

Expression of a Synthetic Gene for Horseradish Peroxidase C in *Escherichia coli* and Folding and Activation of the Recombinant Enzyme with Ca^{2+} and Heme*

(Received for publication, January 16, 1990)

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A synthetic gene encoding horseradish peroxidase isoenzyme C (HRP C) has been synthesized and expressed in *Escherichia coli*. The nonglycosylated recombinant enzyme (HRP C*) was produced in inclusion bodies in an insoluble inactive form containing only traces of heme. HRP C* was solubilized and conditions under which it folded to give active enzyme were determined. Folding was shown to be critically dependent upon the concentrations of urea, Ca^{2+} , and heme and on oxidation by oxidized glutathione. Purification of active HRP C* from the folding mixture gave a peroxidase, with about half the activity of HRP C. Glycosylation is thus not essential for correct folding and activity. The C-terminal and N-terminal extensions to HRP identified previously in cloned cDNA sequences are also not required for correct folding. However, Ca^{2+} appears to play a key role in folding to give the active enzyme. The overall yield of purified active enzyme was 2–3%, but this could be increased by reprocessing material that precipitated during folding.

Peroxidases (1, 2) are a group of heme-containing hydrogen peroxide-utilizing enzymes that act on a wide variety of substrates and have diverse biological roles. Much effort (cf. Ref. 3) has gone into investigations aimed at establishing the structural features of peroxidases that distinguish them from other heme proteins and provide the basis for their distinctive properties. The peroxidase family of enzymes shows amino acid sequence homology (4–7), and three members or groups that have received particularly detailed attention are yeast cytochrome-c peroxidase, horseradish peroxidase (HRP),¹ and

the peroxidases of white rot fungus (7, 8). The first-named enzyme is the only peroxidase for which high resolution structural information is available (9) from x-ray crystallography; the second is the subject of this paper. A common feature of the peroxidases is the strong conservation of amino acids in the regions of the histidine residues on either side of the heme binding pocket. Limited sequence homology between HRP C and cytochrome-c peroxidase has been used (10) for simple modeling of the structure of the former enzyme.

Peroxidase action, as long established (cf. Ref. 2) for HRP, involves $2e^-$ oxidation of the enzyme by hydrogen peroxide, to give a species known as Compound I. This then reverts to the resting state, via two successive $1e^-$ reactions with reducing substrate molecules, the first yielding a second enzyme intermediate, Compound II. Much effort has gone into establishing the structures of Compounds I and II, as this is obviously crucial to understanding the catalytic mechanism. For cytochrome-c peroxidase, the intermediates have been studied in detail by x-ray crystallography (11) and by a variety of spectroscopic methods (eg. Ref. 12) and by using site-directed mutagenesis (13) to replace selected amino acid residues at the active center of the protein. For HRP, only spectroscopic studies (cf. Ref. 14) have so far been possible. There are well established differences between the two enzymes in the structures of their Compound I. For cytochrome-c peroxidase (cf. Ref. 15), iron is in the ferryl ($\text{Fe}^{\text{IV}}=\text{O}$) state and an unpaired electron resides on an amino acid residue, now identified (12) as Trp-191. In HRP, in contrast, ferryl iron is magnetically coupled (cf. Ref. 14) to a porphyrin π -radical cation.

As isolated from plants, HRP has a carbohydrate content of 18% (2) and consists of more than 30 multiforms (10). Isolation of the dominant isoenzyme (HRP C) requires careful purification (16). HRP C is a monomeric glycoprotein of 308 residues with eight oligosaccharide side chains. It has a calculated protein M_r of 33,922 (17) and has been shown (18, 19) to contain up to 2 mol of calcium ions/mol protein, although only one of these appears (20, 21) to be important for maintaining the structure and function of the enzyme. In contrast, cytochrome-c peroxidase has not been reported to bind calcium.

HRP finds important practical uses in clinical work, in enzyme immunoassays, and in histological chemistry as a "reporter" enzyme. Recently chemiluminogenic assays (22, 23) have been developed, having potential for greater sensitivity than is shown by conventional chromogenic assays.

It would be of considerable interest to probe the functioning of HRP C by varying amino acid residues in the heme environment. To this end, a system for the expression and over-

* This work was supported by the Science and Engineering Research Council through a Collaborative Research Award under the Molecular Recognition Initiative. A preliminary report of some of this work has been published (49) and was presented as a poster at the Biochemical Society Meeting, Guildford, United Kingdom, July 1989, Communication Abstr. 107. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J05552.

¹ The abbreviations used are: HRP, horseradish peroxidase (unspecified variant); apoHRP, horseradish peroxidase apoenzyme (whether folded or unfolded) and lacking heme, Ca^{2+} , or both; HRP C, horseradish peroxidase, isoenzyme C, glycosylated plant enzyme; HRP C*, unglycosylated recombinant peroxidase; TB, terrific broth; TBE, Tris/borate/EDTA (Ref. 26); TEMED, N,N,N',N' -tetramethylethylenediamine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; MES, 4-morpholine-ethanesulfonic acid; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonate); FPLC, fast protein liquid chromatography.

production of an active recombinant peroxidase, amenable to site-directed mutagenesis, is required. A bonus from such work is the possibility that an unglycosylated recombinant HRP C might yield crystals, suitable for x-ray analysis, more readily than does the plant enzyme. In this paper, we describe the construction of a synthetic gene encoding HRP C, based on the amino acid sequence of the mature protein published by Welinder (24) and confirmed in the sequence of one of three cDNA clones subsequently isolated by Fujiyama *et al.* (25). We describe expression of the gene in *Escherichia coli*, solubilization of the inactive enzyme from inclusion bodies, and its folding to give the active enzyme, HRP C*.

EXPERIMENTAL PROCEDURES

DNA manipulations, growth media, and buffers (unless individually specified) were as described by Manniatis *et al.* (26).

Oligodeoxyribonucleotides—The oligonucleotides depicted in Fig. 1 were synthesized by automated solid phase phosphoramidite chemistry. They were de-protected and removed from the controlled-pore glass support by incubation in concentrated NH_3 . Typically, 50 mg of controlled-pore glass carrying 1 μmol of oligonucleotide was de-protected by incubation for 5 h at 70 °C in 600 μl of concentrated NH_3 . The supernatant was transferred to a fresh tube, and the oligomer was precipitated with 3 volumes of ethanol. Following centrifugation, the pellet was dried and resuspended in 1 ml of water. The concentration of crude oligomer was then determined by measuring the absorbance at 260 nm.

