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

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

ABSTRACT: We recently reported that the R2 subunit of class I*c* 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 $EX_2HX_{60-180}EX_2H$ 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 Mn^{III}/Fe^{III} 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 O_2 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 $Fe_2^{II/II}$ clusters in the hydroxylase components of bacterial multicomponent monooxygenases (e.g., soluble methane monooxygenase or sMMO[1]) activate O_2 for hydroxylation of unactivated carbon centers (*1*, *2*), and the structurally similar cofactors in plant fatty acyl desaturases (e.g., stearoyl acyl carrier protein Δ^9 desaturase (*3*)) activate O_2 for dehydrogenation reactions. A particularly well-studied member of this group of proteins is the sMMO hydroxylase (sMMOH), which forms the $Fe_2^{III/III}(\mu\text{-}O_2^{2-})$ and $Fe_2^{IV/IV}(\mu\text{-}O^{2-})_2$ intermediates, **P** and **Q** (*1*, *2*) (Scheme 1F), during its reaction with O_2. **Q** initiates hydroxylation of methane by abstracting hydrogen (*1*, *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 $Fe_2^{III/III}$ 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 $Fe_2^{II/II}$ cluster activates O_2 to oxidize a nearby tyrosine residue by one electron to a stable tyrosyl radical (*4*). An extensively characterized $Fe_2^{III/IV}$ intermediate, **X** (*5*, *6*), oxidizes the tyrosine as it converts to a stable $Fe_2^{III/III}(\mu\text{-}O^{2-})$ 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 thiyl radical, which initiates reduction of the nucleotide by abstracting hydrogen from C-3′ (*7*).

We recently demonstrated that the class I*c* RNR from *Chlamydia trachomatis* employs a different strategy to generate the cysteine thiyl radical in its R1 subunit. Its R2 subunit harbors a stable, heterodinuclear Mn^{IV}/Fe^{III} cofactor (*9*), which has an $S = 1$ ground state as a consequence of antiferromagnetic (AF) coupling between the Mn^{IV} ($S_{Mn} = 3/2$) and high-spin Fe^{III} ($S_{Fe} = 5/2$) sites (*10*). This cluster can generate the cysteine thiyl radical on the R1 subunit as the cluster is reduced to the Mn^{III}/Fe^{III} state (*9*). The Mn^{III}/Fe^{III} cluster exhibits an $S = 1/2$ ground state arising from AF coupling between the Mn^{III} ($S_{Mn} = 2$) and high-spin Fe^{III} ($S_{Fe} = 5/2$) sites. In the presence of the R1 subunit, cytidine 5′-diphosphate (CDP), and adenosine 5′-triphosphate (ATP), the Mn^{III}/Fe^{III} 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

† This work was supported by the National Institutes of Health (GM-55365 to J.M.B.), the Beckman Foundation (Young Investigator Award to C.K.), and the Dreyfus Foundation (Teacher Scholar Award to C.K.).

