Electron Transfer Reactivity of the *Arabidopsis thaliana* Sulphhydryl Oxidase AtErv1*

*Ole Farver, Elvira Vitu, Scot Wherland, Deborah Fass, and Israel Pecht*

From the Departments of Immunology and Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel, the Institute of Analytical Chemistry, University of Copenhagen, 2100 Copenhagen, Denmark, and the Department of Chemistry, Washington State University, Pullman, Washington 99164-4630

The redox reactivity of the three disulfide bridges and the flavin present in each protomer of the wild-type *Arabidopsis thaliana* mitochondrial sulphhydryl oxidase (AtErv1) homodimer has been investigated. Pulse radiolytically produced CO2 radical ions were found to reduce the disulfide bridges to yield disulfide radicals, RSSR**−**. Rates and absorption changes due to formation or decay of RSSR**−** and the flavin quinone, semiquinone, and hydroquinone were measured and analyzed. During the first 100 μs following the pulse, the flavin was reduced to the semiquinone by intramolecular electron transfer from the active site disulfide radical. The semiquinone and the remaining disulfide radicals then reacted by much slower, 40 ms to 40 s, inter-homodimer electron transfer reactions, culminating in reduced flavin and diethiols. The diethiols were then subject to oxidation by enzyme molecules via their intrinsic enzymatic activity, at a rate comparable to the slower intermolecular processes in the 10-s time regime. Mutants of AtErv1 lacking each of the three individual cysteine pairs were studied to determine the involvement of the respective disulfide groups in these reactions. Elimination of the active site disulfide bridge increased the stability of the flavin semiquinone making it a long-lived product. Relevance of these observations to the design and function of the sulphhydryl oxidases is discussed.

Cystine disulfide bridges are important structural elements of proteins and may also function as redox active centers. Formation of disulfide bonds is a crucial process for establishing, maintaining, and controlling protein folding and hence function. Sulphhydryl oxidase (SO) enzymes catalyze the transfer of electron pairs from two cysteine thios to dioxygen or cytochrome c [Fe(III)], yielding a disulfide bridge and hydrogen peroxide (1–2), or cytochrome c [Fe(II)] (3–6). The eukaryotic SOs known to date contain a functional pair of cysteines and a flavin adenine dinucleotide (FAD) in their active sites, which are located within a crevice at the mouth of a four-helix bundle (7–8). Many SOs contain one or more additional disulfides that are also involved in the catalytic cycle, presumably by shuttling electron pairs from substrates to the active site (7, 9–10). Finally, other disulfide bridges having structural or regulatory roles may also be present in these enzymes (11).

One of the eukaryotic SO enzyme families, Erv/ALR, has representatives that function in the ER (11, 12), mitochondria (13), cytosol (14–15), the late secretory pathway (16), or are secreted from the cell (17). The first plant to have its entire genome sequenced, *Arabidopsis thaliana*, contains a mitochondrial SO called AtErv1 (18). The physiological substrates of AtErv1 are not known, but a homologous yeast mitochondrial SO is responsible for the “disulfide trapping mechanism” for retention of proteins in the intermembrane space (IMS) (19). Yeast Erv1 oxidizes IMS proteins with the aid of a protein called Mia40 (20). A Mia40 homolog exists in *A. thaliana*, suggesting that an orthologous oxidative pathway operates in the plant mitochondrial IMS.

AtErv1 is a homodimer, in which each subunit has three disulfides. The proposed catalytic mechanism of AtErv1 assumes the two-electron uptake from protein substrates by the “shuttle” disulfide (Cys-177/Cys-182) present on the flexible C-terminal tail (21), followed by transfer of the electrons to the active site disulfide (Cys-119/Cys-122) and further to the FAD. The flavin then reduces dioxygen (21) to hydrogen peroxide, or cytochrome c [Fe(III)] to cytochrome c [Fe(II)] (3). The third disulfide present in this enzyme (Cys-148/Cys-165) plays a structural role and does not participate in its electron transfer activity.

Flavoproteins can form the necessary interface between two-electron donors and single-electron acceptors (or vice versa), or between two-electron donors and two-electron acceptors, through modulation of the relative stability and reactivity of the quinone, semiquinone, and hydroquinone states of the flavin. In the present study, we have employed the pulse radiolysis method to resolve individual electron transfer (ET) steps potentially related to the catalytic cycle of AtErv1. By this technique, reducing or oxidizing species are produced in the reaction solution by very short pulses (~1 μs) of high-energy (5 MeV) electromagnetic radiation. The ensuing electron transfer reactions are then monitored by absorption spectrometry. In addition to the advantageous time resolution of this method, it...
enables production of reagents with a wide range of reduction potentials (22). A disulfide bridge has been used previously as an entry port for pulse radiolytically produced reduction equivalents in studies of electron transfer through proteins including azurin (23) and ceruloplasmin (24). An elegant example of thorough analysis by pulse radiolysis of a flavin-containing enzyme is the work of Hille on xanthine oxidase (25).

