Manganese(II) Active Site Mutants of 3,4-Dihydroxyphenylacetate 2,3-Dioxygenase from *Arthrobacter globiformis* Strain CM-2[†]

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ABSTRACT: Whereas all other members of the extradiol-cleaving catechol dioxygenase family are iron-dependent, the 3,4-dihydroxyphenylacetate 2,3-dioxygenase (MndD) from *Arthrobacter globiformis* CM-2 is dependent on manganese for catalytic activity. Recently, the endogenous iron ligands of one family member, the 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), were identified crystallographically as two histidines and a glutamic acid [Sugiyama, K., *et al.* (1995) *Proc. Jpn. Acad., Ser. B 71*, 32−35; Han, *et al.* (1995) *Science 270*, 976−980; Senda, T., *et al.* (1996) *J. Mol. Biol. 255*, 735−752]. Though BphC and MndD have low overall sequence identity (23%), the three BphC metal ligands are all conserved in MndD (H155, H214, and E266). In order to determine whether these residues also act as ligands to manganese in MndD, site-directed mutants of each were constructed, purified, and analyzed for activity and metal content. Mutations H155A, H214A, and E266Q yielded purified enzymes with specific activities of <0.1% of that of the wild-type dioxygenase and bound 0.4, 1.8, and 33% of the wild-type level of manganese, respectively. The relatively high level of manganese [with a Mn(II) EPR signal distinctly different from that of the wild-type enzyme] observed for E266Q suggests that the glutamine may act as a weak ligand to the metal. Mutant E266D, which retains the potential metal binding capability of a carboxylate group, exhibited 12% of the wild-type activity in crude extracts, suggesting that Mn remains bound; however, this mutant protein was too unstable to be purified and analyzed for metal content. On the basis of the low activity and metal content of mutant proteins, we propose that the conserved residues H155, H214, and E266 ligate manganese in MndD. As is the case with the superoxide dismutases, the extradiol-cleaving catechol dioxygenases appear to utilize identical coordinating residues for their iron- and manganese-dependent enzymes.

Monocyclic and polycyclic aromatic compounds are biodegraded in the environment to catecholic intermediates which are the targets of bacterial ring cleavage dioxygenases. These dioxygenases catalyze the oxidative cleavage of the aromatic ring by the addition of molecular oxygen to the substrate, leading to aliphatic products which can be further degraded into tricarboxylic acid cycle intermediates (Bayly *et al.*, 1966; Blakely, 1977; Mondello, 1989; Sparins *et al.*, 1974). The ring cleavage dioxygenases are divided into two families on the basis of the position where the ring is opened: intradiol dioxygenases cleave at a point between the two hydroxyl groups (ortho cleavage), while extradiol dioxygenases cleave at a point adjacent to one of the hydroxyl groups (meta cleavage) (Lipscomb & Orville, 1992). The majority of bacterial ring cleavage dioxygenases that have been characterized contain iron as the catalytic metal center; however, there is one report of a magnesium-dependent extradiol dioxygenase (Gibello *et al.*, 1994) and three known manganese-dependent dioxygenases: two from the genus *Arthrobacter* (Qi, 1991; Boldt *et al.*, 1995; Whiting *et al.*, 1996) and one from *Bacillus brevis* (Que *et al.*, 1981). The three manganese-dependent dioxygenases catalyze extradiol cleavage of 3,4-dihydroxyphenylacetic acid (3,4-DHPA[1]) (Figure 1) and have an α_4 holoenzyme structure

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[1] Abbreviations: MndD, Mn(II)-dependent 3,4-dihydroxyphenylacetate 2,3-dioxygenase; BphC, Fe(II)-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase; CTD, catechol 2,3-dioxygenase; IPNS, isopenicillin *N*-synthase; 3,4-DHPA, 3,4-dihydroxyphenylacetic acid; 5-CHMSA, 5-(carboxylmethyl)-2-hydroxymuconic semialdehyde; DHBP, 2,3-dihydroxybiphenyl; KP_i, potassium phosphate; EPR, electron paramagnetic resonance; FPLC, fast protein liquid chromatography; IEC, ion exchange chromatography; CD, circular dichroism; MCD, magnetic circular dichroism; ICP, inductively coupled plasma; ELISA, enzyme-linked immunoabsorbent assay; SDM, site-directed mutant; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; XAS, X-ray absorption spectroscopy; ZFS, zero-field splitting.

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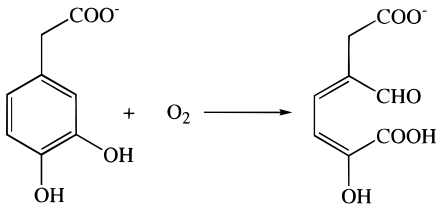

3,4-DHPA 5-CHMSA

FIGURE 1: Reaction catalyzed by MndD, from *Arthrobacter globiformis* strain CM-2.

with two to three atoms of Mn(II) tightly bound (Que *et al.*, 1981; Qi, 1991; Whiting *et al.*, 1996). In those studies, enzyme activity was correlated with the manganese content and EPR spectroscopy indicated that the manganese center was perturbed by the binding of substrates or inhibitors.

