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Cloning and sequencing of chloroperoxidase cDNA*

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ABSTRACT

An oligod-d(T)$_{12-18}$ primed cDNA library has been prepared from *Caldariomyces fumago* mRNA. A clone containing a full-length insert was sequenced on the supercoiled plasmid, pBR322. The complete primary sequence of chloroperoxidase has been derived. We have also determined about 73% of the peptide sequence by amino acid sequencing. The DNA sequence data matches all of the available known peptide sequences. The mature polypeptide contains 300 amino acids having a combined molecular weight of 32,974 daltons. A putative signal peptide of 21 amino acids is proposed from DNA sequence data. The chloroperoxidase gene encodes three potential glycosylation sites recognized as Asn-X-Thr/Ser sequences. Three cysteine residues are found in the protein sequence. A small region around Cys$_{87}$ bears a minimal homology to the active site of cytochrome P$_{450cam}$. No other heme protein homologues can be detected. We propose that Cys$_{87}$ serves as a thiolate ligand to the iron of heme prosthetic group. A rare arginine codon, AGG, is used three times out of twelve in contrast to the very infrequent use of this codon in *E. coli* or yeast.

INTRODUCTION

Chloroperoxidase, an enzyme which is secreted at high levels by *Caldariomyces fumago*, was discovered by Shaw and Hager in 1959 (1). Cultures of *C. fumago* grown on fructose as the sole carbon source routinely accumulate 500 mg per liter of chloroperoxidase as a secreted protein. Under conditions of fructose induction, chloroperoxidase is produced at a purity level of ~85% in the culture filtrate. The mature enzyme is heavily glycosylated and contains a heme prosthetic group (2). It catalyzes peroxidative halogenations involved in the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentenedione) (2,3). The enzyme has been crystallized (2) and the physical and biochemical properties of the enzyme has been studied extensively (2-6). The enzyme exhibits several interesting features. Besides the unusual peroxidative halogenation reactions, the enzyme also has a potent catalase activity (4). In the absence of halide ion the enzyme acts as a peroxidase similar to plant peroxidases, such as horseradish peroxidase (4). Various spectroscopic studies indicate that chloroperoxidase has a very similar heme

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environment to that of the cytochrome P_{450} family of hemoproteins (7-14). Recently, Bangcharoenpaurpong et al., has demonstrated that chloroperoxidase has a sulfur ligand which shows nearly identical properties to that of P_{450cam}, although subtle differences do exist (15). Thus, chloroperoxidase appears to have an enzyme active site which links it to a wide variety of hemoproteins. These characteristics makes it interesting and important to carry out further detailed studies on the enzyme. Therefore we are now in the process of examining potential structure-function relationships of chloroperoxidase to catalase, horseradish peroxidase, and the P_{450} cytochromes. One very effective approach to these problems is to change amino acid residues at the active site of the enzyme by site-directed mutagenesis and observe the changes in catalysis and spectroscopic properties of the mutant enzyme. This experimental approach requires that the DNA coding for chloroperoxidase be cloned, sequenced, and expressed in a suitable vector. The tertiary structure of the enzyme must also be known in order to define potential active site residues. In this paper, we report the cloning and sequencing of complementary chloroperoxidase DNA. We also present the complete amino acid sequence of chloroperoxidase. These results should provide a basic foundation for future work on the expression and the site-directed mutagenesis studies on chloroperoxidase. The sequence data will also greatly facilitate the crystallographic studies on the tertiary structure of the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Chloroperoxidase from *Caldariomyces fumago* strain ATCC 16373 was prepared and assayed as previously described (1). Trypsin and chymotrypsin were from Worthington Biochemicals. *Staphylococcus aureus* V-8 protease, trifluoroacetic acid and phenylisothiocyanate (Sequanal grade) were obtained from Pierce. HPLC solvents were obtained from Burdick and Jackson or J. T. Baker. Sodium dodecyl sulfate (electrophoresis grade) was from Bio-Rad.

