The Manganese Binding Site of Manganese Peroxidase: Characterization of an Asp179Asn Site-Directed Mutant Protein†

Margo Kusters-van Someren,‡ Katsuyuki Kishi, Taina Lundell,§ and Michael H. Gold*

Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291-1000

Received April 10, 1995⊗

ABSTRACT: A site-directed mutant, D179N, in the gene encoding *Phanerochaete chrysosporium* manganese peroxidase isozyme 1 (*mnp*1), was created by overlap extension, using polymerase chain reaction. The mutant gene was expressed in *P. chrysosporium* under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter. The mutant manganese peroxidase (MnP) was purified, and its spectra and MW were very similar to those of the wild-type enzyme. Steady-state kinetic analysis of MnP D179N revealed that the K_m for the substrate Mn^{II} was ~50-fold greater than the corresponding K_m for the wild-type recombinant enzyme (3.7 mM versus ~70 μM). Likewise, the k_{cat} value for Mn^{II} oxidation of the mutant protein was only 1/265 of that for the wild-type enzyme. By comparison, the apparent K_m for H_2O_2 of MnP D179N was similar to the corresponding value of the wild-type MnP. The first-order rate constant for MnP D179N compound II reduction by Mn^{II} was approximately 1/200 of that for the wild-type enzyme. The equilibrium dissociation constant (K_D) for MnP D179N compound II reduction by Mn^{II} was ~100-fold greater than the K_D for the wild-type compound II. In contrast, the second-order rate constant for *p*-cresol reduction of the mutant compound II was similar to that of the wild-type enzyme. These results also suggest that the mutation affects the binding of Mn^{II} to the enzyme and, consequently, the rate of compound II reduction by Mn^{II}. In contrast, the mutation apparently does not have a significant effect on H_2O_2 cleavage during compound I formation or on *p*-cresol reduction of compound II. The results strongly suggest that Asp179 is one of the acidic amino acid ligands in the Mn^{II} binding site of MnP.

Phanerochaete chrysosporium and other white-rot fungi are capable of degrading the plant cell wall polymer lignin (Gold et al., 1989; Kirk & Farrell, 1987; Buswell & Odier, 1987) and a variety of environmentally persistent aromatic pollutants (Bumpus & Aust, 1987; Hammel, 1989; Valli & Gold, 1991; Valli et al., 1992; Joshi & Gold, 1993). When cultured under ligninolytic conditions, *P. chrysosporium* secretes two families of extracellular peroxidases, lignin peroxidase (LiP)[1] and manganese peroxidase (MnP), which, along with an H_2O_2-generating system, comprise the major components of its extracellular lignin-degrading system (Kirk & Farrell, 1987; Buswell & Odier, 1987; Gold & Alic, 1993; Wariishi et al., 1991; Kuwahara et al., 1984; Hammell et al., 1993). Both LiP and MnP depolymerize lignin *in vitro* (Wariishi et al., 1991; Hammel et al., 1993). Moreover, MnP activity is produced by all white-rot fungi known to degrade lignin (Hatakka, 1994; Perie & Gold, 1991; Orth et al., 1993).

MnP has been purified and characterized both biochemically and kinetically (Gold et al., 1989; Gold & Alic, 1993; Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1989, 1992). In addition, the sequences of *mnp* cDNA (Gold & Alic, 1993; Pribnow et al., 1989; Pease et al., 1989) and genomic clones (*mnp1* and *mnp2*) (Gold & Alic, 1993; Godfrey et al., 1990; Mayfield et al., 1994a) encoding two *P. chrysosporium* MnP isozymes have been determined. Spectroscopic studies and DNA sequences suggest that the heme environment of MnP is similar to that of other plant and fungal peroxidases (Glenn et al., 1986; Pribnow et al., 1989; Harris et al., 1991; Banci et al., 1992; Dunford & Stillman, 1976; Mino et al., 1988; Wariishi et al., 1988). Kinetic and spectral characterizations of the oxidized intermediates—MnP compounds I, II, and III—indicate that the catalytic cycle of MnP is similar to that of horseradish peroxidase and LiP (Gold et al., 1989; Glenn et al., 1986; Wariishi et al., 1988, 1992; Renganathan & Gold, 1986). The crystal structure of LiP has been reported (Edwards et al., 1993; Poulos et al., 1993; Piontek et al., 1993), and the structure of MnP recently has been solved (Sundaramoorthy et al., 1994a,b). These structures confirm that the heme environments of MnP and LiP are similar to those of cytochrome *c* peroxidase and other plant and fungal peroxidases (Poulos et al., 1993; Sundaramoorthy et al., 1994b). MnP is unique in its ability to catalyze the one-electron oxidation of Mn^{II} to Mn^{III} (Glenn & Gold, 1985; Glenn et al., 1986; Wariishi et al., 1992) as shown in Scheme 1. The

† This work was supported by Grant MCB 9207997 from the National Science Foundation, Grant DE-FGO6-92ER20093 from the Division of Energy Biosciences, U.S. Department of Energy, and Grant 2-4722-03 from the U.S. Department of Agriculture to M.H.G. T.L. was supported by a fellowship from the Academy of Finland.

* To whom correspondence should be addressed at the Department of Chemistry, Biochemistry, and Molecular Biology, Oregon Graduate Institute of Science & Technology, P.O. Box 91000, Portland, OR 97291-1000. Telephone: 503-690-1076. Fax: 503-690-1464.

‡ Present address: Section of Molecular Genetics of Industrial Microorganisms, Wageningen Agricultural University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands.

§ Present address: Department of Applied Chemistry and Microbiology, University of Helsinki, Viikki, SF-00710 Helsinki, Finland.

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: LiP, lignin peroxidase; MnP, manganese peroxidase; MnP1, manganese peroxidase isozyme 1; *mnp*1, gene encoding MnP1; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; rMnP1, recombinant wild-type manganese peroxidase isozyme 1; SDS, sodium dodecyl sulfate.

0006-2960/95/0434-10620$09.00/0

enzyme-generated Mn^{III} is stabilized by organic acid chelators such as oxalate which also is secreted by the fungus (Wariishi et al., 1992; Kuan et al., 1993). The Mn^{III}–organic acid complex, in turn, oxidizes phenolic substrates such as lignin substructure model compounds (Tuor et al., 1992), synthetic lignin (Wariishi et al., 1991), and aromatic pollutants (Valli & Gold, 1991; Valli et al., 1992; Joshi & Gold, 1993).

