AurF from *Streptomyces thioluteus* and a Possible New Family of Manganese/Iron Oxygenases†

Carsten Krebs,*†§ Megan L. Matthews,† Wei Jiang,§ and J. Martin Bollinger, Jr.*†§

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

Received May 31, 2007; Revised Manuscript Received June 27, 2007

ABSTRACT: We recently reported that the R2 subunit of class Ic ribonucleotide reductase from *Chlamydia trachomatis* contains a heterodinuclear Mn/Fe redox cofactor [Jiang, W., Yun, D., Saleh, L., Barr, E. W., Xing, G., Hoffart, L. M., Maslak, M.-A., Krebs, C., and Bollinger, J. M., Jr. (2007) Science 316, 1188—1191]. The *N*-oxygenase, AurF, from *Streptomyces thioluteus* catalyzes the six-electron oxidation of *p*-aminobenzoate to *p*-nitrobenzoate and contains the EX5HX60—186EX3H sequence motif previously used to identify proteins with non-heme diiron clusters. Two research groups independently obtained evidence for the presence of iron and manganese in preparations of AurF. The electron paramagnetic resonance (EPR) spectrum of purified, resting AurF presented in one of these studies is markedly similar to the spectrum of the MnII/FeIII form of *C. trachomatis* R2. We propose that *S. thioluteus* AurF also may harbor a heterodinuclear Mn/Fe cofactor, which it may use to activate O2 for oxidation of the aryl amine to the nitro compound. Hypothetical proteins encoded in the genomes of several other bacteria have similar sequences and may also be members of this nascent family of oxygen-activating Mn/Fe proteins.

Carboxylate-bridged diiron clusters are employed as enzyme cofactors for a wide variety of oxidative transformations. For example, the FeII clusters in the hydroxylase components of bacterial multicompartment monooxygenases (e.g., soluble methane monooxygenase or sMMO)† activate O2 for hydroxylation of unactivated carbon centers (*I, 2*), and the structurally similar cofactors in plant fatty acyl desaturases (e.g., stearoyl acyl carrier protein Δ9 desaturase (3)) activate O2 for dehydrogenation reactions. A particularly well-studied member of this group of proteins is the sMMO hydroxylase (sMMOH), which forms the FeII(μ-O22−) and FeIIIV(μ-O2−)2 intermediates, P and Q (1, 2) (Scheme 1F), during its reaction with O2. Q initiates hydroxylation of methane by abstracting hydrogen (2). Subsequent or concomitant transfer of a hydroxyl radical equivalent from the diiron center to the methyl group completes the hydroxylation and generates a stable FeIII/III form of the cofactor. The catalytic cycle is completed by reduction of the cofactor by the enzyme’s reductase component (sMMOR) with two electrons harvested from NADH.

The R2 subunit of a conventional class I ribonucleotide reductase (RNR) (e.g., from *Escherichia coli*) becomes catalytically active when its carboxylate-bridged FeII cluster activates O2 to oxidize a nearby tyrosine residue by one electron to a stable tyrosyl radical (4). An extensively characterized FeIIIV intermediate, X (5, 6), oxidizes the tyrosine as it converts to a stable FeIIIV(μ-O2−) cluster (Scheme 1C) (5). In the RNR catalytic reaction, the tyrosyl radical in R2 oxidizes a cysteine residue in the R1 subunit by long-distance (~35 Å), inter-subunit, proton-coupled electron transfer (PCET) (7, 8). This step produces a cysteine thyl radical, which initiates reduction of the nucleotide by abstracting hydrogen from C3′ (7).

We recently demonstrated that the class Ic RNR from *Chlamydia trachomatis* employs a different strategy to generate the cysteine thyl radical in its R1 subunit. Its R2 subunit harbors a stable, heterodinuclear MnIV/FeIII cofactor (9), which has an S = 1 ground state as a consequence of antiferromagnetic (AF) coupling between the MnIV (S = 3/2) and high-spin FeIII (SFe = 5/2) sites (10). This cluster can generate the cysteine thyl radical on the R1 subunit as the cluster is reduced to the MnIII/FeIII state (9). The MnIII/FeIII cluster exhibits an S = 1/2 ground state arising from AF coupling between the MnIII (SMn = 2) and high-spin FeIII (SFe = 5/2) sites. In the presence of the R1 subunit, cystidine 5′-diphosphate (CDP), and adenosine 5′-triphosphate (ATP), the MnIII/FeIII cluster exhibits a sharp electron paramagnetic resonance (EPR) spectrum centered at g = 2 with six groups of resonances arising from coupling to one Mn nucleus
(100% $^{55}$Mn with nuclear spin quantum number $I = 5/2$) (Figure 1). The hyperfine structure of the spectrum, in particular, on the first, fifth, and sixth groups of resonances, is a consequence of the anisotropy of the $A_{\text{Mn}}$ tensor and (to a lesser extent) the $g$ tensor. Spectral simulations allowed these parameters to be determined and revealed pronounced anisotropy of $A_{\text{Mn}}$ [(269, 314, 392) MHz] (9). The observed anisotropy is consistent with the assignment as Mn III ($S_{\text{Mn}}$) (11, 12). The heterodinuclear nature of the cofactor was verified by the observation of hyperfine coupling to $^{57}$Fe ($I = 1/2$) in the EPR spectra (9). Mössbauer experiments revealed that the Fe site is in the high-spin Fe III state (9).

