Superoxide Reactivity of Rubredoxin Oxidoreductase (Desulfoferrodoxin) from *Desulfovibrio vulgaris*: A Pulse Radiolysis Study

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Rubredoxin oxidoreductase (Rbo), also called desulfoferrodoxin,[1−4] is a homodimeric non-heme iron protein found in some sulfate-reducing bacteria and archaea. Rbo has recently been implicated as both a superoxide dismutase (SOD)[5] and a superoxide reductase (SOR).[6,7] The latter activity has been proposed to represent a new paradigm by which anaerobic microorganisms minimize the toxic effects of adventitious dioxygen exposure.[8]

$$2O_2^- + 2H^+ \rightarrow O_2 + H_2O_2 \qquad \text{(SOD-catalyzed reaction)}$$

$$e^- + O_2^- + 2H^+ \rightarrow H_2O_2 \qquad \text{(SOR-catalyzed reaction)}$$

The crystal structure of Rbo from *Desulfovibrio desulfuricans*[9] shows two distinct iron sites: Center I is an $[Fe(SCys)_4]$ site, the function of which is presumed to be electron transfer. Center II is a novel square pyramidal $[Fe(NHis)_4(SCys)]$ site, and the published evidence indicates that Center II is the site of reaction with superoxide.[5,7] The reduction potentials of Centers I (2 mV) and II (90−240 mV vs NHE) are such that Rbo as-isolated is pink (Rbo_{pink}) with a ferric Center I and ferrous Center II.[2,3] Rbo_{pink} can be oxidized to a gray form (Rbo_{gray}) in which both centers are ferric.[7]

A related protein, SOR from *Pyrococcus furiosus*,[8] contains a Center II homologue as the only metal site. X-ray crystal structures of *P. furiosus* SOR show a carboxylate from a glutamate residue occupying the coordination site opposite to the cysteine thiolate ligand in the six-coordinate ferric form, but not the five-coordinate ferrous form.[10] In the Rbo crystal structure, the analogous carboxylate from glutamate residue E47 is >10 Å away from the Center II iron.[8] This glutamate residue is conserved in all known Rbos, and SORs.[11−14] This report addresses the aforementioned structural and functional ambiguities of Rbo Center II by employing pulse radiolysis to study the reaction of superoxide with *D. vulgaris* Rbo and an E47A-mutated Rbo.[15,16]

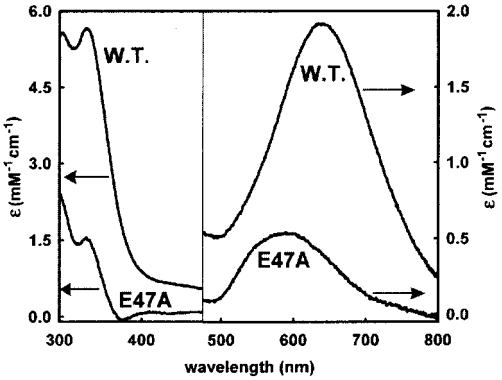

Figure 1. Difference absorption spectra (~23 °C) of wild type (W.T.) or E47A [Rbo_{gray} − Rbo_{pink}], in which Rbo_{gray} was generated by aerobic reaction of ~34 μM Rbo_{pink} Center II with xanthine (0.4 mM)/xanthine oxidase (10 μg) in 50 mM 3-(*N*-morpholino)propanesulfonate at pH 7.3. Supporting Information shows the corresponding absolute absorption spectra.

Centers II in both wild type and E47A Rbo_{pink} could be oxidized to form Rbo_{gray} by reaction with a xanthine/xanthine oxidase superoxide-generating system.[5,7] Difference absorption spectra (Figure 1) generated by subtracting the initial Rbo_{pink} spectrum from that of the corresponding superoxide-oxidized protein clearly show that the E47A mutation perturbs ferric Center II, but not Center I. The wild-type Rbo_{gray} − Rbo_{pink} difference absorption spectrum shows the previously reported feature at 647 nm (ϵ_{647} ~1900 M^{-1} cm^{-1}) due to ferric Center II.[2,3,5,7] The corresponding feature in the difference spectrum of E47A Rbo is blue shifted to approximately 590 nm and less intense (ϵ_{590} ~ 500 M^{-1} cm^{-1}). These results suggest that E47 interacts with the iron atom of ferric Center II, most likely by carboxylate ligation.[17]

