Deciphering Structural and Functional Roles of Individual Disulfide Bonds of the Mitochondrial Sulfhydryl Oxidase Erv1p*\textsuperscript{[S]}

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Erv1p is a FAD-dependent sulfhydryl oxidase of the mitochondrial intermembrane space. It contains three conserved disulfide bonds arranged in two CXXC motifs and one CX\textsubscript{16}C motif. Experimental evidence for the specific roles of the individual disulfide bonds is lacking. In this study, structural and functional roles of the disulfides were dissected systematically using a wide range of biochemical and biophysical methods. Three double cysteine mutants with each pair of cysteines mutated to serines were generated. All of the mutants were purified with the normal FAD binding properties as the wild type Erv1p, showing that none of the three disulfides are essential for FAD binding. Thermal denaturation and trypsin digestion studies showed that the CX\textsubscript{16}C disulfide plays an important role in stabilizing the folding of Erv1p. To understand the functional role of each disulfide, small molecules and the physiological substrate protein Mia40 were used as electron donors in oxygen consumption assays. We show that both CXXC disulfides are required for Erv1 oxidase activity. The active site disulfide is well protected thus requires the shuttle disulfide for its function. Although both mutants of the CXXC motifs were individually inactive, Erv1p activity was partially recovered by mixing these two mutants together, and the recovery was rapid. Thus, we provided the first experimental evidence of electron transfer between the shuttle and active site disulfides of Erv1p, and we propose that both intersubunit and intermolecular electron transfer can occur.

Disulfide bonds play very important roles in the structure and function of many proteins by stabilizing protein folding and/or acting as thiol/disulfide redox switches. The process of disulfide formation is catalyzed by dedicated enzymes in vivo (1–4). Erv1p is a FAD-dependent sulfhydryl oxidase located in the \textit{Saccharomyces cerevisiae} mitochondrial intermembrane space (4–6). It is an essential component of the redox regulated Mia40/Erv1p import and assembly pathway used by many of the cysteine-containing intermembrane space proteins, such as members of the “small Tim” and Cox17 families (7–10). Upon import of a Cys-reduced substrate, Mia40 interacts with the substrate via intermolecular disulfide bond and shuttles a disulfide to its substrate. Although oxidized Mia40 promotes disulfide bond formation in the substrates, Erv1p functions in catalyzing reoxidation of the reduced Mia40 and/or release of the substrate (11–13).

The common features for the FAD-dependent sulfhydryl oxidases are that the enzymes can catalyze the electron transfer from substrate molecules (e.g. protein thiols) through the non-covalent bound FAD cofactor to molecular oxygen or oxidized cytochrome c (14). The sulfhydryl oxidases can be divided into three groups: Ero1 enzymes, multidomain quiesin sulfhydryl oxidases, and single domain Erv (essential for respiration and vegetative growth)/ALR proteins. The yeast Ero1p and the mammalian homologues (Ero1α and Ero1β) are large flavoenzymes present in the ER with at least five disulfide bonds, but only two of the disulfide bonds are conserved. The conserved cysteines are essential for the catalytic activity of Ero1p forming the active site CXXC and shuttle disulfide CX\textsubscript{16}C, respectively (15, 16). Furthermore, nonconserved disulfide bonds have been shown recently to be important in regulating the activity of both yeast and mammalian Ero1 (17–19). The second group of oxidases, the multidomain quiesin sulfhydryl oxidases, have important functions in higher eukaryotes (14, 20). Quiesin sulfhydryl oxidases consist of an Erv/ALR module fused to one or more thioredoxin-like domains with two conserved CXXC motifs in the Erv/ALR module. Quiesin sulfhydryl oxidase enzymes are found in many subcellular and extracellular locations, but not in mitochondria. Instead, single domain Erv/ARL enzymes of the third group are found in the \textit{7} mitochondrial of many eukaryotic cells (21). Erv1p belongs to this single domain Erv/ARL family, which includes the human mitochondrial ARL, plant AtErv1, and yeast Erv2p of the ER lumen.

