High-Pressure-Assisted Reconstitution of Recombinant Chloroperoxidase†

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ABSTRACT: An expression vector containing a T7 promoter and an OmpA signal sequence followed by the DNA sequence of mature chloroperoxidase from the fungus *Caldariomyces fumago* has been transformed into *Escherichia coli*. This construct gave high-level expression of apochloroperoxidase when induced with isopropyl thiogalactopyranoside. The nonglycosylated apoenzyme was secreted into periplasmic space. The recombinant apochloroperoxidase was expressed at a level representing about 2% of the total cellular protein. Before conversion to holoenzyme, the apochloroperoxidase was denatured in 8 M urea and partially purified by DEAE chromatography. Maximum yields of holoenzyme were obtained when the denatured apochloroperoxidase, dissolved in a refolding buffer containing iron protoporphyrin IX, calcium ions, and oxidized glutathione, was subjected to high pressure (207 MPa) at −12 °C and then allowed to refold at atmospheric pressure and room temperature. The recombinant holoenzyme was characterized by absorption and CD spectroscopy and tested for halogenation and peroxidation activity. The yield of active holochloroperoxidase was about 5% when high-pressure treatment was used as part of the reconstition process. In the absence of pressure treatment, holoenzyme was formed at about the 1% level. The holochloroperoxidase preparations which resulted from high-pressure treatment showed, upon return to atmospheric pressure, a considerably higher content of native-like secondary structure compared to the nonpressurized preparations. These experiments show that active recombinant chloroperoxidase molecules can be produced, and prove that glycosylation is not a mandatory requirement for chloroperoxidase refolding.

Chloroperoxidase (CPO)[1] is a heavily glycosylated monomeric hemoprotein secreted by the filamentous fungus *Caldariomyces fumago* (Morris & Hager, 1966). The molecular weight of CPO is 42 000. Iron protoporphyrin is bound to the active site of the enzyme through cysteine ligation (Dawson & Sono, 1987; Sono et al., 1991; Blanke & Hager, 1988). CPO is a very versatile enzyme that can catalyze halogenation, dehydrogenation, demethylation, and oxygen insertion reactions (Thomas et al., 1970; Kedderis et al., 1980; Libby et al., 1982). Recently, it has been found that chloroperoxidase is a highly effective catalyst for the enantioselective epoxidation of a variety of simple olefins (Allain et al., 1993; Colonna et al., 1993) and sulfides (Colonna et al., 1990). CPO possesses a potent catalase activity (Thomas et al., 1970) and shares spectral properties and chemical reactivities with the P-450 cytochromes (Kedderis et al., 1986; McCarthy & White, 1983; Ortiz de Mantellano et al., 1987; Kobayashi et al., 1986, 1987; Sono et al., 1984, 1986; Hollerberg & Hager, 1973; Champion et al., 1973, 1975, 1976; Dawson et al., 1976, 1983, 1988). Thus, it is of interest to understand the active site structure−function relationships that confer on this enzyme its broad functional specificity. Development of a heterologous expression system for CPO offers an approach to site-specific mutagenesis studies for exploring these structure−function relationships. Reconstitution of recombinant CPO represents a special challenge since related heavily glycosylated heme proteins have proven to be notoriously difficult to refold into an active conformation (Pappa & Cass, 1993).

In this paper, we describe the construction of an expression vector and the expression of the CPO gene in *Escherichia coli*. The purification of the apoenzyme CPO and its reconstitution and refolding in the presence of heme to give active chloroperoxidase are described.

MATERIALS AND METHODS

The pET plasmid and expression system were from Novagen (Madison, WI); molecular weight standards were from Bio-Rad Laboratories (Richmond, CA); recombinant DNA enzymes were obtained from Bethesda Research Labs (Bethesda, MD), or from New England Biolabs (Beverly, MA), and were used according to the supplier's protocol. DNA manipulations, growth media, and buffers (unless individually specified) were as described by Sambrook et al. (1989).