Amino acid sequence analysis has shown that the intradiol and extradiol dioxygenases are evolutionarily distinct (Durham *et al.*, 1980; Harayama & Rekik, 1989; Hofer *et al.*, 1993). There now appears to be at least three phylogenetically distinct families of extradiol dioxygenases (Asturias *et al.*, 1994). The manganese-dependent 3,4-DHPA 2,3-dioxygenase from *Arthrobacter globiformis* strain CM-2 (MndD) has been placed in the major extradiol dioxygenase family on the basis of overall sequence homology as well as the fact that it contains 14 out of the 18 amino acids that are most highly conserved across that dioxygenase family (Boldt *et al.*, 1995). MndD shares, on average, only 23% amino acid sequence identity with all other members of its extradiol dioxygenase family, for example, BphC from *Burkholderia cepacia* LB400 (Figure 2). An exception to this is the Fe-dependent 3,4-DHPA 2,3-dioxygenase from *Brevibacterium fuscum*, with which MndD has 78% identity (Y.-Z. Wang and J. D. Lipscomb, personal communication).

Elucidation of the metal center structure and reaction mechanism of the Fe(II)−extradiol dioxygenases has lagged behind that of the Fe(III)−intradiol dioxygenases, largely due to the fact that the former are colorless and EPR silent, thus complicating spectroscopic studies (Lipscomb & Orville, 1992). Nonetheless, over the past 15 years, a variety of spectroscopic techniques, including Mössbauer analysis (Tatsuno *et al.*, 1980), XAS (Shu *et al.*, 1995), MCD (Mabrouk *et al.*, 1991), and EPR of Fe(II)−nitrosyl adducts (Arciero & Lipscomb, 1985; Arciero *et al.*, 1986), have been employed in studies of the catechol 2,3-dioxygenase (CTD) from *Pseudomonas putida*. Taken together, these studies reveal a metal center in the resting enzyme that consists of a high-spin Fe(II) ion in a square pyramidal geometry, coordinated by three endogenous amino acid residues and two exogenous solvent molecules. In the presence of catechol, the Fe(II) remains pentacoordinate, but these solvent molecules are displaced by bidentate coordination of the substrate catechol. Further exposure to nitric oxide results in a six-coordinate metal center, indicating the presence of the probable dioxygen binding site.

Recently, the structure of the Fe(II)-dependent extradiol-cleaving dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC), was determined crystallographically (Sugiyama *et al.*, 1995; Han *et al.*, 1995; Senda *et al.*, 1996). Three amino acids, H146, H210, and E260 (numbered with respect to BphC from *B. cepacia* LB400), along with two solvent molecules, were found to serve as ligands to the Fe(II) center in a square pyramidal geometry as predicted by the spectroscopic studies (see Figure 3). More recently, the crystal structures of the anaerobic Fe(II)−ES complex (S. Han and J. T. Bolin, personal communication) and the Fe(III)−ES complex (Senda *et al.*, 1996) have confirmed the bidentate binding of the substrate via the vicinal hydroxyl groups.

Sequence alignments show that the three amino acids identified as metal ligands in BphC are conserved throughout the major family of extradiol-cleaving dioxygenases, including MndD (Figure 2) (Boldt *et al.*, 1995; Eltis & Bolin, 1996). In order to determine whether the Mn(II) center in MndD also utilizes these residues as ligands, we have constructed and expressed four conserved site-directed mutations: E266D, E266Q, H155A, and H214A. We have fully purified and quantified the metal content of the latter three. On the basis of these results, we propose that H155, H214, and E266 do, in fact, act as ligands to Mn(II) in MndD.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Both wild-type (WT) and mutant MndD proteins were expressed from the *lacZ* promoter of pUC18 (Norrander *et al.*, 1983) in *Escherichia coli* DH5α. All clones were grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1989) containing 30 μg/mL ampicillin in plates and 15 μg/mL ampicillin in liquid media. Cells were grown at 37 °C except where growth at a lower temperature is specified.

Site-Directed Mutagenesis. Site-directed mutagenesis was done using the Transformer mutagenesis kit from CLONTECH (Palo Alto, CA). Mutagenic primer design was done using the PRIMER 2 program (Scientific and Educational Software, State Line, PA) to create primers with good hybridization profiles. Primers were synthesized by IDT Technologies (Coralville, IA). All mutants were constructed using plasmid pYB2 (Boldt *et al.*, 1995), containing a 2.0 kb *Sma*I fragment, carrying *mndD*, cloned into pUC18. Potential mutants were screened either by looking for changes in the restriction enzyme pattern that corresponded to a particular codon change or by direct DNA sequencing. Mutants identified by the former method were confirmed by DNA sequencing.