The Erv/ARL enzymes are characterized by a highly conserved central catalytic core of ~100 amino acids, which includes an active site CXXC motif (Cys\textsubscript{130}–Cys\textsubscript{133} for Erv1p), CX\textsubscript{16}C disulfide bond (Cys\textsubscript{159}–Cys\textsubscript{176} for Erv1p), and residues involved in FAD binding (Fig. 1A). Based on the partial crystal structure data of Erv2p (22) and AtErv1 (23), the catalytic core of Erv proteins contains a four-helix bundle forming the non-covalent FAD-binding site with the active site CXXC in close proximity to the isoalloxazine ring of FAD. In addition, the long range CX\textsubscript{16}C disulfide bond of the Erv proteins brings the short fifth helix to the four-helix bundle in proximity to the adenine ring of FAD (Fig. 1A). Thus, the CX\textsubscript{16}C disulfide bond is pro-

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fide bonds are essential for Erv1p function (22). The hypothesized shuttle disulfide of Erv2p is located closely to the isoalloxazine ring of FAD. The N and C termini were labeled as N and C, respectively. The structure was generated using Pymol program. B, schematic of the primary structure of yeast, plant, and human sulfhydryl oxidase with the conserved Cys motifs. The conserved central catalytic core regions are shown as black bars, and the nonconserved regions are in gray.

posed to play a structural role in stabilizing the FAD binding and/or protein folding, but direct experimental evidence to verify the roles is lacking. Apart from the catalytic core, the other parts of the proteins seem flexible and unfolded. Importantly, all members of the Erv/ALR family have at least an additional disulfide bond located in the nonconserved N- or C-terminal region to the catalytic core (Fig. 1B), which is hypothesized as a shuttle disulfide based on the partial crystal structure of Erv2 (22). The hypothesized shuttle disulfide of Erv2p CXC and AtErv1 CX2C are located in the C terminus, but Erv1p (Cys30–Cys33) and ALR have a CXXC shuttle disulfide located N-terminal to the catalytic core. Furthermore, structural and chemical data have suggested that Erv/ARL enzymes form homodimer or oligomers in the presence or absence of intermolecular disulfide bonds (5, 23, 24).

Yeast mitochondrial Erv1p contains a total of six Cys residues forming three pairs of disulfide bonds (residues 30–33, 130–133, and 159–176) as described above. Previous studies with single Cys mutants showed that although all three disulfide bonds are essential for Erv1p function in vitro, only Cys33 was required for the oxidase activity of Erv1p in vitro (24). The conclusion that only Cys130–Cys133 disulfide is required for Erv1p oxidase activity in vitro was based on a study using the artificial substrate DTT2 as the electron donor. Abnormal color changes were observed for some of the single Cys mutants of Erv1p in the previous study that were probably caused by protein misfolding or formation of non-native disulfides because of the presence of a redox active but unpaired Cys. It is clear that Cys130–Cys133 is the active site disulfide; however, experimental evidence for the role of Cys30–Cys33 disulfide is lacking, and the specific role played by the unique CX16C motif of Erv proteins is unknown.

In this study, we dissected the structural and functional roles of all three individual disulfides of Erv1p systematically. To avoid misfolding via unpaired Cys, three double Cys mutants of Erv1p were generated with each of the disulfides mutated to serines. All three mutants were successfully purified with the normal FAD binding properties of the wild type (WT) Erv1p. Various biophysical and biochemical methods were used to study the folding and oxidase activity of the WT and Erv1p mutants. Both artificial and the natural substrate (Mia40) of Erv1p were used as electron donors to understand the functional mechanism of Erv1p. Our results show that both the first (Cys30–Cys33) and second (Cys130–Cys133) disulfides are essential for Erv1 oxidase activity in vitro. Although none of the three disulfides are essential for FAD binding, the third disulfide (Cys159–Cys176) plays an important role in stabilizing the folding of Erv1p. More importantly, this study provided direct experimental evidence to show that Cys30–Cys33 functionally acts as a shuttle disulfide passing electrons to the active site Cys130–Cys133 disulfide. Moreover, the electron transfer seems to occur through both intersubunit and intermolecular interactions.

EXPERIMENTAL PROCEDURES

Materials—4-acetamido-4’-maleimidystilbene-2,2’-disulfonic acid (AMS) and tris(2-carboxyethyl)phosphine (TCEP) were obtained from Molecular Probes (Invitrogen). EDTA was from BDH Co, and all other chemicals were obtained from Sigma at the highest grade. A peptide corresponding to 14 C-terminal residues of Erv1p was used to raise antibodies in rabbit against Erv1p (Eurogentec Ltd.).

