subunit by two-dimensional difference

imaging. However, it was obvious that the main protrusions making up domains 1 and 2 in the complete complex were also clearly visible in complex I purified from the deletion strain (data not shown). Therefore, we concluded that the observed differences in the matrix arm are the result of the missing 51 kDa and 24 kDa subunits.

To further confirm the appropriate size of the missing domain and to determine the possible location of other subunits in the peripheral arm, we fitted the X-ray model of a hydrophilic fragment of the peripheral arm of complex I from *T. thermophilus* (Sazanov and Hinchliffe 2006), (PDB accession code 2FUG). Bacterial complex I contains at least 14 subunits, which are viewed as the minimum set of subunits required for the bioenergetic function of complex I. While these subunits are not identical across species, they exhibit high homology scores between prokaryotic and eukaryotic complex I. The hydrophilic fragment of *T. thermophilus* contains 7 of these subunits and in addition one subunit that is not found in complex I from most bacteria.

Figure 4a shows a superposition of the subcomplex and the holoenzyme. When the X-ray model is placed into this structure with the Nqo1 (51 kDa) and Nqo2 (24 kDa) subunits positioned in domain 1, it becomes obvious that the missing mass quite accurately corresponds to the combined mass of the two bacterial subunits (figure 4b).

In our earlier three-dimensional reconstruction, several major domains could be identified in the matrix arm of complex I (figure 3). Domain 1 is surrounded by the three major domains 2, 4 and 5. The X-ray model of the hydrophilic fragment contains essentially three major domains. Without an anchor point, fitting a model of three spheres into a model of four spheres would provide 24 possible positions. The location of the two subunits that we have identified now provides an anchor point. After fixing domain 1, only six configurations remain possible for placing the X-ray model into the 3D electron microscopy structure. We explored all six configurations (table 1). For all fitting experiments, the X-ray model was first positioned by hand and then, when possible, refined with the fitting option of the Chimera software package. The fitting algorithm in Chimera maximizes the overlap between the atoms in the X-ray structure and the density in the EM structure. It is a steepest descent algorithm and finds the closest local maximum.

The top views of fits 1–5 are shown in figure 5. Position 6 was tried but obviously did not fit at all and was discarded. For all fits, the 51 kDa and 24 kDa subunits were placed into domain 1. Fit 1 (figure 5.1 and figure 6 left column) was carried out relative to our highest resolution structure (volume 8_2, 17 Å resolution, in Radermacher et al. 2006). In this fit, the 51 kDa (Nqo1) and 24 kDa (Nqo2) subunits fill domain 1. The 49 kDa (Nqo4) and 30 kDa (Nqo5) subunits occupy domain 2 with the helix H1 of PSST reaching into domain 3. The approximate distance of the iron-sulfur cluster N2 to the top of the membrane arm is 60 Å (figure 7a).

In fit 2 (figure 5.2 and figure 6, left column), we used the lower resolution (24 Å) structure (presented in figure 6 in Radermacher et al. 2006). While this fit by Chimera leaves the major part of the flavoprotein subunits in domain 1, a portion at the periphery becomes unoccupied. The 49 kDa (Nqo4) and 30 kDa (Nqo5) subunits are in domain 3 with the helix H1 of PSST just reaching into the membrane arm. The approximate distance of the iron-sulfur cluster N2 to the top border of the membrane arm is 35 Å (figure 7b). When this placement is used as a starting position for fitting the X-ray model to the higher resolution structure of complex I, it moves back to the position obtained by fit 1. Conversely, when fit 1 is used as a starting position for fitting to the lower resolution EM structure the X-ray structure moves to the position of fit 2. In both fits, 1 and 2, the 75 kDa (Nqo3) subunit, corresponding to NUAM in *Y. lipolytica*, occupies domain 5, from which the connection to the central and distal membrane protuberances emanates (Radermacher et al. 2006).

In position 3, the PSST helix H1 protrudes out from the front of the peripheral arm. While this protrusion is not a possible position, bending of this helix could move it closer to domain 6, which would provide other proteins for contact. The 75 kDa subunit occupies domain 2 and the 49 kDa subunit domain 5. The distance from Iron sulfur cluster N2 to the membrane arm is 53 Å. Even though it is difficult to interpret, position 4 is interesting because the PSST helix attaches to domain 6, and this is the only position that assigns any mass to this region. In fit 4 the 75 kDa subunit also occupies domain 2 and the 49 kDa subunit domain 4. Domain 5 remains empty. The distance between iron sulfur cluster N2 and the membrane arm is 40 Å. Position 5 would result in the helix H1 of PSST protruding out of the back surface of the hydrophilic arm of complex I, with the 49 kDa subunit occupying domain 2 and the 75 kDa subunit in domain 4. Also here domain 5 remains empty. The distance of iron sulfur cluster N2 to the membrane arm is 81 Å.

