Progress Report for DOE Grant No. DE-FG02-87ER13690 entitled "Characterization of Lignin Peroxidases from *Phanerochaete chrysosporium*"

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Papers published and in preparation during the granting period.

During the granting period, we published 11 papers in refereed journals, 1 chapter in Methods in Enzymology , and 7 book chapters. One manuscript is in review and 4 are in the process of being written. Five of the reprints are provided in the appendix. Listed below are the titles and authors of the papers.

Manuscripts in refereed journals

1) Pease, E. A., Andrawis, A. and Tien, M.(1989)"Manganese-Dependent Peroxidases from *Phanerochaete chrysosporium*" J. Biol. Chem. 246, 13531-13535.
2) Cai, D. and Tien, M. (1989) "On the Reactions of Lignin Peroxidase Compound III (Isozyme H8)" Biochem. Biophys. Res. Commun. 162, 464-469.
3) Andrawis, A., Pease, E. A., Kuan, I.-C., Holzbaur, E. L. F. and Tien, M. (1989) "Characterization of Two Lignin Peroxidase Clones from *Phanerochaete chrysosporium*" Biochem. Biophys. Res. Commun. 162, 673-680.
4) Farrell, R. L., Murtagh, K. E., Tien, M. Mozuch, M. D. and Kirk, T. K. (1989) "Physical and Enzymatic Properties of Lignin Isozymes from *Phanerochaete chrysosporium*" Enzyme Microb. Technol. 11, 322-328.
5) Kuan, I.-C. and Tien, M. (1989) "Phosphorylation of Lignin Peroxidases from *Phanerochaete chrysosporium*: Identification of Mannose-6-Phosphate" J. Biol. Chem. 264, 20350-20355.
6) Millis, C. D., Cai, D., Stankovich, M. T. and Tien, M. (1989) "Oxidation-Reduction Potentials and Ionization States of Extracellular Oxidases from the Lignin-Degrading Fungus *Phanerochaete chrysosporium*" Biochemistry 28, 8484-8489.
7) Cai, D. and Tien, M. (1990) " Characterization of the Oxycomplex of Lignin Peroxidases from *Phanerochaete chrysosporium*: Equilibrium and Kinetics Studies" Biochemistry 29, 2085-2091.
8) Tien, M. and Myer, S. B. (1990) "Selection and Characterization of Mutants of *Phanerochaete chrysosporium* Exhibiting Ligninolytic Activity under Nutrient-Rich Conditions" Appl. Environ. Microbiol. 56, 2540-2544.
9) Kirk, T. K., Tien, M., Kersten, P. J., Kalyanaraman, B., Hammel, K. E., and Farrell, R. L. (1990) "Lignin Peroxidases from Fungi: *Phanerochaete chrysosporium*" Methods in Enzymology 188, 159-171.
10) Cai, D. and Tien, M. (1991) "Lignin Peroxidase of *Phanerochaete chrysosporium*: Evidence for an Acidic Ionization Controlling Activity" J. Biol. Chem., in press.
11) Orth, A. B., Denny, M. and Tien, M. (1991) "Overproduction of Lignin-Degrading Enzymes by an Isolate of *Phanerochaete chrysosporium*" Appl. Environ. Microbiol., in press.
12) Banci, L., Bertini, I., Turano, P., Tien, M. and Kirk, T. K. (1991) "A Proton NMR Investigation into the Basis for the Relatively High Redox Potential of Lignin Peroxidase" Proc. Natl. Acad. Sci. USA, in press.