Plasmid Construction. Plasmid pET-22b (Studier et al., 1990) was used to express a slightly modified chloroperoxidase gene (substitution of an Asp for a Glu residue at the +1 codon in the N-terminus). A synthetic OmpA signal (Grayeb et al., 1984) sequence was inserted into the pET-22b plasmid between the *Nde*I and *Bam*HI sites. The cDNA encoding a full-length chloroperoxidase, previously cloned in this laboratory (Fang, 1986), was mutated by site-directed mutagenesis using the procedure of Wells et al. (1985). The colonies containing the mutated sequence were identified by hybridization using the mutagen oligonucleotide as the

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[1] Abbreviations: CPO, chloroperoxidase; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; LB, Luria−Bertani medium; EDTA, ethylenediaminetetraacetic acid; GSSG, oxidized glutathione; GSH, glutathione.

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radioactive probe. The +1 codon of mature form chloroperoxidase was modified from GAG to GAT; thus, the amino acid residue of the mature form chloroperoxidase was altered from Glu to Asp, and a *Bam*HI site was generated. Following subcloning and DNA sequencing, the expression plasmid, pECAE, was constructed by ligation of *Bam*HI/*Hin*dIII-cleaved vector DNA with the subcloned 1.2 kb fragment which encodes the mature form CPO gene.

Expression of Chloroperoxidase in E. coli. BL21 (DE3) cells, transformed with the pECAE expression plasmid, were grown to an OD_{600} value of 0.8−1.0 in LB medium containing 100 μg/mL ampicillin at 37 °C. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5−1.0 mM, and the culture was grown for a further 20 h at 30 °C. The cells were harvested and subjected to lysozyme treatment and sonication. The cell pellet from a 1 L culture was suspended in 50 mL of 20 mM Tris-HCl (pH 8.0), 10 mM EDTA, 20 mg of lysozyme (Sigma), and 100 μg of DNase, and 50 μL of 0.5 mM phenylmethanesulfonyl fluoride was added to the cell suspension. After 1 h incubation on ice, the cell lysate was sonicated 3 times (30 s treatments) and centrifuged at 12000*g* for 10 min at 4 °C (Smith et al., 1990). The pellet was suspended in 50 mL of 0.5% Triton X-100, 1 mM EDTA, and 10 mM Tris-HCl buffer, pH 8.0, and centrifuged. This washing procedure was repeated until a solid pellet was obtained. The pellet was dissolved in 2 mL of 8 M urea, 1 mM EDTA, and 20 mM potassium phosphate buffer, pH 6.5. This procedure gave a urea-solubilized apoCPO protein that was about 70% pure, as judged from SDS−PAGE analysis. After centrifugation at 12000*g* for 10 min, the soluble fraction was diluted into 10 mL of 8 M urea in 20 mM Bis-Tris buffer, pH 6.5, and purified on a DEAE-Sephadex A-50 column that had been equilibrated with the same buffer. The DEAE column was washed with the same buffer until the OD_{280} dropped below 0.1. The column was then eluted using a linear gradient of 0−70 mM ammonium sulfate in 8 M urea, 20 mM Bis-Tris buffer, pH 6.5, with a flow rate of 1.5 mL/min. Peak fractions between 70 and 80 which contained the apoCPO protein were pooled.

Preliminary Reconstitution. The partially purified apoCPO was diluted to 100 μg/mL in refolding medium, which contained 20 mM potassium phosphate buffer, pH 6.5, 10 μg/mL bovine hemin (Sigma, type I), 1 mM GSSG, and 1 mM DTT. The solution was left to stand for 48 h at 4 °C. Precipitated protein was then removed by centrifugation.

Pressure-Assisted Refolding. Small aliquots of the CPO supernatant fraction obtained in the preliminary reconstitution previous step were transferred to a series of 100 mM buffers (potassium phosphate, potassium citrate, potassium acetate, or potassium phthalate) in pH range from 2 to 6.8. In a parellel set of experiments, apoCPO which had been prepared by heme removal (ethanol precipitation at pH 2, −20 °C) was used to prepare a similar set of wild-type apoCPO solutions. A 10-fold excess of iron protoporphyrin IX was added to wild-type apoCPO samples. Native holoCPO with its heme intact was also used for preliminary pressure experiments. All the samples were pressurized with gradually increased hydrostatic pressure to 207 MPa in 34.5 MPa increments, and then the temperature was lowered to −12 °C in 2 °C steps. The other conditions and equipment used for the pressure experiments are described by Paladini and Weber (1981). Progression of the reaction was monitored by UV−VIS spectroscopy, and in selected samples, the reaction also was followed by right-angle fluorescence spectroscopy using a Greg PC fluorometer (ISS, Inc., Champaign, IL). Emission spectra were collected between 300 and 400 nm. Absorption spectra were collected from 240 to 700 nm after a 10 min equilibration of the sample at each pressure and temperature. $CaCl_2$, DTT, GSH, and GSSG were used separately and in combination as additives in the potassium citrate test solutions. After optimizing refolding conditions, larger scale preparations of reconstituted CPO were prepared in a similar high-pressure bomb in 0.8 mL Eppendorf tubes. After pressure release, the samples were recovered from the bomb and tested for halogenation activity using the MCD assay (Hager et al., 1966). Selected samples were also further checked for peroxidative activity and their UV-CD spectra were collected. UV−Vis spectra were also recorded on samples in which excesses of heme had been removed by repeated washing on Centricon 30 filters or by molecular sieving on a Sephadex G-25 column.