The present study illustrates the applicability of the method to proteins containing multiple disulfides, both structural and redox active. As strong, one-electron donors, the disulfide radicals produced by pulse radiolysis may be non-physiological compared with dithiols that donate two electrons, but they necessarily produce the semiquinone state of the flavin. The semiquinone would also be formed when a one-electron acceptor, cytochrome c [Fe(III)] (known as a physiological oxidant), reacts with the fully reduced flavin. A variety of opportunities for intra- and intermolecular ET exist between disulfide radicals and the flavins in the AtErv1 dimer, between the disulfide radicals themselves, and between different reduction states of the flavins, as well as with dithiols that are produced by the pulses. The pulse radiolysis method enables determination of the rates of these processes, thereby providing insights into the reactivities of the available sites. The amplitudes of the absorbance changes provide the amounts of the species that are formed and hence also their relative stabilities. Results of the present studies yielded insights into possible inter- and intramolecular electron transfer processes that take place during the reductive half-cycle of this enzyme.

**MATERIALS AND METHODS**

Site-directed mutants were generated using the Quickchange kit (Stratagene). Sequences of mutagenic oligonucleotides can be provided upon request. The AtErv1 coding region was fully sequenced for all mutants. AtErv1 and its mutants were expressed and purified as described for enzymatic assays (21) and then dialyzed against 10 mM phosphate buffer, pH 7.0, with 200 mM sodium formate.

All experiments were performed under anaerobic conditions between 9 and 13 °C at pH 7.0 in nitrous oxide-saturated solutions containing 200 mM formate and 10 mM phosphate. AtErv1 dimer concentrations were in the range from 2.5 to 6 μM. All protein concentrations and respective rate constants are reported as protomer concentrations. Pulse widths of 0.75–1.0 μs were used. Under these conditions, pulse widths of 1.0 μs produce ~15 μM CO₂ radicals, as determined by independent dosimetry measurements. Experiments were done using a 1-cm Spectrosil® cuvette, using either one or three light passes, which result in an overall optical path-length of either 1 or 3 cm. The analyzing light beam was produced with a 150-watt xenon lamp together with a Bausch & Lomb double grating monochromator. Appropriate optical filters with cut-off at 285 or 385 nm were used to reduce photochemical and light scattering effects. The data acquisition system consisted of a Tektronix 390 A/D transient recorder attached to a PC. The temperature of the reaction solutions in the cuvette was controlled by a thermostating system and continuously monitored by a thermistor attached to the cuvette. Reactions were generally performed under pseudo-first order conditions, with typically a 10-fold excess of protein’s redox-sites over reducing radicals. In each experiment 2,000 data points were collected, divided equally between two different time scales. Usually the processes were recorded over at least three half-lives. Each kinetic run was repeated at least four times. The data were analyzed by fitting to a sum of exponentials using a non-linear least squares program written in MATLAB®.

**RESULTS**

The main primary products of interaction of high-energy electromagnetic radiation with aqueous solutions are hydrated electrons (eₐq) and OH radicals. These are rather nonselective agents, but they can be converted to milder, more specific ones such as CO₂ radicals in N₂O-saturated aqueous solutions containing formate ions by the following reaction sequence in Reaction.

\[ e_{aq} + N_2O + H_2O \rightarrow OH + OH^- + N_2 \]

\[ OH + HCO_3^- \rightarrow CO_2 + H_2O \]

**Reaction Scheme 1**

In dilute protein solutions, the CO₂ radicals will either react with thermodynamically and sterically accessible redox centers, or decay by dimerization to form oxalate, and the reactions can be followed spectrophotometrically. Table 1 lists the wave-lengths monitored and the protein extinction coefficient changes for all possible reactions.

**Wild type (WT) AtErv1**—Each subunit of WT AtErv1 contains four potential redox centers: an FAD and three disulfide bridges. The FAD-proximal, active site disulfide is 3.4 Å (cys-teine sulfur to isoalloxazine C4) from the FAD. The distal, shuttle disulfide is on a flexible polypeptide segment that could not be resolved in electron density maps obtained from x-ray crystallography of AtErv1 (21). This segment of the enzyme may access conformations that place the shuttle disulfide anywhere between van der Waals contact and ~30 Å from the active site disulfide of the second subunit of the dimer, as estimated from the extensibility of the polypeptide chain. The third, structural disulfide is found ~15 Å from the active site disulfide and the FAD isoalloxazine (Fig. 1).