Book Chapters Published

1) Holzbaur, E. L., Andrawis, A., and Tien, M. (1990) "Molecular Biology of Lignin Peroxidases from *Phanerochaete chrysosporium*", in Molecular Industrial Mycology: Systems and Applications (Leong, S.A. and Berka, R., eds.) Marcel Dekker, New York, in press.
2) Tien, M. and Cai, D. (1990) "Lignin-Degrading Peroxidases from *Phanerochaete chrysosporium*" in Biological Oxidation Systems (Reddy, C. C., Hamilton, G. A, and Madyastha, K. M., eds.) Volume 1, pp. 433-452, Academic Press, NY.
3) Pease, E. A. and Tien, M. (1990) "Lignin-Degrading Enzymes from the Filamentous Fungus *Phanerochaete chrysosporium*" in Biocatalysts for Industry (Dordicke, J., ed.) Plenum Publishing Corporation, NY, in press.
4) Andrawis, A., Pease, E. A. and Tien, M. (1989) "Extracellular Peroxidases of *Phanerochaete chrysosporium*: cDNA Cloning and Expression" in Biotechnology in Pulp and Paper Manufacturing, Applications and Fundamental Investigations (Kirk, T. K. and Chang, H. m., eds.) Butterworth Publishing. Chapter 61, pp. 601-614.
5) Cai, D. and Tien, M. (1990) "Lignin Peroxidase: Catalysis, Oxycomplex and Heme-Linked Ionization" American Chemical Society, in press.
6) Orth, A. B., Pease, E. A. and Tien, M. (1991) "Properties of Lignin-Degrading Peroxidases and Their Use in Bioremediation" in Biological Degradation and Bioremediation Technologies (Chaudry, G. R., ed.) Discordes Press. in press.
7) Cai, D. and Tien, M. (1991) "Lignin-Degrading Peroxidases of *Phanerochaete chrysosporium*" Proceeding of an international workshop on Lignin Biodegradation and Practical Utilization. June 27-30, Trieste, Italy.

Manuscripts submitted

1) Pease, E. A., Aust, S. D. and Tien, M. (1991) "Heterologous Expression of Active Mn Peroxidase from *Phanerochaete chrysosporium* in Insect Tissue Culture" Biochem. Biophys. Res. Commun.

Descriptive Summary of Research Accomplishments

Much of the work summarized below is described in greater detail in the enclosed reprints and prepints. To avoid redundancy between the progress report, reprints, preprints and grant proposal, I will not go into great detail here but will refer the reader to the accompanying papers for a more detailed description of the work. Our long term objectives is to elucidate the role and mechanism of the various isozymes in lignin biodegradation. Consequently, we have an interest in defining their complexity, regulation and mechanism of action.

Electrochemical studies on lignin and Mn peroxidases. The work described here is the first and only electrochemical study of the lignin and Mn peroxidases. This study was performed to investigated the structural aspects which confer the lignin and Mn peroxidases with their high reactivity. The experimentally determined redox potential of the Fe^{3+}/Fe^{2+} couple for the lignin peroxidase isozymes

H1, H2, H8 and H10 are very similar, near -130 mV (Table I of Millis *et al.*, enclosed). The redox potential for the Mn peroxidase isozymes H3 and H4 are similar to each other (- 88 mV and - 95 mV, respectively) and are more positive than the lignin peroxidases. The higher redox potential for the Fe^{3+}/Fe^{2+} couple is consistent with the heme active site of these fungal peroxidases being more electron deficient. This is in keeping with the ability of these enzymes to oxidize substrates of higher oxidation-reduction potential. These results would suggest a Compound I or II intermediate more electron deficient and consequently of higher oxidation-reduction potential.

To investigate the accessibilty of the heme active site to the substrate which is oxidized [veratryl alcohol and Mn (II)], we investigated whether these substrates had any affect the redox potential of the heme. The E_{m7} value for the lignin and Mn peroxidases are not affected by their respective substrates, veratryl alcohol and Mn (II). These results suggest that substrates do not directly interact with the ferric heme-iron as axial ligands. This is consistent with the present model for peroxidase catalysis. Suicide inhibitor (1) and nmr studies (2) indicate that the heme-iron of horseradish peroxidase (HRP) is not fully accessable to bulky substrates and suggest that the oxidation of substrates occur at the periphery of the heme.