For gel purification a sample (equivalent to 1 ml with A_{260} 10) of the crude oligonucleotide was dried and resuspended in 15 μl of marker dye (90% formamide (de-ionized), 10 mM Tris, 10 mM borate, 1 mM EDTA, 0.1% bromophenol blue). The samples were heated at 90 °C for 1 min and then loaded on to a 1.2-mm-thick denaturing polyacrylamide gel with 1.6-mm wide slots. The gel was prepared from a stock of 15% acrylamide, 0.6% bisacrylamide, and 7 M urea in 1 $\times$ TBE (26) and was polymerized with 0.1% ammonium persulfate and 0.025% TEMED. The gel was pre-run for 1 h. The samples were run at 1500 V for 4–5 h. The bands were visualized by UV shadowing, and those corresponding to the full-length product were cut out and transferred to micro test tubes. The oligomers were eluted from the gel slice by soaking overnight in a solution containing 0.5 M ammonium acetate, 0.01 M magnesium acetate, and 0.1% SDS. The elutes were then transferred to fresh tubes and the oligomer precipitated with 3 volumes of ethanol at –70 °C for 15 min. The precipitate was collected by centrifugation in an Eppendorf Microfuge for 10 min, the pellet washed in 80% ethanol, the purified oligomer dried, redissolved in 1 ml of water, and finally filtered through a 0.45- μm microfilter. The concentration of purified product was measured by determining A_{260} .

Ligation and Cloning of Oligonucleotides—The desired gene sequence was divided into a front-half and a back-half of 501 and 474 base pairs, respectively. Both halves were designed with a common *Xho*I site to allow for the complete gene to be assembled with a single cloning step. The front- and back-halves of the gene were divided into 24 and 22 oligodeoxyribonucleotides (oligomers), respectively, as depicted in Fig. 1. The division was such as to provide 7-base cohesive ends, after annealing complementary pairs of oligomers to form linkers. The end points of the oligomers were chosen to minimize the potential for inappropriate ligation of oligomers at the assembly stage.

All the oligomers with the exception of the 5'-terminal oligomers BB279 and BB302 for the front-half, and BB303 and BB324 for the back-half were then phosphorylated with T4 polynucleotide kinase, to provide them with a 5' phosphate group, as required for the ligation step. Complementary oligomers were then annealed and the oligomers ligated together by T4 DNA ligase, as depicted in Fig. 1. The ligation products were separated on a 2% low gelling temperature gel, and the bands corresponding to the front- and back-halves of the horseradish peroxidase gene were cut out and extracted from the gel. The purified fragments were then ligated separately to *Eco*RI/*Hind*III-cut DNA of the plasmid vector pUC18. The ligated products were transformed into HW87 and plated on L-agar plates (26) containing 100 $\mu\text{g ml}^{-1}$ ampicillin.

Nucleotide Sequence Analyses and Final Assembly of HRP C* Gene—Colonies containing potential clones were then grown in L-broth (26) containing ampicillin at 100 $\mu\text{g ml}^{-1}$ and plasmid DNA isolated. Positive clones were identified by direct dideoxy sequence

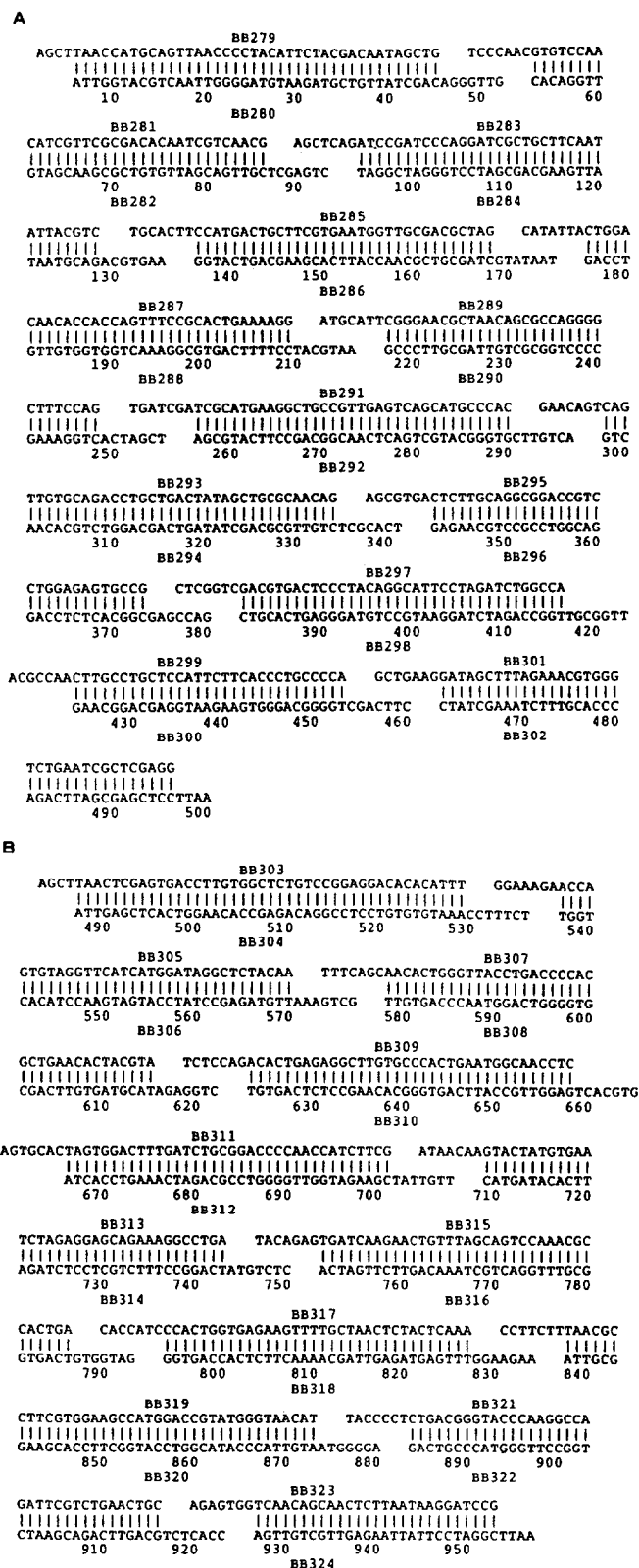


FIG. 1. A, design of oligomers for synthetic horseradish peroxidase gene: 5'-half. B, design of oligomers for synthetic horseradish peroxidase gene: 3'-half.

