FTIR Study of the Thermal Denaturation of Horseradish and Cytochrome *c* Peroxidases in D_2O[†]

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ABSTRACT: Fourier transform infrared (FTIR) spectroscopy was employed to examine the thermal denaturation of the Fe(III), Fe(II), and Fe(II)−CO forms of cytochrome *c* peroxidase and horseradish peroxidase in phosphate buffer at pD 7.0. The amide I′ regions of the deconvolved spectra are consistent with predominantly α-helical secondary structure around room temperature, but the α-helical absorption of the two peroxidases differs significantly; bands assigned to α-helical components occur at 1659 and 1649 cm^{-1} in horseradish peroxidase and at 1652 and 1637 cm^{-1} in cytochrome *c* peroxidase. The thermal denaturation mechanisms of the peroxidases also vary. All three forms of cytochrome *c* peroxidase retain their secondary structure up to 50 °C, when bands characteristic of aggregation (1616 and 1684 cm^{-1}) appear in the amide I′ region, and above 55 °C rapid loss of secondary structure is accompanied by enhanced aggregation. In horseradish peroxidase, on the other hand, the Fe(III) and Fe(II) states exhibit dissimilar denaturation mechanisms. Slow, gradual alteration of secondary structure is observed for Fe(III) horseradish peroxidase on heating, and polypeptide unfolding appears to be complete around 90 °C, without aggregation. In Fe(II) and Fe(II)−CO horseradish peroxidase, aggregation bands appear at ∼55 °C, signaling the onset of denaturation. Frequency shifts in the ν(CO) bands above room temperature reveal that conformational changes in the heme cavity *precede* global conformational changes in cytochrome *c* peroxidase but *not* in horseradish peroxidase. The reduction in amide II intensities, due to peptide H−D exchange on heating the peroxidases in D_2O, indicates the formation above room temperature of partially unfolded states with increased solvent accessibility but intact secondary structures.

Recently the crystal structures of a number of heme peroxidases have been determined. These include cytochrome *c* peroxidase (CCP)[1] (Finzel et al., 1984), lignin peroxidase (LIP) (Poulos et al., 1993), *Arthromyces ramosus* peroxidase (ARP) (Kunishima et al., 1994), *Coprinus cinereus* peroxidase (CIP) (Petersen et al., 1994), pea cytosolic ascorbate peroxidase (APX) (Patterson et al., 1995), and manganese peroxidase (MnP) (Sundaramoorthy et al., 1994). Their three-dimensional structures reveal that these six peroxidases possess strikingly similar secondary and tertiary structures, which is surprising given their low sequence homology (e.g., 20% between CCP and either ARP or LIP) (Poulos, 1993; Kunishima et al., 1994). Such observations support the grouping of these enzymes into the plant peroxidase superfamily (Welinder, 1992), which comprises structurally homologous, low molecular weight peroxidases (35−45 kDa) from plant, bacterial, and fungal sources. Furthermore, comparison of the X-ray structures of the mammalian peroxidases, myeloperoxidase (Zeng & Fenna, 1992; Fenna et al., 1995) and prostaglandin H synthase (Picot et al., 1994), with those of CCP and LIP revealed conserved structural elements around the heme, leading to the proposal of a peroxidase fold (Garavito et al., 1994).

Horseradish peroxidase isoenzyme C (HRP) is the most studied heme peroxidase (Dunford, 1991), although an X-ray structure is not yet available for this isoenzyme (Henriksen et al., 1995). However, the secondary and tertiary structure of HRP is expected to be very similar to those of ARP, CIP, and LIP since these enzymes share a number of structural features (Welinder, 1992; Poulos, 1993; Poulos & Fenna, 1994; English & Tsaprailis, 1995). The common structural elements include proximal and distal Ca^{2+} binding sites, four disulfide bridges, and a number of N-glycosylation sites. In addition, a binding site close to the heme for reducing substrates has been identified in plant and fungal peroxidases (Veitch et al., 1994; Kunishima et al., 1994; Petersen et al., 1994; Poulos et al., 1993).

Although CCP and APX have similar secondary and tertiary structures to the other peroxidases in the plant peroxidase superfamily, they do not possess disulfide bridges, bound Ca^{2+}, or glycosylation sites. Therefore, an examination of the effects of these stabilizing elements on the conformational stabilities of structurally related proteins is of interest. In this study, Fourier transform infrared (FTIR) spectroscopy is used to compare the secondary structures of CCP and HRP in their Fe(II) and Fe(III) forms as a function of temperature. The amide I′ regions of the IR spectra of the proteins in D_2O at pD 7.0 were examined to determine

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[1] Abbreviations: APX, pea cytosolic ascorbate peroxidase; ARP, *Arthromyces ramosus* peroxidase; CCP, cytochrome *c* peroxidase; CIP, *Coprinus cinereus* peroxidase; HRP, horseradish peroxidase; LIP, lignin peroxidase; Mb, myoglobin; MnP, manganese peroxidase.

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the secondary structure of the proteins, and the amide II region was employed to probe polypeptide unfolding by monitoring peptide N−H ⇌ N−D exchange (Susi & Byler 1986; Surewicz et al., 1993; van Stokkum et al., 1995).