Studies on lignin peroxidase compound I formation. This study demonstated that the mechanism of lignin peroxidase compound I formation is similar to other peroxidases and that the ionization controlling activity is shifted to a very acidic value. A unique property of the lignin peroxidases is their low pH optimum for catalysis. Furthermore, pH has no affect on lignin (3) and Mn peroxidase compound I formation (4). This is in contrast to all other peroxidases which exhibit decreased rates of compound I formation at low pH. Poulos and Kraut (5) proposed that the His of the distal pocket functions as an acid/base catalyst and must be deprotonated to catalyze compound I formation. This would suggest that either the mechanism for lignin peroxidase is different from other peroxidases or alternatively, an identical mechanism dependent on a residue with a much lower pKa value (e. g. below 2). We investigated the possibility of such an ionization with competitive binding studies. This type of study has been used by Lambeir *et al.* (6) to demonstrate the presence of an active site ionizable group with a pKa below 2 for chloroperoxidase. We used a similar technique with chloride and lignin peroxidase to demonstrate the presence of an active site ionization (see enclosed preprint by Cai and Tien). The results demonstrate that chloride inhibits lignin peroxidase catalysis by binding to the H_2O_2 binding site. Similar to $NaNO_3$ and chloroperoxidase, if the same ionizable group affects both chloride binding and compound I formation in lignin peroxidase, the binding of chloride should shift the equilibrium toward the protonated state causing a chloride-concentration-dependent increase in the apparent pKa.

$$EH\cdot Cl \underset{Cl^-}{\overset{K_{d,app}^{Cl}}{\rightleftharpoons}} EH \underset{H^+}{\overset{K_a}{\rightleftharpoons}} E \xrightarrow[k_1]{H_2O_2} \text{compound I}$$

A complete pH profile was constructed for each chloride concentration and the pKa,app calculated according to Equation 10 (see Cai and Tien, enclosed) using non-linear regression. These experimental results fully support the prediction that the pKa,app increases as chloride concentration increases, and are consistent with the presence of an acidic ionizable group in lignin peroxidase which affects compound I formation and is not detectable in the absence of chloride. By fitting this plot to Equation 9

(see Cai and Tien, enclosed) using non-linear regression, the intrinsic values for pKa and the K_d for chloride are calculated: pKa = 1.0 ± 0.3 and KCl_d = 100 ± 60 μM. The estimated pKa value is within the range for an Asp residue, which is found in the lignin peroxidase active site (7). The pKa for the carboxyl side chain of a free aspartic acid is normally equal to 3.9. The lowering of pKa by 3 units can be achieved by the formation of a salt bridge between aspartate and a positively charged group. These findings suggest that the mechanism of compound I formation is similar to other peroxidases (i.e. an ionizable group is needed) except that the pKa for this group is highly acidic.

Studies on post-translational modification of lignin and Mn peroxidases. We discovered in the granting period that the lignin peroxidases were phosphorylated (Kuan and Tien, J. Biol. Chem). The lignin peroxidase isozymes (H2, H6, H8 and H10) with the exception of isozyme H1 are phosphorylated. The Mn-dependent peroxidases do not appear to be phosphorylated. No phosphate was detected in lignin peroxidase isozyme H1 and Mn-dependent peroxidase isozymes H3, and H4 while approximate one molecule of phosphate per molecule of protein was found for isozymes H2, H6, H8 and H10. Analysis of products from partial acid hydrolysis indicated that no radioactivity co-migrated with either standard phosphoserine or phosphothreonine. A radioactive compound migrated slower than phosphothreonine and did not correspond to phosphotyrosine. The radioactivity, however, did co-migrate with sugar phosphate standards mannose 6-P and glucose 6-P. The presence of mannose 6-P was detected by its isomerization by mannose 6-P isomerase to yield fructose 6-P, which is then metabolized by a series of reactions to yield NADPH. N-glycanase which hydrolyzes asparagine-linked oligosaccharides from glycoproteins, was able to remove the phosphate moiety from the lignin peroxidases. These results indicate that the mannose-6-phosphate is contained on an asparagine-linked oligosaccharide. Amino acid sequences, derived from nucleotide sequencing of cDNA clones, also identified N-glycosylation sites which follow the general rule of Asn-X-Thr/Ser in isozymes H2 (8), H8 (7) and H10 (8).

The discovery that the lignin peroxidase isozymes contain mannose 6-P raises the possibility that they are (at some point) targeted to go through the lysosomes. Cytochemical studies actually support such a possibility. Recent study using immuno-gold labeling demonstrated that intracellular lignin peroxidase was most frequently observed to be enclosed with inner membranes (multivesicular vesicles). An earlier cytochemical study by Garcia *et al.* (9) using immuno-gold complexes also localized intracellular lignin peroxidase inside membrane-like structures or endocytic compartments.