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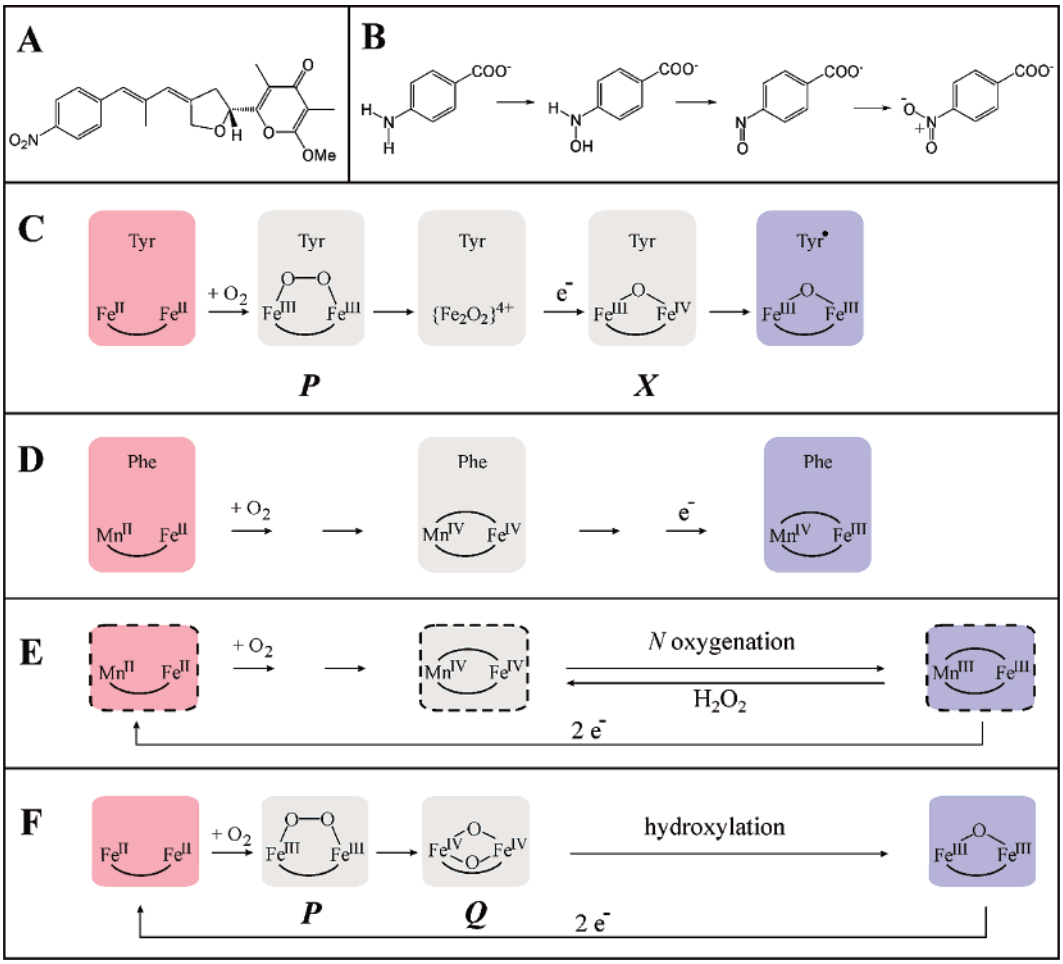
[1] Abbreviations: PAB, *p*-aminobenzoate; PNB, *p*-nitrobenzoate; EPR, electron paramagnetic resonance; PCET, proton-coupled electron transfer; RNR, ribonucleotide reductase; sMMO, soluble methane monooxygenase; sMMOH, the hydroxylase component of sMMO; sMMOR, the reductase component of sMMO; ACP: acyl carrier protein; AF: antiferromagnetic; CDP: cytidine 5′-diphosphate; ATP: adenosine 5′-triphosphate; MBP: maltose binding protein; LB: Luria Bertani.

10.1021/bi701060g CCC: $37.00
Published on Web 08/24/2007

Scheme 1: Structure of Aureothin (**A**), the Conversion of PAB to PNB Catalyzed by AurF (**B**), and Comparison of the Proposed Mechanisms of Oxygen Activation by *E. coli* R2 (**C**), *C. trachomatis* R2 (**D**), *S. thioluteus* AurF (**E**), and of sMMO (**F**)[a]



[a]The reactant complexes are shaded in red, the stable product states are in blue, and reaction intermediates are in gray. The states shown in (**C**), (**D**), and (**F**) have been experimentally characterized. The states in (**E**) are hypothetical.

(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 $\mathbf{A}_{Mn}$ tensor and (to a lesser extent) the **g** tensor. Spectral simulations allowed these parameters to be determined and revealed pronounced anisotropy of $\mathbf{A}_{Mn}$ [(269, 314, 392) MHz] (*9*). The observed anisotropy is consistent with the assignment as Mn^{III} ($S_{Mn} = 2$) (*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. 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 $Fe_2^{III/III}$ form of the *C. 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 *bis*-(*μ*-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. thioluteus* may provide the first example of such a Mn/Fe-dependent oxygenase.