Mutagenesis and Protein Preparations—Cysteine to serine mutants of Erv1 were created using QuikChange site-directed mutagenesis with Pfu DNA polymerase (Stratagene) and pET-24a(+) harboring the wild type complete Erv1 gene as template (5). All of the constructs were verified by DNA sequencing. Sequences of mutagenic oligonucleotides can be provided upon request. The Erv1p-His6 proteins were expressed in the Escherichia coli Rosetta-gamiTM 2 (Novagen) and purified using His tag affinity beads followed by fast protein liquid chromatography using Superdex75 column as described previously (25). Concentrations of the WT and Erv1p mutants

2 The abbreviations used are: DTT, 1,4-dithiothreitol; AMS, 4-acetamido-4’-maleimidystilbene-2,2’-disulfonic acid; TCEP, tris(2-carboxyethyl)phosphine; WT, wild type; Tricine, N-[2-hydroxy-1,1-bis( hydroxymethyl)ethyl]glycine; PK, proteinase K.
were calculated using the molar extinction coefficients determined in this study as listed in Table 1. Mia40c (amino acids 284–403), the C-terminal domain of Mia40, was cloned into pGEX 4T-1 vector (GE Healthcare), expressed in the E. coli Rosetta-gami™ 2, and purified using GST affinity beads as described previously (11). The partially reduced Mia40c (Mia40c-pR) was prepared by incubation with 0.5 mM TCEP for 20 min at room temperature, followed by gel filtration using a Superdex75 column to remove TCEP. The protein had the same redox state as that described in Ref. 11. The concentration was determined based on a 5,5'-dithiobis(nitrobenzoic acid) assay for the free thiol groups. All of the experiments were carried out under aerobic conditions at 25 °C in buffer AE (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA), unless indicated otherwise.

**AMS Assays**—At various time points, protein aliquots were removed from reaction solutions and added to nonreducing gel sample buffer containing excess amount of AMS (10 mM) for 30 min in the dark at room temperature as described before (26). AMS interacts with free thiols of reduced proteins covalently, but not disulfide bonds. Each bound AMS molecule increases the molecular mass of the protein ~0.5 kDa. Different redox states of the proteins were analyzed by 16% Tricine-SDS-PAGE under nonreducing conditions.

**Determination of the Extinction Coefficients**—Absorption spectra of Erv1p and its mutants were recorded using a Cary 300 spectrophotometer from 250 to 700 nm, at 1-nm intervals, using a 1-cm path length quartz cuvette. The extinction coefficients and the percentage of enzyme-bound FAD for the WT and mutant Erv1p were calculated based on a molar extinction coefficient of 11.3 mm⁻¹ cm⁻¹ at 450 nm for free FAD and 72.68 mm⁻¹ cm⁻¹ for Erv1p at 275 nm as reported previously (27). FAD was released from the proteins by the addition of 1% SDS.

**Circular Dichroism**—CD analysis was performed using a JASCO J810 spectropolarimeter with a 1-mm path length quartz cuvette. Far-UV CD spectra were measured at 25 °C with 300 μl of 10 μM proteins as described previously (28). Each spectrum represents an average of four scans from 200 to 260 nm at 0.2-nm intervals with the spectra for buffer alone subtracted. Thermal denaturation was measured at 222 nm, at 1 °C intervals over 5–90 °C with temperature increase of 1 °C/min.

**Protease K Digestion**—20 μl of 5 μM Erv1p and its mutants were incubated with 50 μg/ml protease K at 25 °C for 30 min, followed by inhibition of the protease activity by the addition of 10 μM phenylmethylsulfonyl fluoride for 10 min. Then the samples were analyzed by 16% Tricine-SDS-PAGE and Western blotting with antibody raised with peptide of the C terminus of Erv1p. Mock controls were treated in exactly the same manner.

**Oxygen Consumption Assay**—Oxygen consumption of Erv1 was measured using a Clarke-type oxygen electrode (Hansatech Instrument Ltd.) at 25 °C as described before (25). For measurements with DTT and TCEP as electron donors, 1 or 2 μM Erv1 as indicated in the text was pre-equilibrated at 25 °C followed by the addition of 10 μM DTT or 3.5 mM TCEP to initiate the reaction. For measurements with Mia40c-pR as substrate, 50 μM freshly prepared Mia40c-pR was pre-equilibrated at 25 °C followed by the addition of 1 μM Erv1 to catalyze the reaction.

**Mass Spectrometry Analysis**—The WT Erv1 (5 μM) was incubated with 0 or 50 μM freshly prepared Mia40c-pR for ~10 s, and then the reaction was stopped by the addition of nonreducing SDS-PAGE sample buffer containing 4 mM iodoacetamide. The proteins were separated by nonreducing SDS-PAGE. The bands corresponding to Erv1 were excised and digested with AspN. The peptides were analyzed by mass spectrometry on a Bruker matrix-assisted laser desorption ionization time-of-flight using a positive reflection method.