Scheme 1

$$MnP + H_2O_2 \rightarrow MnPI + H_2O \quad (1)$$

$$MnPI + Mn^{II} \rightarrow MnPII + Mn^{III} \quad (2)$$

$$MnPII + Mn^{II} \rightarrow MnP + Mn^{III} + H_2O \quad (3)$$

To study structure/function relationships in MnP via site-directed mutagenesis, an expression system is required for the efficient production and purification of large quantities of enzyme. We recently developed a homologous expression system for MnP isozyme 1 (MnP1) (Mayfield et al., 1994b). In this system, the *P. chrysosporium* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoter is used to drive expression of the *mnp* gene during the primary metabolic growth phase when endogenous MnP is not expressed. This expression system produces recombinant MnP1 (rMnP1) in amounts which are comparable to the endogenous MnP1 produced by the wild-type strain (Mayfield et al., 1994b). Furthermore, biochemical characterization suggests that the rMnP1 is very similar to the wild-type enzyme (Mayfield et al., 1994b). In this study, Asp179, one of the potential ligands for manganese binding in MnP (Sundaramoorthy et al., 1994b; Johnson et al., 1993), was converted to Asn179 using overlap extension PCR. Kinetic and spectroscopic properties of the purified D179N mutant protein were investigated.

MATERIALS AND METHODS

Organisms. *P. chrysosporium* wild-type strain OGC101, auxotrophic strain OGC107-1 (Ade 1), and prototrophic transformants were maintained as described previously (Alic et al., 1990). *Escherichia coli* XL1-Blue and DH5αF′ were used for subcloning plasmids.

Oligodeoxyribonucleotides. Four oligonucleotides were used for site-directed mutagenesis of Asp179 of *mnp1* (Pribnow et al., 1989; Godfrey et al., 1990). Oligonucleotide $B1_{norm}$ is a 17-mer corresponding to *mnp1* positions 554–570 (Godfrey et al., 1990). Oligonucleotide $D1_{rev}$ is complementary to the *mnp1* sequence over nucleotide positions 1156–1140. Oligonucleotides $N179_{norm}$ and $N179_{rev}$ are 28-mers and are partly overlapping: $N179_{norm}$ spans nucleotides 841–868, and $N179_{rev}$ is complementary to nucleotides 858–830. Oligonucleotides were synthesized at the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR. $N179_{norm}$ and $N179_{rev}$ contain the preferred (Ritch & Gold, 1992) codon and anticodon, respectively, for Asn, AAC replacing GAC, which encodes Asp179.

Site-Directed Mutagenesis by PCR. A 554-bp *Bpu*1102I–*Dra*III fragment containing the D179N mutation was generated by overlap extension (Ho et al., 1989) using the polymerase chain reaction (PCR). Amplification of the first two fragments (Figure 1) was achieved using 30 ng of pGM1 [the *gpd–mnp* construct subcloned in pUC18 (Mayfield et al., 1994b)] as target DNA, 400 μM of each dNTP, 1 unit of Deep Vent polymerase (New England Biolabs), and a 1-μM aliquot of either oligonucleotides $B1_{norm}$ and $N179_{rev}$ or oligonucleotides $N179_{norm}$ and $D1_{rev}$ in a total volume of 50 μL. Samples were overlaid with mineral oil, heated for 2 min at 94 °C, and subjected to 25 cycles of denaturation (1 min at 94 °C), annealing (0.5 min at 55 °C), and extension (1 min at 72 °C), followed by a final incubation for 5 min at 72 °C in a thermocycler (Ericomp). The reaction products of both reactions were analyzed by electrophoresis on a 1% agarose gel; fragments were visualized by ethidium bromide under UV light, and those of the correct size were excised. The DNA was purified by GeneClean (BIO101). These two fragments which partially overlap were combined and used as template DNA in a third reaction with oligonucleotides $B1_{norm}$ and $D1_{rev}$ under the conditions described above, but without pGM1 DNA (Figure 1). The final PCR product was analyzed by agarose gel electrophoresis, excised, purified by GeneClean, and digested with *Bpu*1102I and *Dra*III (New England Biolabs).

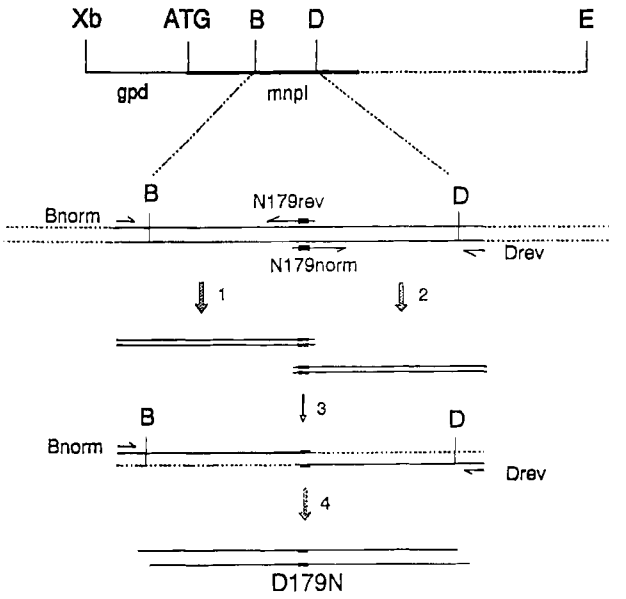

FIGURE 1: Site-directed mutagenesis strategy. In the map of the construct, the thin solid line indicates the *gpd* promoter fragment, the thick solid line indicates the *mnp1* coding region, and the dashed line represents the noncoding 3′ region of the *mnp1* gene. Xb, *Xba*I; ATG, translation start codon; B, *Bpu*1102I; D, *Dra*III; E, *Eco*RI. 1, PCR using oligonucleotides B_{norm} and $N179_{rev}$; 2, PCR using oligonucleotides $N179_{norm}$ and D_{rev}; 3, PCR by overlap extension with oligonucleotides B_{norm} and D_{rev}; 4, Digestion with *Bpu*1102I and *Dra*III.