Aureothin (Scheme 1A) is a metabolite from *S. 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*-

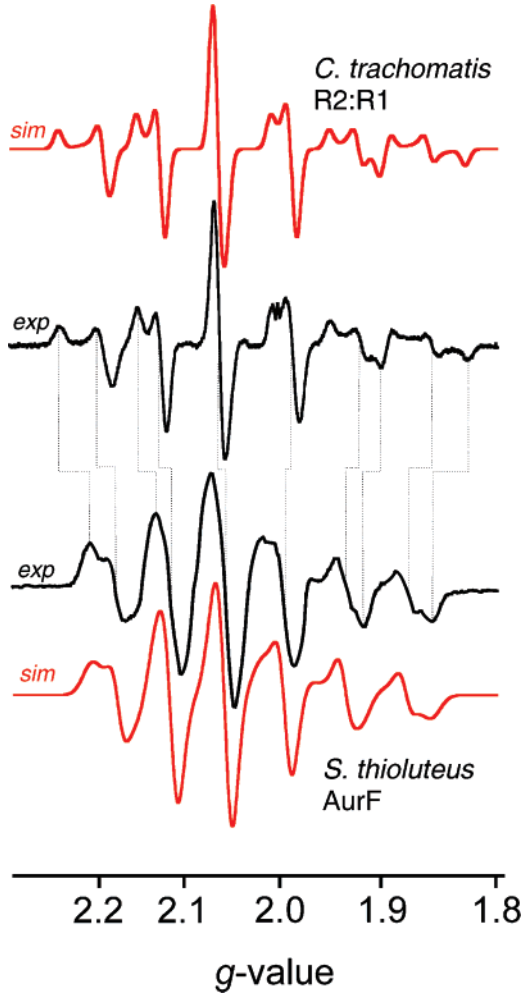


FIGURE 1: Comparison of the X-band EPR spectra of the Mn^{III}/Fe^{III} cluster of the *C. trachomatis* Mn^{III}/Fe^{III}-R2·R1·CDP·ATP complex (top) (*9*) and the oxidized form of *S. thioluteus* AurF (bottom, reproduced from ref 17 with permission of the publisher, Copyright 2000 Wiley-VCH). The corresponding peak positions are indicated by dotted lines. Experimental spectra are shown in black, and simulations according to the parameters quoted in the text are shown in red.

nitrophenyl compounds (*16*, *21*), making it potentially useful in biotechnology.

AurF is a metalloenzyme, but the nature of the cofactor is presently not known. Hertweck and co-workers noted that a maltose binding protein (MBP)−AurF fusion protein, which was shown to exhibit *N*-oxygenase activity *in vivo* and *in vitro*, contains significant amounts of manganese and iron in a ~20:1 ratio (*16*). The authors suggested that "AurF employs manganese as an oxygen-activating cofactor" and that a "Mn-oxo species" may be the oxygenating intermediate (*16*). By contrast, Zhao and co-workers reported that AurF is a carboxylate-bridged diiron enzyme (*17*). This assessment was based on the fact that the protein has the $EX_2HX_{60-180}EX_2H$ sequence hallmark of the diiron-carboxylate proteins (*23*, *24*) and their observation of an EPR signal with *g*-values (1.94, 1.79, and 1.70) similar to those of AF-coupled $Fe_2^{II/III}$ clusters observed in other carboxylate-bridged $Fe_2^{II/III}$ proteins (*25*, *26*) upon dithionite reduction of recombinant AurF isolated from *E. coli* grown in Fe-supplemented minimal medium. Interestingly, Zhao and co-workers also reported the EPR spectrum of oxidized AurF grown in Luria Bertani (LB) medium and noted that it exhibits an EPR signal associated with manganese (*17*). Specifically, they noted that the spectrum resembled those of "a mononuclear Mn^{IV} species" (*27*) and *E. coli* "ribonucleotide reductase enzyme containing a $Fe^{III}-Mn^{III}$ 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* Mn^{III}/Fe^{III}-R2·R1·CDP·ATP complex (*9*) (Figure 1).[2] The overall splitting of the signals of the putative Mn^{III}/Fe^{III}-AurF is smaller, implying that the absolute magnitude of $\mathbf{A}_{Mn}$ is less. However, a similar hyperfine pattern is observed (indicated by dotted lines), revealing $\mathbf{A}_{Mn}$ to be anisotropic also in the putative Mn^{III}/Fe^{III}-AurF. Our estimates for the $\mathbf{A}_{Mn}$- and **g**-tensors obtained by simulation of the published spectrum [$\mathbf{A}_{Mn}$ = (210, 270, 322) MHz; **g** = (2.030, 2.014, 2.015)[3]] are consistent with the hypothesis that the Mn^{III} site is AF-coupled to a high-spin Fe^{III} site, yielding the $S = 1/2$ ground state. Although the magnitude of $\mathbf{A}_{Mn}$ of AurF is less than that of $\mathbf{A}_{Mn}$ of the *C. trachomatis* Mn^{III}/Fe^{III}-R2·R1·CDP·ATP complex, it is very similar to $\mathbf{A}_{Mn}$ of the Mn^{III} site of the $Mn_2^{III/IV}$ 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 ($\mathbf{a}_{Mn}$): $\mathbf{a}_{Mn}$ = (−158, −205, −213) MHz for catalase and $\mathbf{a}_{Mn}$ = (−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 Mn^{III}/Fe^{III}-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 Mn^{II}/Fe^{II} complex could form a Mn^{IV}/Fe^{IV} intermediate similar to that detected in the *C. trachomatis* R2 activation reaction (Scheme 1D) (*9*, *13*). The Mn^{IV}/Fe^{IV} intermediate could oxidize the substrate by two electrons (either as *N*-oxygenation or dehydrogenation), resulting in the resting Mn^{III}/Fe^{III} state, which, we propose, is the state that gives rise to the EPR

