Structure of Yeast Sulfhydryl Oxidase Erv1 Reveals Electron Transfer of the Disulfide Relay System in the Mitochondrial Intermembrane Space*

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Received for publication, June 24, 2012, and in revised form, August 19, 2012. Published, JBC Papers in Press, August 21, 2012, DOI 10.1074/jbc.M112.394759

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Background: Mia40 is regenerated by the sulfhydryl oxidase Erv1 in the disulfide relay system.
Results: Crystal structures of the Erv1 core domain and full length of Erv1 were determined.
Conclusion: The Erv1 N-terminal amphipathic helix is critical for electron transfer from Mia40 to the core redox center of Erv1.
Significance: This is the first structural snapshot of the electron transfer process in Mia40-Erv1 disulfide relay system.

The disulfide relay system in the mitochondrial intermembrane space drives the import of proteins with twin CX9C or twin CX9C motifs by an oxidative folding mechanism. This process requires disulfide bond transfer from oxidized Mia40 to a substrate protein. Reduced Mia40 is reoxidized/regenerated by the FAD-linked sulfhydryl oxidase Erv1 (EC 1.8.3.2). Full-length Erv1 consists of a flexible N-terminal shuttle domain (NTD) and a conserved C-terminal core domain (CTD). Here, we present crystal structures at 2.0 Å resolution of the CTD and at 3.0 Å resolution of a C30S/C133S double mutant of full-length Erv1 (Erv1FL). Similar to previous homologous structures, the CTD exists as a homodimer, with each subunit consisting of a conserved four-helix bundle that accommodates the isoalloxazine ring of FAD and an additional single-turn helix. The structure of Erv1FL enabled us to identify, for the first time, the three-dimensional structure of the Erv1NTD, which is an amphipathic helix flanked by two flexible loops. This structure also represents an intermediate state of electron transfer from the NTD to the CTD of another subunit. Comparative structural analysis revealed that the four-helix bundle of the CTD forms a wide platform for the electron donor NTD. Moreover, computational simulation combined with multiple-sequence alignment suggested that the amphipathic helix close to the shuttle redox enter is critical for the recognition of Mia40, the upstream electron donor. These findings provide structural insights into electron transfer from Mia40 via the shuttle domain of one subunit of Erv1 to the CTD of another Erv1 subunit.

Formation of correct disulfide bonds is important for the structure and function of most proteins. In eukaryotic cells, the intermembrane spaces (IMS) of mitochondria and endoplasmic reticulum are the two major locations for the introduction of disulfide bonds (1). The IMS has a dedicated disulfide relay system to introduce disulfide bonds into the small cysteine-rich substrate proteins (2), such as small Tim proteins and copper chaperone Cox17, which are nuclearily encoded and cytosolically synthesized (3, 4). These substrate proteins are characterized by a relatively low molecular mass in the range of 8–17 kDa and a conserved motif of cysteine pairs. These are twin CX9C or twin CX9C motifs that are crucial for the import of preprotein and accumulation of mature proteins in the IMS (5–7). Newly synthesized unfolded substrate proteins would pass through the translocase of the outer membrane and form a mixed disulfide bonded intermediate with Mia40 (mitochondrial intermembrane space import and assay/oxidoreductase 40) in the IMS (8–10). Mia40 is a conserved oxidoreductase that is soluble in mammals and plants but membrane-anchored in fungi (9, 11). It harbors an conserved redox-active motif of -CPC-CX9C-CX3C-C (8, 11), using a CPC site to form an intermolecular disulfide bond with substrate proteins (12, 13). In the disulfide exchange reaction, a disulfide bond is introduced into the substrate protein, accompanied by the release of the reduced Mia40, which is reoxidized/regenerated to a functional state by the sulfhydryl oxidase Erv1 (essential for respiration and viability/FAD-linked sulfhydryl oxidase 1) (2, 14). Thereafter, the reduced Erv1 passes the electron to either cytochrome c or molecular oxygen (15–18). Together, Mia40 and Erv1 are two essential components of the disulfide relay system that is of crucial importance for mitochondrial biogenesis (2, 19, 20). The FAD-linked sulfhydryl oxidase Erv1 (EC 1.8.3.2) is essential for the respiration and vegetative growth of the yeast (21, 22). A number of Erv1 homologs have been characterized in plants (23), mammals (24, 25), and double-stranded DNA viruses (26, 27). The mammalian homologs are called augmenters of liver regeneration (ALRs). All Erv1/ALR family members

* This work was supported by Ministry of Science and Technology of China Project 2012CB911000.