Oligo-d(T) cellulose, type II, was from Collaborative Research. Oligo-$d(T)_{12-18}$ was from P-L Biochemicals. Dideoxy and deoxy nucleoside triphosphates were from Phamacia. RNase and *r*-Prep A were from Promega Biotec. Reverse transcriptase for cDNA synthesis was from Life Sciences. Reverse transcriptase for DNA sequencing, calf intestine alkaline phosphate, T4 polynucleotide kinase and ultra pure urea were from Boehringer Mannheim. RNase H, T4 DNA polymerase, terminal deoxynucleotidyltransferase and pBR322

dG-tailed at the Pst I site were from Bethesda Research Laboratories. Colony/Plaque Screen, Gene Screen Plus, and ^{32}P orthophosphate were from New England Nuclear. Restriction enzymes were from BRL, Phamacia, or Boehringer Mannheim. Alpha-^{32}P-dATP(≈800 Ci/mmol) was from Amersham.

Proteolytic Cleavages

Digestions were performed at 37°C for 15 hours at a protease to chloroperoxidase ratio of 1:50 in filter-sterile solutions. The reactions were stopped by lyophilization. Trypsin and chymotrypsin were used in 1% ammonium bicarbonate. *S. aureus* V-8 protease was used in a buffer containing 50 mM ammonium bicarbonate and 4 M urea. Restricted tryptic cleavage at arginine residues was accomplished after phenlthiocarbamylation of lysine side chains by Edman's reagent. In this procedure, 50 nmol of dried chloroperoxidase were added to 50 µl of a precoupling buffer consisting of ethanol/triethylamine/water (2:1:1 v/v/v). This mixture was dried to a film. Fifty microliters of a coupling solution consisting of ethanol:triethylamine:phenylisothiocyanate:water (7:1:1:1 v/v/v/v) were then added and the derivatization allowed to proceed for 30 minutes at 25°C. The reaction mixture was then dried under vacuo. The derivatized protein was purified by reverse phase HPLC before trypsin treatment. Mild acid cleavage at aspartyl residues was performed in 0.1% trifluoroacetic acid at 100°C for 3 hours. Samples were then lyophilized or directly injected onto the reverse phase HPLC column.

Separation and Analysis of Peptides

Peptide separation by reverse phase HPLC was achieved using a Vydac 214 TP 54 C4 analytical column (250 x 4.6 mm) and a Beckman model 332 gradient chromatograph operated at a flow-rate of 1.5 ml/min. Mobile phase A was 0.1% aqueous trifluoroacetic acid, wherease mobile phase B consisted of 0.1% trifluoroacetic acid in a mixture of acetonitrile/isopropanol (2:1 v/v). All peptides were completely eluted with a linear gradient from 0 to 80% mobile phase B. Peptides were detected at 217 nm, collected manually, and dried under vacuo. Before application to the sequencer's filter, they were redissolved in 30 microliters of 0.1% sodium dodecyl sulfate. Peptide sequencing was performed on an Applied Biosystems model 470 A gas-phase protein sequencer, using programs and chemicals supplied by the manufacturer. Phenylthiohydantoin amino acids were analyzed by reverse phase HPLC as previously described (2).

Purification of Poly A(+) RNA

Poly A(+) RNA was purified from *C. fumago* as previously described (16).

Construction of a cDNA Library of C. fumago and Isolation of cDNA Clones

Double stranded cDNA was synthesized from the polyA-RNA using oligo-$d(T)_{12-18}$ as a primer according to the method described by Gubler and Hoffman (17), except that 50 mM KCl was included for the first strand synthesis, and E. coli DNA ligase and NAD were omitted from the reaction mixture for the second strand synthesis. Double stranded cDNA was tailed with d(C) residues and annealed to d(G)-tailed pBR322. E. coli strain RR1 was transformed with this annealing mixture according to the method developed by Mandel and Higa (19). Transformants were selected on LB plates containing 15 µg/ml tetracycline. To screen the library, about 3200 clonies were grown on Colony/Plaque $Screen_{TM}$ membranes. These colonies were lysed and the membranes were dried according to the direction provided by the manufacturer. A previously reported oliognucleotide probe (29mer) (16) was used in the colony hybridization (20) and Southern analysis (21).