Both HRP and CCP contain noncovalently bound protoporphyrin IX heme which forms stable CO adducts in the Fe(II) form (Coletta et al., 1986; Miller et al., 1990). The CO adducts have been examined extensively by vibrational spectroscopy around room temperature (Barlow et al., 1976; Smith et al., 1983; Evangelista-Kirkup et al., 1986; Uno et al., 1987, Smulevich et al., 1987). In this work we have investigated the effects of CO binding on the overall thermal stability of HRP and CCP. Furthermore, by monitoring the CO stretching vibrations, which occur between 1900 and 1970 cm^{-1}, localized structural changes in the heme pocket during thermal denaturation were independently probed.

Recently, the FTIR spectra of different redox (Schlereth & Mäntele, 1992) and ligation states of myoglobin (Mb) and hemoglobin (Causgrove & Dyer, 1993; Gregoriou et al., 1995) have been examined in detail. However, the results presented in this paper constitute the first report of peroxidase secondary structure determination by FTIR spectroscopy. Structural and thermal denaturation studies carried out using other techniques, such as circular dichroism (CD) and calorimetry, are compared with the FTIR results.

EXPERIMENTAL PROCEDURES

Materials. Grade I, salt-free, lyophilized horseradish peroxidase (HRP) (EC 1.11.1.7) was obtained from Boehringer Mannheim and used without further purification. Recombinant cytochrome *c* peroxidase from *Escherichia coli* (CCP) (EC 1.11.1.5) was isolated following a previously described procedure (Fishel et al., 1987). The buffer (pD 7.0) was prepared by dissolving 0.1 M sodium phosphate salts (Mallinckrodt) in 99.9% D_2O (Aldrich), and research grade CO gas was purchased from Matheson or Union Carbide. Spectra were recorded on a Nicolet FTIR spectrometer (Model 8210E) equipped with a deuterated triglycine sulfate (DTGS) detector and purged with dry air from a Balston dryer. The IR cell consisted of a temperature-controlled cell mount (Model 118−1, Wilmad) and 13- × 2-mm CaF_2 windows separated by a 50-μm Teflon spacer (Wilmad). The IR cell temperature was controlled to within ±0.5 °C using a thermostat (Omega) and monitored with a thermocouple placed in close proximity to the CaF_2 windows.

Methods. Lyophilized Fe(III)HRP and Fe(III)CCP were dissolved in 0.1 M sodium phosphate buffer (pD 7.0) to a final concentration of 2 mM. All protein solutions were allowed to stand at room temperature until no further change due to H−D exchange was observed in the amide I′ and amide II bands. Preliminary trials demonstrated that once the initial H−D exchange was complete (≤15 min), no further change in the amide II bands occurred over a period of 4 h at room temperature. The Fe(II)HRP and Fe(II)CCP solutions were prepared by adding ∼1 μL of a saturated sodium dithionite solution to 0.1 mL of the solutions containing the Fe(III) proteins. To form the CO adducts, CO gas was bubbled over the Fe(III)HRP and Fe(III)CCP solutions for 5 min before dithionite addition. Approximately 7 μL of sample solution was pipetted onto one of the CaF_2 windows. The cell was immediately assembled and placed in the IR instrument, and the cell temperature controller was programmed to increase the temperature by 5 °C every 30 min. After a 15-min interval at each temperature setting to allow for thermal equilibration, the IR spectrum of the sample was collected. Each spectrum is an average of 512 scans recorded at 4-cm^{-1} resolution. Resolution enhancement of the amide bands was carried out as described previously (Kauppinen et al., 1981), employing a HWHH of 13 cm^{-1} and a *K* factor of 2. The start and end frequencies were 1500 and 1800 cm^{-1}. The ν(CO) bands were plotted without any resolution enhancement.

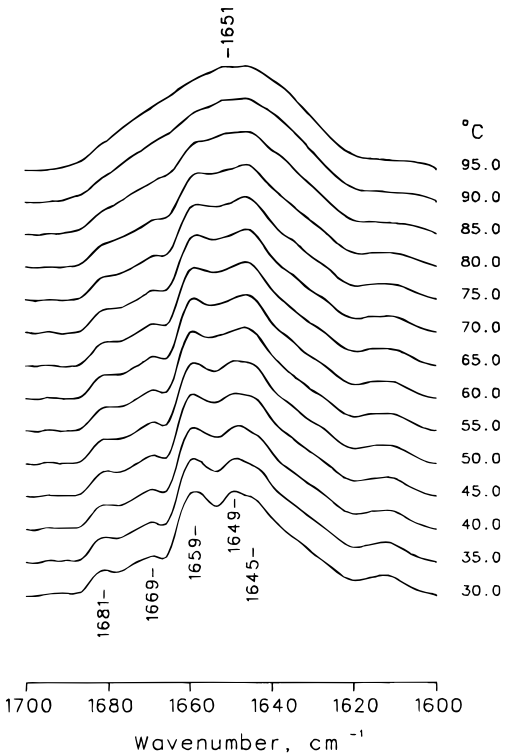

FIGURE 1: Deconvolved spectra in the amide I′ region of 2 mM Fe(III)HRP in 0.1 M phosphate buffer in D_2O at pD 7.0 vs temperature. The solution of HRP was allowed to equilibrate for 15 min at each temperature before a new spectrum was recorded. Each spectrum is the average of 512 scans recorded at 4-cm^{-1} resolution in a 50-μm path length cell.