[2] The spectrum reported for the oxidized form of *S. thioluteus* AurF grown on LB medium was recorded with a microwave frequency (9.06 GHz) different than for the spectrum of the *C. trachomatis* Mn^{III}/Fe^{III}-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 Mn^{III} sites of the proposed Mn^{III}/Fe^{III} cluster of AurF and of the $Mn_2^{III/IV}$ cluster of catalase have spin coupling coefficients of −4/3 and +2 for the $S_{Total} = 1/2$ states, respectively, in the strong exchange coupling limit ($J \gg D$).

[5] The EPR spectrum of the proposed Mn^{III}/Fe^{III} cluster might well be perturbed by the binding of substrate or product, as seen for the case of *C. trachomatis* R2.

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gi|41322768      ---------- -----MREEQ PHLATTWAAR GWVEEEGIGS ATLGRLVRAW PRRAAVVN-- -KADILDEWA  52
gi|111019967     ---------M IIDDLQCSDA DGLVHLPGLP SFDPADEAEN AVISRLAGNW HRRSTVK--- --RDEPDLDE  56
gi|111023067     ---------- ---------- MTPQKTPSLP EYDPSDPVES AVVSRLARNW GQRATVK--- --KPEPDLDA  45
gi|92114812      ---------- ---------- ----MYTATD PFAPARESE- -ILARLTDNW NQRVTVR--- --KARLDLSD  39
gi|108762941     MRRQTPPARL QTRNDSPLQL KADDGALVTQ PLSGDSSAAR RFLTRLGPHW ARQAAVK--- --NKEPSLTE  65
gi|118733227     ---------- ---------- -------MNL DISRTDDAVR DALKKLSLLW KSRAAVN--- -QDLPDYCGL  39
gi|84353437      ---------- ---------- ---------- ------MTTA VTYVSHVDRW EARATVR--- ----SRPRRI  27
gi|77461577      ---------- ---------- ---------- ---MSALAPK PVFQFTLSDW NTRASVRTSP HDYHLPDNVQ  37
                                                                          *      *

gi|41322768   53 DYDTLVPDYP LEIVPFAEHP LFLAAEPHQR QRVLTGMWIG YNERVIATEQ LIAEPAFDLV MHGVFPGSDD 122
gi|111019967  57 LFDAGRADYP EGIVPFRDHP TWQAMPDAMR SRLLSWAWIA YNRHTVIAEQ RVANPAFALV MDGEFPGLGG 126
gi|111023067  46 LFDLDKQDYP NDLLPFADHD RFLGMREEQR NQLRAWAWIA FNKNVMDIEQ YVVNPGFDLV AHDALDTGLG 115
gi|92114812   40 YYDTSAPDFP PSMVPFWDDP RFQAIDEAEK RRVLAGAWIN YNEKTIFVED KVINPLCSLL MKGALPGVDD 109
gi|108762941  66 LLEPAKPDFA ERLLPFRDHP TYQGLDEAMK RRVLSCGWLI YNERVVRVEL DVVNPVCNDV LLSKLPGATS 135
gi|118733227  40 AFDPARQDFT ASLLPFRDHQ AWVEAPQHLR DQCLSYAWGI YNLKTIYIEC NVVTPACEDI IKTPPPSANR 109
gi|84353437   28 VEDDELRYYP LERQPLCAHP AIVDAGDAVR DFVLLQSFYK YIQDVIIFET EIVNATALRI ARGRFAHPFP  97
gi|77461577   38 EQLETRHWFP PAFLPYLAHP SIEAAGRSML HRLTARHLVH FLDYTTLLEH RIVNRAVETI VHGELPVAVP 107
                              *                                     *

