A Long-Lived, Substrate-Hydroxylating Peroxodiiron(III/III) Intermediate in the Amine Oxygenase, AurF, from *Streptomyces thioluteus*

Victoria Korneeva Korboukh,[†,‡] Ning Li,[‡] Eric W. Barr,[‡] J. Martin Bollinger, Jr.,*[,†,‡] and Carsten Krebs*[,†,‡]

Departments of Chemistry and Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received August 1, 2009; E-mail: ckrebs@psu.edu; jmb21@psu.edu

AurF from *Streptomyces thioluteus* utilizes O_2 as oxidant to effect the six-electron oxidation of *p*-aminobenzoate (pABA) to *p*-nitrobenzoate (pNBA), a building block of the antibiotic aureothin.[1] This conversion entails three successive two-electron oxidations via *p*-hydroxylaminobenzoate (pHABA) and *p*-nitrosobenzoate intermediates.[2,3] Sequence analysis and the crystal structure of AurF showed that it has a carboxylate-bridged dinuclear metal center, which was presumed to activate O_2 for the oxidation reactions. Following initial controversy as to the identities of the metal ions in the functional form(s),[2−7] Zhao and co-workers recently established that the diiron form is competent to convert pABA to pNBA in the presence of O_2 and a reducing system.[8] Here we demonstrate that the $Fe_2^{II/II}$ cluster in AurF reacts with O_2 in the absence of substrate to form a remarkably stable ($t_{1/2} \approx$ 7 min at 20 °C) adduct with spectroscopic properties characteristic of a peroxo-$Fe_2^{III/III}$ complex. The intermediate complex decays rapidly ($t_{1/2} \approx 0.005$ s at 20 °C) when mixed with stoichiometric pABA, implying that it is competent for at least the first oxidation in the three-step sequence. The nearly quantitative (>80%) conversion of pABA to pNBA upon addition of <0.3 equiv of the amine substrate to a solution of the intermediate suggests that the complex might effect all three steps in the sequence. The results establish a new reactivity of nonheme diiron-peroxide intermediates: arylamine oxygenation.

Diiron AurF was isolated from overproducing *Escherichia coli* cells as described in the Supporting Information. The UV/visible absorption spectrum of the protein (Figure 1, blue trace) has a band at ∼360 nm similar to those exhibited by other proteins with μ-oxo-$Fe_2^{III/III}$ clusters, wherein it has been assigned to an oxo-to-iron charge transfer transition.[9] The 4.2-K/53-mT Mössbauer spectrum of AurF isolated from cultures supplemented with $^{57}FeSO_4$ (>95% ^{57}Fe) can be simulated as two quadrupole doublets with parameters typical of high-spin Fe^{III} [$\delta_1 = 0.54$ mm/s, $\Delta E_{Q,1} = -1.86$ mm/s; $\delta_2 = 0.48$ mm/s, $\Delta E_{Q,2} = 0.80$ mm/s] and relative contributions to the experimental spectrum of 78% (orange line) and 22% (green line), respectively (Figure 2A). The large $|\Delta E_{Q,1}|$ of 1.86 mm/s is typical of μ-oxo-$Fe_2^{III/III}$ clusters, consistent with the absorption spectrum and the crystal structure.[8] The complex with smaller ΔE_Q could have one or more μ-hydroxo bridge(s). Mössbauer spectra collected in externally applied fields of 5 T and 8 T (Figure 2E and 2F) can be simulated well with the same parameters and the assumption of a diamagnetic ground state. The $S = 0$ ground state arises from antiferromagnetic coupling between the two high-spin Fe^{III} sites.

Treatment of the protein with 1 equiv of sodium dithionite (DT) bleaches the band at 360 nm in the absorption spectrum (Figure 1, red trace) and produces a new quadrupole doublet with parameters ($\delta = 1.24$ mm/s, $\Delta E_Q = 3.06$ mm/s) typical of high-spin Fe^{II} (Figure
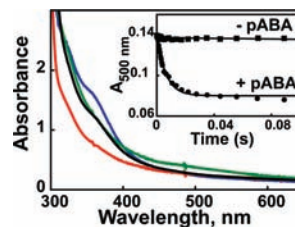
Figure 1. Absorption spectra of a sample of AurF subjected to DT reduction and O_2 reoxidation: as-isolated protein (0.40 mM in 50 mM HEPES pH 7.5 and 10% glycerol buffer; blue line); after treatment with 1 equiv of DT for 10 min at 20 °C (red line); after addition of 0.2 volume of cold oxygenated buffer ($[O_2]_{final}$ = 0.38 mM, green line), and after addition of pABA (0.92 mM final; black line). Inset: Kinetic traces after mixing DT-reduced AurF (0.64 mM) at 21 °C with an equal volume of O_2-saturated buffer, allowing the reaction to proceed for 0.1 s, and then mixing the resultant solution with an equal volume of anaerobic buffer either lacking pABA (■) or containing 1 equiv pABA (●). The solid lines are simulations with parameters quoted in the text.