When an anaerobic, N₂O-saturated, 10 μM (protomer) solution of WT AtErv1 was subjected to CO₂ radical ions, a fast absorption increase was monitored at 340 nm (Fig. 2A) with a

| Process/wavelength | 340 nm | 406 nm | 453 nm | 580 nm |
|--------------------|--------|--------|--------|--------|
| SS + CO₂⁻ → SS⁻   | +5.0   | +10.0  | +6.2   | 0.0    |
| Q + CO₂⁻ → SQ     | 0.0    | −3.4   | −6.1   | +1.5   |
| SQ + CO₂⁻ → HQ    | 0.0    | −1.0   | −3.4   | −1.8   |
| SS⁻ + Q → SQ + SS | −5.0   | −13.4  | −12.3  | +1.5   |
| SS⁻ + SQ → HQ + SQ| −5.0   | −11.0  | −9.6   | −1.8   |
| S⁻ S⁻ + CO₂⁻ → S⁻ S⁻ | −10.0 | −10.0  | −6.2   | 0.0    |
| S⁻ S⁻ + SQ → S⁻ S⁻ + HQ| 0.0 | −4.4  | −9.5   | −0.3   |
| SQ + SQ → Q + HQ   | 0.0    | +2.5   | +2.7   | −3.3   |
| SS⁻ + SS⁻ → S⁻ S⁻ + SS⁻ | −10.0 | −20.0  | −12.4  | 0.0    |

**TABLE 1**

Protein extinction changes for various processes

SS, disulfide; SS⁻, disulfide radical; S⁻ S⁻, two electron reduced, dithiolate or dithiol form of the disulfide; CO₂⁻, carbon dioxide radical; Q, oxidized, quinone, form of the flavin; SQ, one electron reduced, semiquinone, form of the flavin; HQ, two electron reduced, hydroquinone, form of the flavin.
On the same time scale as the initial fast process monitored at 340 nm, an absorption increase was observed at 580 nm (Fig. 2B), characteristic for the formation of FAD SQ \((\Delta \epsilon_{580} = 1,500 \text{ M}^{-1} \text{ cm}^{-1})\). The observed rate constant of \((2.0 \pm 0.5) \times 10^4 \text{ s}^{-1}\) was also protein concentration-dependent and similar to that of RSS*R\(^-\) formation. SQ formation may be due to either direct reduction by CO\(_2\) radicals or reduction mediated by the active site disulfide. Considering the proximity of these two redox centers, the latter process is expected to take place in a very fast time domain that cannot be resolved by our technique. The reduction yield of this phase was calculated to be \(-3.3 \mu M\) per pulse, i.e. the ratio between RSS*R\(^-\) and FAD SQ produced was \(-1.7\). The 580-nm absorption decayed with an observed rate constant of \(3.0 \pm 1.0 \text{ s}^{-1}\). Thus, FAD SQ is relatively unstable in this enzyme and may either dismutate intermolecularly (SQ + SQ → Q + HQ) \((\Delta \epsilon_{580} = -3,300 \text{ M}^{-1} \text{ cm}^{-1})\) or be reduced further by an RSS*R\(^-\) radical in another dimer. However, the former process should give rise to a net absorption increase at 453 nm, which is not observed (vide infra). Therefore, the process is likely to be reduction of the SQ by RSS*R\(^-\).

A fast absorption increase was also observed at 453 nm (Fig. 2C), with a rate constant similar to those observed at 340 nm and 580 nm (Table 2). The rates of these fast phases are protein concentration-dependent and thus reflect bimolecular events (i.e. between two enzyme homodimers). As both disulfide radicals and FAD absorb at 453 nm, the observed absorption changes have composite contributions: FAD reduction to SQ causes an absorption decrease \((\Delta \epsilon_{453} = -6,100 \text{ M}^{-1} \text{ cm}^{-1})\), whereas the disulfide radical formation exhibits an absorption increase with a similar extinction value \((\Delta \epsilon_{453} = +6,200 \text{ M}^{-1} \text{ cm}^{-1})\). The observed net absorption increase at 453 nm indicates that more disulfide bridges than FAD molecules were reduced. Assuming that the absorption increase at 453 nm is due to partitioning of reduction equivalents between FAD and disulfides, this conclusion agrees with the above calculations based on the absorption changes observed for disulfide radical formation at 340 nm and SQ formation at 580 nm. The same slow absorption decay observed at 340 nm and 580 nm was also observed at 453 nm with a rate constant \(2.7 \pm 0.3 \text{ s}^{-1}\), in accordance with the concomitant slow decay of both disulfide radicals and SQ \((\Delta \epsilon_{453} = -9,600 \text{ M}^{-1} \text{ cm}^{-1})\).