DNA Sequencing on Supercoiled Plasmid

DNA sequencing was performed on supercoiled plasmids using the Sanger dideoxy sequencing technique (22). Templates were prepared according to the mini plasmid preparation described in Molecular Cloning (23). The procedure developed by Zagursky et al. (24) was used with the following exceptions. About 6-fold more primer was used for each sequencing reaction, the ethanol precipitation step was carried out at ice temperature and the concentrations of nucleotides were optimized for the double strand sequencing reaction.

Oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer model 380A. Originally, crude primer products were purified by trityl-specific reverse phase HPLC before being used in sequencing reactions (25). Later a simpler procedure was developed. A 100 µl aliquot of crude primer dissolved in concentrated ammonium hydroxide was directly dried in a Speed Vac. The resulting pellet was mixed vigorously with 1 ml of 70% ethanol on a Vortex agitator at room temperature. The pellet was recollected by centrifugation in a microfuge for 5 min at room temperature. After removal of the supernatant fraction, the pellet was dried briefly and dissolved in an appropriate volume of water. This oligonucleotide solution was used without further purification. The quality of sequence data obtained using these crude primer preparations was equivalent to those obtained using HPLC purified primers.

Maxam-Gilbert Sequencing

A small portion of the DNA at the 5' end of the message complement strand was sequenced by the Maxam-Gilbert technique (26). The chloroper-

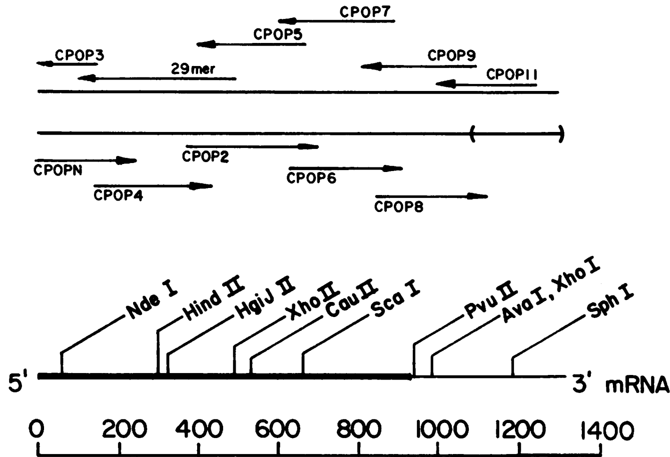

Figure 1. Sequencing strategy and restriction map of the cDNA insert in pGFx79. In the top panel, lines and arrows represent sequences and directions of sequences obtained by Maxam-Gilbert method. The sequence obtained using the 29mer as primer is indicated. In the bottom panel, unique type II restriction sites are indicated. The cDNA insert is shown in the direction of mRNA synthesis. In order to carry out Maxam-Gilbert sequencing, pGFx79 was cleaved with PstI, and the cDNA fragment was labeled with ^{32}P at the 5' ends, and then cleaved with PvuII after inactivation of T4 polynucleotide kinase. The smaller fragment was used for Maxam-Gilbert sequencing.

oxidase cDNA insert, generated by cleaving pGFx79 with PstI, was labeled with ^{32}P at the 5' ends. This labeled insert was then cleaved with PvuII, which generates 2 fragments. The smaller fragment (approximately 400 bp) was used for sequencing.

Preparation of M13 Templates

M13 templates for dideoxysequencing were prepared according to the method developed by Messing (27).