Construction of pAGM4. The *Bpu*1102I–*Dra*III fragment containing the D179N mutation was first subcloned into pGM1, containing unique *Bpu*1102I and *Dra*III sites, replacing the wild-type *mnp1* fragment with the mutant fragment, to generate pGM4. The entire *Bpu*1102I–*Dra*III fragment was analyzed by double-stranded DNA sequencing using the four oligonucleotides described above, and a Sequenase Version 2.0 DNA sequencing kit (USB) with a 7-deaza-dGTP labeling mix and termination mixes (USB). Subsequently, the 4.0-kb *Xba*I–*Eco*RI fragment of pGM4 containing the *gpd* promoter and the mutated *mnp*1 gene was subcloned into pOGI18 (Godfrey et al., 1994), generating pAGM4. The presence of the D179N mutation in pAGM4 was confirmed by double-stranded DNA sequencing of the appropriate sequence, using oligonucleotide $B1_{norm}$ as a primer.

Transformation of Phanerochaete chrysosporium. *P. chrysosporium* strain Ade1 (Gold et al., 1982; Alic et al., 1987) was transformed as described previously (Alic et al., 1990), using 1 μg of pAGM4 as the transforming DNA. Thirty-five transformants were transferred to minimal slants (Gold et al., 1982) to confirm adenine prototrophy and, subsequently, were assayed for MnP activity using the *o*-anisidine plate assay as described (Mayfield et al., 1994b). The six transformants showing the strongest activity by the plate assay were purified by fruiting as described (Alic et al., 1987), and the progeny again were assayed for MnP activity by the plate assay.

Production of the D179N Mutant Protein. Transformant strain D179N-6 was maintained on 2% malt agar slants and grown from a conidial inoculum at 37 °C in 20-mL stationary cultures in 250-mL Erlenmeyer flasks for 2 days. The medium was as described elsewhere (Kirk et al., 1978) except that it was supplemented with 2% glucose, 12 mM ammonium tartrate, and 20 mM dimethyl succinate (pH 4.5). The mycelial mat from one flask was homogenized for 20 s in a blender and used to inoculate a 2-L Erlenmeyer flask containing 1 L of the medium described above, except that in the large cultures the pH was adjusted to 6.5 and sodium succinate was used instead of dimethyl succinate. Cultures were grown at 37 °C on a rotary shaker at 200 rpm for 60 h. The MnP mutant protein is produced under primary metabolic conditions when endogenous *mnp* genes are not expressed (Mayfield et al., 1994b), enabling purification of the mutant protein.

Purification of the MnP D179N Mutant Protein. The MnP D179N mutant protein was purified by the protocol described for the wild-type recombinant MnP1 (Mayfield et al., 1994b). The extracellular fluid from 6 L of 60-h-old cultures was concentrated to 200 mL and dialyzed against 20 mM sodium acetate (pH 6.0) at 4 °C, using a hollow fiber filter system (10 000 MW cut-off; Amicon). Chromatography was as described (Mayfield et al., 1994b) and included DEAE-Sepharose column chromatography, Cibacron blue 3GA agarose column chromatography, and fast-protein liquid chromatography using a Mono Q column (Pharmacia).

SDS–PAGE and Western Analysis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a 12% Tris–glycine gel system (Laemmli, 1970) and a Mini-Protean II apparatus (Bio-Rad). The gels were stained with Coomassie blue. For Western (immunoblot) analysis, proteins were electroblotted (Sambrook et al., 1989) onto nitrocellulose (Micron Separations, Inc.), and MnP protein was detected as described (Pribnow et al., 1989; Mayfield et al., 1994a).

Enzyme Assay and Spectroscopic Procedures. MnP activity was measured by following the formation of Mn^{III}–malonate at 270 nm as previously described (Wariishi et al., 1992), except that the final concentration of $MnSO_4$ in the reaction mixture was increased to 5.0 mM. UV absorption spectra of the various oxidation states of MnP D179N, as well as rMnP1, were recorded at room temperature using a Shimadzu UV-260 spectrophotometer. The enzyme was maintained in 20 mM potassium succinate or malonate buffer, pH 4.5. The ionic strength of the buffers was adjusted to 0.1 M using K_2SO_4. Enzyme concentrations were determined at 406 nm using an extinction coefficient of 129 mM^{-1} cm^{-1} (Glenn & Gold, 1985). MnP D179N compound I was prepared by mixing 1.0 equiv of H_2O_2 with the native enzyme. Compound II was prepared by the successive addition of 1.0 equiv of H_2O_2 and 1.0 equiv of Mn^{II} or ferrocyanide.

Kinetic Analyses. To determine apparent K_m and k_{cat} values of the mutant enzyme for Mn^{II} and H_2O_2, 1/initial velocity versus 1/[substrate] was plotted at fixed concentrations of H_2O_2 or Mn^{II}. Reaction mixtures contained MnP D179N (10 μg/mL), $MnSO_4$ (0.5–5.0 mM), and H_2O_2 (0.02–0.1 mM) in 50 mM sodium malonate, pH 4.5. Kinetic measurements with rMnP1 were carried out as described (Mayfield et al., 1994b). The reduction of MnP compound II (2 μM) in 20 mM potassium malonate, pH 4.5, or 0.5–5 mM potassium oxalate, pH 4.6 (ionic strength 0.1 M, adjusted with K_2SO_4), was followed at 406 nm, which is the Soret absorbance maximum of the native enzyme (see Results). Compound II of MnP D179N and wild-type MnP1 were freshly prepared for each experiment, and the reaction was initiated by adding reducing substrate, Mn^{II} or *p*-cresol, in at least 10-fold excess. All of the kinetic traces displayed single-exponential character from which pseudo-first-order rate constants were calculated. Several substrate concentrations were used, and plots of pseudo-first-order rate constants versus substrate concentration were obtained.

Chemicals. DEAE-Sepharose CL-6B, Cibacron blue 3GA agarose, and H_2O_2 (30% solution) were obtained from Sigma. *p*-Cresol was obtained from Aldrich. The concentration of the H_2O_2 stock solution was determined as described (Cotton & Dunford, 1973). *p*-Cresol was purified by thin-layer chromatography before use. All other chemicals were reagent grade. Solutions were prepared using deionized water obtained from a Milli Q purification system (Millipore).