analysis of the plasmid DNA by using the 17-base universal primer, a reverse sequencing primer complementary to the opposite strand on the other side of the polylinker, and some of the oligomers employed in the assembly of the gene that served as internal primers. One front-half and one back-half clone were subsequently re-sequenced on both strands to confirm that no mutations were present. The complete gene was then assembled by isolating the 466-base pair

XhoI *EcoRI* fragment from a back-half clone and ligating it to a front-half clone that had also been digested with *EcoRI* and *XhoI*. The identity of the final construct was confirmed by restriction analysis and subsequent complete resequencing. The complete sequence is shown in Fig. 2.

Expression of HRP C* in *E. coli*—The synthetic gene was excised from pUC18-HRP at the *NdeI* and *BamHI* sites and recloned into

the expression vector pGC517, to give plasmid pSD18, in which HRP expression was driven from the *tac* promoter. Tight regulation of *tac* expression was ensured by using a host *E. coli* K₁₂ strain (HW1110) which carried a chromosomal *lacI^r* mutation (HW1110 is derived from W3110).

An overnight culture of *E. coli* HW1110 (pSD18) (10 ml of L-broth) was inoculated into 1.5 liters of terrific broth, containing 100 $\mu\text{g ml}^{-1}$ ampicillin. One liter of terrific broth contained: Bacto-tryptone (24 g), Bacto-yeast extract (24 g), glycerol (4 ml), KH_2PO_4 (17 mmol), and K_2HPO_4 (72 mmol). Cultures were grown for 10 h at 37 °C with vigorous shaking (A_{600} 1.7–1.8), before induction with 0.5 mM isopropyl- β -D-thiogalactoside and grown for a further 18 h to a final A_{600} of 2.9–3.1. Cultures were harvested and resuspended in 30 ml of Buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at –20 °C.

Isolation and Extraction of Unfolded apoHRP C*—To 10 ml of bacterial suspension (equivalent to 500 ml of culture) was added 20 mg of lysozyme (Sigma), 100 μg of DNase I, phenylmethanesulfonyl fluoride to 0.5 mM, and EDTA to 10 mM. After 30 min on ice, lysed bacteria were sonicated (MSE ultrasonic disintegrator MK2) for 3 $\times$ 30 s. After centrifugation at 10,000 $\times g$ for 30 min, the pellet (approximately 50% pure HRP C* as judged by SDS-PAGE) was resuspended in 10 ml of Buffer A (see above) containing 2 M urea, homogenized by hand, and re-sedimented at 10,000 $\times g$ for 30 min. The pellet was washed two further times with Buffer A containing 2 M urea before finally being dissolved in 2 ml of Buffer A containing 8 M urea. The residual pellet was re-sedimented and extracted with a further 2 ml of 8 M urea buffer, centrifuged as above, and the two 8 M urea supernatants pooled. This gave a urea-solubilized apoHRP C* preparation that was >80% pure, as judged by SDS-PAGE.

Reduction of Disulfides and Further Purification of Reduced ApoHRP C*—ApoHRP C* (2.2 mg ml^{-1}) solubilized in 8 M urea (4 ml) was brought to 30 mM DTT and left at 30 °C for 1 h under N_2 . A 4-ml sample of the reduced protein was applied to a 2.5 $\times$ 100-cm column of Sephacryl S-200, equilibrated with Buffer A (see above) containing 6 M urea and 5 mM DTT. Gel filtration was carried out at 22 °C at a flow rate of 50 ml/h. ApoHRP C* was eluted just beyond the void volume, and fractions containing >90% pure HRP C*, as determined by SDS-PAGE, were pooled, rapidly frozen in liquid N_2 , and stored at –20 °C.

Excess DTT was removed by passing a 2-ml sample through a 9-ml Sephadex G-25 column (Pharmacia LKB Biotechnology Ltd., PD-10) equilibrated with Buffer A (see above) containing 6 M urea and 1 mM DTT. Protein-containing fractions (0.5 ml) were pooled; these were identified by transferring 10- μl aliquots to a microtiter plate and adding 200 μl of diluted ($\times$ 5) Bio-Rad protein assay reagent (Bio-Rad Laboratories, München, Germany; catalog No. 500-0006).

Small-scale Assay of Folding and Heme Incorporation—The following procedure was used for initial optimization of folding conditions (Figs. 5 and 6). Purified reduced urea-treated apoHRP C* (8 μg in 20 μl of 6 M urea, containing 1 mM DTT) was added to 180 μl of folding medium. The composition of the folding medium was varied (see legends) but for maximum activity contained: 50 μl 6 M urea in Buffer A (see above), 10 μl of 0.1 M CaCl_2 , 5 μl of 0.2 mM bovine hemin (Sigma, Type II) in Buffer A (without EDTA), 110 μl of Buffer A, and 5 μl of 28 mM oxidized glutathione.

After 1 h at 22 °C, development of peroxidase activity was monitored by transferring 5- μl aliquots of folding mixtures to the wells of a microtiter plate. The assay was started by the addition of 100 μl of a solution containing 0.4 mg of o-phenylenediamine in 50 ml of phosphate-citrate buffer, pH 5.0 (51 mM Na_2HPO_4 , 24 mM citric acid), and 0.35 mM H_2O_2 and stopped after 5 min at 20–25 °C by the addition of 40 μl of 3 M H_2SO_4 . A_{490} values of the contents of wells were recorded on a microtiter plate reader (MR700, Dynatech) and expressed as relative units.