**RESULTS**

**Characterization of the WT and Three Double Cys Mutants of Erv1p**—To understand the roles of individual disulfide bonds of Erv1, three double Cys mutants of Erv1p with the Cys residues corresponding to each of the three disulfides mutated to serines were generated. They are named as C30S/C33S, C130S/C133S, and C159S/C176S, respectively, in the rest of the report. All three mutants were successfully purified with the same method and yellowish color as that of the WT Erv1p. No abnormal color was observed for any of the mutants. The UV-visible spectrum of the WT Erv1p shows a characteristic bound FAD spectrum with a maximum absorbance at 460 nm and a shoulder peak at ~485 nm (supplemental Fig. S1). The absorption maximum was ~10 nm blue-shifted to 450 nm upon the addition of 1% SDS (data not shown), the same wavelength as that of free FAD confirming the release of cofactor FAD. The same protein-bound FAD spectrum as that of the WT Erv1p was observed for C30S/C33S and C159S/C176S mutants (supplemental Fig. S1), but a slightly blue-shifted spectrum with the maximum at 453 nm was obtained for C130S/C133S (supplemental Table S1). It is consistent with the fact that the active site Cys₁³₀–Cys₁³₃ disulfide is located proximal to the isoalloxazine ring of FAD and the mutation changes bound-FAD absorption slightly. The molar extinction coefficients for the bound FAD in the WT and all three double Cys mutants were determined (see “Experimental Procedures”) to be 11.9, 11.1, 12.1, and 11.9 mm⁻¹ cm⁻¹ at the corresponding wavelength of the absorption maximum (supplemental Table S1). These values are similar to each other and to that of other members of Erv/ALR family. The same FAD-binding yield of ~93% was obtained for the WT and all the mutants. Taken together, these results show that all three double Cys mutants were correctly folded and with FAD bound at a molar ratio of 1:1 as that of the WT. None of the three individual disulfide bonds of Erv1p is essential for FAD binding.

**Structural Roles Played by Individual Disulfide Bonds of Erv1p**—It was shown that Cys₁³₀ and Cys₁³₃ are involved in formation of an intermolecular disulfide bonded dimer and oligomers (5, 24). Thus, the oligomerization state of the double Cys mutants was investigated using reducing and nonreducing SDS-PAGE. For all the proteins except C30S/C33S mutant, a fraction of ~20% proteins migrated slowly on the nonreducing gel with an apparent molecular weight corresponding to a dimer (data not shown). The result is consistent with the previous observation (5, 24). Next, the effect of these mutations on the overall conformation of Erv1p was investigated using far-UV CD spectra. The WT and all of the mutants have a
similar spectrum profile with a conformation dominated by α-helical structures as expected (data not shown). However, an intensity decrease was observed for C30S/C33S mutant. It may be due to the absence of intermolecular disulfide bonded dimer, or the Cys30–Cys33 disulfide may be important for the overall folding of the non-FAD-binding N-terminal segment.

To understand the possible structural role played by each disulfide, thermal denaturation of the WT and Erv1p mutants was studied using CD at 222 nm. As shown in Fig. 2A, the WT Erv1p is stable against heat denaturation with a melting temperature (Tm) of 68 °C. The N-terminal double Cys mutant (C30S/C33S) had no apparent effect on the overall stability of Erv1p. In contrast, both the core domain double Cys mutants (C130S/C133S and C159S/C176S) had a clear effect on the stability of Erv1p, with a Tm of 52 °C for C130S/C133S and 38 °C for C159S/C176S (Fig. 2A). Mutation of the Cys159–Cys176 disulfide alone resulted in a decrease of 30 °C in Erv1p Tm. A fraction of the C159S/C176S mutant was unfolded at the physiological temperature and as low as 25 °C. Thus, our results show that the Cys159–Cys176 disulfide plays a key role in stabilizing the overall folding of Erv1p.

Next, the effects of the individual disulfides on the stability of Erv1 were confirmed by proteinase K (PK) digestion analysis (Fig. 2B). After incubation of the WT and the mutants with or without the presence of 50 μg/ml PK at 25 °C for 30 min, the samples were digested by Western blotting using antibody against the C terminus of Erv1p. In the presence of PK, the WT and all three double Cys mutants were degraded (Fig. 2B). Although a stable C-terminal fragment of ~15 kDa was clearly observed for the WT and C30S/C33S mutant, the intensity of the same fragment was very weak for C130S/C133S and C159S/C176S mutants, and no other bands were detected. Thus, the results of PK digestion are consistent with those of the thermal denaturation study.