The novel cofactor in $C.\,\text{trachomatis}$ R2 is generated by reaction of the Mn II/Fe II -R2 complex with O$_2$ (9). During this reaction, a Mn IV/Fe IV intermediate accumulates almost quantitatively (Scheme 1D) and decays by reduction of the Fe IV site (13). On the basis of the X-ray crystallographic results on the Fe$_2$II/III form of the $C.\,\text{trachomatis}$ R2 protein, which revealed the presence of two bridging oxygenic ligands (putatively, two hydroxides) (14), we proposed that the Mn IV/Fe IV intermediate has a $\text{bis-(\mu-oxo)}$ “diamond core” structure, as was also (and originally) proposed for Q in sMMOH (15). The demonstration of a possible heterodinuclear (Mn/Fe) homologue of Q raised the possibility that, similar to the oxidative versatility of the diiron proteins, Mn/Fe enzymes that carry out oxygenase reactions (as opposed to the oxidase reaction in R2) might exist. Indeed, observations published by Hertweck and co-workers (16) and Zhao and co-workers (17) suggest that the N-oxygenase, AurF, from $S.\,\text{thioluteus}$ may provide the first example of such a Mn/Fe-dependent oxygenase.

Aureothin (Scheme 1A) is a metabolite from $S.\,\text{thioluteus}$ (18) with antifungal, antitumoral, and insecticidal activities (19). Its $p$-nitrophenyl group is derived from $p$-nitrobenzoate (PNB) (20), which is produced via oxidation of $p$-aminobenzoate (PAB) by AurF. Both O-atoms incorporated into the nitro group originate from O$_2$ (Scheme 1B) (21). It was proposed that three successive two-electron oxidations produce $p$-hydroxylaminobenzoate, $p$-nitrosobenzoate, and finally the PNB product (17, 21, 22). The hydroxylamine intermediate was demonstrated, establishing that the first reaction is an $N$-oxygenation (22). $^{18}$O$_2$ labeling studies of the conversion of this intermediate to PNB showed that the second reaction is formally a dehydrogenation reaction, presumably yielding the not-yet-detected $p$-nitrosobenzoate intermediate (17). AurF catalyzes oxidation of a variety of other para-substituted anilines to the corresponding $p$-
medium and noted that it exhibits an EPR signal associated with manganese (17). Specifically, they noted that the spectrum resembled those of “a mononuclear MnIV species” (27) and *E. coli* “ribonucleotide reductase enzyme containing a FeIII—MnIII center” (28). They reasoned that Mn was taken up in lieu of Fe and tested the idea by producing AurF in *E. coli* grown in minimal medium supplemented with Mn. The resulting AurF was found to contain 2 Mn per protein but to lack enzymatic activity in vivo.

The EPR spectrum of “oxidized AurF−” grown on LB medium (Figure 1A of ref (17)) is similar to that of the *C. trachomatis* MnIII/FeIII-R2-R1-CDP-ATP complex (9) (Figure 1).2 The overall splitting of the signals of the putative MnIII/FeIII-AurF is smaller, implying that the absolute magnitude of $A_{Mn}$ is less. However, a similar hyperfine pattern is observed (indicated by dotted lines), revealing $A_{Mn}$ to be anisotropic also in the putative MnIII/FeIII-AurF. Our estimates for the $A_{Mn}$ and $g$-tensors obtained by simulation of the published spectrum [$A_{Mn} = (210, 270, 322)$ MHz; $g = (2.030, 2.014, 2.015)^{2}$] are consistent with the hypothesis that the MnIII site is AF-coupled to a high-spin FeIII site, yielding the $S = 1/2$ ground state. Although the magnitude of $A_{Mn}$ of AurF is less than that of $A_{Mn}$ of the *C. trachomatis* MnIII/FeIII-R2-R1-CDP-ATP complex, it is very similar to $A_{Mn}$ of the MnIII site of the MnIIIV cluster of catalase (11), after correcting for the different spin coupling coefficients of the two systems using standard methods (29).4 This analysis yields the following intrinsic hyperfine tensors ($a_{Mn}$): $A_{Mn} = (-158, -205, -213)$ MHz for catalase and $a_{Mn} = (-158, -203, -242)$ MHz for AurF.

