The interaction of oxygen with iron-containing proteins is important in many biological processes, including transport, metabolism, respiration, and cell protection. The reaction of oxygen or its reduced derivatives, superoxide and hydrogen peroxide, with iron enzymes often involves short-lived iron-peroxide intermediates along the reaction cycle (1, 2).

Iron-peroxide intermediates are central in the reaction cycle of many iron-containing biomolecules. We trapped iron(III)-(hydro)peroxo species in crystals of superoxide reductase (SOR), a nonheme mononuclear iron enzyme that scavenges superoxide radicals. X-ray diffraction data at 1.95 angstrom resolution and Raman spectra recorded in crystallo revealed iron-(hydro)peroxo intermediates with the (hydro)peroxo group bound end-on. The dynamic SOR active site promotes the formation of transient hydrogen bond networks, which presumably assist the cleavage of the iron-oxygen bond in order to release the reaction product, hydrogen peroxide.

Heme-based peroxidases, catalases, and many oxygenases promote heterolytic cleavage of the peroxide oxygen-oxygen bond to form high-valence reactive iron-oxo species. In contrast, other iron enzymes, such as superoxide reductase (SOR) (3, 4), are fine-tuned to cleave the iron-oxygen bond and avoid the formation of potentially harmful iron-oxo species. Although the
protein, the metal configuration, and the solvent environment have been shown to play a role, the mechanisms by which iron-peroxide intermediates are processed are not fully understood (1, 2). Despite pioneering studies on hemoproteins (5–7), structural data revealing peroxide species in nonheme mononuclear iron enzymes have remained scarce (8). We have developed an approach in which kinetic crystallography (9) was assisted by “in crystallo” Raman spectroscopy (10) to characterize (hydro)peroxo species in SOR. SOR is found in some air-sensitive bacteria and archaea and converts the toxic superoxide anion radical (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) via a one-electron reduction pathway: O$_2^−$ + 2H$^+$ + SOR(Fe$^{2+}$) → H$_2$O$_2$ + SOR(Fe$^{3+}$) (3, 4, 11). The SOR catalytic domain displays an immunoglobulin-like fold (12, 13) encompassing an iron atom coordinated to four equatorial histidines and one axial cysteine, thus bearing structural resemblance to the ubiquitous cytochromes P450s. However, contrary to P450s, the ferrous ferrous structural data described below reveal a series of end-on iron(III)-(hydro)peroxo species involved in tight hydrogen bond networks (Fig. 1) and allow us to propose a mechanism for protonation step have remained elusive (14). Investigations of various SOR adducts (12, 14, 15), pulse-radiolysis studies (16–18), and resonance Raman spectroscopy experiments (19, 20) have suggested an-inner sphere catalytic mechanism involving the formation of transient iron(III)-(hydro)peroxo species. As described for similar enzymes (2, 21), protonation steps play a crucial role in governing the chemistry that occurs at the SOR active site (11, 16, 17). In SOR from the sulfate-reducing bacterium Desulfopallidatus baarsii (4, 12), a first iron(III)-peroxo intermediate has been proposed to be rapidly protonated (in ~100 μs), forming a more stable iron(III)-hydroperoxo species (17). A second protonation then occurs, possibly promoted by a water molecule (22), and yields the H$_2$O$_2$ product through a dissociative mechanism in which Glu$^{15}$ ultimately binds to the oxidized enzyme (13, 17). Thus, SOR avoids heterolytic cleavage of the O-O bond, preventing the formation of oxo-ferryl compounds. To date, the structure of the iron-peroxide species that can be accommodated within the SOR active site and the mechanism governing the decisive second protonation step have remained elusive (11).