Spectroscopy. All routine optical absorption measurements were performed using either the Cary 219A or the Hewlett Packard 8451A spectrophotometers. Spectra were collected at 20−24 °C. The CD measurements were collected on an Instruments S.A./Jobin-Yvon CD VI spectropolarimeter (Longjumen Cedex, France) interfaced with an AT&T 6386 WGS computer. The intensities were calibrated using an aqueous solution of (+)-10-camphorsulfonic acid. All CD spectra were obtained at room temperature.

Additional Procedures. The SDS−PAGE analyses of protein samples were run on the Phastsystem (Pharmacia−LKB, Piscataway, NJ). Proteins were blotted from the Phast gels onto Immobilon P membranes using the Phast transfer module according to the manufacturer's protocols. Blots were developed using ABC-peroxidase immunostaining kits (Pierce, Rockford, IL). The preliminary antibody was rabbit polyclonal IgG prepared from the serum of CPO-injected rabbits (Nuell, 1988). Diaminobenzidine was used as the peroxidase substrate (Young, 1989).

RESULTS AND DISCUSSION

Construction of Expression Plasmid. A full-length cDNA encoding the chloroperoxidase gene from *Caldariomyces fumago* has been previously cloned in this lab. Initial attempts to express this gene as a mature protein using the pET-12b plasmid in *E. coli* BL21 (DE3) yielded very low levels of recombinant protein which could only be detected by Western blotting (data not shown). Since the CPO mRNAs in this kind of construct should have been protected by secondary structure at both the 5′ and 3′ ends (Deng et al., 1990), the low level of expression was probably not due to low levels of mRNA. Rather, it seemed more probable that proteolytic degradation of the recombinant protein was the primary cause for the low-level production of apoenzyme. In order to avoid proteolysis, we deemed it desirable to secrete the protein into periplasmic space. Periplasmic expression experiments were carried out with three different constructs using the signal peptides, OmpA, PelB, and OmpT, fused to the N-terminal coding region of the protein. The CPO cDNAs containing the PelB and OmpT signal peptide coding sequences were subcloned into plasmids pET-12b and pET-22b and a modified pET-22b vector in which

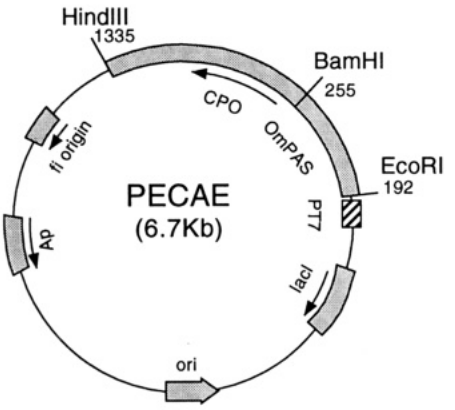

FIGURE 1: Construction of plasmid pECAE. The expression plasmid pECAE was prepared by inserting a synthetic oligonucleotide comprising the OmpA signal peptide sequence into plasmid pET-22b between the *Nde*I and *Bam*HI sites. This insertion replaced the PelB leading sequence in the original expression vector. The mature CPO gene was excised from PGFMB1 by digestion with *Bam*HI and *Hin*dIII, followed by ligation with the altered pET-22b plasmid. The finished plasmid, pECAE, encodes the mature CPO coding region and the OmpA leader sequence. Further details concerning the construction of pECAE are given under Materials and Methods.