Taken together, CD and PK digestion studies show that the C-terminal region of Erv1p was folded and resistant to PK digestion but not the N terminus. Therefore, although the N-terminal disulfide Cys30–Cys33 has no effect on the stability of Erv1p, both of the central core disulfides play a role in stabilizing the folding of Erv1p, especially the Cys159–Cys176 disulfide.

The Effects of the Individual Disulfides on the Oxidase Activity of Erv1p—The effects of individual disulfide bonds on the sulfhydryl oxidase activity of Erv1p were studied using oxygen consumption assays. First, the commonly used reducing agent DTT was employed as the electron donor with and without the presence of the WT or mutant Erv1p. As shown in Fig. 3A,
higher oxidase activity (4–8 d determined to be 0.8 s−1). The initial rates for WT and C159S/C176S were 0.1 and 0.5 s−1, respectively, less than that reported previously (24, 27). The Km values were 10 mM for the WT Erv1p (24). For the C159S/C176S mutant, a decreased Km was observed (Fig. 3A). Taken together, these results showed that using DTT as substrate, only Cys130–Cys133 disulfide is required for the oxidase activity of Erv1p, confirming that it is the active site disulfide.

**Both Cys30–Cys33 and Cys130–Cys133 Disulfides Are Required for the Oxidase Activity of Erv1p toward Its Physiological Substrate**—Previous yeast genetic studies demonstrated that all six Cys residues of Erv1p were required for its function in vivo (24). Therefore, we asked whether all three disulfides are essential for the oxidase activity toward its native substrate protein Mia40. To this end, a functional C-terminal domain of Mia40, Mia40c (residues 284–403), was expressed and purified as reported previously. Mia40c contains all the six conserved Cys residues of CPC-C-X4-C-X5-C of the protein. It has been shown that the CPC motif is the redox active site of Mia40, which can be selectively reduced and act as an electron donor for Erv1p (11). Thus, the partially reduced Mia40c (Mia40c-pR), with the Cys of CPC in the reduced form and C-X4-C motifs in the oxidized form, was prepared and used as an electron donor for Erv1p. Oxygen consumption of 50 μM Mia40c-pR in the presence of 1 μM the WT Erv1p (curve a), C30S/C33S (curve b), C130S/C133S (curve c), and C159S/C176S (curve d), respectively. B, AMS assay of the redox state change of the WT and Erv1p mutants. The proteins were detected by Western blotting with antibody against Erv1p. D, mass spectrometry analysis of Erv1p before (panel a) and after (panel b) incubated with Mia40c-pR for 10 s. The peptides contain Cys30 and Cys133 in the oxidized (1548.69 Da) or reduced and alkylated (1664.88 Da) forms were shown.

**FIGURE 4. Oxygen consumption and AMS assay of Mia40c-pR oxidation catalyzed by the WT and Erv1p mutants.** A, oxygen consumption profiles of 50 μM Mia40c-pR in the presence of 1 μM the WT Erv1p (curve a), C30S/C33S (curve b), C130S/C133S (curve c), and C159S/C176S (curve d), respectively. B, AMS assay of the redox state change of the WT and Erv1p mutants. The proteins were detected by Western blotting with antibody against Erv1p. D, mass spectrometry analysis of Erv1p before (panel a) and after (panel b) incubated with Mia40c-pR for 10 s. The peptides contain Cys30 and Cys133 in the oxidized (1548.69 Da) or reduced and alkylated (1664.88 Da) forms were shown.

**TABLE 1**

Oxygen consumption kinetic parameters for the WT and Erv1p mutants

| Electron donor | Erv1p | kcat | Km | kcat/Km |
|---------------|-------|------|----|---------|
| 10 mM DTT     | WT    | 1.3 ± 0.1 | 57 ± 4 | 2.3 ± 0.2 × 10^4 |
|               | C30S/C33S | 1.5 ± 0.1 | 62 ± 5 | 2.4 ± 0.2 × 10^4 |
|               | C130S/C133S | <0.1 | 87 ± 8 | 9.2 ± 0.2 × 10^3 |
| 3.5 mM TCEP   | WT    | 1.1 ± 0.1 | 27 ± 3 | 4.1 ± 0.3 × 10^4 |
|               | C30S/C33S | <0.1 | 87 ± 8 | 9.2 ± 0.2 × 10^3 |
|               | C130S/C133S | <0.05 | 87 ± 8 | 9.2 ± 0.2 × 10^3 |
|               | C159S/C176S | 0.7 ± 0.1 | 18 ± 2 | 3.9 ± 0.3 × 10^4 |