gi|41322768  123 PLIRKSVQQA IVDESFHTYM HMLAIDRTRE LR-KISERPP QPELVTYRRL RRVLADMPEQ WERDIAVLVW 191
gi|111019967 127 QDMDIALAQA MVDEQYHTLM HINASALTRR KRGNPFPDSA LPESHTSRTH RRLRAHAAER WQRSLTTLAF 196
gi|111023067 116 DTFAVAVHQA MVDEQYHTLM HLNASAVTRR QRGWAMPNNA LPDVLTLRRQ RAATADAADP RKRAITTFAF 185
gi|92114812  110 TVSKKAIAQT QVDEQFHILM CLEVCDCARR QHR--LQDLH IPTPLLGQRM DASLAKAENE REYALIRMAY 177
gi|108762941 136 QAARESMAQA LVDEAYHILL VVRACRLTRE QRG--LEDLR LPVVGVVRRM HARQASCAEA WQRDLVQLMT 203
gi|118733227 110 ALLQDVMSQA LLDEALHTRM SIMACNYIYE MRG--LAPLD YADFNLVGWR NGVLAQCGAE WERRLARFAI 177
gi|84353437   98 FACRQDAMTV VIDEDYHAYV AMDYLRQVEE ATG--IAPLP PNSEIELSRA IPRAVERVGA QYRDGMELLC 165
gi|77461577  108 APMKTAALQL YTDEGYHALF SSQVAEQIAS ----LYAIAG RPVMPRRITR MNLLIARTGQ EQRPLACFLL 173
                            **  *

gi|41322768  192 GAVAETCINA LLALLARDAT IQPMHSLITT LHLRDETAHG SIVVEVVREL YARMNEQQRR ALVRCLPIAL 261
gi|111019967 197 ATVSEISINA YLDLLADDHD IQVVNSTTAK LHNRDEYCHA SISDEMAKLV YDVLDPVKRR FFLDMLVAGL 266
gi|111023067 186 MTVAEISISS YLDLISENDV IQPVNRATVR LHNRDEYCHA SIADDIAGVV YDTLSPHDRR HFLDGLVDAM 255
gi|92114812  178 ATVAEMTINV FLKRLSQDRS IQPLNRLNTE LHRQDEASHA AIFGEIARAV YERLGAGQQA IFRAHLLRAI 247
gi|108762941 204 GVATEMCISR YLSLLSTASE IQAFNRVTTA LHQQDEASHV DLFGTLARDV FNALEPVQQE FVREILPLPW 273
gi|118733227 178 ACASETLITD YLKTMAEDRS IQPICHEVTR THAVDEWSHS SVFSSVATDI VQGLSNRERS YLRSIILKTV 247
gi|84353437  166 VAISENTVTA EVAAFSRDAT LKRSVKGVMA DHLADEGRHS AFWINLVKLY WSEIDEPARL ALGEGLPCFL 235
gi|77461577  174 GFVSETIIAR ELLDVCRDSL VSGVN-DMLR DHLTDEARHS RYFTEVFHYL WLHLNPRQRT FTATTLLDIL 242
                     *                                  *  **  *