Crude Extract Preparation. E. coli clones were grown to an optical density of 0.9−1.2 at A_{600} and harvested by centrifugation at 10000*g* for 10 min at 4 °C. Cells were resuspended in 10 mL of 50 mM KP_i (pH 8.0) per gram of wet weight. Cells were broken by two passages through a French pressure cell at a pressure of 18 000 PSI. A few flakes of DNase I were added to the broken cell suspension, which was then centrifuged at 15000*g* at 4 °C for 25 min. The supernatant was collected and used as the crude extract in enzyme activity assays and ELISAs. Crude extracts were stored at −80 °C.

ELISA Quantitation of MndD in Crude Extracts. WT and mutant MndD protein was quantified in *E. coli* DH5α crude extracts using an ELISA. Briefly, crude extracts were bound overnight to Costar 5590 microtiter plates (Fischer Scientific, Pittsburgh, PA) at 4 °C. The primary antibody was the anti-manganese-dependent dioxygenase polyclonal antibody raised in rabbits against purified MndD from *Arthrobacter* Mn-1 (Olson *et al.*, 1992). The polyclonal antibodies have been shown to cross-react with MndD (Olson *et al.*, 1992; Boldt

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MndD   PDIVRCAYME IVVTDLAKSR EFYVDVLGLH VTEEDENTIY LRSLEEFIHH    62
BphC   MSIRSLGYMG FAVSDVAAWR SFLTQKLGLM EAGTTDNGDL FRIDSRAWRI    50

MndD   NLVLRQGPIA AVAAFAYRVK SPAEVDAAEA YYKELGCRTE RRKEGFTKGI   112
BphC   AVQQGEV..D DLAFAGYEVA DAAGLAQMAD KLKQAGIAVT TGDASLARRR    98

MndD   G..DSVRVED PLGFPYEFFY ETEHV.ERLT QRYDLYSAGE ....LVRLDH   155
BphC   GVTGLITFAD PFGLPLEIYY GASEVF.EKP FLPGAAVSG. FLTGEQGLGH   146

MndD   FNQVTPDVPR GRAYLED.LG FRVSEDIKDS D...GVTYAA WMHRKQTVHD   201
BphC   FVRCVPDSDK ALAFYTDVLG FQLSDVIDMK MGPDVTVPAY FLHCNERHHT   196

MndD   TALTGGN.GP RMHHVAFATH EKHNIIQICD KMRPCASATG SNGPRPAPVS   250
BphC   LAIAAFPLPK RIHHFMLEVA SLDDVGFAFD RVDADG.LIT STLGRHT.ND   244

MndD   NAFYLYILDP DGHRIEIYTQ DYYTGDPDNP TITWDVHDNQ RRDWWGNPVV   300
BphC   HMVSFYASTP SGVEVEYGWS ARTVDR.... ..SWVVVRHD SPSMWGHKSV   288

MndD   PSWYTEASLV LDLDGNPQPV IVREEKSEMA VTVGAEPSPT PAR         343
BphC   R....DKAAA RNKA                                        298
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FIGURE 2: Sequence alignment of BphC, from *Burkholderia cepacia* LB400, and MndD, from *A. globiformis* CM-2. The pairwise comparison shown is derived from a multiple-sequence alignment derived from the data in Eltis and Bolin (1996). The residues in bold indicate amino acids targeted for site-directed mutagenesis in this paper (H42, H155, H214, and E266).

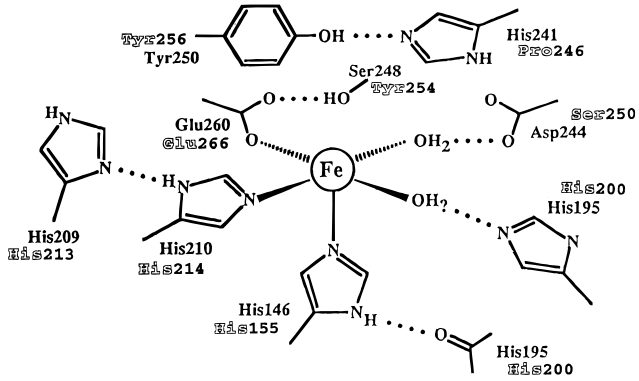

FIGURE 3: Schematic diagram of the Fe(II) active site in BphC, from *B. cepacia* LB400, based on the crystal structure. Amino acid names in solid font represent residues in BphC, and those in outline font represent the corresponding residues from MndD.

et al., 1995). The secondary antibody was goat anti-rabbit alkaline phosphatase conjugate (BIORAD, Hercules, CA) with *p*-nitrophenyl phosphate used as the alkaline phosphatase substrate (Sigma). The amount of the alkaline phosphatase reaction product, *p*-nitrophenol, was measured at 405 nm with a microplate reader. Purified MndD from *Arthrobacter* Mn-1 alone, or added to *E. coli* DH5α (pUC18) crude extract to constitute 3% of the total protein, was used in each experiment to construct a standard curve. Standard curves were very similar with or without added *E. coli* crude extract. Standard curves were consistently hyperbolic, showing saturation kinetics as the protein binding capacity of the microtiter plate well was reached, and giving a straight line from a double-reciprocal plot. Nonlinear standard curves are commonly observed in ELISAs, and linearization methods are the most common method of data analysis (Parker, 1990; Rodbard, 1974).