RESULTS

Expression and Purification of Mutant Protein. The presence of the D179N mutation was confirmed by double-stranded DNA sequencing of the altered *Bpu*11021I–*Dra*III restriction fragment in pGM4 and in the complete transformation vector pAGM4. Out of 35 selected transformants, 6 displayed readily detectable MnP activity on the plate assay. These six transformants expressed extracellular mutant MnP protein within 3 days of growth in liquid high-carbon, high-nitrogen shake cultures, as verified by Western (immunoblot) detection (data not shown), under conditions in which the wild-type enzyme was not expressed. The amount of mutant protein secreted by the pAGM4 transformants was similar to the level of recombinant wild-type MnP1 (rMnP1) reported previously (Mayfield et al., 1994b). However, the six transformant strains displayed only slow Mn^{II}-oxidizing activity as assayed by monitoring the formation of Mn^{III}–malonate complex (Wariishi et al., 1992). MnP D179N from transformant 6 was purified using anion-exchange, blue agarose, and Mono Q chromatography as described (Mayfield et al., 1994b). As shown in Figure 2, one major peak and a later eluting minor peak were separated on Mono Q column chromatography. The major peak eluted essentially at the same time as rMnP1 when the latter was chromatographed under identical conditions (Mayfield et al., 1994b). Furthermore, as shown in Figure 2 inset, the molecular mass (46 kDa) of the predominant form of MnP D179N was identical to that of rMnP1 and wild-type MnP.

Spectral Properties of MnP D179N. Figure 3 shows the absorption spectra of the native, compound I, and compound

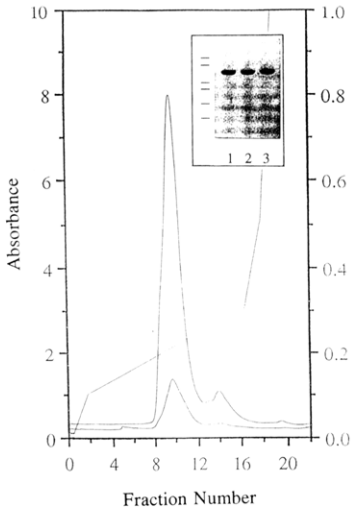

FIGURE 2: Mono Q anion exchange chromatography of the MnP D179N protein. The column was equilibrated with 10 mM sodium acetate, pH 6.0, and eluted by an increasing sodium acetate (pH 6.0) gradient as shown. Absorbances at 405 nm (top trace) and 280 nm (bottom trace) were measured. MnP activity was measured as described in the text and correlated with the absorbance at 405 nm. Inset: SDS−PAGE of various forms of Mn peroxidase: SDS−PAGE of wild-type MnP1 (lane 1), rMnP1 (lane 2), and MnP D179N (lane 3). 5 μg of each protein was loaded on a 12% Tris/glycine−polyacrylamide gel system (Laemmli, 1970). Proteins were visualized by Coomassie blue staining. Migration of the molecular mass standards (from the top): bovine serum albumin, 66.3 kDa; glutamate dehydrogenase, 55.4 kDa; lactate dehydrogenase, 36.5 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa, as indicated.

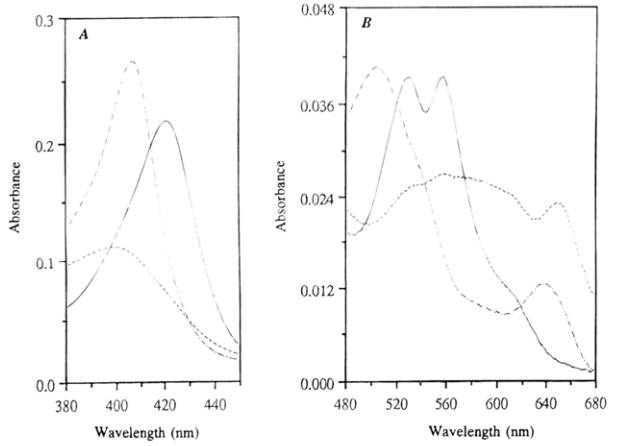

FIGURE 3: Electronic absorption spectra of oxidized states of MnP D179N. Comparison of UV/vis absorption spectra of native MnP D179N (−•−), MnP D179N compound I (- - -), and compound II (—). Spectra were recorded in 20 mM potassium malonate, pH 4.5, at 25 °C. The enzyme concentrations were 2 μM (*A*, Soret spectra) and 4 μM (*B*, visible spectra). MnP D179N compound I (- - -) was prepared by adding 1 equiv of H_2O_2 to native MnP D179N in 20 mM potassium malonate, pH 4.5 (μ = 0.1). MnP D179N compound II (—) was prepared by the successive additions of 1.0 equiv of H_2O_2 and 1.0 equiv of Mn^{II} to the native enzyme in the same buffer.

II states of MnP D179N. Upon the addition of 1.0 equiv of H_2O_2 to the native enzyme, the Soret band at 406 nm rapidly decreased and red-shifted to yield a band at 398 nm. The visible region of compound I displayed a peak at 650 nm with a broad absorption at 530−600 nm. Approximately 40% of compound I was spontaneously converted to compound II in 30 min (data not shown). Upon the addition of 1.0 equiv of Mn^{II} to compound I, a spectrum for compound

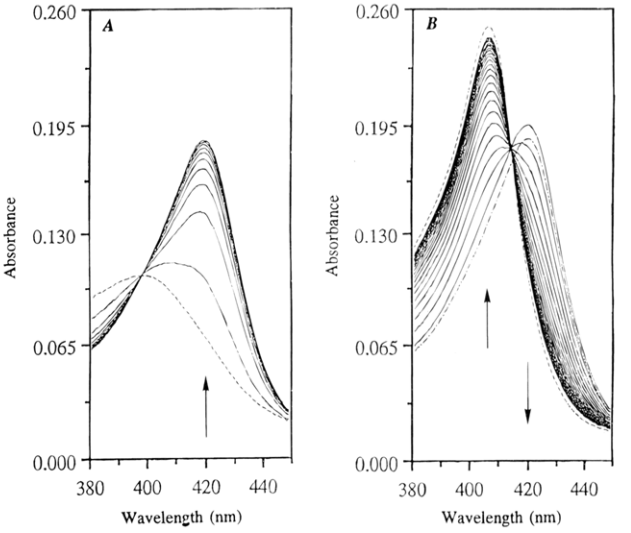

FIGURE 4: Reduction of MnP D179N compounds I and II. Reduction of MnP D179N compounds I (*A*) and II (*B*) with Mn^{II}. (*A*) MnP D179N compound I (- - -) was prepared as described in the legend to Figure 3. Soret spectra were taken at 0.5 min intervals from 0.5 to 5.0 min. Final reactant concentrations: MnP D179N compound I, 2 μM; Mn^{II}, 20 μM (10 equiv). The dashed line represents native MnP D179N (2 μM). Arrows indicate progress of reaction. (*B*) MnP D179N compound II (−•−) was prepared as described in the legend to Figure 3. Spectra were taken at 1 min intervals from 1 to 25 min. Final reactant concentrations: MnP D179N compound II, 2 μM; Mn^{II}, 20 μM (10 equiv). The dashed line represents native MnP D179N (2 μM). Arrows indicate progress of reaction.