gi|41322768  262 EAFAEQDLSA LLLELNAAGI RGAEEIVGDL RSTAGGTRLV RDFSGARKMV EQLGLDDAVD FDFPERPDWS 331
gi|111019967 267 DAFVATDYST WEAIFRIEKV TGWEKMLADV RAEKSGARLV RDYSGLYSLM SDMNVLGDVD FDWGLAVTGK 336
gi|111023067 256 TAFSSNDYST WHTIVDILDL DGGQAMIDDA EHDTRRSALV QDFSGIHKLC KHLGVAGEMS FEWA------ 319
gi|92114812  248 DDFVELDVGF WRVILDHLEI PERHGILQDM TAKSRASRSL RDYGALVKLL RRIGIDEGPS FIFQ------ 311
gi|108762941 274 LWFSEGEADV WRSVLLQLGI PRADVMMDDC IANKLLSANE RAMTDAQKFS EALGIDNIHW DRAAALL--- 340
gi|118733227 248 QMFANNELGA WSTVFTMVGM PHARDILHDV GDSNEIGVYT DSVQSLIDRI GLAGRNLGGS VALVSSVLEE 317
gi|84353437  236 REYLSADLQL QFDRRLIDAL DLPAAERARI ADDMVGAYPI TSQHPMIVNI RQFFRMSGLL DHAPTRAALS 305
gi|77461577  243 EIFFDVDEHW LQESLRGAGI ANTTVREILG TMTTAQARRQ RARAGSLATL SALKKAGFFT EPQNQTLFAR 312


gi|41322768  332 PHTPR- 336
gi|111019967     ------
gi|111023067     ------
gi|92114812      ------
gi|108762941     ------
gi|118733227 318 MQA--- 320
gi|84353437  306 DYL--- 308
gi|77461577  313 AGLIDG 318
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FIGURE 2: CLUSTALW alignment of the amino acid sequences of *S. thioluteus* AurF (gi|41322768) and hypothetical proteins from *Rhodococcus* sp. RHA1 (gi|111019967 and gi|111023067), *Chromohalobacter salexigens* DSM 3043 (gi|92114812), *Myxococcus xanthus* DK 1622 (gi|108762941), *Delftia acidovorans* SPH-1 (gi|118733227), *Burkholderia cenocepacia* PC184 (gi|84353437), and *Pseudomonas fluorescens* PfO-1 (gi|77461577). Conserved residues are shown in boldface. The proposed ligands to the Mn/Fe center are shown in red. Presumed important second-sphere residues are shown in blue.

signal observed by Zhao and co-workers.[5] By analogy to the bacterial multicomponent monooxygenases, the resting state could then be reduced to the Mn^{II}/Fe^{II} 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 Mn^{III}/Fe^{III} cluster by H_2O_2 could directly generate the active Mn^{IV}/Fe^{IV} 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.[6] 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 $\mathbf{E}X_{28-33}\mathbf{DE}X_2\mathbf{H}$ 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

[6] The identified sequences were deposited after the studies by the Hertweck and Zhao groups (*17, 20*).

the aspartate residue preceding each coordinating EX_2H should hydrogen bond with the uncoordinated nitrogen atom of the histidine ligand contributed by the opposite EX_2H 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 should be straightforward. As for the case of *C. trachomatis* R2, neither Fe^{II} nor Mn^{II} should activate preparations of the metal-free (apo) enzymes when added individually, but addition of the two together should yield marked activation.

MATERIALS AND METHODS

Analysis of the EPR Spectrum. The published EPR spectrum of AurF was simulated with the program SimFonia from Bruker (Billerica, MA) according to the second-order-perturbation method.

Sequence Analysis. The amino acid sequence of AurF was used to query the database at NCBI (www.ncbi.nlm.nih.gov) with their BLAST v2.0 program. The results were aligned with CLUSTALW (www.ch.embnet.org/software/ClustalW.html).

NOTE ADDED IN PROOF

While this manuscript was under review, the three-dimensional structure of AurF was reported by Schulz, Hertweck, and co-workers, who concluded from their data that the enzyme is "a di-manganese monooxygenase" (*32*). Anomalous diffraction experiments suggested that the AurF present in the crystals also contained ~ 15% iron. Although their data would suggest that AurF selects manganese in slight preference to iron under the growth and over-expression conditions employed in their study, the authors noted that "it remains conceivable...that the observed activity is caused by the low iron content of AurF," consistent with our hypothesis that the enzyme uses a heterodinuclear cofactor. Interestingly, the conserved histidine residue that precedes the second EX_2H motif (His223 in AurF) provides a ligand to metal site 1 that is not present in other known di-iron oxidases and oxygenases. Sequence alignments presented above and in the Schulz and Hertweck study show that the "extra" His ligand is conserved among all the sequences tentatively assigned here as manganese- and iron-dependent oxygenases. Hertweck and Schulz suggested that this extra His ligand confers specificty for Mn; thus, we would suggest that metal site 1 binds manganese, as we have also proposed for the manganese- and iron-dependent ribonucleotide reductase R2 subunit from *C. trachomatis* (*9*).