One important question pertinent to the catalytic mechanism of this enzyme is identification of the disulfide bridges that are reduced as a result of these initial pulses. Disulfide radicals and SQ were formed at the same, diffusion-controlled rate, so information about how many disulfides are reduced can be derived from the relative amplitudes of this phase monitored at different wavelengths. If all three disulfides and FAD are reduced indiscriminately by CO\(_2\), and the latter is immediately reduced via the active site disulfide radical, we would expect a ratio of 1.0 between the initial yields of RSS*R\(^-\) and SQ, which is not in agreement with the observed net absorption increase at 453 nm. As stated above, we assume that the active site FAD-disulfide complex acts as a single electron accepting site when reduced by CO\(_2\), rapidly forming SQ. If the other two disulfide groups are reduced with equal probability, we would expect a ratio between reduced disulfides and FAD of \(-2:1\). This ratio is
indeed in good agreement with the calculated average ratio of 1.7 \pm 0.2 obtained from analysis of all our data collected for the WT enzyme over the time regime of \( \sim 10 \) s after the pulse.

Each enzyme solution was exposed to a series of pulses to monitor possible changes in reactivity caused by the increased extent of protein reduction. Under the anaerobic conditions employed, 10 \( \mu \)M \( \text{AtErv1} \) may, in principle, take up 80 \( \mu \)M reduction equivalents. Static spectra of the enzyme solutions were routinely measured before, between pulses, and after each set of experiments. Fig. 4 demonstrates that 7.8 \( \mu \)M out of 10 \( \mu \)M FAD were reduced as a result of exposure to thirteen pulses (1.0 \( \mu \)s each, total equivalent to \( \sim 195 \mu \)M \( \text{CO}_2^- \)), based on calculations using absorbance changes monitored at 453 nm (\( \Delta \epsilon_{453} = -9,500 \text{ M}^{-1} \text{ cm}^{-1} \)). The presence of the SQ form of FAD could not be detected in the static WT spectra, suggesting that this form has limited stability.

To distinguish between the reactivities of the three different disulfide groups present in \( \text{AtErv1} \), comprehensive experiments were next performed on mutants where one cystine at a time was replaced by redox inactive amino acid residues. The cysteines participating in the active site and shuttle disulfides were replaced by alanines, and those of the structural disulfide with a valine and a leucine. Results of pulse radiolysis experiments using these three mutants are presented in the following sections.

**Mutant Missing the Structural Disulfide**—Upon reaction with \( \text{CO}_2^- \) radicals, solutions of this mutant also exhibited the fast increase in 340-nm absorption, a change assigned to disulfide radical ion formation (Table 2). The observed rate constants for this bimolecular reaction decreased with the number of reduction equivalents introduced, as observed for other \( \text{AtErv1} \) variants. However, the 340-nm absorption decay amplitude was very small and seen only in the slow time window (\( k_{\text{obs}} 4 \pm 1 \text{s}^{-1} \)), indicating that the disulfide radical dismutation, occurring on a millisecond time range in the WT enzyme, did not take place in this mutant. This observation suggests that the structural disulfide, when present, may be involved in the faster phase of disulfide dismutation.

An absorption increase was monitored at 580 nm, reflecting SQ formation, on the same time scale as the fast absorption decay monitored at 340 nm. The observed rate constant of \( (0.61 \pm 0.03) \times 10^4 \text{ s}^{-1} \) is lower than the other \( \text{AtErv1} \) variants. Still, the amplitudes are comparable, \( \sim 0.01 \text{ cm}^{-1} \). The absorption at 580 nm decreased to zero, but with a much lower rate constant \( (0.03 \pm 0.02 \text{ s}^{-1}) \) than that observed for WT.

In contrast to the other \( \text{AtErv1} \) variants (see also below), a fast absorption decay was monitored at 453 nm with observed rate constant \( (0.9 \pm 0.4) \times 10^4 \text{ s}^{-1} \). Because the only processes occurring at this fast rate are disulfide and FAD reduction by \( \text{CO}_2^- \) (see Table 2), SQ formation must be dominant, possibly...
due to greater FAD exposure. A slow 453-nm absorption decrease with a rate constant 4 ± 1 s⁻¹ is also monitored in this mutant, reflecting RSS'R' decay, in accordance with the observations at 340 nm. Finally, on a 10-s time scale, an absorption increase was observed with a concentration-dependent rate constant, which could reflect intermolecular dismutation of the SQ ($Δε_{343} = +2,700$ M⁻¹ cm⁻¹), in accordance with the absorption change monitored at 580 nm. The ratio of disulfide to FAD reduction yield is 1.2 ± 0.2, i.e. lower here than for WT and the mutant missing the active site disulfide, and comparable or slightly lower than the mutant missing the shuttle disulfide, both discussed below.