Discussion

Our understanding of the pumping mechanism of respiratory complex I is still limited, and identifying its subunit organization is an essential prerequisite to obtain further functional insight. The results presented here show the first direct visualization of subunits (51 kDa and 24 kDa) in a three-dimensional structure of the complete complex I. This visualization was achieved by a comparison of three-dimensional reconstructions of the holoenzyme and of a subcomplex lacking the 51 kDa and 24 kDa subunits. The two subunits were localized in domain 1 in the matrix arm. Removal of subunits from a large macromolecular complex can lead to destabilization, causing structural changes throughout the molecule. This destabilization often results in a sample that exhibits a variety of shapes, some closely resembling the intact molecule, and some showing varying degrees of rearrangements. Large scale conformational changes, for example, were observed during the study of the structure of the 50S ribosomal subunit from *E. coli* when subunits or the 5S rRNA were removed (Carazo et al. 1988) (Radermacher et al. 1990). In the current study, we observed a similar effect in a subset of the data. The variations present can be appreciated in figure 2. In this study, the subset of images representing a structure with close resemblance to the holoenzyme is rather large and comprises more than 45% of the data set. Aside from lacking a mass in domain 1, both the reconstructions from class 1 and class 2 closely resemble the complete enzyme. The differences observed between classes 1 and 2 are within the range that we have observed previously for complex I (Radermacher et al. 2006). The large difference between the structures of the full enzyme and the subcomplex clearly positions the 51 kDa and 24 kDa subunits in the most outer lobe of complex I (domain 1). The 51 kDa subunit contains FMN and the binding site for NADH. NADH is formed in the mitochondrial matrix, and the location we identified puts the 51 kDa subunit in an efficient position for NADH oxidation.

Recently, the X-ray structure of the complex I hydrophilic fragment from *T. thermophilus* has been solved and has provided the structures and relative arrangements of the central seven hydrophilic subunits (Sazanov and Hinchliffe 2006; Sazanov 2007), including the 51 kDa and the 24 kDa subunits localized in our structure. The X-ray models of these two subunits fit perfectly into the electron density of domain 1 defined by our three-dimensional reconstructions. However, the subunits do not possess sufficient asymmetry at lower resolution to make this fit unique. In addition, when the X-ray structure is fitted into the whole complex without the restrictions imposed by the present study there would be 24 possibilities for orientations. The localization of the 51 kDa and the 24 kDa subunits substantially narrows down the 24 possibilities for orienting the X-ray model of the hydrophilic fragment within the 3D structure of the holoenzyme to only 6.

The six possible fits can be further analyzed in the context of all the biochemical, biophysical and structural data available for complex I. Ubiquinone and iron sulfur cluster N2 are separated

by only 12 Å in the active complex as shown by EPR studies (Yano et al. 2005). A combination with earlier EPR studies (Salerno et al. 1979) led to the proposal that the ubiquinone is located 5 Å within the membrane region, and at the same time within 12 Å of iron sulfur cluster N2 (Ohnishi and Salerno 2005). Cross-linking studies in complex I from *Paracoccus denitrificans* have shown that both, PSST and the 49 kDa subunit are adjacent to the membrane arm subunit ND3 (Kao et al. 2004). The cross-linking between PSST and ND3 was carried out with *m*-maleimidobenzoyl-*N*-hydrosuccinimide ester, a cross-linker with a 9.9 Å arm, while the cross-linking of the 49 kDa subunit was carried out with a zero-length cross-linker. However, the crosslinked peptides were not identified. Further information has been obtained from antibody binding experiments in combination with 2D electron microscopy which have placed the 49 kDa (Guénebaut et al. 1997; Zickermann et al. 2003) and 30 kDa subunits within the upper half of the matrix arm. Both studies at the time concluded that the 49 kDa subunit was located in what we have identified as domain 5. The second study used two monoclonal antibodies against the 49 kDa subunit with binding epitopes designated as 49.1 and 49.2 and a commercial his-tag antibody against a his-tag at the C-terminal of the 30 kDa subunit. Of these three antibodies only the binding epitope 49.2 is present in the X-ray model. The C-terminal amino acids on the 30 kDa subunit that carry the his-tag in the *Yarrowia* complex I are missing as well as the amino acids at the N-terminus of the 49 kDa subunit that contain the binding epitope 49.1. In the interpretation of the fitting results, it should also be taken into account that conformational changes in the overall structure can occur not only after subunit removal, but also when fragments are isolated from the whole enzyme. The hydrophilic fragment from *T. thermophilus* complex I falls into the latter category. In the context of the possible sources of variation combined with the currently still limited resolution of the 3D structure of the holo-enzyme, the current fits of the X-ray model can only serve as a best attempt to predict the location of the subunits within the complete enzyme.