SOD activity of Rbo was monitored by the rate of disappearance of superoxide via its absorbance at 260 nm.[18] No enhancement in the spontaneous disproportionation rate was observed in the presence of 1 μM Rbo_{pink} (rate data included as Supporting Information). At pH 7.8, typical SODs enhance the superoxide disproportionation rate constant by 4 orders of magnitude.[19] Our measurements show a limiting catalytic rate constant for Rbo

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(15) Procedures for overexpression, purification, and mutation of recombinant Rbo are described in the Supporting Information. Iron analyses by inductively coupled plasma atomic emission confirmed the presence of ~4 Fe/Rbo homodimer for both wild type and E47A Rbo, as expected for fully occupied Centers I and II. The purified Rbo was concentrated to ~4 mM monomer and transferred into 0.125 mM Tris pH 7.8. Pulse radiolysis experiments were performed using the 2-MeV van de Graaff accelerator at Brookhaven National Laboratory.[16]
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(17) The published Rbo crystal structure,[9] professedly of Rbo_{gray}, is actually likely to be that of Rbo_{pink}, i.e., ferrous Center II, possibly resulting from exposure of Rbo_{gray} to the X-ray source. In our hands *D. vulgaris* Rbo_{gray} slowly autoreduces to Rbo_{pink} at ambient temperature, even in aerobic solutions. The polypeptide loop containing E47 was reported to be highly mobile in the Rbo crystal structure. During oxidation to Rbo_{gray}, movement of this loop would allow coordination of the E47 carboxylate to ferric Center II, as occurs in *P. furiosus* SOR.[10]
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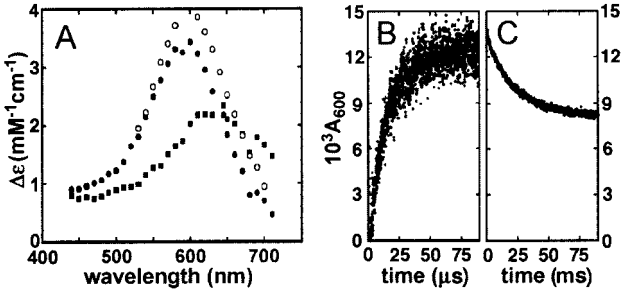


Figure 2. (A) Optical absorption spectra of the 600-nm intermediate (~75 μs after pulse) (●) and final product (■) obtained ~100 ms after pulse radiolysis of Rbo_{pink} (100 μM Center II) solution to generate 1.7 μM superoxide. (○) E47A Rbo 600-intermediate (~75 μs after pulse). (B and C) Time courses for the formation (B) and decay (C) of the 600-nm intermediate following pulse radiolysis of Rbo_{pink} (50 and 25 μM Center II, respectively) solutions to generate 1.7 μM superoxide. Conditions: 0.12 mM Tris, 10 mM formate, 5 μM EDTA at pH 7.78, 25 °C, 2.0-cm optical path.

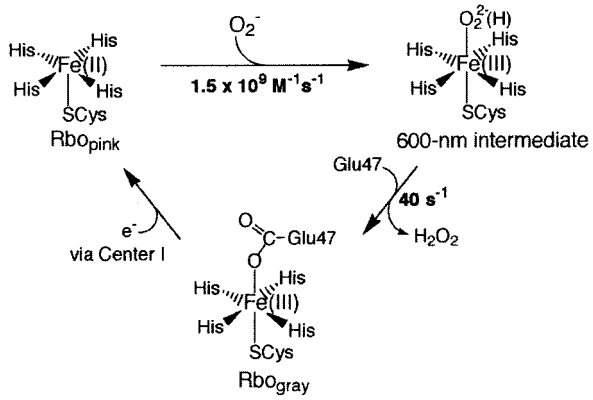
below that for spontaneous superoxide disproportionation ($\sim 10^5$ M^{-1} s^{-1} at ambient temperature and pH 7.8[16]), and, thus, establish that *Rbo is not an SOD*. In the same fashion, E47A Rbo was also shown to be devoid of SOD activity. The iron-coordinated glutamate residue in *P. furiosus* SOR was proposed to obstruct the reaction of the ferric center with superoxide, thereby disfavoring SOD activity.[9] The lack of SOD activity for E47A Rbo does not support such a role for the analogous glutamate residue in Rbo.