Mutant Missing the Active Site Disulfide—This variant also exhibited a fast increase in absorption at 340 nm upon reaction with CO₂. The concentration-dependent observed rate constant (Table 2) implies a second order rate constant of $-2 \times 10^9$ M⁻¹ s⁻¹, again close to the diffusion controlled limit. Thus, in the presence or absence of the active site disulfide, the remaining disulfide groups exhibit similar reactivity toward the pulse-generated radicals. As in the WT enzyme, the 340-nm absorption decreased, although not completely, on an intermediate time scale (rate constant 150 ± 60 s⁻¹) and with an amplitude of $-0.005$ cm⁻¹. At 580 nm, an absorption increase with a rate constant of 2.3 ± 10⁻⁴ s⁻¹ was observed, indicating formation of the FAD SQ at a rate similar to the above 340-nm absorption increase. Such a fast process in the mutant missing the FAD-proximal, active site disulfide suggests that the flavin was reduced directly by the CO₂ radicals. From the absorption increase, we calculate a yield for SQ formation of 2.0 ± 0.2 μm. Unique to this mutant among the AtErv1 variants studied is the observation that the absorption at 580 nm, caused by the SQ formation, does not decay completely even on the longest time scale examined (minutes), suggesting that the SQ is stabilized by this mutation. This conclusion is further supported by static spectra of the ensuing solutions showing that the 580 nm band is observed for at least 10 min after the pulse. In contrast to all other enzyme variants studied here, no absorption changes were observed at either wavelength on the slow time scale (1–10 s) in the mutant lacking the active site disulfide.

Finally, as for WT AtErv1, a net increase was observed in absorption at 453 nm following the pulse, again indicating that more disulfides than FAD were reduced. This absorption decayed at the same rate as that observed for the intermediate processes monitored at both 340 and 580 nm, and the ratio between reduced disulfides and FAD was found to be 2.5 ± 0.2. A ratio of 2.0 is expected for an equal probability of reducing any of the three redox centers.

Mutant Missing the Shuttle Disulfide—In the absence of the shuttle disulfide, the fast formation of RSS'R' radicals was again observed at 340 nm with a second order rate constant of $-4 \times 10^9$ M⁻¹ s⁻¹ (Table 2). As for the structural disulfide mutant, no intermediate phase of radical disappearance in the millisecond time range was observed. Instead, the RSS'R' decayed in a much slower pseudo-first order process (Table 2).

The direct reduction of FAD to the SQ form monitored at 580 nm ($k_{obs} = (1.8 ± 0.8) \times 10^4$ s⁻¹) was 2-fold slower than the disulfide radical formation, and the SQ radicals decayed with a rate constant of 2 ± 1 s⁻¹, i.e. concomitant with the disulfide radical. At 453 nm, a fast absorption increase was observed (Table 2), with amplitudes indicating that the yield of RSS'R' was higher than that of reduced FAD. The ratio between disulfide reduction and SQ formation was calculated to be 1.4 ± 0.2. The 453-nm band decayed below zero with a rate constant of 6 ± 2 s⁻¹, and this negative amplitude suggests that the disulfide radical oxidation proceeds concomitantly with FAD or SQ

---

**TABLE 2**

| Wavelength | Fast time scale (100–400 μs) | Int. time scale (10–40 ms) | Slow time scale (1–40 s) |
|------------|-----------------------------|---------------------------|-------------------------|
|            | $k_{fast}$/s⁻¹ | Amp | $k_{fast}$/s⁻¹ | Amp | $k_{slow}$/s⁻¹ | Amp |
| nm         |                |     |                |     |                |     |
| 1. WT      |                |     |                |     |                |     |
| 340        | (5.7 ± 1.0) \times 10⁴ | + | 290 ± 50 | - | 2.1 ± 0.8 | - |
| 580        | (2.0 ± 0.5) \times 10⁴ | + | None | - | 3.0 ± 1 | - |
| 453        | (2.2 ± 0.2) \times 10⁴ | + | 260 ± 50 | - | 2.7 ± 0.3 | - |
| 580        | (0.61 ± 0.03) \times 10⁴ | + | None | - | 4 ± 1 | - |
| 580        | (0.9 ± 0.4) \times 10⁴ | - | None | - | 0.03 ± 0.02 | - |
| 3. Missing active-site disulfide |                |     |                |     |                |     |
| 340        | (2.3 ± 1.1) \times 10⁴ | + | 150 ± 60 | - | None | - |
| 580        | (2.3 ± 1.0) \times 10⁴ | + | 140 ± 40 | - | None | - |
| 453        | (3.3 ± 0.5) \times 10⁴ | + | 220 ± 80 | - | None | - |
| 4. Missing shuttle disulfide |                |     |                |     |                |     |
| 340        | (3.7 ± 1.3) \times 10⁴ | + | None | - | 5 ± 3 | - |
| 580        | (1.8 ± 0.8) \times 10⁴ | + | None | - | 2 ± 1 | - |
| 453        | (2.5 ± 0.3) \times 10⁴ | + | None | - | 6 ± 2 | - |
reduction. From the static spectrum, we calculated that \( \sim 9.3 \) \( \mu \text{M} \) FAD was reduced to HQ out of the initial 11.2 \( \mu \text{M} \) protein concentration in 15 pulses of 1.0 \( \mu \text{s} \) each.