Large-scale Folding, Heme Incorporation, and Purification of Active HRP C*—Purification of reduced urea-treated HRP C* on Sephacryl S-200 was omitted; the protein was solubilized in 8 M urea, reduced, and transferred into Buffer A containing 6 M urea and 1 mM DTT. The sample (4 ml) was diluted 10-fold into 40 ml of folding medium. The conditions and amounts of protein are summarized in Table I. After 24 h at 22 °C, the folding medium was centrifuged (10,000 $\times g$, 30 min) and the soluble protein (40 ml) dialyzed against two changes of 1.0 l of 20 mM sodium acetate buffer, pH 4.3. Precipitated HRP C* was removed by centrifugation. The soluble peroxidase activity was adsorbed on a 1.0-ml cation exchange column (Mono S HR5/5; Pharmacia LKB Biotechnology Ltd. FPLC system) that had been equilibrated with the same buffer, and eluted with a linear NaCl

M Q L T P T F Y D N S C P N V S N
AAGCTTAACCATGAGTTAACCCCTACATCTACGACAATAGCTGCCAACGTGTCCAA
HindIII HpaI
TTCGAATTGGTACGTCATTTGGGGAGTGAAGATGCTGTTATCGACAGGTTGCACAGGTT
10 20 30 40 50 60
I V R D T I V N E L R S D P R I A A S I
CATCGTTCCGCACAACTCGTCAACAGCTCAGATCCGATCCAGGATCGTGTCTCAAT
NciI SacI SspI
GTAGCAAGCGCTGTGTAGCAGTGTCTGAGTCTAGGCTAGGGTCTAGCGACGAAGTTA
70 80 90 100 110 120
L R L H F H D C F V N G C D A S I L L D
ATTACGCTGCACTTCCATGACTGCTTCGTGAATGGTTGCGACGCTAGCATATTACTGGA
NheI
TAATGCAGAGCTGAAGTACTGACGAAGCACTTACCAACGCTGCGATCGTATAATGACCT
130 140 150 160 170 180
N T T S F R T E K D A F G N A N S A R G
CAACACCACAGTTTCCGCACTGAAAGGATGCATTCCGGGAACGTAACAGCGCCAGGGG
NsiI
GTTGTGGTGGTCAAAGCGTGACTTTTCTACGTAAGCCCTTGCATTGTGCGGGTCCCC
190 200 210 220 230 240
F P V I D R M K A A V E S A C P R T V S
CTTCCAGTGCATCGATCGAAGGCTGCCGTTGAGTCAGATGCCACGACAGTCAG
ClaI/PvuI SphI
GAAAGTCTCAGTACGCTACTTCCGACGGCACTCAGTCTGACGGTCTGTGTCAGTC
250 260 270 280 290 300
C A D L L T I A A Q Q S V T L A G G P S
TTGTGCAGACCTGCTGACTATAGCTCGGCAACAGAGCGTGACTCTTGACGGCGGACCGTC
BspMI FspI RsrII
AACACGCTCGGACGAGTACTGACGCGGTTGTCTGCACTGAGAAGCTCGCGCTGGCAG
310 320 330 340 350 360
W R V P L G R R D S L Q A F L D L A N A
CTGGAGAGTGCCGCTCGGTGCGAGTACCTACAGGCATTCCTAGATCTGGCAACGC
SalI BglII/BalI
GACCTCTCAGGCGAGCGAGCTGCACTGAGGATGTCCGTAAGGATCTAGACCGGTTGCG
370 380 390 400 410 420
N L P A P F F T L P Q L K D S F R N V G
CAACTTGCTGCTCAATTTCTACCTGCCCCAGCTGAAGGATAGCTTTAGAAACGTGGG
PvuII
GTTGAACGGACGAGGTGAAGAGTGGGAGCGGCTGCTATCGAAATCTTTGCACCC
430 440 450 460 470 480
L N R S S D L V A L S G G H T F G K N Q
TCTGAATCGCTCAGTGACCTTTGTGCTCTGTCGGAGACACATTGGAAAGAACCA
XhoI BspMI
AGACTTAGCGAGCTCACTGGAACAGGACAGCGCTCTGTGTGAACCTTTCTTGGT
490 500 510 520 530 540
C R F I M D R L Y N F S N T G L P D P T
GTGTAGGTTTCATCATGGATAGGCTCTCAATTTAGCAACACTGGGTTACCTGACCCAC
BstEII
CACATCCAAGTAGTACCTATCCGAGATGTTAAAGCTGTGTGACCACTGAGCTGGGGTG
550 560 570 580 590 600
L N T T Y L Q T L R G L C P L N G N L S
GCTGAACACTACGTATCTCCAGACACTGAGAGGCTGTGCCCCACTGAATGGCAACCTCAG
SnaBI PflMI
CGACTTGTGATGATAGAGTCTGTGACTCTCCGAACACGGGTGACTTACCGTTGGAGTC
610 620 630 640 650 660
A L V D F D L R T P T I F D N K Y Y V N
TGCACTAGTGGACTTTGATCTGCGGACCCCAACCATCTTCGATAACAGTACTATGTGAA
ApaI/SpeI ScaI
ACGTGATCACTGAACTAGACGCTGGGGTTGGTAGAAGCTATTGTTCATGATACATT
670 680 690 700 710 720
L E E Q K G L I Q S D Q E L F S S P N A
TCTAGAGGAGCAGAAAGGCTGATACAGAGTCAAGAACTGTTTAGCAGTCCAAACGC
XbaI StuI BclI
AGATCTCCTCGCTTTCCGGACTATGTCTCACTAGTCTTGACAACTCGTCAGGTTTGGC
730 740 750 760 770 780
T D T I P L V R S F A N S T Q T F F N A
CACTGACACCATCCCACTGGTGAAGATTTTGCTAACTCTACTCAACCTTCTTTAACGC
BstXI
GTGACTGTGGTAGGGTGACCACTCTTCAAAACGATTGAGATGAGTTTGAAGAAATTGGC
790 800 810 820 830 840
F V E A M D R M G N I T P L T G T O G Q
CTTCGTGAAGCCATGGACCGTATGGGTAACTATCCCTCTGACGGGTACCAAGGCCA
NcoI KpnI
GAAGCACCTTCGGTACCTGGCATAACCATTTGTAATGGGAGAGTGGCCCTGGTTCGGT
850 860 870 880 890 900
I R L N C R V V N S N S * *
GATTCGTGAACCTGACAGTGGTCAACAGCAACTCTTAAGGATCCGAATTC
PstI BamHI EcoRI
CTAAGCAGACTTACGCTCTCACCAGTTGCTGTTGAGAATTATCTCAGGCTTAAG
910 920 930 940 950

FIG. 2. Sequence of the synthetic horseradish peroxidase gene, HRP C*.

based assay (see "Experimental Procedures").