Assays of Enzyme Activity. MndD wild-type and mutant activities were assayed spectrophotometrically by measuring the rate of formation of the extradiol product, 5-CHMSA, at 380 nm as described previously (Whiting *et al.,* 1996). The percent WT specific activities shown in Table 1 were calculated by dividing the measured activity of the mutant by the milligrams of protein as determined by ELISA quantitation and then dividing by the WT specific activity

Table 1: Activity of Manganese-Dependent 3,4-DHPA 2,3-Dioxygenase Site-Directed Mutants[a]

	% total cell protein		% WT specific activity	
protein	18 °C	37 °C	18 °C	37 °C
MndD	3.6	6.0	100	100
H42A	4.1	28	97	30
H155A	3.8	0.6	0.001	ND[b]
H214A	5.1	1.7	0.12	ND[b]
E266D	0.9	0.1	12	0.12
E266Q	3.5	7.7	1.0	0.05

[a] WT and mutant MndD were quantitated in crude extracts using an ELISA. [b] Not determined because the enzyme activity was below the detection limits of the enzyme assay.

which was determined identically. The specific activities of the purified MndD WT and mutants, as shown in Table 3, were calculated using the milligrams of protein as quantitated spectrophotometrically using the extinction coefficient of purified MndD WT ($A_{280}^{0.1\%} = 1.52$) (Whiting *et al.,* 1996).

The steady-state kinetic parameters K_m and k_{cat} for E266Q, as shown in Table 3, were determined as described previously for WT (Whiting *et al.,* 1996), except that higher concentrations of both enzyme (1285 nM) and substrate (15−750 μM) were used.

Western Blot Analysis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and Western blotting were performed using standard techniques (Maniatis *et al.,* 1989). Primary and secondary antibody hybridizations were performed as previously described (Olson *et al.,* 1992).

Purification of Mutant MndD Proteins. The MndD mutant protein E266Q was purified from the *E. coli* DH5α clone using the same purification protocol used to purify WT MndD from *E. coli* DH5α (pYB2) (Whiting *et al.,* 1996). Mutant proteins H155A and H214A were purified using the same protocol except that acetone was added to 40% of the final volume in the second step of purification. For purification, 24 L of each *E. coli* DH5α clone was grown in 2X LB medium supplemented with 200 μM $MnCl_2$ and 100 μg/mL ampicillin. Ampicillin (50 μg/mL) was added when the OD at A_{600} reached 4.7 to maintain selection for the plasmid expressing MndD mutant proteins during growth in

the bioreactor. E266Q was grown at 37 °C, H155A at 23 °C, and H214A at 20 °C. The cells were harvested at A_{600} = 8.0. DEAE and FPLC fractions containing E266Q were identified by assaying for enzyme activity as described earlier, except that 200 μL of each DEAE fraction was used in a reaction volume of 1 mL. Fractions containing H155A or H214A were identified by the presence of a strong band at the same molecular mass as that of purified WT MndD on SDS−PAGE gels. The identity of the purified proteins was confirmed by Western blot analysis. Purified proteins were quantitated as described above.

Metal Quantitation of Purified MndD Mutants. The Mn content of purified H155A, H214A, and E266Q enzymes was determined by EPR spin quantitation and ICP emission analysis. The EPR samples were prepared by concentrating 12−24 mg of purified protein to 300 μL in Centricon-30 concentrators (in 50 mM KP_i buffer at pH 8.0), transferring to quartz EPR tubes, and freezing by slow immersion in liquid nitrogen. An anaerobic EPR sample of E266Q plus the substrate, 3,4-DHPA (22 mM), was also prepared according to methods used previously for the anaerobic WT plus substrate EPR samples (Whiting *et al.*, 1996). The EPR spectra were obtained at X-band (9.2 GHz) using a Varian E-109 spectrometer equipped with an Oxford Instruments ESR-10 liquid helium cryostat. All spectra were taken at 2.5 K as a single 4 min scan from 0 to 8000 G using 0.02 mW power, a 10 G modulation amplitude, and a 100 kHz modulation frequency.

Upon completion of EPR analysis, the samples were thawed and used to prepare the ICP samples as described previously (Whiting *et al.*, 1996). Samples were submitted to the Research Analytical Laboratory (Department of Soil Science, University of Minnesota, St. Paul) for ICP analysis.

Circular Dichroism. Circular dichroism (CD) measurements were made in a 0.1 cm cell in a Jasco J-710 spectropolarimeter. A scan speed of 20 nm/min and a wavelength range of 190−270 nm were used. Protein samples were prepared at a concentration of 0.5 μM in 50 mM KP_i buffer at pH 8.0.