Table 1: Absorbance Maxima (nm) of Native and Oxidized Intermediates of Wild-Type MnP1 and the MnP D179N Mutant

	native	compound I	compound II
wild-type MnP1[a]	406, 502, 632	407, 558, 617 (sh),[b] 650	420, 528, 555
MnP D179N[c]	406, 502, 635	398, 557, 615 (sh),[b] 650	420, 528, 556

[a] Wariishi et al. (1988). [b] Shoulder. [c] This work.

II slowly developed with maxima at 420, 527, and 555 nm. An isosbestic point between compounds I and II was observed at 398 nm (Figures 3 and 4*A*). Compound II was stable for more than 20 min, and only ~10% of the compound II was converted spontaneously to the native enzyme in 1 h (data not shown). However, with the addition of 10 equiv of Mn^{II} to compound II, the enzyme returned slowly to the native state, with an absorbance maximum at 406 nm and an isobestic point at 414 nm (Figure 4*B*). The spectral maxima of the various oxidized states of the D179N protein were essentially identical to those of the wild-type enzyme (Table 1), suggesting that replacement of Asp179 with an Asn did not change the heme environment of the protein significantly.

Kinetic Properties of MnP D179N. The specific activity of MnP D179N for Mn^{II} oxidation was 0.946 μmol mg^{-1} min^{-1} as determined by following the formation of the Mn^{III}−malonate complex at 270 nm (Wariishi et al., 1992). This specific activity was approximately 1/350 of the values obtained for wild-type MnP1 or rMnP1 (341 and 332 μmol mg^{-1} min^{-1}, respectively) (Mayfield et al., 1994b). Under steady-state conditions, linear Lineweaver−Burk plots were obtained over a range of Mn^{II} and H_2O_2 concentrations using 50 mM malonate as the chelator (Wariishi et al., 1992; Mayfield et al., 1994b) (data not shown). The apparent K_m and k_{cat} values for Mn^{II} and H_2O_2 are listed in Table 2. The

Table 2: Steady-State Kinetic Parameters for Wild-Type rMnP1 and MnP D179N Mutant[a]

	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
	Mn^{II}	H_2O_2	Mn^{II}	Mn^{II}
wild-type MnP1[b]	73.2	41.7[c]	3.01×10^2	4.11×10^6
wild-type rMnP1[b]	69.4	39.2[c]	2.89×10^2	4.17×10^6
MnP D179N[d]	3.7×10^3	34.1[e]	1.09	2.95×10^2

[a] Reactions were conducted in 50 mM sodium malonate, pH 4.51. [b] Mayfield et al. (1994b). [c] Apparent K_m for H_2O_2 was determined at 0.5 mM Mn^{II}. [d] This work. [e] Apparent K_m for H_2O_2 was determined at 5.0 mM Mn^{II}.

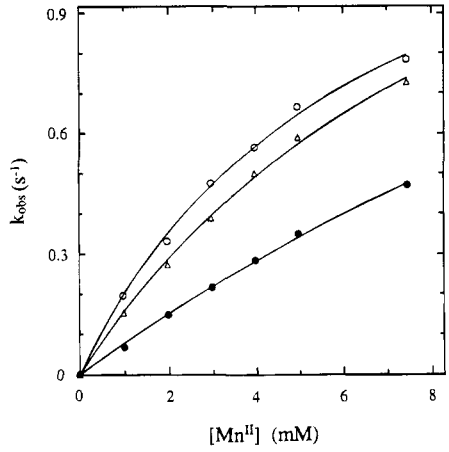

FIGURE 5: Kinetics of reduction of MnP D179N compound II. Reduction of MnP D179N compound II by Mn^{II} in 0.5 (○), 2 (△), and 5 (●) mM potassium oxalate, pH 4.6, $\mu = 0.1$. Each k_{obs} was obtained from the exponential change in absorbance at 406 nm.

apparent K_m for H_2O_2 was the same for both the wild-type MnP1 and MnP D179N. In contrast, the apparent K_m of MnP D179N for Mn^{II} was ~50-fold higher than that for the wild-type MnP1 (Table 2). In addition, the apparent k_{cat} of MnP D179N for Mn^{II} was more than 260 times lower than that for the wild-type MnP1.

The comparative rates of MnP compound II reduction for the mutant and wild-type enzyme also were examined since this is the rate-limiting step in the MnP catalytic cycle (Wariishi et al., 1989; Kuan et al., 1993; Kishi et al., 1994). The reduction was followed at 406 nm under pseudo-first-order conditions using an excess of reducing substrate. The plot of observed pseudo-first-order rate constants versus Mn^{II} concentration leveled off at high Mn^{II} concentrations (Figure 5) (Kishi et al., 1994). This reaction can be explained by a simple binding interaction between the reactants, according to eqs 4–6:

$$\text{compound II} + Mn^{II}\text{–complex} \overset{K_D}{\rightleftharpoons} \text{compound II} - Mn^{II}\text{–complex} \xrightarrow{k} \text{native enzyme} + Mn^{III}\text{–complex} \quad (4)$$

$$k_{obs} = \frac{k}{1 + K_D/[Mn^{II}\text{–complex}]} \quad (5)$$

$$K_D = \frac{[\text{compound II}][Mn^{II}\text{–complex}]}{[\text{compound II} - Mn^{II}\text{–complex}]} \quad (6)$$

where k is a first-order rate constant (s^{-1}) and K_D is a dissociation constant (M). The calculated values are listed in Table 3. With *p*-cresol as the reductant in the conversion of compound II to native enzyme, the plots of pseudo-first-order rate constants versus *p*-cresol concentrations were linear for both wild-type MnP1 and MnP D179N (Figure 6). The second-order rate constants were calculated as 1.94×10^2 and 1.59×10^2 M^{-1} s^{-1} for wild-type MnP1 and MnP D179N, respectively (Table 3).