Oxygen consumption was catalyzed as soon as the WT Erv1p was added. The kcat was determined to be 1.3 ± 0.1 s−1, ~50% higher than that reported previously (24). The Km for molecular oxygen was determined to be 57 μM (Fig. 3B and Table 1). Different effects on the oxidase activity were observed with the three double Cys mutants. As expected, the active site C130S/C133S mutant showed no or very little activity, similar to that of a previous study using single Cys mutants (24). Interestingly, the N-terminal C30S/C33S mutant showed ~15% higher oxidase activity (kcat = 1.5 ± 0.1 s−1) than that of the WT enzyme. In contrast, the previous study with the corresponding single Cys mutants showed only ~30–50% activity of the WT Erv1p (24). For the C159S/C176S mutant, a decreased activity was observed (Fig. 3A), and the kcat and Km values were determined to be 0.8 s−1 and 87 μM, respectively (Table 1). The enzyme specificity, ratio of kcat/Km, was the same (2.3 × 10^4 M−1 s−1) for the WT and C30S/C33S and was similar to that of Erv1 proteins (4–8 × 10^4 M−1 s−1) (29, 30). Taken together, these results showed that using DTT as substrate, only Cys130–Cys133 disulfide is required for the oxidase activity of Erv1p, confirming that it is the active site disulfide.

oxidase activity of Erv1p, both Cys30–Cys33 and Cys130–Cys133 disulfides were required. It seems that less oxygen was consumed (~40 μM) than might be expected given that 50 μM Mia40 was in the reaction. This may partially be due to the fact that the protein was not 100% pure (~95% based on SDS-PAGE), due to errors of concentration as determined by the 5,5′-dithiobis(nitrobenzoic acid) assay, and/or due to the fact that oxygen consumption was very slow after the reaction for 3 min.

Furthermore, changes in the redox state of Erv1p and Mia40c were analyzed in parallel using a SDS-PAGE based AMS thiol-
C159S/C176S mutant (Fig. 4, lanes 10–12). Meanwhile, the redox state of Erv1p was revealed by Western blotting (Fig. 4, lanes 3–5 and 12–14). When C30S/C33S or C130S/C133S mutant was used, Mia40c-pR remained in the reduced form (R), even after 20 min (Fig. 4B, lanes 6–11). The result was observed for the C30S/C33S mutant. Although a slightly increased activity was observed with DTT (Fig. 3A, curve b), little oxygen consumption was detected using TCEP as an electron donor. TCEP is a stronger reducing agent and has a slightly larger size (250 Da) than DTT (154 Da). Comparison between DTT and TCEP shows that the same kind of effects on the Erv1p activity were observed for C130S/C133S (inactive) and C159S/C176S (decreased activity) mutants, respectively (compare Figs. 3A and 5A). However, an opposite result was observed for the C30S/C33S mutant. Although a slightly increased activity was observed with DTT (Fig. 3A, curve b), little oxygen consumption was detected using TCEP as an electron donor (Fig. 5A, curve b). In the absence of Cys30–Cys33 disulfide, TCEP is not a substrate for Erv1p. Thus, our results show that even TCEP cannot access and reduce the active site disulfide of Erv1 directly, suggesting the active site disulfide is well protected and Cys30–Cys33 disulfide has no obvious effect on Erv1p activity and thus is not required for Erv1p oxidase function.