the PelB signal sequence was replaced by a synthetic OmpA signal sequence (Figure 1). All of the resulting plasmid−host expression systems stringently regulated high levels of CPO expression upon proper induction of the T7 promoter. The BL21 (DE3) strain of *E. coli* transformed with the pECAE plasmid (containing the OmpA signal sequence) produced significant amounts of CPO after 1 h induction with IPTG. Expression in this system reached a maximal level in about 7 h at 37 °C or in 20 h at 30 °C. Continued culture of the induced bacteria beyond 7 h at 37 °C led to an increased amount of short CPO peptides. On the basis of SDS−PAGE analysis, the estimated expression of CPO in this system was 2−5% of total cellular proteins; however, only 5−10% of the CPO molecules were in a soluble form. Most of the apoCPO accumulated in the periplasmic space as insoluble inclusion bodies. No halogenation activity could be detected in the cell-free lysate. In addition, absorption spectroscopy failed to detect heme incorporation into the recombinant CPO.

A small-scale lysate prepared from *E. coli* BL21 (DE3) cells transformed with pECAE was analyzed by SDS−PAGE and Western blotting. This experiment revealed a prominent new protein band that cross-reacted with CPO polyclonal antibody (Figure 3B). The estimated molecular weight of the cross-reacting protein was 40 000. This value corresponds roughly to the predicted molecular weight of non-glycosylated CPO. After purification using a 0.5% Triton X-100 wash step and chromatography on a DEAE ion-exchange column (Figure 2), a single protein band was observed in a 12% SDS−PAGE gel (Figure 3A). Amino acid sequence data on the purified protein confirmed that this protein represented a mature CPO and that correct processing had occurred. An N-terminal sequence of Asp-Pro-Gly-Ser-Gly-Ile-Gly-Tyr was determined by automated Edman degradation. This sequence contains the predicted eight amino acids found at the N-terminal of a mature CPO molecule in which an N-terminal Glu has been replaced with an Asp. The other two expression constructs having the OmpT and PelB signal peptide coding sequences also gave

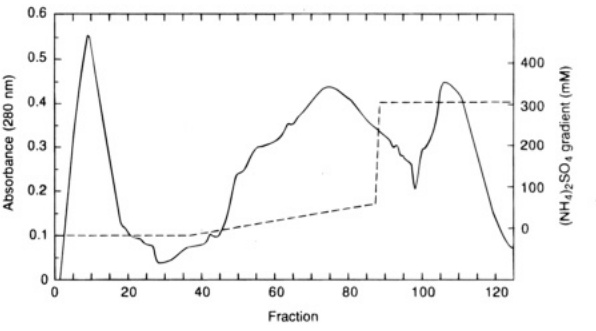

FIGURE 2: Purification of urea-denatured apoCPO from an *E. coli* expression culture. After extensive washing with 0.5% Triton X-100, the inclusion body fraction obtained from a 1 L cell culture was dissolved in 2 mL of 8 M urea, 20 mM Bis-Tris buffer, pH 6.5, and centrifuged. The supernatant fraction was then diluted into 10 mL of the urea−Bis-Tris buffer and loaded onto a 2.5 × 10 cm DEAE-Sephadex A-50 ion exchange column, which had been preequilibrated with the same buffer. Elution was with a linear gradient, 0−70 mM $(NH_4)_2SO_4$ (- - -), at a flow rate of 1.5 mL/min; 1.5 mL fractions were collected. The solid line (−) plots absorbance at 280 nm. Fractions 70 through 80 which contained the purified apoCPO were pooled.

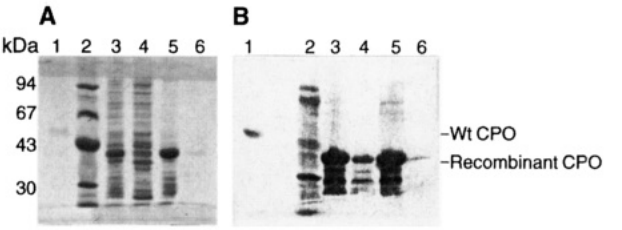

FIGURE 3: SDS−PAGE gel analysis of samples from *E. coli* cell extracts transformed by pECAE. (Panel A) Coomassie-stained SDS−PAGE gel. Lanes: 1, native CPO; 2, biotinylated molecular mass markers; 3, whole cell lysate; 4, supernatant fraction of cell lysate; 5, inclusion bodies; 6, DEAE-purified apoCPO protein. (Panel B) Western blot of SDS−PAGE gel (the biotinylated molecular mass markers gave positive bands in the Western blot). Lanes: same as panel A.

a high level of expression of the CPO gene. SDS−PAGE analysis indicated that both expression plasmids yielded CPO protein at a level of 2−5% of the total cell protein. However, only the construct with the OmpA signal sequence was able to express correctly processed CPO.