DISCUSSION

Although the catalytic cycle of MnP is similar to that of other plant and fungal peroxidases (Gold et al., 1989; Wariishi et al., 1988, 1989; Dunford & Stillman, 1976; Renganathan & Gold, 1986), this enzyme is uniquely capable of oxidizing Mn^{II} to Mn^{III} (Glenn et al., 1986; Wariishi et al., 1989, 1992). The latter, complexed with an organic acid, diffuses from the enzyme to oxidize the terminal phenolic substrate (Glenn et al., 1986; Tuor et al., 1992). This suggests that the primary substrate, Mn^{II}, binds directly to the enzyme. We have shown that free Mn^{II} binds in the vicinity of the heme of the native enzyme with an apparent dissociation constant of 9.6 μM (Wariishi et al., 1992). It is noteworthy that a variety of dicarboxylic chelators, including oxalate and malonate, increased the apparent dissociation constant 5-fold, although a chelator is required for enzymatic activity (Wariishi et al., 1992).

The nature of the Mn^{II} binding site in MnP has been under investigation for several years. Results of reactivity studies with azide and organic hydrazines as suicide inhibitors led Harris et al. (1991) to suggest that the binding of Mn^{II} does not interfere with reactions at the δ-meso position of the heme. However, they suggested that Mn^{II} binds close to the δ-meso position (above or below the plane of the heme). Similarly, based upon the perturbation of the NMR spectrum of MnP upon the binding of Mn^{II}, Banci et al. (1993) suggested a Mn^{II} binding site close to the δ-meso position of the heme. In contrast, Johnson et al. (1993) used homology modeling and energetic considerations to predict the Mn^{II} binding site in MnP. They reported that the most likely binding site consists of the ligands Asp179, Glu35, and Glu39, and one of the heme propionates (Johnson et al., 1993). This would place the Mn^{II} binding site closer to the γ-meso position of the heme. As shown in Figure 7, the recent crystal structure of MnP (Sundaramoorthy et al., 1994b) supports this latter prediction for the binding site.

This binding site is defined by three acidic amino acid ligands, including Asp179, Glu35, and Glu39, and one of the heme propionates. The final two ligands of the hexacoordinate Mn^{II} ion are water molecules. Comparison of the Mn^{II} binding ligands in MnP to corresponding amino acid residues in the LiP crystal structure (Sundaramoorthy et al., 1994b; Johnson et al., 1993) reveals that two of the three anionic residues found in MnP are replaced by noncharged residues in LiP: Ala36 for Glu35 and Asn182 for Asp179. Only Glu39 in MnP is conserved as Glu40 in LiP. In addition, the heme propionate in LiP is not positioned for favorable Mn^{II} binding (Sundaramoorthy et al., 1994b; Johnson et al., 1993). The presence of a single anionic ligand in LiP would not constitute a stable Mn^{II} binding site. Mutation of the proposed Mn^{II} ligands in MnP would aid our understanding of the unique specificity of this peroxidase. Furthermore, a mutation would test whether the proposed Mn^{II} binding site (Sundaramoorthy et al., 1994b) is productive. Herein, we report on the characterization of the site-

Table 3: Kinetic Parameters for Reduction of MnP Compound II[a]

enzyme	chelators (pH) (mM)	substrates	hyperbolic: first-order rate constants (s^{-1})	hyperbolic: equilibrium dissociation constants (M)	linear: second-order rate constant (M^{-1} s^{-1})
wild-type MNPI	oxalate (4.6)[b]				
	1	Mn^{II}	$(2.3 \pm 0.2) \times 10^2$	$(5.6 \pm 1.0) \times 10^{-5}$	
	2		$(2.9 \pm 0.1) \times 10^2$	$(8.7 \pm 0.7) \times 10^{-5}$	
	5		$(5.6 \pm 0.6) \times 10^2$	$(4.4 \pm 0.6) \times 10^{-4}$	
	malonate (4.5)				
	20	*p*-cresol			1.94×10^2
MnP D179N	oxalate (4.6)				
MnP D179N	oxalate (4.6)				
	0.5	Mn^{II}	1.46 ± 0.08	$(6.42 \pm 0.62) \times 10^{-3}$	
	1		1.30 ± 0.07	$(5.64 \pm 0.56) \times 10^{-3}$	
	2		1.70 ± 0.13	$(1.00 \pm 0.12) \times 10^{-2}$	
	5		2.11 ± 0.32	$(2.64 \pm 0.48) \times 10^{-2}$	
	malonate (4.5)				
	20	*p*-cresol			1.59×10^2

[a] Reaction mixtures contained 2 μM MnP. The ionic strength was adjusted to 0.1 M. [b] Kishi et al. (1994).

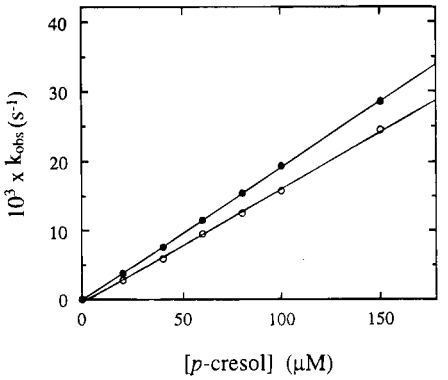

FIGURE 6: Kinetics of reduction of mutant and wild-type MnP compound II by *p*-cresol. Reduction of MnP D179N (○) and wild-type (●) compound II by *p*-cresol in 20 mM potassium malonate, pH 4.5. Each k_{obs} was obtained from the exponential change in absorbance at 406 nm.

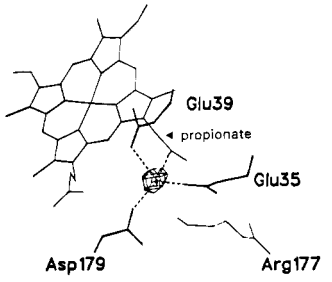

FIGURE 7: Mn^{II} ligands in the binding site of Mn peroxidase (Sundaramoorthy et al., 1994b).

directed mutant D179N, which transforms one of the proposed acidic amino acid ligands in the Mn^{II} binding site.