Table 1. Geometry of the active site. Fe distance from the His plane was defined by the coordinating N atoms of the equatorial histidines in Å. Increasing value indicates an iron position closer to Cys$^{116}$.

| WT-SOR | E114A-SOR reduced | E114A-SOR peroxide intermediates | DFT calculation |
|--------|-------------------|---------------------------------|----------------|
|        | Monomer A        | Monomer B                      |                |
| Fe-S (Å) | 2.4              | 2.4                            | 2.5            |
| Fe from His plane (Å) | 0.4              | 0.4                            | 0.3            |
| Fe-S (Å) | 2.4              | 2.4                            |                |
| Fe-01 (Å) | 2.0              | 126                           | 2.19           |
| Fe-01-02 (°) | 140              | 168                           |                |
| C$\text{S}$-5-01-02 (°) | 132            | 114                           |                |
| Fe from His plane (Å) | 0.4              | 0.3                            | 0.10           |
| Fe-S (Å) | 2.4              | 2.4                            | 2.44           |
| Fe-01 (Å) | 2.0              | 126                           | 1.94           |
| Fe-01-02 (°) | 126              | 123                           |                |
| C$\text{S}$-5-01-02 (°) | 132            | 114                           |                |
| Fe from His plane (Å) | 0.5              | 0.3                            | 0.16           |
| Fe-S (Å) | 2.4              | 2.5                            | 2.6            |
| Fe-01 (Å) | 2.0              | 122                           | 2.49           |
| Fe-01-02 (°) | 122              | 123                           |                |
| C$\text{S}$-5-01-02 (°) | 112            | 99                            |                |
| Fe from His plane (Å) | 0.4              | 0.3                            | 0.11           |

Fig. 1. Structural overview of SOR. The x-ray structure of the SOR-E114A homodimer in the native reduced state is shown as magenta (monomer A) and cyan (monomer B) ribbons with the exception of the LiD loop (residues 45 to 49), which is colored in dark green and orange for monomers A and B, respectively. Reduced and oxidized iron atoms are shown as green and orange balls, respectively. (Inset) The active site of monomer B upon addition of H$_2$O$_2$. The residues coordinating the active iron (His$^{59}$, His$^{69}$, His$^{119}$, His$^{139}$, and Cys$^{116}$) as well as Lys$^{48}$ are represented as sticks. The bound peroxide ligand is shown as a red stick. Water molecules are shown as red balls. In order to support the diatomic nature of the peroxide intermediate, simulated annealed $	ext{F}_\text{obs}$ − $	ext{F}_\text{calc}$ maps omitting the distal or proximal oxygen of the O-O moiety, respectively, were calculated. The two maps are displayed in green (distal) and orange (proximal) at a contour level of 3.0σ.
Mononuclear iron-peroxide complexes are generally obtained by reacting iron(II) with excess H₂O₂ (23). However, to minimize Fenton-driven generation of toxic hydroxyl radicals, we first oxidized crystalline SOR with hexachloroiridate (IV) and then exposed it to H₂O₂ for 3 min before freezing (24). Because the isolation of iron(III)-peroxide complexes is hampered by their high reactivity, crystallographic data were collected with the mutant enzyme E114A (Glu¹¹⁴→Ala¹¹⁴), in which, as described for the E47A (Glu⁴⁷→Ala⁴⁷) variant (19, 20), these intermediates are stabilized (Supporting Online Material (SOM) text). Comparison of the native crystal structures of the wild-type and mutant enzymes revealed that the loss of the E114 side chain does not alter the overall enzyme structure (Table 1).