REFERENCES

1. Wallar, B. J., and Lipscomb, J. D. (1996) Dioxygen activation by enzymes containing binuclear non-heme iron clusters, *Chem. Rev. 96*, 2625−2657.
2. Merkx, M., Kopp, D. A., Sazinsky, M. H., Blazyk, J. L., Müller, J., and Lippard, S. J. (2001) Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins, *Angew. Chem., Int. Ed. 40*, 2782−2807.
3. Fox, B. G., Lyle, K. S., and Rogge, C. E. (2004) Reactions of the diiron enzyme stearoyl-acyl carrier protein desaturase, *Acc. Chem. Res. 37*, 421−429.
4. Stubbe, J. (2003) Di-iron-tyrosyl radical ribonucleotide reductases, *Curr. Opin. Chem. Biol. 7*, 183−188.
5. Bollinger, J. M., Jr., Edmondson, D. E., Huynh, B. H., Filley, J., Norton, J. R., and Stubbe, J. (1991) Mechanism of assembly of the tyrosyl radical-dinuclear iron cluster cofactor of ribonucleotide reductase, *Science 253*, 292−298.
6. Sturgeon, B. E., Burdi, D., Chen, S., Huynh, B. H., Edmondson, D. E., Stubbe, J., and Hoffman, B. M. (1996) Reconsideration of **X**, the diiron intermediate formed during cofactor assembly in *E. coli* ribonucleotide reductase, *J. Am. Chem. Soc. 118*, 7551−7557.
7. Stubbe, J., Nocera, D. G., Yee, C. S., and Chang, M. C. Y. (2003) Radical initiation in the Class I ribonucleotide reductase: long-range proton-coupled electron transfer? *Chem. Rev. 103*, 2167−2202.
8. Nordlund, P., and Reichard, P. (2006) Ribonucleotide reductases, *Annu. Rev. Biochem. 75*, 681−706.
9. 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) A Manganese(IV)/Iron(III) Cofactor in *Chlamydia trachomatis* ribonucleotide reductase, *Science 316*, 1188−1191.
10. Jiang, W., Bollinger, J. M., Jr., and Krebs, C. (2007) The active form of *Chlamydia trachomatis* ribonucleotide reductase R2 protein contains a heterodinuclear Mn(IV)/Fe(III) cluster with $S = 1$ ground state, *J. Am. Chem. Soc. 129*, 7504−7505.
11. Zheng, M., Khangulov, S. V., Dismukes, G. C., and Barynin, V. V. (1994) Electronic structure of dimanganese(II,III) and dimanganese(III,IV) complexes and dimanganese catalase enzyme: a general EPR spectral simulation approach, *Inorg. Chem. 33*, 382−387.
12. Sinnecker, S., Neese, F., and Lubitz, W. (2005) Dimanganese catalase-spectroscopic parameters from broken-symmetry density functional theory of the superoxidized Mn^{III}/Mn^{IV} state, *J. Biol. Inorg. Chem. 10*, 231−238.
13. Jiang, W., Hoffart, L. M., Krebs, C., and Bollinger, J. M., Jr. (2007) A Mn(IV)/Fe(IV) Intermediate in assembly of the Mn(IV)/Fe(III) cofactor of *Chlamydia trachomatis* ribonucleotide reductase, *Biochemistry*, *46*, 8709−8716.
14. Högbom, M., Stenmark, P., Voevodskaya, N., McClarty, G., Gräslund, A., and Nordlund, P. (2004) The radical site in Chlamydial ribonucleotide reductase defines a new R2 subclass, *Science 305*, 245−248.
15. Shu, L., Nesheim, J. C., Kauffmann, K. E., Münck, E., Lipscomb, J. D., and Que, L., Jr. (1997) An $Fe_2^{IV}O_2$ diamond core structure for the key intermediate **Q** of methane monooxygenase, *Science 275*, 515−518.
16. Winkler, R., Richter, M. E. A., Knüpfer, U., Merten, D., and Hertweck, C. (2006) Regio- and chemoselective enzymatic *N*-oxygenation *in vivo*, *in vitro* and in flow, *Angew. Chem., Int. Ed. 45*, 8016−8018.
17. Simurdiak, M., Lee, J., and Zhao, H. (2006) A new class of arylamine oxygenases: evidence that *p*-aminobenzoate *N*-oxygenase (AurF) is a di-iron enzyme and further mechanistic studies, *ChemBioChem 7*, 1169−1172.
18. Hirata, Y., Nakata, H., Yamada, K., Okuhara, K., and Naito, T. (1961) Structure of aureothin, a nitro compound obtained from *Streptomyces thioluteus*, *Tetrahedron 14*, 252−274.
19. Schwartz, J. L., Tishler, M., Arison, B. H., Shafer, H. M., and Omura, S. (1976) Identification of mycolutein and pulvomycin as aureothin and labilomycin respectively, *J. Antibiotics 29*, 236−241.
20. He, J., and Hertweck, C. (2004) Biosynthetic origin of the rare nitroaryl moiety of the polyketide antibiotic aureothin: Involvement of an unprecedented *N*-oxygenase, *J. Am. Chem. Soc. 126*, 3694−3695.
21. Kawai, S., Kobayashi, K., Oshima, T., and Egami, F. (1965) The oxidation of *p*-aminobenzoate to *p*-nitrobenzoate by *Streptomyces thioluteus*, *Arch. Biochem. Biophys. 112*, 537−543.
22. Winkler, R., and Hertweck, C. (2005) Sequential enzymatic oxidation of aminoarenes to nitroarenes via hydroxylamines, *Angew. Chem., Int. Ed. 44*, 4083−4087.
23. Fox, B. G., Shanklin, J., Ai, J., Loehr, T. M., and Sanders-Loehr, J. (1994) Resonance Raman evidence for an Fe-O-Fe center in stearoyl-ACP desaturase. Primary sequence identity with other diiron-oxo proteins, *Biochemistry 33*, 12776−12786.
24. Nordlund, P., and Eklund, H. (1995) Di-iron-carboxylate proteins, *Curr. Opin. Struct. Biol. 5*, 758−766.
25. Fox, B. G., Hendrich, M. P., Surerus, K. K., Andersson, K. K., Froland, W. A., Lipscomb, J. D., and Münck, E. (1993) Mössbauer, EPR, and ENDOR studies of the hydroxylase and reductase components of methane monooxygenase from *Methylosinus trichosporium* OB3b, *J. Am. Chem. Soc. 115*, 3688−3701.