Of the six possible positions (table 1), position 6 could be immediately excluded because there was simply no convincing way to place the structure into this position. Fit 5 is improbable because the amphiphatic helix H1 in PSST protrudes into empty space on the back of the matrix arm. Fit 4 places helix H1 of PSST into domain 6 and is the only fit that places any mass into this small domain. Domain 6 has been observed in all our studies of *Yarrowia* complex I. However, fit 4 contradicts the visualized locations of the antibodies in our previous study (Zickermann et al. 2003) because the end of the 49 kDa subunit that contains the binding epitope for antibody 49.2 (Zickermann et al. 2003) is pointing down to the membrane arm and is buried. This position would leave this epitope inaccessible. Both, fit 5 and fit 4 leave domain 5 empty, which would require this domain to contain accessory subunits, thus making both fits unlikely. In fit 3, helix H1 protrudes into empty space in front of the matrix arm. Fit 3 is reasonable only if PSST can be bent, such that H1 reaches the small domain 6 for protein contact.

We will focus in the following discussion mostly on fit 1, which best fits the antibody binding data (Guénebaut et al. 1997; Zickermann et al. 2003), and fit 2, which is similar to the orientation proposed in (Sazanov and Hinchliffe 2006). Both fits place the 75 kDa subunit in domain 5 pointing towards the proposed tether that connects to the two membrane arm protuberances in our earlier EM structures. Both models suggest that the 75 kDa (NUAM) subunit may be the coupling module between the catalytic center and the postulated conformationally driven proton pumps in the membrane arm connected by the tether that was observed in our earlier study. Domain 1 and 2 are filled in fit 1, while in fit 2 domain 1 is only partially filled and domain 2 remains empty. Fit 2 thus requires that domain 2 would contain accessory subunits absent in the bacterial structure. To accommodate fit 2, domain 1 in the prokaryotic complex would either have to appear smaller than in the eukaryotic enzyme or the relative positions of the major domains in the hydrophilic fragment used for the X-ray crystallographic study would have to be slightly rearranged. The vertical position of the X-ray model within the peripheral arm is dominated by the fit of the 75 kDa subunit into domain 5.

Thus, for example, a small relative movement of the 75 kDa subunit would allow the other subunits to move “upwards”, thus filling domain 1. Helix H1 of PSST is parallel to and just inside the membrane arm in fit 2 and fits vertically into domain 3 in fit 1. Fit 1 shows the best fit to the high resolution electron microscopy structure. The main difficulty of fitting the X-ray model into position 2 for the higher resolution structure is the cavity in the matrix arm. In the higher resolution structure, the peripheral arm connects to the membrane arm in two separate places leaving a void in between. Fit 2 would fill this cavity while fit 1 leaves it open.

In all possible fits, iron-sulfur cluster N2 is outside of the membrane arm, having the shortest distance of 35 Å in fit 2, the largest of 81 Å in fit 5 and an intermediate value of 60 Å in fit 1. In all arrangements, the ubiquinone head group and part of its hydrophobic tail must leave the membrane domain to become reduced by iron sulfur cluster N2. This strongly favors a conformational coupling between ubiquinone reduction and proton translocation. Ubiquinone leaving the membrane contradicts its proposed location 5 Å deep within the membrane bilayer, described in (Onishi and Salerno 2005); the distance of 12 Å between ubiquinone and iron sulfur cluster N2, however, can be accommodated. Cross-linking studies have shown that PSST and the 49 kDa subunits both are linked to the membrane subunit ND3 (Kao et al. 2004). This proximity can be explained by fit 2 and partially by fit 4. For fit 1 one would have to assume that the observed crosslink was between a hydrophilic loop of ND3 extending into the peripheral arm and possibly H1 of subunit PSST. Fit 1 accommodates domain 1 better than fit 2 and is in better agreement with earlier antibody binding studies that localized the 30 kDa (NUGM) and 49 kDa (NUCM) subunits in two-dimensional average images in the upper half of the matrix arm (Guénébaut et al. 1997; Zickermann et al. 2003). The binding experiments of the 30 kDa antibody and the antibody binding to epitope 49.1 could be reconciled with most of the fits, because the missing amino acids in the X-ray model of the hydrophilic fragment could be of sufficient length to explain the positions found. However, epitope 49.2 is present in the X-ray model and is located at the distal end of the 49 kDa subunit. The location visualized for this antibody can only be reconciled with fit 1. While fit 1 represents the most likely position, the remaining discrepancies can only be resolved by further biophysical and structural studies.