Preparation and Reconstitution of Recombinant CPO. A crude lysate from a 1 L culture of pECAE in BL21 (DE3), prepared by the lysozyme/EDTA method, was used to isolate apoCPO for reconstitution. Typically, the Triton X-100 wash step yielded a 15 mg pellet of the apoenzyme from a 1 L culture. After the apoCPO pellet was dissolved in 8 M urea and centrifuged, the apoCPO was judged to be 70−80% pure on the basis of SDS−PAGE analysis. The denatured recombinant apoCPO was subsequently purified on a DEAE ion-exchange column and diluted into refolding buffer in the presence of at least a 5 M excess of hemin. After 48 h incubation at 4 °C, chlorination activity assays indicated that approximately 1% of the denatured apoenzyme had been converted to enzymatically active holoenzyme. This apo−holoenzyme mixture was used for the combined high-pressure−low-temperature treatment which promoted additional reconstitution.

Reconstitution of Recombinant ApoCPO Using High Hydrostatic Pressure. Previous studies have shown that native CPO undergoes an irreversible conversion to an enzymatically inactive 420 nm absorbing species at alkaline

pH values above pH 7 (Hollenberg & Hager, 1973). However, preliminary studies on the influence of hydrostatic pressure on native, wild-type CPO have shown that the conversion of the 450 nm (ferrous enzyme−carbon monoxide complex) to a 420 nm absorbing species can be reversible (Blanke, personal communication). This latter observation is consistent with the finding that cytochrome P-450 also undergoes a reversible interconversion between the native 450 nm absorbing species and a denatured 420 nm form under the influence of high pressure (Marden & Hui Bon Hoa, 1987). The high-pressure-induced denaturation−renaturation reaction which restores heme to the active site of native CPO can be readily followed by measuring absorption changes in the Soret band (400 nm) and also by fluorescence spectroscopy. In addition to detecting the conversion of the native enzyme to the 420 nm species, monitoring of the Soret band can also detect the loss of heme from the heme pocket. Heme bound at the active site of CPO has a very high extinction coefficient (75 000 M^{-1} cm^{-1} at 400 nm). Free heme has an extinction coefficient which is an order of magnitude lower. Thus, the Soret band can serve as a marker of denaturation with heme still bound to the enzyme (CPO-420) and also can measure the progress of the dissociation of heme and its subsequent reassociation with the enzyme to re-form its active site conformation.

A variety of different conditions were tested using wild-type holoCPO in order to optimized CPO refolding and renaturation after high-pressure treatment. CPO was found to be most stable in terms of enzymatic activity in 100 mM potassium citrate buffer, pH 4.5. A similar stability was achieved in potassium phosphate buffer. The poorest survival rates were found in phthalate buffer over the entire pH range and at the extreme pH values (2−2.4 and 6−6.5) in all other buffers. At high pressure (207 MPa) and room temperature, complete denaturation of native CPO to CPO-420 can be achieved as evidenced by the shift in Soret absorbance from 403 to 420 nm. However, only small amounts of heme dissociate from the enzyme at room temperature and high pressure. The maximum dissociation of heme from the active site of wild-type CPO under these conditions (pH 4.5, citrate, 207 MPa) was only in range of 10−15%. In contrast, lowering the temperature to −12 °C significantly promotes dissociation of the heme prosthetic group. Figure 4 records difference spectra taken between native CPO (reference cell) and CPO which was pressurized for time periods up to 4 h at −12 °C. The curve labeled 0 time represents the first high-pressure spectrum which was recorded as soon as the pressure reached −12 °C and 207 Mpa. Partial conversion of the enzyme to the 420 nm absorbing species had already occurred prior to this initial recording as evidenced by the shift in the Soret absorbance to longer wavelengths. The difference spectra taken after 3 and 4 h indicate that approximately 50% of the heme dissociates from the enzyme at −12 °C. Upon return to atmospheric pressure, the native enzyme preparations which had been subjected to the high-pressure−low-temperature cycle yielded holo-active enzyme in the range of 80−90%. Subsequent experiments also showed that the lower temperature had little deleterious effect on the reassociation of heme with recombinant apoCPO.