RESULTS

Analysis of MndD Site-Directed Mutants in Crude Extracts. When WT and mutant MndD clones were grown at 37 °C, all site-directed mutant (SDM) *E. coli* clones exhibited less than 6% of the WT specific activity, except H42A, a mutation involving a nonconserved histidine that served as our control, which exhibited 30% of the WT specific activity (Table 1). The apparent stability of the SDMs relative to WT MndD, measured as a percent of the total cell protein in *E. coli* crude extracts, varied considerably with H42A and E266Q being comparably stable, H155A and H214A somewhat less stable, and E266D highly unstable (Table 1).

In an attempt to obtain larger quantities of more stable proteins, all clones were grown at 18 °C and the dioxygenase specific activities of their crude extracts were determined (Table 1). Mutant E266D was still very unstable, but a substantial increase in its percent WT specific activity from 0.12 to 12% was observed. MndD from mutant H155A was present at a 6-fold higher level when the clone was grown at 18 °C. Its activity was measurable, but 10^5 times lower than that of WT. H214A and E266Q had a stability similar to that of WT MndD but retained only 0.12 and 1.0% of the WT specific activity, respectively.

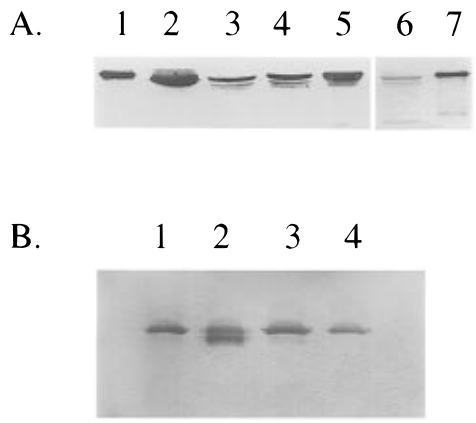

FIGURE 4: (A) Western blot analysis of WT and mutant MndD proteins. Purified WT MndD (lanes 1 and 7). The denoted *E. coli* DH5α crude extract (150 μg) was used in lanes 2−6: H42A (lane 2), H155A (lane 3), H214A (lane 4), E266Q (lane 5), and E266D (lane 6). (B) Western blot analysis of purified WT and mutant proteins: H155A (lane 1), H214A (lane 2), E266Q (lane 3), and WT MndD (lane 4).

Verification of WT and Mutant MndD in E. coli DH5α Clones. Because E266D was present at such low levels, corresponding to less than 1% of the total cell protein (Table 1), a Western blot was used to determine the presence of the MndD protein product in crude extracts of each mutant grown at 18 °C (Figure 4A). Anti-manganese dioxygenase polyclonal antibodies identified an immunologically cross-reacting protein of the same size as purified MndD in each mutant. The intensities of the MndD band in each mutant, in general, correlated with the ELISA quantitation of MndD present in crude extracts. To determine if the low level of MndD present in mutant E266D resulted in the production of an insoluble protein, the amount of WT and mutant MndD present in the insoluble fraction of each *E. coli* DH5α clone was determined by scanning laser densitometry of solid phase ELISA dot blots prepared from the pellets of crude extract preparations. E266D did not contain a significant amount of the dioxygenase in the insoluble pellet fraction of its crude extract (data not shown).

Purification of Mutant Proteins H155A, H214A, and E266Q. To increase protein yield, the clones expressing mutations H155A and H214A were grown at 23 and 20 °C, respectively. Purification of H155A yielded 12.7 mg of protein from 96 g wet weight of cells (132.3 μg/g), with no detectable enzyme activity. The purification of H214A yielded 112 mg of protein from 101 g wet weight of cells (1108 μg/g) and also had no detectable enzyme activity. Without activity to monitor, fractions containing H155A or H214A were identified as 39 kDa proteins by SDS−PAGE. Fractions containing a strong band at the same molecular mass as purified WT MndD were selected for further purification. The identity of the purified proteins as MndD was confirmed by Western blot analysis (Figure 4B). The lower band present in the H214A lane is a frequently observed degradation product of MndD.

Mutant protein E266Q, which constituted 7.7% of the total cell protein when the *E. coli* DH5α clone was grown at 37 °C, was purified and yielded 272 mg of protein from 93 g wet weight of cells (2924 μg/g) (Table 2). This yield is comparable to the amount of MndD obtained from the WT clone (Whiting *et al.*, 1996). The identity of the final purified E266Q protein as MndD was confirmed by Western blot

Table 2: Purification of MndD from Mutant E266Q

purification step	total activity[a] (units)	total protein[b] (mg)	specific activity (units/mg)	purification factor	% yield activity
crude extract	7.75	7110	0.0012	1	100
acetone extraction	2.40	2930	0.00082	0.68	31
DEAE IEC	2.37	477	0.0050	4.2	31
Mono Q FPLC	2.82	272[c]	0.01	8.3	36

[a] A unit of enzyme activity was defined as the amount that oxidizes 1 μmol of 3,4-DHPA to 5-CHMSA per minute in 50 mM KP_i buffer at pH 8.0 and 23 °C ($\epsilon_{380} = 42\,700\ M^{-1}\ cm^{-1}$). [b] Protein concentration determined using the Bradford assay and BSA standard. [c] Protein concentration determined using the MndD extinction coefficient ($A_{280}^{0.1\%} = 1.52$).