The asymmetric unit in SOR-E114A crystals contains four monomers, denoted A to D. Upon soaking with H₂O₂, diffraction data to 1.95 Å resolution (24) (table S1) revealed elongated features in the electron density maps that are consistent with the formation of end-on (η¹) peroxide species in monomers B, C, and D (Figs. 1 and 2), whereas monomer A did not react. To verify the chemical nature of the observed species, we developed a Raman spectrometer to analyze cryocooled crystals under conditions identical to those used for x-ray data collection (24, 25) (fig. S1). Upon H₂O₂ treatment, two ¹⁸O isotope-sensitive main bands at ~567 cm⁻¹ and ~838 cm⁻¹ appeared in the Raman spectra of SOR crystals (Fig. 3). Although these bands probably involve the coupling of a number of vibrational modes, they fall within the expected range for v(Fe-OOH) and v(O-O) frequencies of iron-peroxide species, respectively (26) (SOM text). Importantly, they are not specific to the crystalline phase, because they also appeared with solution samples similarly treated with hydrogen peroxide (SOM text). In addition, Raman spectra from crystals exposed to x-rays (27) showed the same signature as unexposed crystals, ruling out the possibility of substantial photo-alteration during data collection if it is known that the solvent-exposed SOR active site is sensitive to reduction by x-ray-induced photo-electrons (12). Overall, in crystallo Raman spectra strongly suggested the buildup of iron-peroxide species in the crystal. To assess the protonation state of these species, we performed density functional theory (DFT) calculations (SOM text) on model SOR active sites based on the x-ray structures determined in this work. In monomers B and D, these calculations favor high-spin η¹ hydroperoxo species that are protonated at the distal oxygen, consistent with pulse-radiolysis studies that suggested rapid protonation of the SOR iron-peroxo species even at the basic pH (pH = 9) used in our work (17). In monomer C, an η¹ species is also favored, but its protonation state cannot be firmly established.

Fig. 2. Structure of the SOR-peroxide intermediates. Stereo views of the peroxide-bound SOR active sites in monomers C, B, and D are shown in (A), (B), and (C), respectively. Final 2Fobs − Fcalc maps (blue, contoured at 1.0σ), simulated annealed Fobs − Fcalc maps omitting the peroxy moiety and associated water molecules (green, contoured at 4.5σ), and simulated annealed Fobs − Fcalc maps omitting only Lys⁴⁸ (orange, contoured at 3.5σ) are shown, overlaid on the refined models of the SOR-peroxide intermediates. Hydrogen bonds and iron coordination are shown as blue and black dashed lines, respectively.
moiety only interacts with the active iron, and the LID loop displays a “locked-open” conformation, possibly because of weak crystal lattice contacts (SOM text). This LID conformation prevents Lys68 from interacting with the hydroperoxo moiety, leaving the side chain of this residue in a disordered state. In contrast, in monomer B, the LID loop is found “locked closed,” and Lys68 facilitates a tight hydrogen bond network around the distal oxygen of the peroxide moiety that also includes two water molecules (Wat10 and Wat11) (Fig. 2). The positively charged amino group of Lys68 (SOM text) attracts the peroxide ligand, presumably inducing a stretch of the S1-Cys116-Fe-O-O moiety that may further weaken the Fe-O bond. In monomer D, the side chain of Lys68 slightly rotates away from the hydroperoxo moiety, and the two water molecules Wat10 and Wat11 are still observed, together with a third molecule (Wat12) that may play a stabilizing role. However, Wat10 now forms a hydrogen bond with the amino group of Ala45, whereas Wat11 moves slightly toward the iron so that it interacts with both proximal and distal oxygen atoms of the hydroperoxo moiety. Wat11 is therefore in a favorable position to donate a proton to the proximal oxygen atom, allowing the formation and release of hydrogen peroxide. This is a crucial step that differentiates SOR from heme enzymes where protonation occurs at the distal oxygen, liberating water and oxo-ferryl species (2). Simultaneously, a combination of subtle rearrangements of the iron-coordinating histidines shifts the iron into the plane defined by the four equatorial coordinating nitrogens (Table 1) (28). This conformation possibly facilitates access of Wat11 to the metal and the proximal oxygen.