**DISCUSSION**

This study aimed at resolving the individual inter- and intramolecular reduction steps of SO to obtain insights into the reactivity of the different redox centers of the enzyme using AtErv1 as a model case for the Erv/ALR family. This study benefited from the ability of AtErv1 mutants that lack specific disulfides to fold and bind FAD. In addition, this enzyme and its mutants could be obtained by recombinant expression in *Escherichia coli* with high yields. For comparison, a mutant of the non-homologous SO Ero1 lacking the active site disulfide could not be obtained.

Scheme 1 below illustrates the different processes initiated upon reaction of AtErv1 with CO\(_2\) radicals. Introducing a pulse into an anaerobic solution of WT AtErv1 leads to several reactions occurring at close to diffusion controlled rates. With excess of protein redox sites over pulse-generated radicals, the probability of reducing the same molecule by two electrons in one pulse is low. Thus, in WT AtErv1, the initial reduction could, in principle (yet depending on relative exposure) occur at any one of the four redox centers: structural-, catalytic-, shuttle-disulfide, or FAD. FAD reduction can be monitored at 580 nm, while disulfide reduction can be followed at 340 nm where the Q/SQ spectra have an isosbestic point. Because of the limited solvent exposure of the isoalloxazine ring and its very close proximity to the active site disulfide, it is expected that an active site disulfide radical transfers the electron to the flavin, and the observed absorption amplitudes support the notion. However, reduction of the active site disulfide and the neighboring flavin are not resolved by our method, so both are monitored as formation of SQ in the same time regime as RSS\(^+\) formation.

We can estimate that the intramolecular ET rate constant from a disulfide radical, e.g. the shuttle disulfide radical, to the flavin would be 1 \( s^{-1} \) at a separation distance of 25 Å, and much faster if the shuttle disulfide moves nearer to the active site. We would expect to resolve any intramolecular ET processes that occur in the time window from hundreds of nanoseconds to the milliseconds period in which intermolecular reactions are observed. The fact that we do not observe intramolecular ET indicates that such reactions are slower than the observed intermolecular processes. The only other study that addresses the internal ET rate for this enzyme family is that of Farrell and Thorpe (6). They interpret the formation of the stable semiquinone form of the SO ALR (human), produced upon two-electron reduction by dithiothreitol, as occurring due to single-electron intramolecular ET from the HQ to the Q present in the second subunit of the dimer. This ET process must occur at least as fast as turnover of the enzyme, \( \sim 0.1 \) \( s^{-1} \). In contrast, the SQ produced in WT AtErv1 by single electron reduction is unstable and decays by an intermolecular process as discussed below.

Decay of the 340-nm absorption \( \sim 100 \) \( s^{-1} \) and lack of changes at 580 nm on this time scale suggest that reduction of the shuttle or structural disulfide is followed by dismutation. Intra-homodimer dismutation of two disulfide radicals is less likely than interhomodimer dismutation, since the observed rates of this process depend on the overall protein concentration. The results obtained with WT and the different AtErv1 mutants show that dismutation takes place only between structural and shuttle disulfide radicals. As these radicals are not produced at the same rate (Table 2), we therefore expect that the structural disulfide radicals will be the dominating species. Faster production of the structural disulfide radicals compared with the shuttle disulfide radicals could explain the pseudo-first order behavior expressed in the exponential decay shown in Fig. 2A, right panel. The remaining 340 nm absorption decays on a slower time scale, concomitant with absorption decreases at both 453 and 580 nm, having rate constants of \( \sim 2-3 \) \( s^{-1} \). The negative absorption change at 453 nm clearly demonstrates that SQ FAD does not disappear by dismutation, as this would have caused an absorption increase. Thus, the slow process is assigned to a reaction between enzyme molecules that contain an RSS\(^+\) radical and those containing a SQ, forming AtErv1 molecules with fully reduced FAD (HQ) and disulfides in accordance with the observed absorption changes at 580 nm. It is noteworthy, however, that an analogous reaction, electron transfer from a fully reduced shuttle disulfide to the flavin, takes place intramolecularly in a step proposed as part of the catalytic mechanism. The shuttle or structural dithiols produced by the 100 \( s^{-1} \) dismutation mentioned above could be further oxidized intermolecularly by electron transfer to the FAD in another dimer, in a process whereby one molecule acts as a substrate for another. Indeed, the slow concentration dependent absorbance decrease at 453 nm \( \sim 2-3 \) \( s^{-1} \) to below the base line, with no concomitant change observed at 340 nm, is indicative of this latter process and consistent with reported turnover numbers for this enzyme (19). Each pulse leads to progressive accumulation of reduced flavin. Thus, the protein state prior to each additional pulse does not include disulfide radical or thiol species; see Scheme 1.