Fluorescent spectroscopic measurements on native CPO before and while the enzyme was under 207 MPa pressure also confirmed the conclusion that high pressure promotes

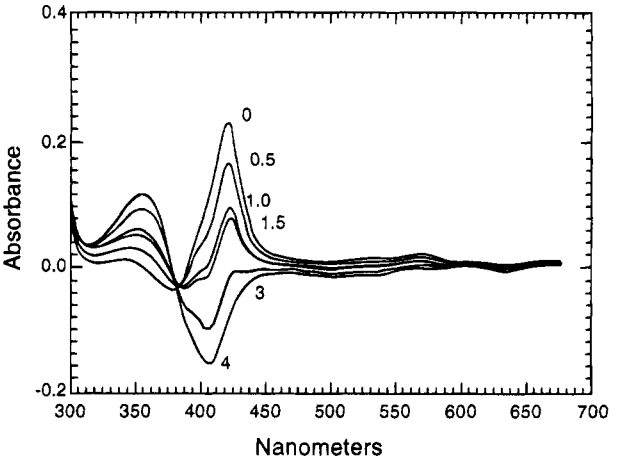

FIGURE 4: Pressure-induced dissociation of heme from native CPO. Native wild-type CPO, 3 μM, in potassium citrate buffer, pH 4.5, was subjected to 207 MPa pressure at −12 °C for 4 h. As soon as the pressure reached 207 MPa, difference spectra were recorded. The reference cuvette contained unpressurized native CPO. The initial recording was made as soon as the sample reached −12 °C at 207 MPa and is labeled 0 h. Subsequent recording were made at 0.5, 1, 1.5, 3, and 4 h.

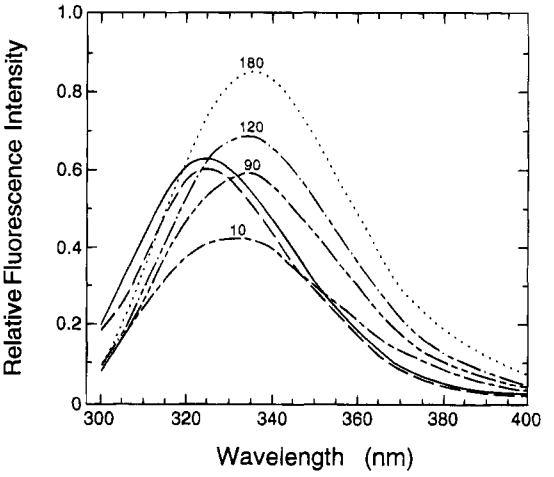

FIGURE 5: Fluorescence emission spectra of wild-type CPO during and after pressure treatment. Native wild-type CPO samples in 100 mM potassium citrate buffer, pH 2.2, were pressurized (207 MPa) in a bomb and excited at 293 nm. Spectra were collected from 300 to 400 nm at 0 time (—), and after 10 (— - —), 90 (— - - —), 120 (— • - —), and 180 min (• • •). The dashed line (— —) records the fluorescence emission spectra after the enzyme samples had been returned to atmospheric pressure. Under the high-pressure conditions, a red shift (from 322 to 334 nm) and an increase in the fluorescence intensity of Trp were observed. Upon return to atmospheric pressure, the maximum fluorescence emission shifted back to 322 nm.

the dissociation of heme from the heme pocket of CPO. Fluorescence emission from tryptophan residues situated near the heme prosthetic group in hemoproteins is quenched by resonance energy transfer to the heme molecule (Bucci et al., 1988; Hirsch, 1994). Using an excitation wavelength of 293 nm, fluorescence emission spectra between 300 and 400 nm were recorded for native CPO at atmospheric pressure and for CPO at 207 MPa (Figure 5). The results clearly indicate that high pressure promotes a red shift in the fluorescence spectra from 322 to 334 nm and there is an observable increase in tryptophan fluorescence intensity. In addition to detecting the loss of heme from the heme pocket, these result indicate that one or more of the five tryptophan

residues in native CPO have moved from a hydrophobic to a more polar environment (Bucci et al., 1988; Weber, 1987).