The atomic coordinates and structure factors (codes 4E0H and 4E0I) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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2 The abbreviations used are: IMS, intermembrane space; NTD, N-terminal shuttle domain; CTD, C-terminal core domain; Erv1FL, C30S/C133S double mutant of the full-length Erv1; RMSD, root mean square deviation; ALR, augmenter of liver regeneration.
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share a conserved core domain harboring a CXXC motif (the core redox center), juxtaposed with FAD and involved in redox reactions. To date, the structures of core domains have been determined for human ALR (28, 29), Arabidopsis thaliana Erv1 (30), Rattus norvegicus ALR (31), and Saccharomyces cerevisiae Erv2 (32). All exist as a homodimer, with each subunit composed of a four-helical bundle that accommodates the isoalloxazine ring of FAD with an additional single-turn helix.

In addition to the conserved core redox center, Erv1/ALR proteins, except for the viral homologs, possess another cysteine pair. This is at the N-terminal domain (NTD) in fungi and mammals and at the C-terminal segment in plants (33). Genetic studies demonstrated that the N-terminal CXXC motif of yeast Erv1 was required for in vivo functions (34). In fact, the NTD of yeast Erv1 is necessary and sufficient for interaction with Mia40. Moreover the N-terminal cysteine pair is required for the formation of a mixed disulfide intermediate with Mia40. Thus, the N-terminal redox center (16, 20, 33, 35).

To gain insights into the structural basis of this electron transfer process, we determined the structure of the CTD at 2.0 Å resolution and the structure of the C30S/C133S double mutant for in vivo functions (34). In fact, the NTD of yeast Erv1 is necessary and sufficient for interaction with Mia40. Moreover the N-terminal cysteine pair is required for the formation of a mixed disulfide intermediate with Mia40 (35). Because of its role in forwarding electrons from Mia40 to the C-terminal core domain (CTD), the NTD is termed the shuttle domain, and the CXXC motif at the NTD is termed the shuttle redox center (16, 20, 33, 35).

EXPERIMENTAL PROCEDURES

Overexpression and Purification of Erv1 and Mutants—The coding sequences of the intact yeast Erv1/YGR029W and the C-terminal core domain (Asp86–Glu189, designated as Erv1CTD) were amplified by PCR using S. cerevisiae S288c genomic DNA as the template and cloned into a pET28a-derived vector, respectively. The constructs add a hexahistidine tag to the N terminus of the recombinant protein, which were derived vector, respectively. The constructs add a hexahistidine tag to the N terminus of the recombinant protein, which were derived vector, respectively.

Equilibration with binding buffer (20 mM Tris-HCl, pH 8.0, 200 mM NaCl). The target protein was eluted with 300 mM imidazole buffer and further loaded onto a Superdex 75 column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 8.0, 200 mM NaCl (20 mM sodium citrate, pH 5.38, 50 mM NaCl for Erv1CTD). Fractions containing the target protein were pooled and concentrated to 10 mg/ml by ultrafiltration (Millipore; 10-kDa cut-off). The purity of proteins was estimated by SDS-PAGE, and the proteins were stored at −80 °C.

RESULTS AND DISCUSSION

Overall Structure of the Highly Conserved Erv1CTD—The yeast Erv1 has two domains. The highly conserved CTD follows a flexible N-terminal domain (20). We first determined the structure of Erv1CTD, which is from Asp86 to Asp189. Each asymmetric unit consists of one Erv1CTD molecule, which adopts an α-helix core domain structure. For example, the root mean square deviation (RMSD) between Erv1CTD and human ALR is only 0.69 Å over 104 Ca atoms. Erv1CTD consists of a four-helix bundle (helices a1–a4) and an additional single-turn helix α5.
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core redox center with a total interface area of 880 Å² (450 Å² for NTD and 430 Å² for CTD). This interface has a typical area for redox protein complexes because of their short-lived interactions. In addition to the intersubunit disulfide bond, five hydrogen bonds are involved in stabilizing the conformation of NTD (Fig. 3C). In particular, the carbonyl oxygen of Ser⁵² and Asn²⁴ forms hydrogen bonds with the amide nitrogen of Val⁵⁷, respectively. The amide nitrogen of Leu⁶ forms a hydrogen...
bond with Asp\textsuperscript{86}. O\textsubscript{62}, whereas Arg\textsuperscript{31} - N\textsubscript{1} makes a hydrogen bond with the carbonyl oxygen of Pro\textsuperscript{84}. These four hydrogen bonds close to the shuttle redox center form a network of seven residues, five of which contribute with the main chain atoms, whereas residues Arg\textsuperscript{31} and Asp\textsuperscript{86} donate their side chain atoms. Sequence alignment showed that Arg\textsuperscript{31} and Asp\textsuperscript{86} are conserved in Erv1/ALR and homologs (data not shown). In addition, Asp\textsuperscript{24} - O\textsubscript{62} forms a hydrogen bond with Trp\textsubscript{132} - N\textsubscript{1} of the CTD. Moreover, hydrophobic contacts between a hydrophobic patch (Val\textsuperscript{87} and Trp\textsubscript{132}) of the CTD and the complementary side (Ile\textsuperscript{21}, Ile\textsuperscript{22}, and Thr\textsuperscript{35}) of the NTD also contribute a part to the interface (Fig. 3D).