A question emerging from results using the WT enzyme is whether the disulfide radical dismutation occurs between

---

\(^5\) D. Fass, unpublished observations.
structural disulfides, shuttle disulfides, or both. Results of experiments using the different mutants help in answering these questions. The mutant missing the structural disulfide shows markedly lower rate constants for the processes occurring at the fast time scale. Thus the structural disulfide is either particularly reactive, or its loss causes enough structural change to alter the reactivity of the other disulfides and the flavin. The absence of the intermediate (≈100 s\(^{-1}\)) dismutation phase of the RSS*R\(^{-}\) radicals in the structural disulfide mutant indicates that in the WT this process occurs either between two structural disulfide radicals or between structural and shuttle disulfide radicals. Because this process is not observed in the mutant missing the shuttle disulfide either, but is observed in the mutant missing the active site disulfide, we conclude that in the WT enzyme dismutation occurs between the shuttle and the structural disulfides. The thiols produced will then transfer the electrons to the active site.

The decay of the 453-nm absorption with a rate constant of ≈3–5 s\(^{-1}\) is still observed in the mutant missing the structural disulfide, demonstrating that the shuttle RSS*R\(^{-}\) reduces the FAD SQ, with the same efficiency as in the WT protein. A very slow absorption increase (≈0.01 s\(^{-1}\)) is also observed here, and the only process that may produce such an increase at 453 nm is a SQ dismutation reaction. Indeed, a concomitant absorption decrease with the same rate constant is observed at 580 nm, confirming this notion. It is noteworthy that all the RSS*R\(^{-}\) radicals of this mutant disappear by reducing the FAD SQ, and no dismutation is observed at 340 nm.

In the mutant missing the shuttle disulfide, an initial fast decrease of the 453-nm absorption is expected (see Table 1) as for the mutant missing the structural disulfide. However, this decrease is not observed, indicating that disulfide(s) are preferentially reduced in this mutant, in accordance with the slower rate of SQ formation monitored at 580 nm. It is not clear how ET from the reduced structural disulfide to the active site takes place, but as it is concentration dependent, it is presumably intermolecular. As for the mutant missing the structural disulfide, no dismutation is observed.

The absorbance changes monitored at 340 nm in the mutant missing the active site disulfide demonstrate that the remaining disulfides are reactive. The RSS*R\(^{-}\) produced in an essentially diffusion-controlled process partially disappears with a ≈100 s\(^{-1}\) rate constant. Based on the above results of experiments using the structural and shuttle mutants, we can now assign this process to dismutation of structural and shuttle disulfide radicals. SQ formation is observed at 580 nm at the same rate as the diffusion controlled formation of RSS*R\(^{-}\). Unique to the active site disulfide mutant, however, this absorption does not decay to zero even on the longest time scales examined (minutes). Thus, when the active site cysteines were replaced by alanines, the SQ became a long-lived entity. Indeed, in the static spectra recorded between and after exposure to the pulses, the SQ characteristic band is present. A role for the active site disulfide in destabilizing the SQ form of AtErv1 might have evolved to favor production of hydrogen peroxide over free superoxide radicals as final product, and to favor two-electron reduction of dioxygen to hydrogen peroxide over single-electron reduction of cytochrome c.

Characterization of disulfide reactivity in the AtErv1 SO has implications for the Erv/ALR family members that function outside the intracellular compartments typically associated with disulfide formation. In particular, poxviruses and related large double-stranded DNA viruses encode Erv/ALR enzymes, which are expressed in the cytosol of infected cells (26–27). These enzymes oxidize cysteines in poxvirus structural proteins, a process essential for assembly of infectious particles. Unlike eukaryotic SOs, the poxvirus versions lack the structural and shuttle disulfides and possess only the active site, FAD-proximal disulfide. The role of the shuttle disulfide may be filled.
Electron Transfer Reactivity of AtErv1

by additional viral proteins that have di-cysteine motifs and act in conjunction with the SO enzymes (27). The structural disulfide, however, is replaced by a cluster of aromatic residues. A structural disulfide may lack utility in the cytoplasm, where mechanisms exist to ensure reduction of disulfides. Present pulse radiolysis results show that the structural disulfide of AtErv1 is accessible to reducing agents, and therefore differs from the disulfides introduced into the downstream targets of the poxviral sulfhydryl oxidation pathway, which are buried (28) and may be protected from direct reduction by cytosolic agents such as reduced thioredoxin or glutathione.