Figure 2A shows optical absorption spectra (440−710 nm at 10-nm intervals) and time courses obtained on microsecond and millisecond time scales during reaction of Rbo_{pink} with substoichiometric levels of superoxide generated by pulse radiolysis. An intermediate exhibiting an absorption centered at 600 nm ($\epsilon_{600} \sim 3500$ M^{-1} cm^{-1}) is fully formed by ~60 μs after the superoxide pulse (Figure 2B). This intermediate accumulates at a nearly diffusion-controlled rate (1.5×10^9 M^{-1} cm^{-1}) that is first order in both superoxide and Rbo concentrations (0.5−5 and 25−100 μM, respectively). The 600-nm intermediate then decays in a first-order process (40 s^{-1}) (Figure 2C) that is independent of superoxide concentration to yield a final spectrum ($\lambda_{max} \sim 650$ nm, $\epsilon_{650} \sim 2300$ M^{-1} cm^{-1}) closely resembling that of Center II in Rbo_{gray} (Figure 1).

The extinction coefficients listed for the 600-nm intermediate and final product are based on the initial concentration of substoichiometric superoxide generated by pulse radiolysis and assumes that all such superoxide reacts with Rbo_{pink}. It can also be reasonably assumed that the absorption spectrum of the final pulse radiolysis product is that of Center II in Rbo_{gray}. If so, then the close agreement between the corresponding extinction coefficients indicates a 1:1 molar stoichiometry between superoxide generated and Center II reacted, i.e., a one-electron process, as expected for the SOR-catalyzed reaction.

An essentially identical 600-nm intermediate forms at approximately the same diffusion-controlled rate upon reaction of E47A Rbo with superoxide (Figure 2A). The E47A 600-nm intermediate decays on the same time scale as for wild type, but in a more complicated fashion. Nevertheless, the final spectrum

Scheme 1



obtained via pulse radiolysis (not shown) closely resembles the E47A difference absorption spectrum (Figure 1).

An SOR cycle for Rbo Center II is proposed in Scheme 1. We formulate the 600-nm intermediate as a ferric-(hydro)peroxo species. This formulation is consistent with optical absorption spectra of synthetic ferric-alkyl(hydro)peroxo and peroxo complexes, which typically exhibit peroxo-to-iron charge-transfer absorptions with λ_{max} 520−615 and ϵ 500−2200 M^{-1} cm^{-1}.[20] The absorption intensity of the 600-nm intermediate is likely due to a combination of peroxo → Fe(III) and cysteinyl sulfur → Fe(III) charge transfer. Protonation of the coordinated (hydro)peroxo by the incoming E47 side chain would lead to release of hydrogen peroxide and Rbo_{gray}, which we propose has a six-coordinate ferric Center II.[17] Reinsertion of an electron via Center I (from an unidentified exogenous donor) would lower the affinity of E47 for iron, leading to regeneration of the five-coordinate ferrous Center II. The nearly diffusion-controlled initial reduction of superoxide by ferrous Center II is consistent with the presumed superoxide scavenging function of Rbo.[21] SOR-type enzymatic turnover of Rbo, however, may be critically dependent on subsequent steps of the cycle, such as protonation of the ferric-(hydro)peroxo complex.[22] Our results suggest a mechanism for SOR activity of Rbo involving formation and decay of a ferric-peroxo species.

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Supporting Information Available: Rbo expression, purification and mutation procedures, plots of superoxide dismutation rate constants in the presence and absence of Rbo, UV−vis absorption spectra of wild type and E47A Rbo (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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