We recently demonstrated the homologous expression of recombinant MnP isozyme 1 (rMnP1) of *P. chrysosporium* (Mayfield et al., 1994b). In this system, the expression of the *mnp*1 gene is under the control of the *P. chrysosporium gpd* gene promoter. This allows expression of rMnP1 during the primary metabolic phase of growth when the endogenous MnPs are not expressed. Both the spectral and kinetic properties of the recombinant enzyme were very similar to wild-type MnP1, indicating that this expression system is suitable for conducting structure/function studies of MnP by site-directed mutagenesis. Overlap extension PCR was used to create the D179N mutation. The mutant gene in pOGI18 was transformed into the Ade1 strain of *P. chrysosporium* as described (Mayfield et al., 1994b; Alic et al., 1990). The MnP D179N protein was purified as described (Mayfield et al., 1994b). The Mono Q chromatographic profile suggests that one major heme protein is produced (Figure 2).

The D179N protein is essentially identical to the wild-type enzyme with respect to chromatographic properties and molecular weight (Figure 2), suggesting that this mutation does not lead to gross conformational changes in the protein. Furthermore, the native D179N mutant protein exhibits essentially identical spectral features to those of wild-type MnP (Figure 3, Table 1) (Wariishi et al., 1988). The spectra of the catalytic intermediates, compounds I and II, of the mutant protein also are essentially identical to those of the wild-type MnP intermediates, except that the Soret band of MnP D179N compound I was red-shifted 9 nm with a peak at 398 nm (Figure 3, Table 1). This difference may be explained by the possible contamination of wild-type MnP compound I preparations with compound II (Wariishi et al., 1988). The latter has a Soret peak at 420 nm (Table 1). The spectral similarities of the mutant MnP D179N and the wild-type MnP strongly suggest that the heme environment of MnP is not affected significantly by this mutation.

In contrast to the minimal effect on the UV/vis spectral properties, the mutation D179N had a dramatic affect on the steady-state kinetic properties of the enyzme. Both the specific activity and the turnover number (k_{cat}) for Mn^{II} oxidation by the MnP D179N protein decrease considerably in comparison to the wild-type enzyme (1/350 and 1/260, respectively). Moreover, the apparent K_m for Mn^{II} of MnP D179N is 50 times higher than that for the wild-type MnP (Table 2). This suggests that Asp179 is one of the ligands in the Mn^{II} binding site and that the Mn^{II} binding site predicted by modeling and crystallographic results is the productive site (Sundaramoorthy et al., 1994b; Johnson et al., 1993).

In comparison, the mutation does not affect the apparent K_m for H_2O_2. This suggests that the affinity of D179N for H_2O_2 and probably the rate of compound I formation in the mutant are not altered. These results suggest that the environment of the amino acid residues thought to be involved in the formation of compound I, including the distal His, distal Arg, and proximal His, has not been altered in this mutant.

Since the reduction of compound II to the native enzyme is the rate-limiting step in the MnP catalytic cycle (Wariishi et al., 1988, 1989; Kuan et al., 1993; Kishi et al., 1994), the

rate of MnP D179N compound II reduction by Mn^{II} was examined. In potassium oxalate (0.5−5 mM, pH 4.6), the plot of k_{obs} versus Mn^{II} concentration is hyperbolic (Figure 5), indicating a binding interaction between Mn^{II} and MnP. The reduction of wild-type MnP compound II also exhibits saturation kinetics in the presence of low concentrations of oxalate (1−5 mM) but at much lower concentrations of Mn^{II} (Table 3) (Kishi et al., 1994). At 1 mM oxalate, the equilibrium dissociation constant for the wild-type enzyme is $\sim 5.6 \times 10^{-5}$ M while that for the D179N mutant is 5.6×10^{-3} M. Thus, the binding constant for the mutant is 100× lower. This strongly suggests that D179 is a Mn^{II} ligand. In contrast to the kinetics observed for the reduction of MnP compound II by Mn^{II}, the reduction of compound II by *p*-cresol obeys second-order kinetics and is irreversible for both wild-type MnP1 and MnP D179N (Figure 6). The rate of reduction of wild-type MnP1 compound II by *p*-cresol is similar to that of MnP D179N compound II. In comparison, the first-order rate constant for Mn^{II} reduction of wild-type compound II in 1 mM oxalate is approximately 200 times that for the reduction of MnP D179N compound II (Table 3), whereas the dissociation constant for Mn^{II} is approximately 1/100 of the value obtained for MnP D179N. These results strongly suggest that the low reactivity of MnP D179N for Mn^{II} oxidation is a consequence of the decreased binding affinity of Mn^{II} for the mutant protein. However, the results do not rule out an additional effect on the electron transfer rate. The results also suggest that *p*-cresol binds to the enzyme at a site other than the Mn^{II} binding site. A recent molecular modeling study proposes that *p*-cresol binds to horseradish peroxidase at a site on the distal side of the heme adjacent to Phe68 (Banci et al., 1994). This site is distinct from the Mn^{II} binding site shown in Figure 7.

In conclusion, the results of this study demonstrate that changing Asp179 to Asn significantly affects the oxidation of Mn^{II}, most probably by decreasing the affinity of the enzyme for Mn^{II}. In contrast, neither the apparent K_m for H_2O_2 nor the rate of compound II reduction by *p*-cresol is significantly affected by this mutation. These results strongly support the assignment of the Mn^{II} binding site shown in Figure 7 (Sundaramoorthy et al., 1994b; Johnson et al., 1993). This site consists of Asp179, Glu39, Glu35, a heme propionate, and two water molecules. The coordination of the Mn^{II} at this site is octahedral, which is typical of Mn^{II} coordination complexes (Demmer et al., 1980). Current views envision electron transfer pathways in proteins through covalent bonds (Onuchic & Beratan, 1990). The structure of this site suggests that the electron may be transferred from Mn^{II} to the porphyrin via the heme propionate ligand, using a nearly continuous σ-bonded path. Additional mechanistic, structural, and mutagenesis studies will be required in order to elucidate further the electron transfer pathway in this system.

REFERENCES

Alic, M., Letzring, C., & Gold, M. H. (1987) *Appl. Environ. Microbiol. 53*, 1464−1469.

Alic, M., Clark, E. K., Kornegay, J. R., & Gold, M. H. (1990) *Curr. Genet. 17*, 305−311.

Banci, L., Bertini, I., Pease, E. A., Tien, M., & Turano, P. (1992) *Biochemistry 31*, 10009−10017.

Banci, L., Bertini, I., Bini, T., Tien, M., & Turano, P. (1993) *Biochemistry 32*, 5825−5831.

Banci, L., Carloni, P., & Savellini, G. G. (1994) *Biochemistry 33*, 12356−12366.