Lysosomal targeting of extracellular enzymes is not unprecedented in lower eukaryotes. The utilization of lysosomes as a mechanism of protein secretion has been proposed for lower eukaryotes (10). Dimond and coworkers (11) have characterized the secretion of numerous lysosomal enzymes from *Dictyostelium.* Two such enzymes, α-mannosidase and β-glucosidase are both glycosylated with mannose 6-P-containing mannose-rich oligosaccharides, targeted for lysosomes and eventually secreted into the extracellular fluid. Because mannose 6-P is frequently utilized as a signal for lysosomal targeting, we suggest that lignin peroxidases are contained in lysosome-like structures prior to secretion.

Isolation of Mn peroxidase cDNAs and studies on its regulation. This study resulted in the isolation and characterization of a Mn peroxidase cDNA. The characterization of the cDNA has provided

valuable information of Mn peroxidase structure and regulation. The cDNA clone λMP-1 encoding a Mn peroxidase was isolated from a λgt 11 library (see Pease *et al.*, enclosed). The complete nucleotide sequence was determined. The sequenced N-terminus (AVXPDGTWVTNAAXXAFIPLA; underline denoting mismatches) was found in the deduced amino-acid sequence thus unequivocally demonstrating that this clone encodes a Mn peroxidase. The cDNA clone is 1312 bp long, possessing a 35-bp 5' and a 129-bp 3' untranslated sequence. Translation starting at this ATG codon predicts a preprotein of M_r 40,070 containing a 24 amino-acid predominantly hydrophobic leader sequence. Because the enzyme is secreted extracellularly, a leader sequence is expected. Cleavage of this leader peptide, ending at Glu-Ser, yields the mature protein with a predicted M_r of 37,711. This is lower than the experimentally-determined M_r of ~46,000. The M_r of the native protein determined from SDS-PAGE is most likely anomalously high due to glycosylation (12).

With the isolated cDNA, we were able to study the expression of the Mn peroxidase-specific mRNAs. Previous reports have indicated that the Mn peroxidases are produced during secondary metabolism. Expression of Mn peroxidase mRNA was detected by Northern blot analysis using randomly-primed λMP-1 cDNA insert as a hydridization probe. Poly(A)$^+$ RNA was isolated from day 1- 6 tissue of nitrogen-limited cultures and analyzed for the Mn peroxidase mRNAs. The probe hybridized to a 1.3 kb band indicating that λMP-1 is full length. The intensity of the 1.3 kb band parallels both the activity and protein appearance. The expression of the Mn peroxidases is similar to the lignin peroxidases in that both are expressed solely during secondary metabolism. However, the response to the onset of secondary metabolism is regulated differently between the two. Expression of Mn peroxidase was maximal on day 3 whereas lignin peroxidase was maximal on day 5.

The primary structure of the Mn-dependent peroxidase shows homology with the lignin peroxidase. The homology of the nucleotide and the deduced amino-acid sequence between λMP-1 and λML-1 (encoding isozyme H8) is 58% and 65%, respectively. The homology between these two peroxidases is most conserved at active site residues. Peroxidases contain two His residues which are proposed to be essential for activity. One of the His residues (proximal) is the axial ligand of the heme, the other His (distal) is located in the distal pocket. Residues in the distal pocket have been proposed to participate in peroxide cleavage to form compound I (5). Assuming a similar tertiary structure between the Mn and lignin peroxidases and others, a high degree of homology in residues flanking the proximal and distal His are observed. Residues near the distal His (distal pocket), Arg42, Asp47 and Leu43, are also conserved. The results would suggest similarity in the tertiary structure of these peroxidases despite a low percentage of homology in nucleotide sequence.

There is a region where the homology between the amino-acid sequence of Mn-dependent peroxidase and lignin peroxidases H8 and H2 approaches 90%. This region is located between the distal and proximal histidine of each isozyme, and consists of 14 hydrophobic amino acids (residues 108-122). Conservation of these residues would imply a functional significance. However, this sequence does not appear to be highly conserved among other peroxidases thus it may only be significant in the lignin degrading family of peroxidases.