The Active Site Cys130–Cys133 Disulfide Is Well Protected and Can Be Activated by Cys30–Cys33 Disulfide through Intermolecular Electron Transfer—To understand whether Cys130–Cys133 was required to act as a shuttle disulfide and whether that requirement was due to the fact that the active site disulfide was not accessible, we repeated the oxygen consumption experiments using TCEP as an electron donor. TCEP is a stronger reducing agent and has a slightly larger size (250 Da) than DTT (154 Da). Comparison between DTT and TCEP shows that the same kind of effects on the Erv1p activity were observed for C130S/C133S (inactive) and C159S/C176S (decreased activity) mutants, respectively (compare Figs. 3A and 5A). However, an opposite result was observed for the C30S/C33S mutant. Although a slightly increased activity was observed with DTT (Fig. 3A, curve b), little oxygen consumption was detected using TCEP as an electron donor (Fig. 5A, curve b). In the absence of Cys30–Cys33 disulfide, TCEP is not a substrate for Erv1p. Thus, our results show that even TCEP cannot access and reduce the active site disulfide of Erv1 directly, suggesting the active site disulfide (Cys130–Cys133) is buried and well protected. The oxygen consumption curves of TCEP oxidation were further analyzed, and the $k_{cat}$ values were determined to be 1.1 and 0.7 s$^{-1}$ for the WT and C159S/C176S, respectively. The $K_m$ values for oxygen were 27 $\mu$M for the WT and 18 $\mu$M for C159S/C176S (Table 1). A similar substrate specificity of 4 $\times$ 10$^6$ M$^{-1}$ s$^{-1}$ was obtained, which was $\sim$2–4 times higher than that determined by using DTT (Table 1). Thus, TCEP is a better electron donor than DTT in terms of activation specificity. It is consistent with the finding that DTT not only can reduce Cys30–Cys33, but also Cys130–Cys133 and maybe even Cys159–Cys176; thus some enzyme activity may be lost during the assays using DTT.

In summary, oxygen consumption and AMS assays showed that both CXXC disulfides (Cys30–Cys33 and Cys130–Cys133) are essential for Erv1p oxidase activity toward the physiological substrate Mia40c. The CX16C disulfide has no obvious effect on Erv1p activity and thus is not required for Erv1p oxidase function.
Next, to confirm that Cys$^{30}$–Cys$^{33}$ can act as a shuttle disulfide, we performed the activity assay using TCEP as an electron donor in the presence of a total of 2 μM enzyme(s): WT Erv1, C30S/C33S, or C130S/C133S alone, or a mixture (preincubated for ~5 min) of the two mutants (1 μM each), respectively. As shown in Fig. 5B, although both mutants alone were inactive, the mixture of these two mutants showed a clear Erv1p oxidase activity. The rate of oxygen consumption was ~0.36 μM s$^{-1}$, which was ~24% that of the WT enzyme (1.5 μM s$^{-1}$) at the same condition (Fig. 5B). The result confirms that Cys$^{30}$–Cys$^{33}$ disulfide of a C130S/C133S mutant can activate C30S/C33S mutant via intersubunit or intermolecular electron transfer. If formation of a heterodimer between the two mutants is necessary for the activity, the activity will increase with the time of incubation. Interestingly, no obvious difference in the levels of activity was observed between 1, 5, and 20 min of incubation, but a slightly decreased activity was obtained when the proteins are preincubated for 2 h (Fig. 5C). Thus, these results suggest that heterodimer formation is not required. To confirm our finding, we carried out the experiments by incubating TCEP with one of the mutants first and followed by addition of the second mutants (Fig. 5D, curves b and c). As shown in Fig. 5D, the same rate of oxygen consumption as that of preincubated proteins (curve a) was obtained as soon as the second mutant was added in both cases. Taken together, we demonstrated experimentally for the first time that Cys$^{30}$–Cys$^{33}$ functionally acts as a shuttle disulfide passing electrons to the active site Cys$^{130}$–Cys$^{133}$ disulfide, and furthermore the electron transfer may occur between two different molecules via intermolecular interactions.

**DISCUSSION**

In this study, the structure and functional roles of all three individual disulfides of the yeast mitochondrial sulfhydryl oxidase Erv1p were analyzed using site-directed mutagenesis coupled with various biochemical and biophysical techniques. We show that whereas none of the three disulfides is essential for FAD-binding to Erv1p, they all play an important role *in vitro*. The specific roles played by each disulfide were deciphered systematically.