Bumpus, J. A., & Aust, S. D. (1987) *BioEssays 6*, 166−170.

Buswell, J. A., & Odier, E. (1987) *CRC Crit. Rev. Biotechnol. 6*, 1−60.

Cotton, M. L., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 582−587.

Demmer, H., Hinz, I., Keller-Rudex, H., Koeber, K., Kottelwesch, H., & Schneider, D. (1980) in *Coordination Compounds of Manganese* (Schleitzer-Rust, E., Ed.) 8th ed., Vol. 56, pp 1−185, Springer-Verlag, New York.

Dunford, H. B., & Stillman, J. S. (1976) *Coord. Chem. Rev. 19*, 187−251.

Edwards, S. L., Raag, R., Wariishi, H., Gold, M. H., & Poulos, T. L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 750−754.

Glenn, J. K., & Gold, M. H. (1985) *Arch. Biochem. Biophys. 242*, 329−341.

Glenn, J. K., Akileswaran, L., & Gold, M. H. (1986) *Arch. Biochem. Biophys. 251*, 688−696.

Godfrey, B. J., Mayfield, M. B., Brown, J. A., & Gold, M. H. (1990) *Gene 93*, 119−124.

Godfrey, B. J., Akileswaran, L., & Gold, M. H. (1994) *Appl. Environ. Microbiol. 60*, 1353−1358.

Gold, M. H., & Alic, M. (1993) *Microbiol. Rev. 57*, 605−622.

Gold, M. H., Cheng, T. M., & Mayfield, M. B. (1982) *Appl. Environ. Microbiol. 44*, 996−1000.

Gold, M. H., Wariishi, H., & Valli, K. (1989) *ACS Symp. Ser. 389*, 127−140.

Hammel, K. E. (1989) *Enzyme Microb. Technol. 11*, 776−777.

Hammel, K. E., Jensen, K. A., Jr., Mozuch, M. D., Landucci, L. L., Tien, M., & Pease, E. A. (1993) *J. Biol. Chem. 268*, 12274−12281.

Harris, R. Z., Wariishi, H., Gold, M. H., & Ortiz de Montellano, P. R. (1991) *J. Biol. Chem. 266*, 8751−8758.

Hatakka, A. (1994) *FEMS Microbiol. Rev. 13*, 125−135.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., & Pease, L. R. (1989) *Gene 77*, 51−59.

Johnson, F., Loew, G. H., & Du, P. (1993) in *Plant Peroxidases: Biochemistry and Physiology* (Welinder, K. G., Rasmussen, S. K., Penel, C., & Greppin, H., Eds.) pp 31−34, University of Geneva, Geneva, Switzerland.

Joshi, D., & Gold, M. H. (1993) *Appl. Environ. Microbiol. 59*, 1779−1785.

Kirk, T. K., & Farrell, R. L. (1987) *Annu. Rev. Microbiol. 41*, 465−505.

Kirk, T. K., Schultz, E., Connors, W. J., Lorenz, L. F., & Zeikus, J. G. (1978) *Arch. Microbiol. 117*, 277−285.

Kishi, K., Wariishi, H., Marquez, L., Dunford, H. B., & Gold, M. H. (1994) *Biochemistry 33*, 8694−8701.

Kuan, I.-C., Johnson, K. A., & Tien, M. (1993) *J. Biol. Chem. 268*, 20064−20070.

Kuwahara, M., Glenn, J. K., Morgan, M. A., & Gold, M. H. (1984) *FEBS Lett. 169*, 247−250.

Laemmli, U. K. (1970) *Nature 227*, 680−685.

Mayfield, M. B., Godfrey, B. J., & Gold, M. H. (1994a) *Gene 142*, 231−235.

Mayfield, M. B., Kishi, K., Alic, M., & Gold, M. H. (1994b) *Appl. Environ. Microbiol. 60*, 4303−4309.

Mino, Y., Wariishi, H., Blackburn, N. J., Loehr, T. M., & Gold, M. H. (1988) *J. Biol. Chem. 263*, 7029−7036.

Onuchic, J. N., & Beratan, D. N. (1990) *J. Chem. Phys. 92*, 722−733.

Orth, A. B., Royce, D. J., & Tien, M. (1993) *Appl. Environ. Microbiol. 59*, 4017−4023.

Pease, E. A., Andrawis, A., & Tien, M. (1989) *J. Biol. Chem. 264*, 13531−13535.

Perie, F. H., & Gold, M. H. (1991) *Appl. Environ. Microbiol. 57*, 2240−2245.

Piontek, K., Glumoff, T., & Winterhalter, K. (1993) *FEBS Lett. 315*, 119−124.

Poulos, T. L., Edwards, S., Wariishi, H., & Gold, M. H. (1993) *J. Biol. Chem. 268*, 4429−4440.

Pribnow, D., Mayfield, M. B., Nipper, V. J., Brown, J. A., & Gold, M. H. (1989) *J. Biol. Chem. 264*, 5036−5040.

Renganathan, V., & Gold, M. H. (1986) *Biochemistry 25*, 1626–1631.
Ritch, T. G., Jr., & Gold, M. H. (1992) *Gene 118*, 73–80.
Sambrook, S. J., Fritch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sundaramoorthy, M., Kishi, K., Gold, M. H., & Poulos, T. L. (1994a) *J. Mol. Biol. 238*, 845–858.
Sundaramoorthy, M., Kishi, K., Gold, M. H., & Poulos, T. L. (1994b) *J. Biol. Chem. 269*, 32759–32767.
Tuor, U., Wariishi, H., Schoemaker, H. E., & Gold, M. H. (1992) *Biochemistry 31*, 4986–4995.
Valli, K., & Gold, M. H. (1991) *J. Bacteriol. 173*, 345–352.
Valli, K., Wariishi, H., & Gold, M. H. (1992) *J. Bacteriol. 174*, 2131–2137.
Wariishi, H., Akileswaran, L., & Gold, M. H. (1988) *Biochemistry 27*, 5365–5370.
Wariishi, H., Dunford, H. B., MacDonald, I. D., & Gold, M. H. (1989) *J. Biol. Chem. 264*, 3335–3340.
Wariishi, H., Valli, K., & Gold, M. H. (1991) *Biochem. Biophys. Res. Commun. 176*, 269–275.
Wariishi, H., Valli, K., & Gold, M. H. (1992) *J. Biol. Chem. 267*, 23688–23695.

BI9507995