The results in Fig. 5 show that generation of activity was critically dependent on the urea concentration, 2 M being optimal. The levels of activity generated were low and developed slowly over 18 h (data not shown), but were strictly dependent on the HRP C* polypeptide (Fig. 5) and, as is discussed below, on Ca^{2+} and heme.

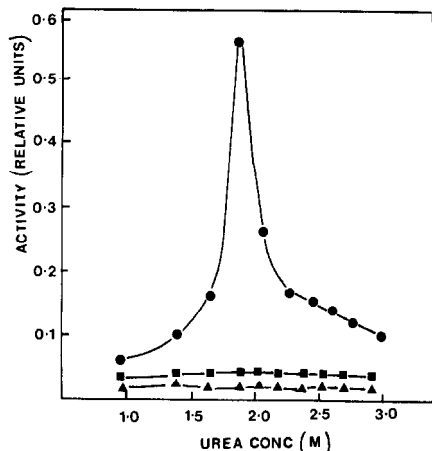


FIG. 5. Optimum urea concentration for folding of recombinant HRP C*. The small-scale refolding assay (see "Experimental Procedures") was used to determine the optimum urea concentration. Reduced apoHRP C* was extracted into 6 M urea, containing 1 mM DTT, and diluted 10-fold into aliquots (200 μl) of a refolding medium containing 0.5–6.0 M urea in Buffer A, 30 μg HRP C* ml^{-1} , CaCl_2 (5 mM), and bovine hematin (Sigma) (5 μM). Relative activities were measured in a microtiter plate-based peroxidase assay, by using 5- μl aliquots of folding mixture. The 6 M urea extracts used were derived from induced cultures of HW1110 (pSD18 vector + HRP gene) (●) and HW1110 (pGC517 vector plasmid only) (■). ▲, contained no protein (urea only).

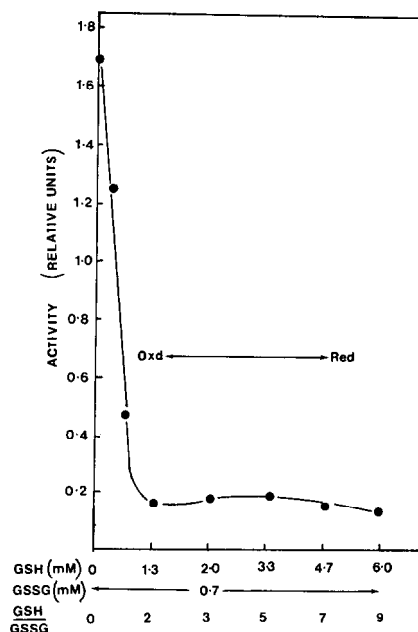


FIG. 6. Requirement for an oxidizing redox couple for folding of recombinant HRP C*. The small-scale folding assay was used to determine the effect of varying the ratio of reduced to oxidized glutathione in the folding medium on the generation of activity. The concentration of reduced glutathione (GSH) was varied from 0 to 6 mM, whereas the concentration of oxidized glutathione (GSSG) was kept constant at 0.7 mM. In addition, 0.1 mM DTT was present throughout. Other conditions were as specified for Fig. 5. Relative activities were measured in the microtiter plate-based peroxidase assay.

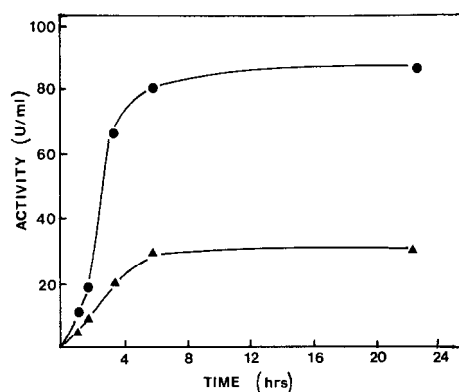


FIG. 7. Time course for folding of recombinant HRP C* at different enzyme concentrations. Samples (5–20 μl) were removed from the folding medium at intervals during folding experiments (as described in the legend to Table I) and assayed for peroxidase activity by using pyrogallol as substrate. ●, 200 μg ml^{-1} HRP C*; ▲, 100 μg ml^{-1} HRP C*.

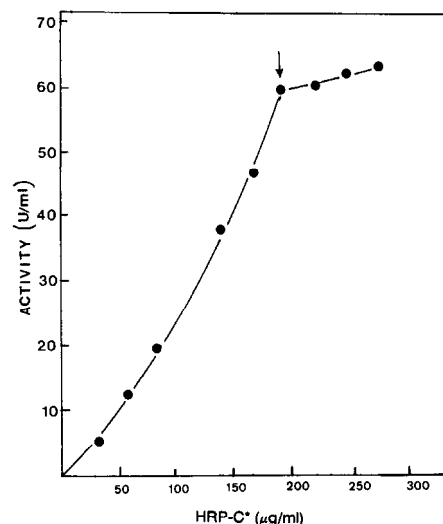


FIG. 8. Effect of protein concentration on the folding of recombinant HRP C*. Folding conditions and measurement of peroxidase activity were as described in Table I, except that the concentration of reduced HRP C* in the medium was varied between 0 and 275 μg ml^{-1} . The arrow indicates the maximum concentration for optimum folding.

HRP contains four intramolecular disulfide bonds (24). The experiment of Fig. 5 was carried out without addition of oxidizing agents. However, both the rate of development and the level of the activity generated were considerably enhanced, when oxidized glutathione was added, to facilitate disulfide bond formation (data not shown). The experiment of Fig. 6 shows that (in contrast to the situation with some proteins, e.g. Ref. 37) even small additions of reduced glutathione to the system, which already contained 0.1 mM DTT, were inhibitory. Folding appeared complete after 6 h (Fig. 7), although further small increases in activity (approximately 10%) were possible up to 24 h later. All the above experiments were carried out at 20–25 °C; additional studies at 4 and 37 °C indicated that the extent of folding to give HRP activity was essentially independent of temperature.

The effect of protein concentration on folding was investigated in the experiment depicted in Fig. 8. Total activity generated in the folding medium increased steadily with increasing concentrations of reduced HRP C*, up to a concentration of about 200 μg ml^{-1} , but there was little further increase beyond this point.