We thus propose that AurF harbors a heterodinuclear Mn/Fe cluster. This proposal allows the seemingly contradictory observations by the Zhao and Hertweck groups to be reconciled. The hypothesis is supported by the following facts: (i) the EPR spectrum of “oxidized AurF−” grown on LB medium is similar to that of the MnIII/FeIII-R2-R1-CDP-ATP complex of *C. trachomatis* RNR (9); (ii) both groups provided evidence for the presence of iron and manganese in their preparations of AurF (16, 17); and (iii) expression of AurF under (presumably) Fe-limited conditions results in inactive dimanganese enzyme (17).

A plausible mechanism for the AurF-catalyzed reaction(s) is shown in Scheme 1E. Activation of O$_2$ at the MnIII/FeIII complex could form a MnIV/FeIV intermediate similar to that detected in the *C. trachomatis* R2 activation reaction (Scheme 1D) (9, 13). The MnIV/FeIV intermediate could oxidize the substrate by two electrons (either as O$_2$-oxygenation or dehydrogenation), resulting in the resting MnIII/FeIII state, which, we propose, is the state that gives rise to the EPR spectrum.

---

2 The spectrum reported for the oxidized form of *S. thiolus* AurF grown on LB medium was recorded with a microwave frequency (9.06 GHz) different than for the spectrum of the *C. trachomatis* MnIII/FeIII, R2-R1-CDP-ATP complex (9.45 GHz). Therefore, the spectra are plotted with $g$ as the abscissa.

3 To account for the shape of the EPR spectrum of AurF, we assumed an anisotropic line width of (30, 30, 20) G. Simulations with isotropic line width could reproduce the positions of the peaks but could not match their shapes as well.

4 The MnIII sites of the proposed MnIII/FeIII cluster of AurF and of the MnIVIII cluster of catalase have spin coupling coefficients of $-4/3$ and $-2$ for the $S_{rad} = 1/2$ states, respectively, in the strong exchange coupling limit ($J > D$).

5 The EPR spectrum of the proposed MnIII/FeIII cluster might well be perturbed by the binding of substrate or product, as seen for the case of *C. trachomatis* R2.
signal observed by Zhao and co-workers. By analogy to the bacterial multicomponent monooxygenases, the resting state could then be reduced to the MnII/FeII form by an NAD(P)H-dependent reductase component. Interestingly, hydrogen peroxide was shown to support turnover of the aforementioned MBP-AurF fusion protein when it was immobilized on chromatography resin (16). Oxidation of the MnIII/FeIII cluster by H2O2 could directly generate the active MnIV/FeIV intermediate, circumventing the reduction step. This so-called "peroxide shunt" was also previously demonstrated for sMMO (30). Our proposed mechanism incorporates the most important feature of the Hertweck hypothesis—the presence of high-valent Mn in the key oxidant (16)—but contrasts with the Zhao hypothesis that AurF is a diiron protein, which would be expected to employ a mechanism similar to the one shown in Scheme 1F (17).

Sequence database searches revealed a number of hypothetical proteins with similarity to S. thioluteus AurF. None of these proteins has been assigned a function. An alignment of a subset of these sequences reveals several conserved amino acids (Figure 2). They include the two EX28-33DE motifs (23) (bold faced residues are strictly conserved) previously recognized by Zhao and co-workers (17). As in the diiron-carboxylate proteins, the glutamate and histidine residues should be ligands to the dinuclear cluster, whereas...
the aspartate residue preceding each coordinating EX$_2$H should hydrogen bond with the uncoordinated nitrogen atom of the histidine ligand contributed by the opposite EX$_2$H motif (14, 31).

In conclusion, we propose that AurF from *S. thioluteus* and the hypothetical proteins with related sequences may be Mn/Fe-dependent oxygenases. Testing this hypothesis and the hypothetical proteins with related sequences may add Mn/Fe-dependent oxygenases. Hertweck and Schulz suggested that the “extra” His ligand is conserved among all the expression conditions employed in their study, the authors present in the crystals also contained Mn$_{III}$/Fe$_{III}$ cluster with S = 1 ground state, *J. Am. Chem. Soc.* 129, 7504–7505.

**REFERENCES**