Characterizing the heme active site by ^1H NMR spectroscopy. This study has provided valuable

information on the active site structure of lignin peroxidase. It has also provided information on the structural basis for the heme reactivity. ^{1}H NMR spectroscopy is a powerful tool for characterizing the active sites of heme proteins, and has shed light on structure-function relationships for several heme proteins (13). La Mar and coworkers have shown that this technique is well suited for many hemeproteins. We examined lignin peroxidase by ^{1}H NMR spectroscopy, and to compare the findings with those described in the literature for both HRP and cytochrome c peroxidase (CcP) in an attempt to relate structure with reactivity (see Banci *et al.*, enclosed).

We have specifically assigned 15 of the NMR signals and proposed tentative assignments for an additional four. Furthermore, 12 signals of the high spin form are collected into sets. These assignments were based on 1D NOE, NOESY and COSY. The data indicate a large degree of similarity between the lignin peroxidase and HRP spectra, however, some important differences are observed. Most significant are the shifted values for the protons of the proximal His. Considerable attention has been focused on the role of H-bonding of the proximal His in controlling heme electron density and reactivity. The strength of the imizadole H-bonding to neighboring amino acids has a significant influence on the imidazolate character of the axial His. In peroxidases where the high oxidation state of iron is stabilized, greater imidazolate character has been suggested and this in turn is believed to be caused by stronger H-bonding. In contrast, the Fe (II) state is stabilized in the globins, and less imidazolate character is observed, which is related to weaker H-bonding. In CcP, the only peroxidase for which the 3-D structure is known (Poulos 1980), the proximal His is strongly H-bonded to an Asp residue (Asp 235), whereas in myoglobin, it is weakly H-bonded to the carbonyl of the peptide backbone.

From model compound studies, the extents of the upfield shift of Hε1 proton of the proximal His have been proposed to be related to the imidazolate character of the His ring (14). This is an important issue because the basicity of the proximal His is believed to be one of the major factors that determine the redox potential of the heme. Indeed, the most relevant differences in the spectra of lignin peroxidase-CN and HRP-CN and CcP-CN are the shift values of the signal of the proximal His. Hε1 is less upfield-shifted, than they are in HRP-CN (15) and CcP-CN (16). It is interesting to note that the shift of the Hε1 in the cyanide adducts is inversely proportional to the redox potential (E^o Fe^{3+}/Fe^{2+}) of the native proteins in the series metmyoglobin, lignin peroxidase, CcP and HRP (see Fig. 5 of Banci *et al.*, enclosed). The relationship between the chemical shifts of the Hε1 proton and the redox potential would suggest that lignin peroxidase has less imidazolate character than HRP and CcP.

In conclusion, the NMR study has shown the fundamental similarity between lignin peroxidase, HRP and CcP. However, small differences are noted by ^{1}H-NMR spectroscopy which can be related to the differenet reactivity; in particular: *i)* the shift of the proximal His ε1 proton is related to the Fe^{3+}/Fe^{2+} redox potential and tentatively, to the imidazolate character; *ii)* the proximal His is oriented differently than in the other 2 peroxidases; and *iii)* a residue, possibly the Arg in the distal site is closer to the distal His.

Heterologous Expression of Lignin and Mn Peroxidases. This study is the first to demonstrated expression of *active* recombinant lignin and Mn peroxidase. Therefore, it represent a major technical

breakthrough. During the past granting period we made numerous attempts at expressing active lignin and Mn peroxidase in prokaryotic expression systems with little success. However, we have been able to express active recombinant lignin and Mn peroxidase in the eukaryotic insect tissue culture expression system. This work was in collaboration with Dr. Joe Li who first expressed at lignin peroxidase H8 (17). The recombinant protein is the same apparent molecular weight as the native enzyme and is fully active without *in vitro* protein folding.

Using the baculovirus system, we have been able to express lignin peroxidase isozymes H2 and H6. The recombinant enzymes are not yet purified; however, our data indicates that both are active. We have also been able to express active recombinant Mn peroxidase isozyme H4 (see the proposal for more details). The activity is dependent upon added Mn and H_2O_2. With a heterologous expression system, we are now prepared to initiate site-directed mutagenesis studies on these enzymes.