26. Davydov, R., Kuprin, S., Gräslund, A., and Ehrenberg, A. (1994) Electron paramagnetic resonance study of the mixed-valent diiron center in *Escherichia coli* ribonucleotide reductase produced by reduction of radical-free protein R2 at 77 K, *J. Am. Chem. Soc. 116*, 11120−11128.
27. Mukherjee, S., Weyhermüller, T., Bothe, E., Wieghardt, K., and Chaudhuri, P. (2004) Dinuclear and mononuclear manganese(IV)-radical complexes and their catalytic catecholase activity, *Dalton Trans.*, 3842−3853.
28. Pierce, B. S., Elgren, T. E., and Hendrich, M. P. (2003) Mechanistic implications for the formation of the diiron cluster in ribonucleotide reductase provided by quantitative EPR spectroscopy, *J. Am. Chem. Soc. 125*, 8748−8759.
29. Bencini, A., and Gatteschi, D. (1990) *EPR of Exchange Coupled Systems*, Springer-Verlag, Berlin.
30. Andersson, K. K., Froland, W. A., Lee, S. K., and Lipscomb, J. D. (1991) Dioxygen independent oxygenation of hydrocarbons by methane monooxygenase hydroxylase component, *New J. Chem. 15*, 411−415.
31. Rosenzweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1993) Crystal structure of a bacterial non-heme iron hydroxylase that catalyzes the biological oxidation of methane, *Nature 366*, 537−543.
32. Zocher, G., Winkler, R., Hertweck, C., and Schulz, G. E. (2007) Structure and action of the *N*-oxygenase AurF from *Streptomyces thioluteus*, *J. Mol. Biol.*, DOI: 10.1016/j.jmb.2007.06.014.

BI701060G