In this report, we provide direct experimental evidence to show that the N-terminal Cys$^{30}$–Cys$^{33}$ disulfide is required to shuttle electrons from the physiological substrate Mia40 to the active site Cys$^{130}$–Cys$^{133}$ disulfide. Although most *in vitro* studies on the function of FAD-dependent sulfhydryl oxidases have used a model substrate (e.g. DTT, reduced lysozyme, or *E. coli* thioredoxin), in this study, we used both artificial substrates (DTT and TCEP) and the physiological substrate, partially reduced Mia40, to address the functional mechanism of Erv1p. By comparison between the results, we are able to show that the active site disulfide Cys$^{130}$–Cys$^{133}$ is accessible to DTT but not to TCEP and Mia40; thus, in terms of biological relevance, TCEP is a better small molecule than DTT as substrate for Erv1p studies. C30S/C33S mutant was more active than the WT toward DTT oxidation but was enzymatically inactive toward Mia40c and TCEP oxidation. Together with the fact that TCEP is a stronger reductant and larger (250 Da) than DTT (154 Da), our results suggest that the active site disulfide is highly protected. It is not accessible to Mia40 and even to TCEP. The standard redox potential for Erv1p active site disulfide (Cys$^{130}$–Cys$^{133}$) has been determined to be of ~150 mV (27). The redox potentials for the mitochondrial intermembrane space, GSH, and DTT were determined to be ~255, ~240, and ~330 mV, respectively (31, 32), and an even lower value for TCEP was predicted (33). Accordingly, the active site disulfide of Erv1p would be thermodynamically unstable in the intermembrane space or against any of above thiol reducing reagents if it was not well protected. The inaccessible nature of the active site disulfide can also explain why DTT is a poor substrate for all members of Erv/ALR and Ero1 families in general. Our conclusion is also in agreement with the finding that GSH is not a good substrate for Erv1p (data not shown) and other Erv enzymes, because GSH is a weaker reductant than DTT and larger (307 Da) than TCEP. Thus, we provided direct experimental evidence for the current hypothesis that Cys$^{30}$–Cys$^{33}$ is required to serve as a shuttle disulfide transferring electrons from substrate to active site Cys$^{130}$–Cys$^{133}$ disulfide.

More importantly, the Erv1p oxidase activity was partially recovered after mixing of the two individually inactive mutants, C30S/C33S and C130S/C133S (Fig. 5, B–D). An optimum of 50% of the WT Erv1p activity is expected for a perfect mixing and reassembled C30S/C33S-C130S/C133S Erv1. Because all known Erv/ALR enzymes form stable dimer or oligomers, the rate of dissociation is expected to be slow and probably in a time scale of min or even hours. Moreover, our results show that the enzyme activity was recovered rapid to ~25% that of the WT Erv1p. Although we cannot exclude the possibility that the subunits rapidly scramble, our results suggest that this recovered activity was via intermolecular rather than intersubunit reaction. It seems likely that in the cells Cys$^{30}$–Cys$^{33}$ can shuttle electrons to Cys$^{130}$–Cys$^{133}$ through both intersubunit and intermolecular reactions and maybe even via intrachain shuttle. Similarly, for Ero1p a mechanism of both intra- and interchain electron transfer has been suggested (34).

Next, our study also suggests that Cys$^{30}$–Cys$^{33}$ disulfide and/or the flexible N-terminal region may also play a role in regulating the accessibility of the active site disulfide through a conformation change induced by interacting with Mia40 and thiol/disulfide exchange. Based on the CD measurements, a clear conformation change was observed for the N-terminal C30S/C33S mutant (data not shown). It suggests that a conformation change may occur in the flexible N-terminal region upon reduction of Cys$^{30}$–Cys$^{33}$ disulfide. Although the oxidase activity can be regained by mixing the active site C130S/C133S mutant with C30S/C33S, no enzyme activity was recovered by mixing C130S/C133S with Erv1-ΔN (a N-terminal 72-amino acid deleted Erv1) (data not shown). Thus, the interactions involving Erv1p N-terminal regions may play an important role during electron transfer from the shuttle disulfide to the active site disulfide and mediating the accessibility of the active site disulfide. In this way, the oxidase activity of Erv1p can be regulated effectively and specifically while circumventing or limiting nonspecific and harmful oxidation of protein thiols. Recently, a similar regulatory role played by a disulfide was demonstrated for both Ero1p and Ero1e (17–19). These regulatory disulfide bonds are among the nonconserved disulfides.
of Erv1p proteins. Although Erv1p has only three conserved disulfides, the location and the spacing between the two Cys residues of the shuttle disulfides (Cys130–Cys133, Cys30–Cys33, and Cys159–Cys176) of the C terminus contribute to the overall stability of Erv1p. The well protected active site disulfide Cys130–Cys133 requires a shuttle disulfide to transfer electrons from substrate, and both intersubunit and intermolecular transfer can occur.

Furthermore, this report provided direct evidence to show that CXCXnC disulfide plays an important role in the overall stability of Erv1p. Based on our thermal denaturation and limited proteinase digestion studies, both disulfides (Cys130–Cys133 and Cys159–Cys176) of the C terminus contribute to the overall stability of Erv1p. The well protected active site disulfide Cys130–Cys133 requires a shuttle disulfide to transfer electrons from substrate, and both intersubunit and intermolecular transfer can occur.

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