Studies on compound III (oxycomplex) stability. We have discovered that the oxycomplex of lignin peroxidase is very stable. Characterizing this complex has revealed valuable information on the reactivity of the heme active site. Oxycomplex is readily formed by mixing ferroperoxidase with O_2 gas, air, or an O_2-containing buffer. Its decompostion results in formation of ferric enzyme. We found that the oxycomplex of lignin peroxidase isozyme H1 is more stable than that of HRP, whereas the oxycomplex of isozyme H8 is more stable than isozyme H1. The stability of these oxycomplex was such that the reversibility of the O_2 binding to ferroperoxidase of isozymes H1 and H8 was observed by CO-trapping.

Numerous factors have been identified which affect O_2 binding to hemeproteins and heme model compounds. Again, particular attention has been given to the role of the axial His in O_2 binding. The observation that the axial His is H-bonded has led to the hypothesis that the extent of H-bonding increases the basicity of the axial His. The basicity has a major effect on the electron density of the heme. Increased basicity of the axial ligand *i)* stabilizes the higher oxidation states of the heme iron; *ii)* increases O_2 affinity, presumably by decreasing the dissociation rate of O_2; *iii)* polarizes the covalent Fe(II)-O_2 bond to cause the transfer of electron density from the iron to the oxygen imparting the oxycomplex with greater $Fe^{3+}\cdot O_2^-$ character; this can eventually promote the oxidation of the heme iron by O_2 to form higher valence intermediates.

Reactions of the oxycomplex. Much controversy exist on the reactions of lignin peroxidase H_2O_2. Our work with compound III (oxycomplex) is in contrast with many of the observations made by Wariishi and Gold (20). These workers reported that the formation of lignin peroxidase compound III can lead to inactivation of the enzyme. They also reported that lignin peroxidase compound III readily reacts with veratryl alcohol (a substrate made by ligninolytic cultures of *P. chrysosporium*) to form resting enzyme and superoxide. Later, Wariishi *et al.*(21) modified their mechanism and proposed the formation of a new peroxidase intermediate, compound III*. Compound III* is similar to compound III except that it is reportedly formed from the reaction of compound III (oxycomplex) with H_2O_2. Wariishi *et al.* (21,22) proposed that it was a complex of compound III with H_2O_2. Evidence for the formation of compound III* comes mainly from stopped-flow rapid scan experiments (54) where a new intermediate in the reaction of compound II with H_2O_2. Their modified mechanism now states that it is compound

III* that reacts with veratryl alcohol to form resting enzyme and superoxide. Again, veratryl alcohol is not oxidized from this reaction but only serves to displace the superoxide:

$Fe^{3+}O_2^-$ + veratryl alcohol -------> Fe^{3+} + O_2^- + veratryl alcohol

Evidence for the above reaction comes from the lack of veratraldehyde formation in single turnover experiments (cyanide was added to trap the ferric enzyme and prevent further reaction of ferric enzyme with the excess H_2O_2 and detection of superoxide in these single turnover experiments from its reaction with tetranitromethane. In contrast, we showed that compound III does not react with veratryl alcohol because no spectral changes were observed upon addition of veratryl alcohol to compound III (23). However, by the addition of only 1 equivalence of H_2O_2, compound III is quickly converted back to resting enzyme with concomitant formation of veratraldehyde as demonstrated by increase in 310 nm absorbance. Addition of another equivalence of H_2O_2 resulted in a further increase in veratraldehyde formation. It has also been shown that cyanide inhibits veratraldehyde formation. Aust and coworkers (24) have shown that cyanide is an inhibitor of veratryl alcohol oxidation because it is a substrate of lignin peroxidase. Furthermore, other researchers have shown that cyanide is a peroxidase substrate (25). It is thus not surprising that veratraldehyde is not formed in the presence of cyanide. Finally, the use of tetranitromethane for detection of superoxide is problematic. We have recently discovered that tetranitromethane reacts with compound III *directly* to produce the reduced product analogous to the reaction with superoxide. Thus it is not surprising that tetranitromethane is reduced in these reaction mixtures.

Topics proposed in the original proposal but not completed in the granting period.