Since the pressure denaturation of native CPO was essentially fully reversible (90−100% recovery of chlorinating activity) over a broad pH range (2.5−6.0) when carried out in the presence of potassium phosphate or potassium citrate buffers, the effect of high pressure on the reconstitution of apo-recombinant enzyme was explored. ApoCPO preparations which had been purified by DEAE chromatography and had been partially reconstituted at atmospheric pressure by reaction with a 5-fold excess of heme were subjected to high pressure. When the pressure reached 207 MPa, the temperature was gradually lowered at an approximate rate of 3 °C per 10 min to the 0−10 °C range and finally 2 °C per 10 min to the −12 °C temperature. After a 4 h period, the pressure was released, and the sample was returned to atmospheric pressure at room temperature. Partial reconstitution of native CPO took place upon release of pressure. The formation of the 420 nm CPO species from the partially reconstituted recombinant CPO (partially reconstituted at atmospheric pressure) under the influence of high pressure is shown in Figure 6a. The characteristic 420 nm absorption band is formed when the pressure is elevated to 138, 173, and 207 MPa. The effect of low temperature on the dissociation of heme from the partially reconstituted enzyme is shown by the disappearance of Soret absorbance at 400 and 420 nm (Figure 6b). A series of experiments compared the recovery of catalytic activity using high pressure at room temperature and high pressure at low temperature in the reconstitution protocol. These experiments showed that high pressure and low temperature gave the best results.

The chemical components of the refolding buffer which were identified as being required or which stimulated the high-pressure−low-temperature reconstitution of recombinant apoCPO are shown in Table 1. Oxidized glutathione is required for reconstitution and undoubtedly plays an essential role in promoting the formation of the single disulfide bond in native CPO which has been shown to be required for catalytic activity. The presence of calcium ions was found to be critical for the stability of reconstituted CPO; however, calcium ions did not have a pronounced influence on the incorporation of heme into the apoenzyme. Absorption measurements indicated that reconstituted CPO prepared both from wild-type and from recombinant enzyme in the absence of calcium ions bound heme at their active sites but rapidly reconverted to the inactive 420 nm species and precipitated from solution.

Further support for the correctness of the high-pressure-induced refolding process and the detection of an increase in ordered structure in the reconstituted holoenzyme came from CD spectroscopic studies. Comparison of the far-UV CD spectra of the native CPO preparations with unpressurized reconstituted enzyme and with high-pressure-reconstituted CPO preparations revealed that only the pressure-treated reconstituted enzyme preparations showed substantial increase in α-helical structure (Figure 7).

The use of high pressure also helped to overcome another notorious problem associated with the incorporation of heme into apoCPO, namely, the low solubility of heme compounds at the acidic pH values which were necessary in order to maintain CPO stability. The high-pressure conditions used in the reconstitution experiments promoted the dissociation of heme aggregates into soluble monomers. Then after pressure release, the unincorporated heme molecules aggregated and precipitated from solution.