Table 1. Oligonucleotide Primers

29mer	ATTTCGTTCATGTC$^{T}_{G}$GCGTAGTCGAAGTG
CPOP2	GACCACTCCTTCTC
CPOP3	GTTCAGAGCTGGGCA
CPOP4	TGCCCAGCTCTGAAC
CPOP5	TTCCAGTATTTCCACC
CPOP6	GGTGGAAGTATTGGTT
CPOP7	TGGGGTCATGGTG
CPOP8	CACCATGACCCCA
CPOP9	GGAATATCTCGATG
CPOP11	TCGTGCAATCGGAA
CPOPN	GCCGCCCTCCCTCA

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CTAAAGCATCTGGCTCACAACAAGCCTCTGCTCATTCATTCGCTCTAAGTCTTTGAATCT

GTGATTCTTCGATACTTCTCTCGCT ATG TTC TCC AAG GTC CTT CCC TTC
                          MET Phe Ser Lys Val Leu Pro Phe
                                                        1
GTG GGA GCG GTT GCC GCC CTC CCT CAC TCC GTC CGT CAG GAG CCT
Val Gly Ala Val Ala Ala Leu Pro His Ser Val Arg Gln Glu Pro
                             10
GGC TCC GGC ATT GGC TAC CCA TAC GAC AAC AAC ACC CTG CCA TAT
Gly Ser Gly Ile Gly Tyr Pro Tyr Asp Asn Asn Thr Leu Pro Tyr
         20                                      30
GTC GCC CCA GGT CCT ACC GAC TCT CGT GCT CCT TGC CCA GCT CTG
Val Ala Pro Gly Pro Thr Asp Ser Arg Ala Pro Cys Pro Ala Leu
                             40
AAC GCT CTT GCC AAC CAC GGT TAC ATT CCT CAC GAT GGC CGT GCC
Asn Ala Leu Ala Asn His Gly Tyr Ile Pro His Asp Gly Arg Ala
         50                                      60
ATC AGC AGG GAG ACC CTC CAG AAC GCT TTC CTC AAC CAC ATG GGT
Ile Ser Arg Glu Thr Leu Gln Asn Ala Phe Leu Asn His Met Gly
                             70
ATT GCC AAC TCC GTC ATT GAG CTT GCT CTG ACC AAC GCC TTC GTC
Ile Ala Asn Ser Val Ile Glu Leu Ala Leu Thr Asn Ala Phe Val
         80                                      90
GTC TGC GAG TAC GTT ACT GGC TCC GAC TGT GGT GAC AGC CTT GTC
Val Cys Glu Tyr Val Thr Gly Ser Asp Cys Gly Asp Ser Leu Val
                            100
AAC CTG ACT CTG CTC GCC GAG CCC CAC GCT TTC GAG CAC GAC CAC
Asn Leu Thr Leu Leu Ala Glu Pro His Ala Phe Glu His Asp His
        110                                     120
TCC TTC TCC CGC AAG GAT TAC AAG CAG GGT GTC GCC AAC TCC AAC
Ser Phe Ser Arg Lys Asp Tyr Lys Gln Gly Val Ala Asn Ser Asn
                            130
GAC TTC ATC GAC AAC AGG AAC TTC GAT GCC GAG ACC TTC CAG ACC
Asp Phe Ile Asp Asn Arg Asn Phe Asp Ala Glu Thr Phe Gln Thr
        140                                     150
TCT CTG GAT GTC GTT GCA GGC AAG ACC CAC TTC GAC TAT GCC GAC
Ser Leu Asp Val Val Ala Gly Lys Thr His Phe Asp Tyr Ala Asp
                            160
ATG AAC GAG ATC CGC CTT CAG CGC GAG TCC CTC TCC AAC GAG CTT
Met Asn Glu Ile Arg Leu Gln Arg Glu Ser Leu Ser Asn Glu Leu
        170                                     180
GAC TTC CCC GGT TGG TTC ACC GAG TCC AAG CCA ATC CAG AAC GTC
Asp Phe Pro Gly Trp Phe Thr Glu Ser Lys Pro Ile Gln Asn Val
                            190
GAG TCT GGC TTC ATC TTC GCC CTT GTC TCT GAC TTC AAC CTG CCC
Glu Ser Gly Phe Ile Phe Ala Leu Val Ser Asp Phe Asn Leu Pro
        200                                     210
GAC AAC GAT GAG AAC CCT CTG GTT CGC ATT GAC TGG TGG AAG TAC
Asp Asn Asp Glu Asn Pro Leu Val Arg Ile Asp Trp Trp Lys Tyr
                            220
TGG TTC ACC AAC GAG TCC TTC CCA TAC CAC CTC GGC TGG CAC CCC
Trp Phe Thr Asn Glu Ser Phe Pro Tyr His Leu Gly Trp His Pro
        230                                     240
CCG TCT CCA GCC AGG GAG ATC GAG TTC GTC ACC TCC GCC TCC TCC
Pro Ser Pro Ala Arg Glu Ile Glu Phe Val Thr Ser Ala Ser Ser
                            250
GCT GTC CTG GCT GCC TCT GTC ACC TCT ACT CCA TCT TCC CTT CCA
Ala Val Leu Ala Ala Ser Val Thr Ser Thr Pro Ser Ser Leu Pro
        260                                     270
TCC GGT GCC ATC GGC CCA GGT GCC GAG GCT GTC CCT CTC TCC TTC
Ser Gly Ala Ile Gly Pro Gly Ala Glu Ala Val Pro Leu Ser Phe
                            280
GCC TCC ACC ATG ACC CCA TTC CTC CTC GCC ACC AAT GCT CCT TAC
Ala Ser Thr Met Thr Pro Phe Leu Leu Ala Thr Asn Ala Pro Tyr
        290                                     300
TAC GCC CAG GAC CCA ACT CTC CGG CCC CAA CGA CAA GCG TGA GCC
Tyr Ala Gln Asp Pro Thr Leu Arg Pro Gln Arg Gln Ala stop