Table 3: Comparison of Purified H155A, H214A, and E266Q with Purified WT MndD

	H155A	H214A	E266Q	WT MndD[a]
kinetic properties				
specific activity (u/mg)	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$	0.006	9.1
K_m (μM)	–	–	90	7
k_{cat} (min^{-1})	$<2 \times 10^{-3}$	$<2 \times 10^{-3}$	0.92	1400
metal content				
Mn/holoenzyme (EPR)	ND[b]	ND[b]	1.1	3.2
Mn/holoenzyme (ICP)	<0.004	0.02	1.2	3.0
Fe/holoenzyme (ICP)	0.004	0.06	0.13	0.4−1.0

[a] Data from Whiting *et al.* (1995). [b]No Mn EPR signal was detected.

analysis (Figure 4B). Steady-state kinetic analysis of purified E266Q (Table 3) showed that E266Q had a K_m of 90 μM, 13-fold higher than that of WT MndD. The k_{cat} for purified E266Q was 0.92 min^{-1}, only 0.07% of the k_{cat} for WT MndD.

Circular dichroism experiments were done with purified mutant proteins to determine if any of the single amino acid changes significantly disrupted the overall folding of MndD. No significant differences in secondary structure relative to the WT were detected for H155A, H214A, or E266Q.

Quantitation of Mn Bound by Purified H155A, H214A, and E266Q. Purification of H155A, H214A, and E266Q allowed for determination of the manganese content of these proteins. No Mn(II) signal was observed in the EPR spectra of the purified MndD from mutants H214A (Figure 5C) and H155A (Figure 5D), indicating that Mn(II) is not bound. This is consistent with ICP analyses which showed only 0.02 and <0.004 Mn/holoenzyme associated with purified H214A and H155A, respectively (Table 3). Although when greatly expanded the EPR did show weak signals due to Fe(III), ICP revealed neither iron, nor any other metal, present in a significant quantity. These results are consistent with the loss of a residue responsible for binding Mn in H155A and H214A.

ICP analysis of E266Q showed the presence of 1.2 Mn/holoenzyme with 0.13 Fe/holoenzyme. Thus, E266Q binds less Mn and less Fe than the WT MndD (Table 3). EPR spectroscopy in Figure 5B shows that E266Q exhibited a signal at $g = 2.09$, with 6-fold hyperfine splitting characteristic of Mn(II) (nuclear spin, $I = {}^5/_2$) (Reed & Markham, 1984). In agreement with ICP analysis, quantitation of this signal revealed that E266Q only contained 1.1 Mn/holoenzyme (Table 3). This is 30% of the Mn/holoenzyme present in WT MndD, which contains 3.0 Mn/holoenzyme (Whiting *et al.*, 1996). These results are consistent with the loss or change of a Mn(II) ligand.

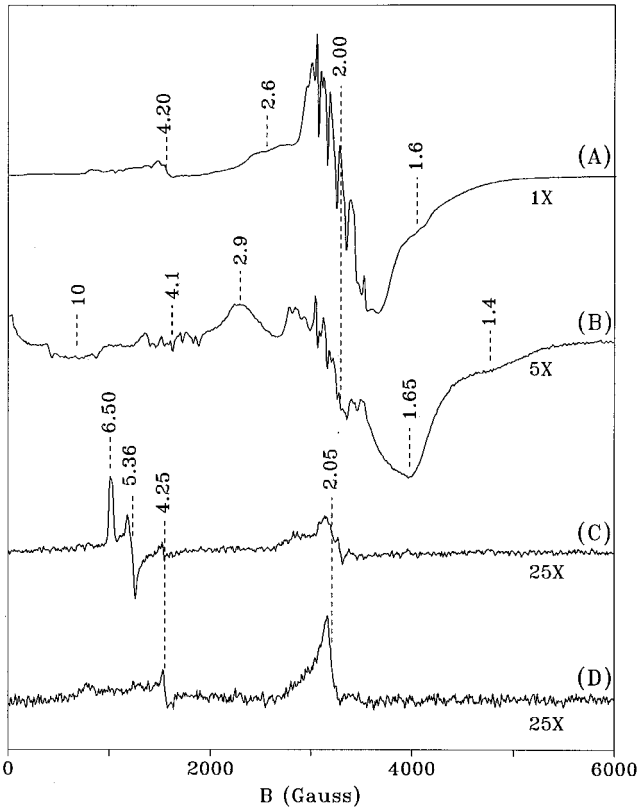

FIGURE 5: EPR spectra of (A) 0.27 mM purified WT MndD; (B) 0.26 mM purified E266Q, with 5× magnification of the *y*-axis; (C) 0.51 mM purified H214A, with 25× magnification of the *y*-axis; and (D) 0.27 mM purified H155A, with 25× magnification of the *y*-axis. The temperature was 2.5 K, the power 0.02 mW at 9.23 GHz, and the modulation amplitude 10 G. All spectra have been normalized to correct for differences in enzyme concentration and instrumental gain.