The overall structure of CTD' of Erv1FL subunit B is quite similar to that of subunit A and the isolated CTD, with RMSD of 0.32 and 0.69 Å over 104 Ca atoms, respectively. However, when approaching the NTD, helix \( \alpha 3 \) (Cys\textsuperscript{130} - Glu\textsuperscript{143}) at the core redox center rotates outwards against the isoalloxazine ring of FAD at an angle of \(-6.0^\circ\) along its C terminus (Fig. 3E). In addition, the other three helices of the four-helix bundle also shift slightly outwards. These conformational changes lead to a wider bundle mouth to interact with the approaching NTD. The segments that locate on the top of the four helices of CTD' constitute a platform that plays a crucial role in the NTD interaction.

Although the NTD of subunit A is cross-linked to the CTD of subunit B, the traceable segment Glu\textsuperscript{14} - Ser\textsuperscript{47} exhibits relatively high B factors. This structural flexibility is considered necessary for recognition of both Erv1CTD and Mia40 (29). Moreover, the linker between the shuttle redox center and the core domain displays a much higher flexibility, and most of the linker could not be traced in the electron density map. This highly flexible linker enables the shuttle redox center to easily flip between the Erv1CTD and Mia40.

A Putative Binding Model between Erv1 and Mia40—After determining the structure of Erv1NTD, we attempted but failed to obtain a crystal of Erv1NTD in complex with Mia40 by a similar cross-linking strategy. Alternatively, we simulated a model of the Mia40-Erv1NTD complex using the program HADDOCK (45), based on our Erv1FL structure and the previously reported structure of Mia40 (Protein Data Bank code 2ZXT, without the maltose-binding protein tag). This was driven by interaction restraints between the active site residues of Erv1 and Mia40, as defined by the program WHISCY (46). Among the 15 output clusters, the cluster of lowest energy with eight members satisfied the best interaction restraints and had the largest buried solvent-accessible interface area of \(~1050\) Å\textsuperscript{2} (595 Å\textsuperscript{2} for Erv1NTD and 455 Å\textsuperscript{2} for Mia40). The overall backbone RMSD of 0.5 ± 0.3 Å for the eight members indicated that...
the model of Mia40-Erv1NTD was somewhat reliable. In the model, helix α0 makes extensive interactions with the hydrophobic cleft of Mia40 (Fig. 4A). This cleft is composed of a cluster of highly conserved hydrophobic residues Met302, Phe315, Phe318, and Met337 (Fig. 4B). These residues are also involved in recognizing the CXXC and CX3C substrates (10), suggesting that Mia40 uses the same site to bind both protein substrates and the electron acceptor Erv1. Notably, this model is in accordance with the previous report that Erv1 competitively binds to the substrate-binding site on Mia40 (29).

The docked interface on Erv1NTD that comprises hydrophobic residues Leu36, Leu37, and Phe39 (Fig. 4B) is in agreement with the results of Banci et al. (29). They used NMR titration to determine that the CRACVDFKTWM segment of ALR (homologous to the Erv1 CRSCNTL36L37DF39Q segment) is critical for recognition of Mia40. Using mutagenesis in combination with complementation assays, they confirmed that the hydrophobic residues downstream of the Erv1 shuttle redox center (Leu36, Leu37, and Phe39) play a vital role in complex formation with Mia40 in vitro and in vivo. These residues on the amphipathic helix α0, as shown in the structure of Erv1FL, are solvent-exposed before being recognized by Mia40. Once fitted into the hydrophobic cleft of Mia40, helix α0 will enable Cys33 of Erv1NTD to come as close as ~6.3 Å to Cys298 of Mia40. With slight conformational changes, these two cysteine residues can form a transient mixed disulfide bond.