Conclusion

We have localized the 51 kDa (NUBM) and 24 kDa (NUHM) flavoprotein subunits in the three-dimensional structure of complex I from *Y. lipolytica*. The subunits are unambiguously localized at the most outer lobe of the peripheral arm of the enzyme. By fitting the X-ray model of the hydrophilic fragment of complex I from *T. thermophilus*, we propose locations for the main hydrophilic subunits in the peripheral arm of complex I from *Y. lipolytica*. All possible fits place the ubiquinone reduction site outside the membrane arm, which would require at least parts of ubiquinone to leave the membrane domain during catalysis. In our preferred fit (fit 1), electron transfer occurs in the outer part of the peripheral arm of complex I and conformational changes required for an active proton transport could be transferred through the 75 kDa subunit via a tether to the proton pumping subunits at the distal end of the membrane arm.

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Figure 1.

The structure of the subcomplex was calculated from a random conical data set. Shown is a tilt pair. left: 0° image, right: tilt image, angle 55°. Tilt axis vertical. Scale bar 100 nm.

Figure 2. Surface representation of the three-dimensional reconstructions of the five classes of the subcomplex data. Class 1 and 2 show the volumes with the least differences to the complete enzyme, calculated from a total of 45% of the data. Classes 3–5 show additional conformation changes. Scale bar 10 nm.

Figure 3.

a) Surface representation of the three-dimensional structure of the subcomplex, b) surface representation of the holoenzyme. The numbering of the domains follows the conventions used in the original publication of the holoenzyme structure (Radermacher et al. 2006). The major domain missing in the subcomplex is domain 1. c) added to indicate the domains not visible in a and b. Scale bar 10 nm.

Figure 4.

a) overlay of surface of the holoenzyme (magenta), the subcomplex (grey) and the ribbon presentation of the X-ray structure from (Sazanov and Hinchliffe 2006), rear view. b) Surface of the subcomplex and surface of the bacterial Nqo1 (51 kDa) and Nqo2 (24 kDa) subunits from the X-ray structure, represented as surface, front view. Scale bars 10 nm.

Figure 5.

The five possible fits of the X-ray model into the EM structure. For better visibility the ribbon representation of the X-ray model is shown in panel 0) Indicated are the different subunits (color coded), the location of iron-sulfur cluster N2 and, marked by “*”, the loop that contains the binding epitope for antibody 49.2 in (Zickermann et al. 2003). The numbering of the panels follows the numbering of the fits as described in the text and in table 1. Scale bar 10 nm (applicable to panels 1–5)

Figure 6.

Views of fit1 (left column) and fit 2 (right column). The fits are shown with the mesh data of the EM structure that fit best: the 17 Å structure in fit 1 and the 24 Å structure for fit 2. a) rear view, b) front view, c) different views for each of the two fits, for better visibility. H1 amphiphatic helix in PSST. Scale bar 100 Å, applies to panels 1–5.

Figure 7.

Location of the iron sulfur clusters within the holoenzyme. a) positions for fit 1, b) positions for fit 2. In fit 1 (a) the distance between iron-sulfur cluster N2 and the upper border of the membrane arm is about 60 Å. In fit 2 (b) this distance is about 35 Å. Scale bar 10 nm.

Table 1

List of the possible six positions of the X-ray model of the hydrophilic fragment of the bacterial complex I into the 3D electron microscopy structure of complex I from *Y. lipolytica*. The table lists the domains as indicated in the EM structure versus the major subunits in the three domains of the X-ray structure. Indicated are the positions of helix H1 in PSST relative to the domains in the peripheral arm and the distance of iron-sulfur cluster N2 measured relative to the top border of the membrane arm. The last two rows indicate if the fit was possible to either the higher or lower resolution structure.

Domain	Fit 1	Fit 2	Fit 3	Fit 4	Fit 5	Fit 6
1	51 kDa	51 kDa	51 kDa	51 kDa	51 kDa	51 kDa
2	49 kDa	-	75 kDa	75 kDa	49 kDa	-
5	75 kDa	75 kDa	49 kDa	-	-	49 kDa
4	-	49 kDa	-	49 kDa	75 kDa	75 kDa
Helix Position:	domain 3	bottom of domain 4	protruding in front	at domain 6	protruding in back	no fit
N2 Distance	60 Å	35 Å	52 Å	40 Å	81 Å	
Fit to 17Å structure	yes	no	yes	no	yes	
Fit to 24 Å structure	no	yes	slight adjustment	moves down	no	