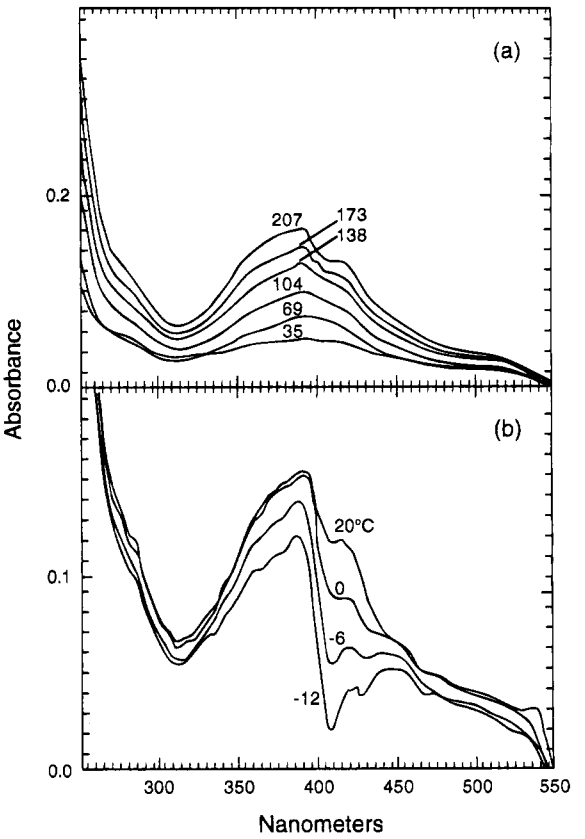

FIGURE 6: Changes in the absorption difference spectra of recombinant apoCPO reconstituted with heme during its incubation under high pressure (a) and low temperature (b). Recombinant CPO was partially reconstituted as described under Materials and Methods. In panel a, the partiall reconstituted enzyme preparation in potassium citrate buffer, pH 4.5, was subjected to gradually increased pressure at 20 °C, and UV−Vis difference spectra were collected at the starting, final, and intermediate points (69, 104, 138, and 173 MPa) using the unpressurized enzyme preparation as the reference sample. In panel b, the temperature was gradually lowered from room temperature to −12 °C after the enzyme preparation had been pressurized to 207 MPa. Again, UV−Vis difference spectra were collected, this time using the room temperature enzyme preparation as the reference sample. Spectra were recorded at 20, 0, −6, and −12 °C.

Table 1: Requirements for the Pressure-Assisted Reconstitution of Recombinant Chloroperoxidase[a]

additions	pressure treated	rel chlorination act. (%)
(1) GSSG, $CaCl_2$	+	100
(2) $CaCl_2$	+	74
(3) none	+	42
(4) DTT	+	40
(5) GSSG	+	33
(6) GSSG, $CaCl_2$, DTT	+	9
(7) GSSG, $CaCl_2$	−	20

[a] Recombinant apoCPO was incubated under 207 MPa pressure and at −12 °C for 4 h in 100 mM potassium citrate buffer, pH 5.5, in the presence of 5 molar excess of iron protoporphyrin IX. Where indicated, the reaction mixtures were supplemented with 10 mM $CaCl_2$, 1 mM DTT, and 1 mM GSSG. After pressure release, the chlorinating activity of the partially reconstituted holoenzyme was assayed and compared to the unpressurized preparation.

Peroxidase versus Halogenation Activity in Reconstituted CPO. Reconstituted enzyme preparations were routinely

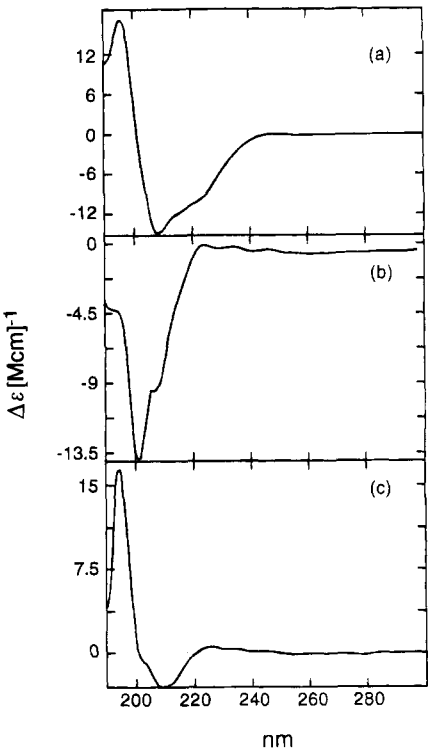

FIGURE 7: Comparison of UV (190−320 nm) CD spectra of wild-type (native) CPO and partially reconstituted CPO before and after high-pressure treatment. Panel a records the CD spectrum of native CPO. Panel b records the CD spectrum of apoCPO which was partially reconstituted at atmospheric pressure as described under Materials and Methods. Panel c records the CD spectrum of reconstituted CPO when the apoCPO preparation was incubated with a 5 molar excess of iron protoporphyrin IX, 10 mM $CaCl_2$, and 1 mM GSSG in 100 mM potassium citrate buffer, pH 5.8, in a pressure bomb at 207 MPa and −12 °C for 1 h. CD spectrum in panel c was collected after pressure release.

assayed for classical peroxidation as well as halogenation activity. Surprisingly, many preparations showed high peroxidative activity but little or no halogenation activity. We interpret this finding to mean that many incorrectly refolded molecules retained the ability to bind heme in a manner which created an active site for peroxidation but these molecules did not posses the thiolate ligation necessary for the creation of the halogenation active site.

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