2B). These observations indicate that the $Fe_2^{III/III}$ protein is reduced to the $Fe_2^{II/II}$ form by DT treatment.

Reaction of $Fe_2^{II/II}$ AurF with O_2 produces a new broad absorption band centered at ∼500 nm ($\varepsilon_{500} \approx 0.5$ mM^{-1} cm^{-1}; Figure 1, green trace). Formation of the 500-nm-absorbing species is complete within 0.01 s at ∼0.6 mM O_2 and 20 °C (Figure S1). The species does not decay noticeably on the 100-s time scale of the SF absorption experiment. The 4.2-K/53-mT Mössbauer spectrum of the protein following vigorous stirring of the $Fe_2^{II/II}$ complex for ∼2 min under ∼2 atm of O_2 (Figure 2C) shows that the diiron cluster is almost quantitatively oxidized. In addition to small amounts of the subspectra of $Fe_2^{II/II}$ (5%, purple line) and μ-oxo-$Fe_2^{III/III}$ (18%, orange line) complexes, the spectrum reveals several new sharp peaks, which can be simulated as two quadrupole doublets with similar parameters. The parameters [$\delta_1 = 0.54$ mm/s, $\Delta E_{Q,1} = -0.66$ mm/s (49%, blue line) and $\delta_2 = 0.61$ mm/s, $\Delta E_{Q,2} = 0.35$ mm/s (33%, red line)] are typical of high-spin Fe(III).[10] Spectra collected in external fields of 5 T and 8 T (Figure 2G and 2H)[11] confirm that the new state has $S = 0$ ground state(s), due to antiferromagnetic coupling of two high-spin Fe^{III} sites. Because it is an $Fe_2^{III/III}$ cluster derived from the $Fe_2^{II/II}$ enzyme by treatment with O_2, we assign this state as peroxo-$Fe_2^{III/III}$. The ∼1.5:1 intensity ratio of the two quadrupole doublets suggests the presence of two or more isomeric complexes, perhaps in equilibrium (as has been observed in the reactive intermediate states of other iron enzymes).[12−14] Mössbauer spectra of samples of the peroxide state frozen after subsequent incubation at 20 °C show that it is stable for minutes in the absence of substrate ($t_{1/2} \approx 7$ min) and decays to the μ-oxo-$Fe_2^{III/III}$ species (Figure S2).

The reactivity of the peroxo-$Fe_2^{III/III}$ state was examined by stopped-flow experiments in which the $Fe_2^{II/II}$ protein was allowed to react with O_2 for 100 ms and the resultant peroxo-$Fe_2^{III/III}$ state was then mixed

with the substrate, pABA, or (as a control) buffer. The 500 nm band of the peroxo-$Fe_2^{III/III}$ state, stable in the absence of pABA (inset to Figure 1, ■), decays rapidly ($k_{obs} = 150 \pm 20$ s^{-1}) upon exposure to 1 equiv of the substrate (inset to Figure 1, ●).

The 4.2-K/53-mT Mössbauer spectra of samples prepared by oxygenation of $Fe_2^{II/II}$ AurF (Figure 2C, replotted in Figure 2D as solid line for clarity) and subsequent treatment of the oxygenated sample with 0.3 equiv of pABA for 1 min (Figure 2D, hashed marks) confirm the reactivity of the peroxo-$Fe_2^{III/III}$ state toward the substrate. The features of the intermediate complex(es) decay, and those of the μ-oxo-$Fe_2^{III/III}$ cluster develop. Analysis of the small-molecule components of reactions performed similarly (described in Supporting Information) showed that, at these low pABA/AurF ratios of ≤0.3, the substrate is converted to pNBA with a yield of >80%.[15]
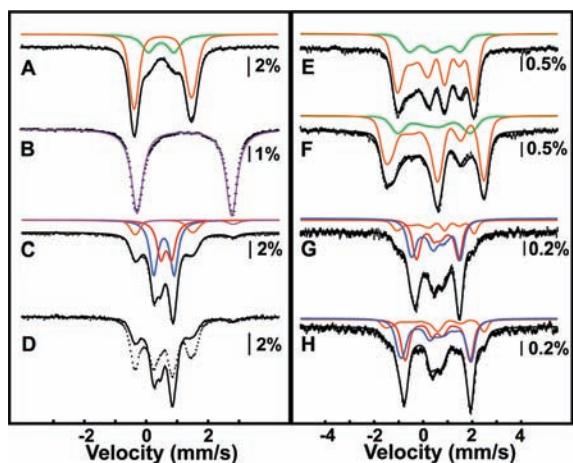
Figure 2. 4.2-K Mössbauer spectra (hashed marks) of as-isolated AurF (A, E, F); DT-reduced AurF (B); DT-reduced AurF exposed to 2 atm O_2 for 2 min, and then either directly frozen for Mössbauer analysis (C) or further treated with 0.3 equiv pABA prior to being frozen for analysis (D); and DT-reduced AurF treated with a slight excess of O_2 from an O_2-saturated buffer solution (G, H). Spectra were collected in external fields of 53 mT (A−D), 5 T (E, G), or 8 T (F, H). The solid lines in A−C are simulations with parameters quoted in the text. The solid line in D is the experimental spectrum from C replotted to illustrate the spectral changes. The solid lines in E−H are spin Hamiltonian simulations with parameters in the text, asymmetry parameters $\eta = -0.4$ (orange), $\eta = 1$ (green), $\eta = 1.4$ (red), $\eta = 0.3$ (blue), and the assumption of $S = 0$ ground states for all species.