In addition to reduced affinity for Mn(II), the loss or change of a ligand may also result in changes in the electronic properties of any bound Mn(II), which should appear as changes in the EPR spectrum relative to that of the WT. E266Q exhibited several signals at $g = 1.4$, 2.9, 4.1, and 10, in addition to the strong isotropic signal at $g = 2.0$, which were not present in the WT spectrum (Figure 5A). The appearance of lower- and higher-field EPR signals indicated an increased zero-field splitting (ZFS) of the E266Q Mn(II) center, relative to that of the WT Mn(II) center (Reed & Markham, 1984), suggesting a change in the coordination environment of the Mn(II). These observations further support the idea that E266 serves as a Mn(II) ligand in MndD.

Although no activation of WT MndD could be achieved using exogenously supplied Mn(II) [or Fe(II)] (Whiting *et al.*, 1996), it seems reasonable, on the basis of its apparently weaker coordination environment, that E266Q could be activated in such a manner. No such activation of E266Q was observed in the presence of added $MnSO_4$ or $MnCl_2$ (up to 5 mM).

DISCUSSION

The first identified Mn-dependent extradiol-cleaving dioxygenase had two characteristics that distinguished it from the more prevalent Fe(II)-dependent enzymes: a six-line Mn(II) EPR signal and resistance to the Fe(II)-dependent enzyme inactivators, H_2O_2 and CN^- (Que *et al.*, 1981). Like the

better characterized Fe(II)-dependent catechol dioxygenases, it was found to exist in a homotetrameric subunit structure of approximately 160 kDa; however, nothing was known of its evolutionary relationship to the Fe(II) enzymes. The successful cloning and sequencing in *E. coli* of the *mndD* gene from *A. globiformis* CM-2 advanced the idea that, although MndD utilizes Mn(II) as its catalytic metal center (Whiting *et al.,* 1996), its sequence places it in the family of Fe(II)-dependent extradiol-cleaving catechol dioxygenases (Boldt *et al.,* 1995). Most striking is the fact that, while the overall sequence identity to most of its homologs is quite low (20−27%), all six fully conserved possible metal-ligating residues are present in MndD (Boldt *et al.,* 1995). The crystallization and structural analysis of the Fe(II)-dependent extradiol-cleaving dioxygenase BphC has revealed that three (H146, H210, and E260) of these six conserved possible metal ligands do in fact coordinate the active site Fe (see Figure 3) (Sugiyama *et al.,* 1995; Han *et al.,* 1995; Senda *et al.,* 1996).

In the work reported here, we have constructed, expressed, and purified site-directed mutants of the homologous residues in MndD (H155A, H214A, and E266Q). In the case of the two H to A mutations, neither activity nor Mn is detected for the purified enzymes, consistent with the deletion of a metal-ligating residue, although CD analysis reveals a secondary structure very similar to that of WT protein. The purified E266Q mutant also exhibited WT-like secondary structure and drastically attenuated specific activity (only 0.06% of that of WT), yet does bind one Mn(II) ion per homotetramer (as compared to three for WT) in a coordination environment that is significantly perturbed relative to that of WT. The extremely low specific activity (<0.1%) of all three purified mutants, relative to that of the WT enzyme, and the high activity of a nonconserved control mutation (H42A) are clearly consistent with the assignment of H155, H214, and E266 as metal ligands. Site-directed mutagenesis has now been used to identify the metal ligands in a range of metalloenzymes, including soybean lipoxygenase L-1 (Minor *et al.,* 1981; Steczko & Axelrod, 1992), urease (Jabri *et al.,* 1995; Park & Hausinger, 1993), D-xylose (glucose) isomerase (Cha *et al.,* 1993; Jenkins *et al.,* 1992), alkaline phosphatase (Ma & Kantrowitz, 1994), isopenicillin *N*-synthase (IPNS) (Borovok *et al.,* 1996; Tan & Sim, 1996), and manganese peroxidase (Kishi *et al.,* 1996). In all these cases, the mutation of the metal ligands (all of which have now been identified crystallographically) resulted in reduction of activity to less than 1% of that of WT.