Under this heading there are two categories: research topics not presently being pursued and topics still on-going. Many projects are still on-going. Under the category of not being pursued is the study on of suicide inhibitors such as phenylhydrazine. These studies were aimed at defining the accessibility of the active site to the reducing substrate. The conclusions of the study will be provided briefly here. We found that like HRP, lignin peroxidase is also modified at the heme periphery. These results suggest that substrate oxidation occurs at the heme periphery. The study entailed the use of ^{14}C-labelled phenylhydrazine. We found covalent modification of the heme. No radiolabelled was found with products associate with covalent modification near the heme iron but only at the heme periphery. Attempt to elucidate the structure of these products proved to be difficult due to the low yield of products. Subsequently, DePillis *et al.* (26) published a paper will conclusion similar to ours. We found that the results of these studies were of limited value and did not pursue it any further.

References Cited.

1. Ortiz de Montellano, P. R. (1987) Acc. Chem. Res. 20, 289-294.
2. La Mar, G. N., de Ropp, J. S., Smith, K. M., Langry, K. C. (1980) J. Biol. Chem. 255, 6646-6652.
3. Andrawis, A., Johnson, K. A., and Tien, M. (1988) J. Biol. Chem. 263, 1195-1198.

4. Wariishi, H., Dunford, H. B., MacDonald, I. D., and Gold, M. H. (1989) J. Biol. Chem. 264, 3335-3340.
5. Poulos, T. L. and Kraut, J. (1980) J. Biol. Chem. 255, 8199-8205.
6. Lambeir, A.-M., Dunford, H. B., and Pickard, M. A. (1983) J. Inorg. Chem. 19, 291-300.
7. Tien, M. and Tu, C.-p. D. (1987) Nature 326, 520-523; also Nature 328, 742.
8. de Boer, H. A., Zhang, Y. Z., Collins, C., and Reddy, C. A. (1987) Gene 60, 93-102.
9. Garcia, S., Latge, J. P., Prevost, M. C., and Leisola, M. (1987) Appl. Environ. Microbiol. 53, 2384-2387.
10. Burns, R. A., Live, G. P., and Dimond, R. L. (1981) Dev. Biol. 84, 407-416.
11. Freeze, H. H. (1986) Mol. Cell. Biochem. 72, 47-65.
12. Tien, M., Kirk, T. K., Bull, C., and Fee, J. A. (1985) J. Biol. Chem. 261, 16870-1693.
13. Goff, H. M. (1983) in *Iron Porphyrins*, eds. Lever, A. B. P. and Gray, H. B., Addison-Wesley, pp. 237-281.
14. Chacko, V. P. and La Mar, G. N. (1982) J. Am. Chem. Soc. 104, 7002-7007.
15. Thanabal, V. de Ropp, J. S. and La Mar, G. N. (1988) J. Am. Chem. Soc. 110, 3027-3035.
16. Satterlee, J. D., Erman, J. E., De Ropp, J. S. (1987) J. Biol. Chem. 262, 11578-11583.
17. Johnson, T. M. and Li, K.-k. J. (1991) Abstract, ASBC and MB Meeting, Atlanta, GA.
18. Wittenberg, J. B., Noble, R. W., Wittenberg, B. A., Antonini, E., Brunori, M., and Wyman, J. (1967) J. Biol. Chem. 242, 626-634.
19. Millis, C. D., Cai, D., Stankovich, M. T. and Tien, M. (1989) Biochemistry 28, 8484-8489.
20. Wariishi, H. and Gold, M. H. (1989) FEBS Letters 243, 165-168.
21. Wariishi, H., Marquez, L., Dunford, H. B. and Gold, M. H. (1990) J. Biol. Chem. 265, 11137-11142.
22. Wariishi, H., and Gold, M. H. (1990) J. Biol. Chem., 265, 2070-2077.
23. Cai, D. and Tien, M. (1989) Biochem. Biophys. Res. Commun.162, 464-469.
24. Fernando, T., Grover, T. and Aust, S. D. (1991) Arch. Biochem. Biophys., in press.
25. Moreno, S. N., Stolze, K., Janzen, E. G., and Mason, R. P. (1988) Arch. Biochem. Biophys.
26. DePillis, G. D., Wariishi, H., Gold, M. H., and Ortiz de Montellano, P. R. (1990) Arch. Biochem. Biophys. 280, 217-223.

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