The combined absorption and Mössbauer spectroscopic results thus establish that addition of O_2 to the $Fe_2^{II/II}$ cluster of AurF produces a long-lived state that has properties consistent with a peroxo-$Fe_2^{III/III}$ formulation. The stopped-flow data show that the intermediate state reacts rapidly with pABA, implying that it is competent for at least the first oxidation in the three step AurF sequence. The possibility that it is competent for *all three* oxidations seems likely (on the basis of the efficient conversion of limiting pABA to pNBA) but requires more rigorous evaluation.

The spectroscopic properties of the AurF state are different from those of peroxo-$Fe_2^{III/III}$ complexes detected in related diiron oxidases and oxygenases such as ribonucleotide reductase subunit R2,[16] stearoyl acyl carrier protein Δ^9 desaturase,[17] and soluble methane monooxygenase hydroxylase (sMMOH).[18] The intermediates in these proteins have μ-1,2-peroxo[19] (or perhaps μ-(η^2,η^2)-peroxo, for the case of sMMOH[20]) diiron cores. The properties of the AurF complex are more similar to those of a peroxo-$Fe_2^{III/III}$ intermediate recently detected in toluene/o-xylene monooxygenase (ToMO).[21] In this enzyme, the peroxide complex is thought to be attacked as an electrophile by the π-system of the substrate. A similar mechanism for the first step in

the AurF reaction, involving nucleophilic attack of the lone pair of the neutral arylamine on the peroxo electrophile (Scheme 1), seems plausible. An attractive possibility is that the peroxo complexes in ToMO and AurF differ structurally from the canonical μ-1,2-peroxides in ways that activate them as electrophiles. Protonation of the peroxide unit, perhaps with rearrangement to a μ-1,1-bridging geometry, seems likely to have this effect.

The remarkable stability of the AurF intermediate, which permits its preparation in concentration, purity, and physical form (e.g., a transmitting glass) appropriate for most spectroscopic methods, should be a considerable asset in ongoing efforts to define the structure of the complex by a combination of experiments and density functional theory calculations.

Scheme 1. Proposed Mechanism for Hydroxylation of pABA by a Putative μ-1,2 (left) or μ-1,1 (right) Hydroperoxo-$Fe_2^{III/III}$ Intermediate
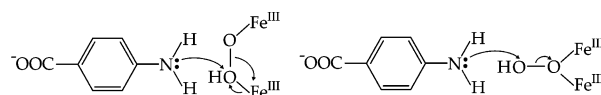
Supporting Information Available: Procedures to produce AurF and quantify pABA and pNBA in reaction samples; stopped-flow absorption and Mössbauer spectra of samples from the reaction of $Fe_2^{II/II}$-AurF with O_2 in the absence of substrate; Mössbauer analysis of the sample used for high-field studies of the peroxo-$Fe_2^{III/III}$ state. This material is available free of charge via the Internet at http://pubs.acs.org.

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(10) An alternative solution of similar quality yields: $\delta_1 = 0.50$ mm/s, $\Delta E_{Q,1} = 0.59$ mm/s (49%) and $\delta_2 = 0.66$ mm/s, $\Delta E_{Q,2} = 0.45$ mm/s (33%); both solutions indicate that the iron sites are high-spin Fe(III).
(11) The preparation and analysis of the 4.2-K/53-mT Mössbauer spectrum of this sample is described in the Supporting Information.
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(15) Experiments were performed both by addition of substrate to intermediate and by oxygenation of reduced enzyme in the presence of pABA. The results were not significantly different.
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