Of particular relevance to this study is IPNS, an Fe(II)-dependent enzyme whose crystal structure was determined with Mn(II) bound in the putative iron site (Roach *et al.,* 1995). The ligand residues included two histidines and an aspartate, analogous to the coordination environment predicted for MndD. The three proposed Fe(II) ligands of IPNS, based on the Mn(II)-substituted crystal structure (H212, H268, and D214), are consistent with the more recent results of site-directed mutagenesis of seven conserved histidines and five conserved aspartic acids (Borovok *et al.,* 1996). Analogous to the results presented here, mutants of the putative ligand residues had no detectable activity, whereas the other mutants retained 5−68% of the WT specific activity. Similarly, site-directed mutagenesis was used to identify the corresponding histidines in IPNS from *Cephalosporium aeremonium* as iron ligands (Tan & Sim, 1996).

Surprisingly, the Mn(II)-substituted IPNS crystal structure also revealed a fourth, more weakly coordinating interaction between the metal and the side chain carbonyl oxygen of a glutamine that is conserved throughout the known IPNSs (Roach *et al.,* 1995). The presence of this coordinating glutamine in IPNS raises the possibility that mutation E266Q of MndD does not fully eliminate the capability of this residue to act as a ligand. This is also supported by the observation that E to Q mutations of Mn(II) ligands in Mn peroxidase still catalyze the oxidation of Mn(II) to Mn(III), albeit at a reduced level (Kishi *et al.,* 1996). The EPR and ICP quantitation results reported here clearly confirm the retention of one atom of Mn(II) per homotetramer, although with a significantly perturbed EPR signal that indicates an altered electronic environment. A viable hypothesis is that the carbonyl oxygen of Q266 replaces the carboxylate oxygen of E266 as a weak third ligand to Mn. Considering that E266Q carries out catalysis with a K_m only 10-fold higher than that of WT, it is reasonable to assume that a third ligand helps to maintain the Mn(II) in a catalytically productive position in the active site. It should be noted that, as with WT, no activation of E266Q could be achieved through the addition of exogenous Mn(II).

The 500-fold lower k_{cat} observed for E266Q underscores the fact that transition-state stabilization demands a much higher degree of structural fidelity for the metal center than the formation of the Michaelis complex. Consistent with this general statement, the EPR spectrum of E266Q remains unchanged upon anaerobic addition of substrate. This is in stark contrast to WT, which under identical conditions exhibited dramatic shifts in its EPR spectrum consistent with direct substrate coordination to the Mn(II) center (Whiting *et al.,* 1996). It has been previously shown that substrate binding to the extradiol-cleaving dioxygenase, protocatechuate 4,5-dioxygenase, activates its Fe(II) center for nitric oxide (and presumably also dioxygen) binding (Arciero *et al.,* 1985). This substrate activation step is proposed to be critical to the extradiol cleavage mechanism of the Fe(II) enzymes (Lipscomb & Orville, 1992). Assuming a similar mechanism exists in the Mn(II)-dependent enzymes, the inability of E266Q to efficiently form such a substrate-activated complex, as suggested by its EPR, may explain its greatly decreased turnover rate.

If E266 is in fact a metal ligand, mutation E266D, which retains the carboxylate moiety (albeit with a slightly shorter tether to the backbone), might be expected to coordinate the Mn(II). Indeed, the negatively charged carboxylate oxygen is a much stronger metal ligand than a glutamine carbonyl oxygen, assuming spatial considerations allow it to interact with the metal. We found that crude extracts of E266D did exhibit 12% of the WT specific activity, suggesting that Mn(II) remains fully ligated in a catalytically productive manner. Unfortunately, the extremely low levels of E266D in crude extracts precluded its purification and subsequent metal content and EPR analysis.

In the cases of H155A and H214A, the problem of low mutant protein levels in crude extracts was overcome by culturing the *E. coli* at 18 °C rather than at 37 °C. However, for their purification, the H155A and H214A cultures were grown in 24 L fermenters at 23 and 20 °C, respectively, and it is unclear if the slightly higher temperature or the purification procedure resulted in the loss of the very low dioxygenase activity present in crude extracts grown at 18

°C. However, a reasonable robustness and overall WT-like structure of the purified mutants can be inferred from their purification by a procedure similar to that of the WT, as well as the similarity of their respective CD spectra.

We have previously shown that both BphC and MndD belong to the major family of extradiol dioxygenases (Boldt *et al.*, 1995). In BphC, residues H146, H210, and E260 are responsible for ligating the Fe and correspond to H155, H214, and E266 in MndD (Figure 3). Our site-directed mutagenesis data supporting the role of H155, H214, and E266 as Mn(II) ligands strengthen the argument for its homology with the major extradiol dioxygenase family. Although the catalytic metals differ between BphC and MndD, there is a precedent for the same amino acids ligating either Mn or Fe in the superoxide dismutases (Lah *et al.*, 1995; Parker & Blake, 1988). Other conserved residues in MndD are predicted to be near the active site, including H200, H213, and Y256 (see Figure 3). Further work is underway to characterize these mutant proteins in order to better predict the role of each residue in ring cleavage of 3,4-DHPA by MndD.

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