These results illustrate the utility of the pulse radiolysis method in monitoring ET processes to and between protein molecules that use disulfides as functional redox centers. The observed redox activities of disulfide bridges and flavin in AtErv1 SO, an enzyme that in vivo catalyzes two-electron oxidation of thiols, resolved several interesting features relevant to this family of enzymes. The ET steps that characterize the reduction of the enzyme observed in this study are predominantly intermolecular. One apparent exception is the reduction of the flavin by its proximal, active site disulfide. The observed instability of the SQ intermediate, except in the mutant missing the active site disulfide, may indicate a preference of WT AtErv1 for two-electron transfers, favoring dioxygen (over cytochrome c) as an oxidizing substrate.

REFERENCES
1. Thorpe, C., Hoober, K. L., Raje, S., Glynn, N. M., Burnside, J., Turi, G. K., and Coppock, D. L. (2002) Arch. Biochem. Biophys. 405, 1–12
2. Gross, E., Sevier, C. S., Heldman, N., Vitu, E., Bentzur, M., Kaiser, C. A., Thorpe, C., and Fass, D. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 299–304
3. Herrmann, J. M., Kauff, F., and Neuhaus, H. E. (2009) Biochim. Biophys. Acta, Mol. Cell Res. 1793, 71–77
4. Bihlmayer, K., Mesecke, N., Terziyska, N., Bien, M., Hell, K., and Hermann, J. M. (2007) J. Cell Biol. 179, 389–395
5. Dabur, D. V., Leverich, E. P., Kim, S. K., Tsai, F. D., Hirasawa, M., Knaff, D. B., and Koehler, C. M. (2007) EMBO J. 26, 4801–4811
6. Farrell, S. R., and Thorpe, C. (2005) Biochemistry 44, 1532–1541
7. Gross, E., Sevier, C. S., Vala, A., Kaiser, C. A., and Fass, D. (2002) Nat. Struct. Mol. Biol. 9, 61–67
8. Gross, E., Kastner, D. B., Kaiser, C. A., and Fass, D. (2004) Cell 117, 601–610
9. Frand, A., and Kaiser, C. A. (2000) Mol. Biol. Cell 11, 2833–2843
10. Raje, S., and Thorpe, C. (2003) Biochemistry 42, 4560–4568
11. Sevier, C. S., Qu, H., Heldman, N., Gross, E., Fass, D., and Kaiser, C. A. (2007) Cell 129, 333–344
12. Gerber, J., Mühlenhoff, U., Hofhaus, G., Lill, R., and Lisowsky, T. (2001) J. Biol. Chem. 276, 23486–23491
13. Hofhaus, G., Stein, G., Polimeno, L., Francavilla, A., and Lisowsky, T. (1999) Eur. J. Cell Biol. 78, 349–356
14. Hagiya, M., Francavilla, A., Polimeno, L., Ichiba, I., Sakai, H., Seki, T., Shimomishi, M., Porter, K. A., and Starzl, T. E. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8142–8146
15. Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12068–12073
16. Mairet–Coello, G., Tury, A., Esnard–Feve, A., Fellmann, D., Risold, P., and Griffon, B. (2004) J. Comp. Neurol. 473, 334–363
17. Ostrowski, M. C., and Kistler, W. S. (1980) J. Biol. Chem. 255, 559–563
18. Levanon, A., Danon, A., and Lisowsky, T. (2004) J. Biol. Chem. 279, 20002–20008
19. Herrman, J., and Köhl, R. (2007) J. Cell Biol. 176, 559–563
20. Mesecke, N., Terziyska, N., Kozany, C., Baumann, F., Neupert, W., Hell, K., and Herrmann, J. M. (2005) Cell 121, 1059–1069
21. Vitu, E., Bentzur, M., Lisowsky, T., Kaiser, C. A., and Fass, D. (2006) J. Mol. Biol. 362, 89–101
22. Farver, O., and Pecht, I. (2007) Prog Inorg. Chem. 55, 1–78
23. Farver, O., and Pecht, I. (1992) J. Am. Chem. Soc. 114, 5764–5767
24. Farver, O., Bendahl, L., Skov, L. K., and Pecht, I. (1999) J. Biol. Chem. 274, 26135–26140
25. Hille, R., and Anderson, R. F. (1991) J. Biol. Chem. 266, 5608–5615
26. Rodríguez, I., Redrejo–Rodríguez, M., Rodríguez, J., Alejo, A., Salas, J., and Salas, M. (2006) J. Virol. 80, 3157–3166
27. Senkevich, T. G., White, C. L., Koonin, E. V., and Moss, B. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 6667–6672
28. Ga, H. P., Garman, S. C., Allison, T. J., Fogg, C., Moss, B., and Garboczi, D. N. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4240–4245