TGCCCCAGCTGCCACCACCTCCATGGCCGTCTTCAAGAACCCATACCTCGAGGCCATTGG

CACCCAGGACATCAAGAACCAGCAGGCTTACGTCAGCTCCAAGGCTGCTGCCATGGCCTC

TGCCATGGCCGCCAACAAGCCCCGCAACCTTTAAGCCCATCTACGACATCGAGATGGTCC

AGCATTCACTGTTCCGAATGTGAATGTGCTACTTAACGACAACACCGACGAAATGCCTTTC

CTCACTACAGCATCAGCATGCAGAGGCGCATTTGCTTTTTCCCGCATAGATCGGCTGGCT

TGGTCACGAGTTTTCCGATTGCACGATTGATTTTATACAATAGAACGATAGACTATAGAC

ATAGAAAAAACTGTATGCATTTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

AAA
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RESULTS AND DISCUSSION

Isolation of the cDNA Clone

A cDNA library of *C. fumago* was prepared according to the method described in Experimental Procedures. Approximately 3200 clones in the library were screened with the specific oligonucleotide probe (29mer) which corresponds to chloroperoxidase amino acid residues 167 to 177 (16). Five positive clones were detected, four of which were verified by Southern blot analysis. The clone containing the longest insert (approximately 1.5 kb) was designated pGFx79 and was used in further experiments.

The cDNA insert of chloroperoxidase on pGFx79 was directly sequenced on the supercoiled plasmid. The oligonucleotide probe used for screening (29mer) was used as the first primer. More primers were then synthesized based on the new sequence information. The sequences of the eleven oligonucleotides used as primers for dideoxy sequencing are shown in Table 1. This strategy was used to sequence the most of the cDNA (see Fig. 1).