Our data highlight the dynamic behavior of the SOR active site on route to product formation (Fig. 4 and movie S1). Monomer C may be viewed as an early state along the reaction coordinate that precedes the conformational rearrangements leading to the protonation of the HOO− adduct. We suggest that this state is sta-
bilized in the crystal because of the locked-open configuration of the LID loop. In contrast, monomers B and D reveal subsequent activated configurations, emphasizing the catalytic role of Lys18. The observation of Wat11 in the immediate environment of the hydroperoxo species in these monomers strongly supports the hypothesis that this water molecule is the proton donor for product formation and release. We propose that Lys48 hydrogen bonds to Wat11 and imports it into the SOR active site in a motion promoted by electrostatic attraction of the positively charged amino group to the hydroperoxo ligand. Once anchored in the vicinity of the proximal oxygen, Wat11 becomes more acidic because of the interaction with the amino group of Lys48. Protonation of the proximal oxygen is probably simultaneous with the cleavage of the iron-oxygen bond, and Wat11 may immediately replace the hydrogen peroxide product in the form of a hydroxide ion until Glu47 binds to the iron, as already suggested (18, 22).

SOR illustrates the key role played by subtle protein motions in enzyme catalysis (29). In crystalline SOR, the flexible LID loop adopts various conformations, suggesting that there is little free energy difference between disordered (entropy-driven) states for this loop and ordered (enthalpy-driven) ones where Lys48 is stabilized by transient H-bonding networks. Our data are consistent with the idea of a breathing of the LID loop that serves to import catalytically competent water molecules into the SOR active site (29). In the crystal, local packing forces may slightly modify the thermodynamic energy balance, selecting different conformations in each monomer.

The structural observations described in this work are obviously not sufficient to entirely account for the specific reactivity of SOR toward breakage of the iron-oxygen bond. Finely tuned electron donation by the trans thiolate ligand (Cys116) is expected to precisely adjust the strength of this bond (11). Furthermore, several lines of evidence indicate that the iron spin state greatly modulates the strength of the iron-oxygen and oxygen-oxygen bonds in iron(III)-peroxide complexes (1, 11, 21, 23). Whereas many heme catalysts that promote cleavage of the O-O bond involve low-spin states of the iron, SOR (19, 20, 30) and the oxygen carrier di-iron hemerythrin (31) are unique in that they involve a high-spin (S = 5/2) iron state (30) (SOM text). Interestingly, SOR and hemerythrin share structural and spectroscopic properties: In oxy-hemerythrin, an end-on iron-peroxide species stabilized by a strong hydrogen bond is also observed (31). In addition, the Raman vibrations measured for SOR and oxy-hemerythrin are relatively similar and imply a weaker Fe-O bond and a stronger O-O bond when compared to low-spin iron-peroxide model compounds known to favor heterolytic cleavage of the O-O bond (26).

The data suggest a possible mechanism for hydrogen peroxide formation, highlighting the role of a key water molecule finely controlled by the enzyme dynamics. The revealed conformational transitions provide a strong basis for further computational and structural investigations of the mechanism of superoxide scavenging by SOR and may facilitate the design of biomimetic catalysts.

References and Notes

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23. Materials and methods are available as supporting material on Science Online.
24. Raman experiments were performed under nonresonant conditions, taking advantage of the large protein concentration found in crystals to enhance the signal-to-noise ratio. In this way, the crystals were sampled homogeneously, and potential light-induced damage or photochemistry that could develop under resonant conditions was avoided.
25. We report the structures of three intermediates in the O2 activation and insertion reactions of an extradiol ring-cleaving dioxygenase. A crystal of Fe2+-containing homoprotocatechuate 2,3-dioxygenase was soaked in the slow substrate 4-nitrocatechol in a low O2 atmosphere. The x-ray crystal structure shows that three different intermediates reside in different subunits of a single homotetrameric enzyme molecule. One of these is the key substrate-alkylperoxo-Fe2+ intermediate, which has been predicted, but not structurally characterized, in an oxygenase. The intermediates define the major chemical steps of the dioxygenase mechanism and point to a general mechanistic strategy for the diverse 2-His-1-carboxylate enzyme family.

Crystal Structures of Fe2+ Dioxygenase Superoxo, Alkylperoxo, and Bound Product Intermediates

Elena G. Kovaleva and John D. Lipscomb*

Aerobic life is possible because O2 must be activated before it will react rapidly with most biological molecules, which prevents indiscriminate oxidation. Oxygenase enzymes have evolved numerous chemical strategies to selectively effect this activation so that...