TABLE I

Isolation and activation of recombinant HRP C*

Steps in the extraction and solubilization from inclusion bodies of inactive apoHRP C* are summarized, followed by data on its folding to give the active holoenzyme and on the final purification of this material. Details are given under "Experimental Procedures." The starting material was 500 ml of terrific broth culture. The inclusion bodies contained 50% apoHRP C*, as indicated by SDS-PAGE. After washing and extraction with urea, the content had increased to 80%. For folding, reduced apoHRP C* (4 ml) in 6 M urea containing 1 mM DTT was diluted to a final volume of 40 ml in the following folding medium (final concentrations): 50 mM Tris-HCl, pH 8.0, 1 mM EDTA (Buffer A), 2 M urea, 5 mM CaCl₂, 5 μ M bovine hemin, 0.7 mM oxidized glutathione, 100 μ M reduced DTT, and 200 μ g apoHRP C*. The final product (see Table II), after FPLC, was homogeneous by SDS-PAGE (see Fig. 10). In a number of similar experiments, the overall yield of active enzyme during folding and FPLC was 2–3%.

Step	Volume ml	Protein mg/ml	Total activity units ^a	Total protein mg	Specific activity units ^a /mg	Purification factor activity	Yield activity
Low-speed pellet containing HRP inclusion bodies	2.1	9.04	ND	19.1	ND		
Pellet, extracted (washed) $\times$ 3 with 2 M urea, then solubilized in 8 M urea	4.0	2.20	ND	8.80	ND		
After 24-h incubation in folding medium	40	0.21	3,120	8.80	355	$\times$ 1	(100%)
After dialysis and centrifugation	42	0.008	3,190	0.336	9,500	$\times$ 27	102%
After FPLC on a Mono S column	3.0	0.057	2,460	0.170	14,500	$\times$ 41	79%

^a Pyrogallol assay (see "Experimental Procedures").

^b ND, not determined.

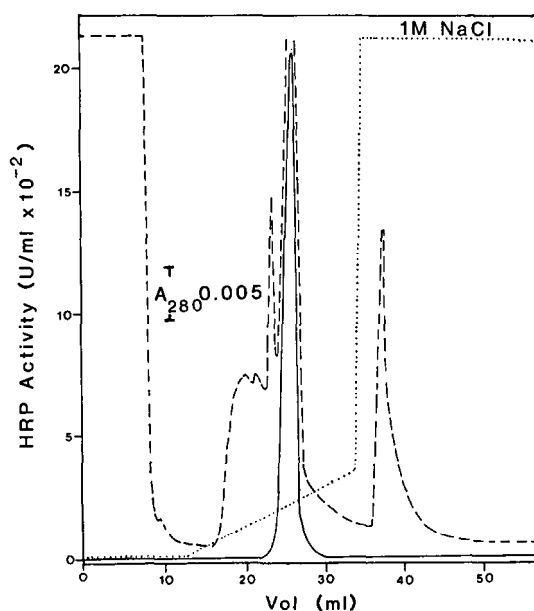


FIG. 9. Purification of active recombinant HRP C* from the folding medium by cation exchange chromatography. An enzyme sample in the folding medium was dialyzed against 20 mM sodium acetate buffer, pH 4.3, and adsorbed to a 1-ml FPLC cation exchange column (Mono S HR5/5), equilibrated with the same buffer. Elution was with a linear NaCl gradient, 0–140 mM, in 20 ml. The amount of protein and the volumes used were those shown in Table I. —, HRP activity; ---, A_{280} ; ····, NaCl concentration.

Having optimized the folding conditions, larger-scale folding and purification of recombinant HRP C* was undertaken. For this work, chromatographic purification of the urea-solubilized apoenzyme was omitted and replaced by chromatography of the final active holoenzyme. Table I shows the results of a typical activation and purification experiment (see legend for a summary of the conditions). Microbodies from a 500-ml culture were prepared and apoHRP C* solubilized and reduced as described under "Experimental Procedures." This gave a preparation that was 80% pure (Fig. 2). Reduced urea-treated denatured apoHRP C* was diluted into the optimized folding mixture. After 24 h at 20–25 °C, a dark red/brown

precipitate had formed; this was inactive. However peroxidase activity was detectable in the soluble fraction, which, after centrifugation, accounted for 4% of the total protein.

It is likely that the inactive precipitate is the result of formation of intermolecular disulfide linkages, since Coomassie-stained nonreducing SDS-PAGE showed the disappearance of monomeric HRP C* and the appearance of high molecular weight HRP C* multimers during the renaturation procedure (data not shown). We found that solubilization of the inactive precipitate in 8 M urea, followed by a further cycle of reduction and folding, allowed the recovery of another 4% of the protein as active soluble HRP C*. The data thus suggest that, under the conditions chosen, the folding pathway of HRP C* proceeds mainly to a conformation that is vulnerable to intermolecular disulfide formation.

Isolation and Characterization of Folded HRP C*—The soluble peroxidase activity obtained in the folding procedure was concentrated and purified by FPLC cation exchange chromatography (Fig. 9; Table I). Active recombinant enzyme was eluted as a single peak, at the same salt concentration (70 mM NaCl) as the plant enzyme, HRP C. It was homogeneous by SDS-PAGE (Fig. 10), responded to HRP antibodies, and migrated as expected from its predicted M_r (34,000), whereas the plant enzyme appeared larger (M_r 42,000), presumably due to its extensive glycosylation (24). This difference in mobility was even more pronounced under nondenaturing PAGE conditions (data not shown).

Specific activity measurements and determination of heme from the intensity of the Soret peak at 402 nm indicate (Table II) that the two enzymes are similar. The recombinant holoenzyme (HRP C*) appears to be as active² as the plant enzyme (HRP C), but the preparation seems to be contaminated with some 40–60% of inactive heme-free enzyme. Changes (not shown) in the absorption spectra confirmed that the refolded recombinant enzyme reacted with peroxide analogously to the wild-type plant enzyme. The spectra for the two enzymes in Fig. 11 are nearly identical and correspond largely to Compound III (2).

Dependence on Heme and Calcium Ions during Activation of HRP C*—The presence of calcium ions in plant HRP C

² See Table II, Footnote c.