Dideoxysequencing of the 5' end of the message complement strand failed, presumably due to the long stretch of poly A in this strand. This segment was sequenced by the Maxam-Gilbert method (26) as shown in Fig. 1. The 3' end portion of the opposite strand was sequenced using a primer which hybridized just 5' to the stretch of poly A residues. Primers hybridizing to the 3' side of the poly A region did not sequence through the poly A region, although these primers could sequence through the oligo-d(C) tail without difficulty.

The sequence from the insert in pGFx79 contained all of the sequence information for mature chloroperoxidase. The insert also contained an in frame sequence coding for 9 amino acids upstream from the N-terminal of the mature enzyme; however a translational start codon, ATG, was not detected. Since we had previously obtained a partial cDNA clone primed with the 29mer (pMA340), further sequence information not covered by pGFx79 was obtained from this clone (16).

Figure 2. Nucleotide Sequence and Translated Amino Acid Sequence of Chloroperoxidase. The arrow marks the N-terminus of the mature enzyme. Three cysteine residues and the positions of the rare codon AGG are underlined. The three potential glycosylation sites are also indicated by a dashed line. The numbering of amino acid residues is shown on the top of the corresponding codons, starting from the first residue in the mature chloroperoxidase.

Analysis of the Overall Structure - The cDNA sequence and the translated amino acid sequence of chloroperoxidase are shown in Fig. 2. The sequence covers the whole translational reading frame. The sequence extends 85 nucleotides to the 5' side of the translational start codon and 380

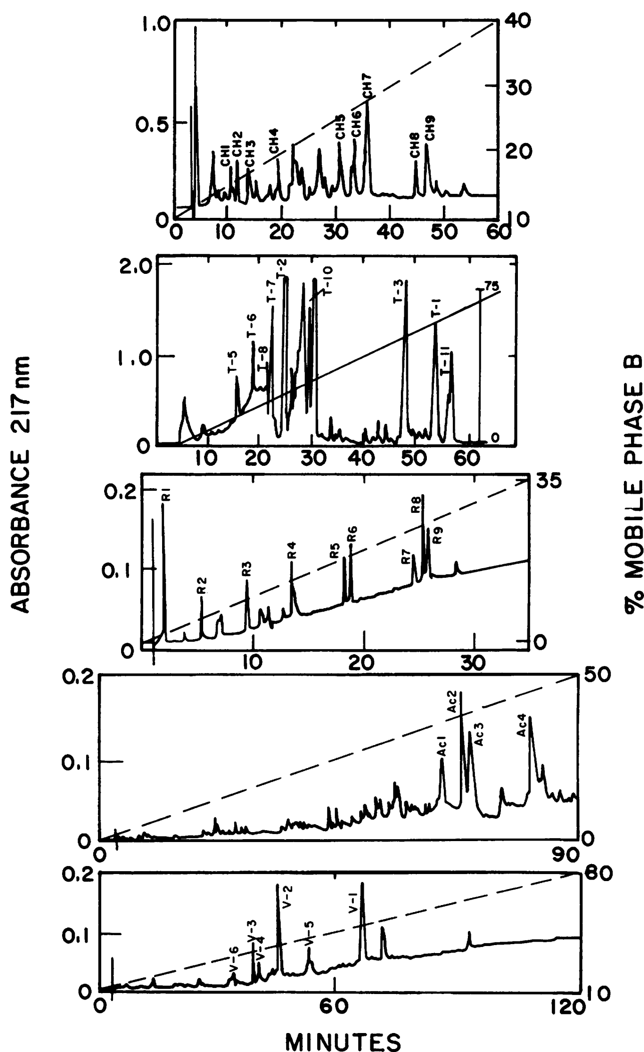

Figure 3. HPLC Isolation of Chloroperoxidase Peptides. Peptides were prepared and chromatographed according to the methods described in "Experimental Procedures". Peptides were prepared by hydrolysis of chloroperoxidase using chymotrypsin (CH), trypsin (T), restricted trypsin cleavage (R), mild acid (Ac), and V-8 protease (V).