Universal Mode of Electron Transfer from Mia40 to Erv1 Shuttle Domain in Animals and Fungi—Erv1 homologs have a highly conserved core domain but variable shuttle domains (33). To find the probable original shuttle domain and its distribution, the sequence of yeast Erv1 was used in a BLAST search against the nonredundant protein sequences database. We chose 10 representatives of various species and compared the residues around the shuttle redox centers (Fig. 5). Both fungi and animals have an N-terminal shuttle domain with a highly conserved redox center (CXXC) but variable linkers between the shuttle and the CTD. Similar to the residues that constitute the amphipathic helix α0 in the yeast Erv1NTD, the corresponding residues in the animal homologs were also predicted to have a high propensity to form a helix (residues FKTWM in human ALR) (29). In yeast and human Erv1/ALR, the hydrophobic residues are somewhat aligned on one side of the amphipathic helix α0, indicating a universal hydrophobic interaction pattern between Mia40 and Erv1/ALR from fungi and animals.

In fungi and animals, the N-terminal shuttle redox center of Erv1/ALR functions as an antenna stretched from the core domain. This antenna is held by the CTD with a flexible linker.
of varied lengths. Of note, the linker is gradually truncated during evolution from lower to higher organisms (Fig. 5). The linker of yeast Erv1 is composed of 38 amino acids, whereas the linker of human ALR is of only 14 amino acids. A shorter linker might be helpful in enhancing electron transfer efficiency from the shuttle to the core redox center.

A Working Model of Electron Transfer Process in the Mia40-Erv1 Disulfide Relay System—Based on our structural analyses and previous reports, we propose a working model of electron transfer in the Mia40-Erv1 disulfide relay system (Fig. 6). To simplify the illustration, we show that only one of two shuttle domains of the Erv1 dimer is working with a molecule of Mia40 during the catalysis cycle. In our model, 1) after the oxidation and release of a given substrate protein, the hydrophobic cleft of the reduced Mia40 is exposed (12). Meanwhile, the amphipathic helix α0 at the shuttle domain of the oxidized Erv1 is recognized by the hydrophobic cleft of Mia40. 2) The shuttle redox center forms an intermolecular disulfide bond with Mia40 (between Mia40-Cys298 and Erv1-Cys33) (12, 20). Consequently, this transient disulfide bond is exchanged upon the attack of Mia40-Cys296 to release oxidized Mia40. Regenerated Mia40 is ready to oxidatively refold another substrate protein. The electron transfer from Mia40 to the shuttle domain of Erv1 requires mechanisms to overcome the thermodynamically unfavorable redox gradient. This might be driven by conformational changes of Erv1NTD at different redox states (17). 3) The reduced shuttle domain of Erv1 swings back and lands on a platform on the core redox center of a neighboring subunit in the same dimer. The conformational changes of the core domain facilitate the formation of an intersubunit disulfide bond between Cys33 and Cys130. 4) The intersubunit disulfide bond is subsequently attacked by Cys30 at the shuttle domain to

FIGURE 5. Multiple-sequence alignment of the shuttle domains of Erv1/ALR proteins from S. cerevisiae (NP_011543.2), G. clavigera kw1407 (EFX00923.1), H. magnipapillata (XP_002163122.1), A. mellifera (XP_001120016.1), S. purpuratus (XP_786637.1), D. rerio (NP_001082855.1), X. laevis (BC_097922.1), G. gallus (XP_414848.2), R. norvegicus (NP_037354.2), and H. sapiens (NP_005253.3). Cysteines of the shuttle redox center are marked with blue stars. Alignments were performed with ClustalW and ESPript.

FIGURE 6. A schematic electron transfer cycle of the Mia40-Erv1 disulfide relay system.
regenerate an oxidized shuttle redox center. Simultaneously, the core redox center of Erv1 is reduced. This process is spontaneously driven by differences in redox potential between the shuttle and core redox centers (17, 20). 5) Electron transfer from the core redox center to FAD does not require a conformational change (the distance from the flavin C4α to the thiol group of Cys133 is ~3.3Å) but must overcome the unfavorable redox gradient (17). This process might be driven by coupling to the efficient downstream electron flow to cytochrome c (47). 6) The reduced FAD efficiently transfers the electron to the most favorable physiological electron acceptor, cytochrome c. Thus, Erv1 is ready for another electron transfer cycle.

Conclusions—This work captured an intermediate structure of the N-terminal shuttle domain cross-linked to the core domain of Erv1 via an introduced disulfide bond between Cys13 of one subunit and Cys130 of another subunit. The frozen position of the highly flexible shuttle domain enabled us to determine a model of electron transfer from the upstream Mia40 to the downstream cytochrome c via a recognition pattern similar to the interaction between Mia40 and its substrate protein. These findings provide for the first time structural insights into the overall Mia40-Erv1 disulfide relay system.

Acknowledgments—We thank the staff at the Shanghai Synchrotron Radiation Facility for the data collection. We are grateful to all the developers of the CCP4 Suite, ESPript, MOLPROBITY, and PyMOL.

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