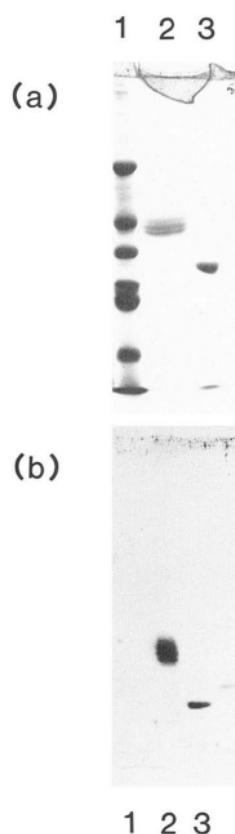


FIG. 10. Electrophoresis of pure recombinant HRP C* and of plant HRP C. Samples (10 μ l) of FPLC column fractions (cf. Fig. 9) were subjected to SDS-PAGE under reducing conditions in the presence of β -mercaptoethanol and then (a) stained with Coomassie Blue or (b) detected in a parallel experiment by immunoblot analysis (see "Experimental Procedures"). Samples were: lane 1, markers (bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (M_r 20,100), and α -lactoglobulin (M_r 14,200)); lane 2, plant HRP C; lane 3, recombinant HRP C*.

has already been noted. Complete removal of Ca^{2+} with 6 M guanidinium chloride and 10 mM EDTA (18) reduced the specific activity by 60% and dramatically increased the thermal lability of the plant enzyme. Retention of 40% of the initial activity after removal of calcium ions was confirmed (19). An extension (21) of these studies, by using nmr and metal ion substitution, indicated that a single calcium ion was required to maintain the protein structure in the vicinity of the heme. The absolute dependence (Fig. 12) of the recombinant enzyme on calcium ions for activation was therefore a most interesting finding and throws light on the nature of the folding pathway. Not only were calcium ions essential for obtaining activity but they appeared to be necessary, also, for the folding of the reduced polypeptide. Thus, if calcium ions were omitted from the folding mixture and 1 mM EDTA was included, no peroxidase activity and, moreover, no soluble protein was formed (data not shown). Clearly, therefore, calcium ions play a key role in the folding of HRP C*.

The conditions required for removing heme from plant HRP C (treatment with acid and butanone) are even more drastic than are those for removal of calcium ions from the enzyme. Activity is restored (38) on adding heme (or a modified heme) back to the apoenzyme. In our folding experiments, the recombinant enzyme required only a 2-fold molar excess of heme for maximal activation (data not shown). Although both heme and calcium ions were present together in the

TABLE II

Comparison of properties of recombinant HRP C* and plant HRP C

A sample of the recombinant enzyme, HRP C*, purified by FPLC (see "Experimental Procedures" and Table I) was compared with the plant enzyme (HRP C; Sigma Grade VI, further purified by FPLC).

Property	Plant enzyme	Recombinant enzyme
Specific activity (units ^a /mg protein ^b)	1150	630 ^c
A_{402}/A_{280}	3.29	2.11
A 1% protein ^b	30.6 ^d	11.8 ^e
A 1 cm, 402		

^a The ABTS assay was used.

^b The bicinchoninic acid protein determination was used, with bovine serum albumin as standard.

^c If specific activity is expressed as units ml^{-1}/A_{402} , the data indicate that the recombinant enzyme was ~ 1.4 times as active as the plant enzyme.

^d This result is in agreement with the theoretical value, calculated from M_r 33,922 (from the amino acid analysis) and ϵ (402 nm) $102 \text{ mm}^{-1} \text{ cm}^{-1}$ (1) of 30.1. However, agreement could be fortuitous, since proteins can differ substantially in the values of $A_{280}/\text{mg} \cdot \text{protein}$ in the bicinchoninic acid protein determination (33) and since the carbohydrate of the enzyme might interfere.

^e The low value could be explained in part if carbohydrate in the plant enzyme was responding as protein in the bicinchoninic acid assay. Glucose is known (33) to give a positive response. However, for HRP C, it is difficult to calculate the extent of such interference precisely.

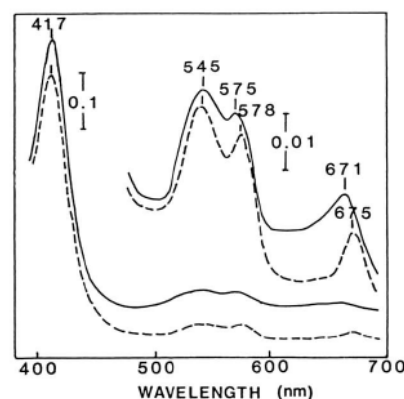


FIG. 11. Absorption spectra of HRP C* and HRP C in the presence of H_2O_2 . An approximately 5-fold molar excess of H_2O_2 was added to samples of plant HRP C and of recombinant HRP C* in 20 mM MES, pH 6.0, and visible absorption spectra were recorded after 1–2 min. ---, recombinant enzyme; —, plant enzyme.

folding mixture, heme, unlike calcium ions, was not absolutely required for folding. Thus, in additional experiments, providing calcium ions were present in the mixture, it was found that heme could be added after dialysis and purification (according to the procedures of Table I) of an apoenzyme, although the recovery of active protein was then only half that shown in the table. These observations support the conclusion that calcium ions are required for HRP C* to form a structure capable of binding heme.

Efficiency of Folding of HRP C*—The efficiency of folding of HRP C* isolated from *E. coli* by our procedure is only 3–4%, in terms of conversion of inactive protein to active enzyme. To understand the reason for this it would be necessary to compare, under identical conditions, the efficiency of folding of the recombinant enzyme with that of the plant enzyme. We carried out only very limited studies in this direction. We subjected a sample of the plant enzyme to a cycle of denaturation, reduction, and refolding. The enzyme was treated with 8 M urea, in the presence of 30 mM DTT and 1 mM EDTA. These conditions proved inadequate to remove more than a proportion of the heme, since on gel