nucleotides beyond the last nucleotide of the first in-frame stop codon, plus a 40 nucleotide long poly A tail. We have also sequenced approximately 73% of the total amino acid sequence of chloroperoxidase. The accuracy and validity of the DNA sequence of chloroperoxidase presented in Fig. 2 was verified by comparison with the amino acid sequence data. The isolation of peptides from various proteolytic treatments is shown in Fig. 3. The subsequent sequencing of the amino acid residues in these peptides gave the sequence data shown in Fig. 4.

The mature chloroperoxidase is composed of 300 amino acid residues. A hydrophobic peptide of 21 amino acid residues is encoded by chloroperoxidase mRNA immediately upstream of the first residue of the mature polypeptide. We propose that this short hydrophobic peptide is a signal peptide cleaved from premature chloroperoxidase during secretion.

Three potential glycosylation sites of the type Asn-X-Thr/Ser are found in the polypeptide sequence. Identification of two of these sites as glycosylation sites has been supported by primary peptide sequencing data. It has been previously shown that chloroperoxidase is a heavily glycosylated protein (2).

Biochemical studies have shown that chloroperoxidase contains a thiolate ligand to the iron atom of the heme prosthetic group. It is believed that this thiol group comes from a cysteine residue (7-15). As

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N-term          <EPGSGIGYPYDDDTLPYVAPGPT

                                             → V-5
T-1/V-5         ETLQNAFLNHMGIANSVIXLALTNAFVVXEYVTGNDXXDXLVXLXL

T-5             QGVANSNDFIDNR

                   → CH-8
T-3/CH-8        ESLSNELDFPGWFTESKPIQNV

                          → AC-3      →T-2  → T-10     → T-6
V-1/R-4         SGFIFALVSDFNLPDNDENPLVRIDWWKYWFTNESFPYHLGWHPPSP-
                            →CH-9          →CGP-2

                 → R-4
                AREIXFVTVANSAVLAANVXXXPXSLPXGAIGP

                             →T-8
V-6/T-8         AVPLXFAXXMXPFLLAXNAPYYAQDP

CH-2            ALANHGYIP

                            →T-7
V-3/T-7         TFQTSLDVVAGKTHFDYADMNEI
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Figure 4. Assignment of Amino Acid Sequences to Chloroperoxidase Peptides.

shown in Fig. 3, chloroperoxidase contains 3 cysteine residues. We assume that two of the cysteine residues form a disulfide bond (29) and the other serves as the thiolate ligand. In chloroperoxidase, cys 87 is the most likely candidate for the thiolate ligand role since there is some homology in this region between cytochrome P_{450}cam and the chloroperoxidase amino acid sequence.

Codon Usage

Codon usage in chloroperoxidase was examined using the Bionet System program. The codon usage in chloroperoxidase was compared to that in 25 *E. coli* genes. A general similarity in the frequency of codon usage between the coding sequence of chloroperoxidase and 25 *E. coli* genes was seen. However, a striking difference exists in the utilization of one of the arginine codons, AGG. Only one out of 345 arginine residues in the 25 *E. coli* genes is encoded by AGG whereas 3 out of 12 arginines in chloroperoxidase are encoded by this rare codon.

Future Experiments

Currently, experiments to express chloroperoxidase in *E. coli* are being conducted. These experiments should produce an unglycoslated enzyme which can serve several purposes. It will be interesting to compare the catalytic activity of the carbohydrate-free enzyme with that of the native enzyme. The carbohydrate-free chloroperoxidase might be a better product to use for X-ray crystallographic studies. In the past, we have always experienced difficulties in obtaining high quality crystals of the native enzyme, presumably due to the heterogeneity resulting from heavy glycosylation.

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