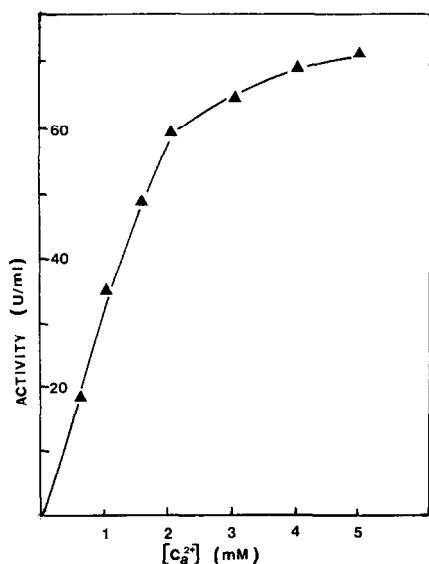


FIG. 12. Requirement of Ca^{2+} for folding of recombinant HRP C*. The folding conditions and measurement of peroxidase activity were as described for Table I, except that the Ca^{2+} concentration was varied from 0 to 5 mM. Note that the medium also contained 1 mM EDTA.

filtration (to remove the urea), much of the color remained with the protein fraction. However, the product was completely inactive, and samples were therefore permitted to refold at a range of different urea concentrations. The other parameters, as described above, were set to those found earlier to be optimal for folding of the recombinant enzyme. In contrast to the low folding efficiency for the latter, 60–70% of the activity of the plant enzyme was restored in this experiment, at an optimum urea concentration of zero.

DISCUSSION

We have constructed and expressed a synthetic HRP C gene in *E. coli*. Similar work on the expression of a synthetic HRP gene, but no data on conversion of the gene product to functional enzyme has been reported recently, independently, by Ortlepp *et al.* (39). Both in the present work and in that of these workers, the gene product appeared in an insoluble inactive form contained within inclusion bodies. This is a problem frequently encountered (40, 41) in expression of heterologous proteins in *E. coli*. However, we have further succeeded in folding the reduced denaturant-solubilized polypeptide in the presence of calcium ions and heme to give active enzyme. Our data show that the recombinant enzyme, which, like all such enzymes produced in *E. coli*, is not glycosylated, is a competent peroxidase, reacting with peroxide analogously to the glycosylated plant enzyme. The rather lower specific activity² and heme content of the purified recombinant enzyme indicate that 100% incorporation of heme has not been achieved.

The sequences of three cDNAs encoding HRP C were published (25) after our gene was constructed. They correspond to proteins having, in comparison with HRP C, extensions at both the N and the C termini. Our data show clearly that neither extension is required for activity of the enzyme, nor is either absolutely required for folding. The N-terminal extensions encoded by two of the cDNAs appear to correspond to signal sequences, characteristic of secreted proteins. The role of the 15-amino acid extension at the C terminus is uncertain. C-terminal sequences of several enzymes have been implicated in protein targeting (42, 43). Alternatively, the C-terminally extended HRP C may represent an inactive pro-

enzyme awaiting proteolytic cleavage to activate it.

Now that active recombinant HRP C* may be prepared, it would be of considerable interest to study in detail the folding pathways of HRP C and HRP C*, and in particular, to determine the sequence in which the calcium ions and the heme are incorporated into the enzyme and the four disulfide bridges are formed, as well as to elucidate the possible role of the glycosyl residues in the folding processes. Although the processes are no doubt complex, our results presented here already provide some important information.

As is shown by the well known rapid spontaneous refolding of reduced and denatured ribonuclease (44) or lysozyme (37), the requirement, with peroxidase, for 8 cysteine residues to be correctly paired to give four disulfide bridges need not be a serious limitation in the folding. Similarly, introducing calcium ions or heme individually into the otherwise complete peroxidase molecule presents no problems. Thus, as noted above, calcium ions may be selectively removed by treatment of HRP C with guanidium chloride and EDTA (18), and heme is liberated by acidification to pH 2 (15). Either constituent may be restored to the enzyme, simply by adding it back, at neutral pH values, to the appropriate apoenzyme form. On the other hand, refolding and reactivation of completely unfolded plant HRP C, with disulfide bridges reduced and heme and calcium ions fully removed, has never, to our knowledge, been described in the literature. Furthermore, the extent of unfolding of the HRP C sample that we studied is uncertain. A detailed comparison of the folding of HRP C* with that of HRP C is not therefore possible at present.

Our data on the folding of the recombinant enzyme, HRP C*, indicate that a strictly defined folding medium is required and that with this and at an apparent equilibrium only 3–4% of the enzyme molecules are in a soluble active native-like state. Insolubility of the remainder of the enzyme indicates that inter- rather than intramolecular disulfide bond formation has occurred. Whether refolding of the completely unfolded plant enzyme would occur more readily is uncertain.³ It differs from the recombinant enzyme in being glycosylated and in having a blocked N terminus, whereas the N-terminal amino acid of the recombinant enzyme is presumably methionine. Whether the lack of glycosylation has any effect on folding is uncertain. There are indications in the literature that, whereas under some conditions (45, 46), glycosylation may have no major effects on the unfolding and refolding of specific proteins, nevertheless under others (46), it may serve effectively to suppress irreversible aggregation of partially unfolded intermediates. It is tempting to speculate on the relation of such aggregation and comparable *in vivo* aggregation (47) of unglycosylated recombinant proteins to that which we observe during the folding of HRP C*.

Our data are more definitive on the role of calcium ions in the folding and emphasise their importance in the structure of peroxidase. The clear implication of our work is that the binding of calcium ions is an obligatory step in the folding pathway that has to take place, at least for the unglycosylated recombinant enzyme, before correct disulfide bridge formation and heme incorporation can be completed. Calcium ions are well known to have important structural roles in many proteins (see, *e.g.* Ref. 48, for recent work); however, so far as we are aware, peroxidase is the only heme protein in which they have been reported as a constituent. It will be particularly

³ The experiment, in which ready restoration of activity occurred to a sample of HRP C treated with 8 M urea, 30 mM DTT, and 1 mM EDTA, could represent no more than restoration of Ca^{2+} and reformation of disulfide links, in a species that retained heme and was only partly unfolded.

interesting to use site-directed mutagenesis to identify the calcium ion binding residues (*cf.* Ref. 10) in the enzyme and to determine whether there is homology in this part of the molecule with cytochrome-*c* peroxidase. Conceivably, calcium ions might play a part in the folding of peroxidases other than HRP, without remaining a constituent of the enzymes in their final folded forms. It will also be important to know whether the Ca^{2+} -binding site in HRP is in any way related to that (8) of the substrate, Mn^{2+} , in the manganese peroxidase of *Phanerochaete chrysosporium*.

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