RESULTS AND DISCUSSION

Thermal Denaturation of the Fe(III) Peroxidases at pD 7.0. Figure 1 shows the deconvolved spectra obtained for Fe(III)HRP in the amide I′ (1700−1600 cm^{-1}) region at pD 7.0 as a function of temperature. At 30 °C, the amide I′ band appears to exhibit at least five components, which are attributed to different secondary structural elements based on literature assignments (Susi & Byler, 1986; Krimm & Bandekar, 1986; Haris & Chapman, 1995). The peaks at 1681 and 1669 cm^{-1} can be assigned to the amide I′ modes of turns and loops. Antiparallel β-sheets would also give rise to a peak at 1681 cm^{-1}; however, since the lower frequency component of the β-sheet vibration, expected between 1640 and 1620 cm^{-1}, is not observed (Figure 1), the peak at ∼1681 cm^{-1} is assumed to arise mainly from turns and loops. Random (or unordered) structure gives rise to the shoulder at 1645 cm^{-1} in D_2O. Peaks between 1650 and 1660 cm^{-1} are generally assigned to α-helical absorption (Susi & Byler, 1986; Krimm & Bandekar, 1986; Haris & Chapman, 1995). Figure 1 shows two peaks at 1659 and 1649 cm^{-1}, indicating that there are two types of α-helices

in Fe(III)HRP. Prestrelski et al. (1991) reported similar splitting of the α-helix absorption of lysozyme and α-lactalbumin and ascribed this to subtle differences in hydrogen bonding or geometry of the helices.

From the relative intensities of the amide I′ peaks, it would appear that the secondary structure of HRP is predominantly α-helical with little, if any, β-sheet, in view of the absence of a defined band between 1640 and 1620 cm^{-1} in Figure 1 (Susi & Byler, 1986; Krimm & Bandekar, 1986; Haris & Chapman, 1995). This conclusion is consistent with the results of CD studies on Fe(III)HRP, which predict 30−40% α-helix with very little β-sheet structure (Strickland et al., 1968). The CD results also predict very little random coil in HRP, but this is not corroborated by the significant absorbance seen in Figure 1 at 1645 cm^{-1}, which is assigned to random structure in D_2O (Susi & Byler, 1986; Haris & Chapman, 1995).

On increasing the temperature (Figure 1), there is a gradual increase in the intensity of the 1645-cm^{-1} shoulder relative to the intensities of the 1659- and 1649-cm^{-1} peaks. This trend can be interpreted in terms of an increase in unordered structure at the expense of α-helical structure at higher temperatures. Alternatively, enhanced H−D exchange on heating could shift the α-helical absorption at 1649 to ∼1645 cm^{-1}, since shifts on the order of 2−5 cm^{-1} have been reported for amide I bands in D_2O (Arrondo et al., 1993). Nonetheless, significant α-helical absorption is observed at 1659 cm^{-1} up to 80 °C, revealing that Fe(III)HRP retains secondary structure at high temperature, consistent with its known thermal stability (Dunford, 1991). The absence in the IR spectrum at 95 °C of bands at 1616 and 1684 cm^{-1}, which have been identified as characteristic of aggregation (Clark et al., 1981; Ismail et al., 1992), indicates that the denatured form of Fe(III)HRP at pD 7.0 does not undergo aggregation or gel formation.

Figure 2 shows the deconvolved spectra obtained for Fe(III)CCP at pD 7.0 as a function of temperature. Comparison of the amide I′ bands of the two peroxidases clearly indicates that their secondary structures are not identical. At 25−30 °C, Fe(III)CCP exhibits only one peak (1652 cm^{-1}) within the normal range (1650−1660 cm^{-1}) for α-helical absorption in proteins. A comparison of the CD spectra of HRP (Strickland et al., 1968) and wild-type CCP from yeast (Sievers, 1978), which exhibits an identical crystal structure to recombinant CCP (Wang et al., 1990), indicates greater β-sheet content in CCP, suggesting that the intense band at 1637 cm^{-1} may arise from β-sheet absorption. However, bands between 1620 and 1640 cm^{-1} have also been assigned to α-helical absorption (Chirgadze et al., 1976; Martinez & Millhauser, 1995; Haris & Chapman, 1995), and the 1637-cm^{-1} band is assigned to α-helical structure based on the X-ray data for CCP, as discussed later. Resolved peaks arising from the absorption by random structure (1645 cm^{-1}) and turns (1663 and 1676 cm^{-1}) are observed in the CCP spectra (Figure 2) in addition to those assigned to α-helical components.

The behavior of the two peroxidases with increasing temperature also differs, indicating different denaturation mechanisms. On heating, Fe(III)CCP retains its secondary structure up to 50 °C (Figure 2), but there is an onset of aggregation between 50 and 55 °C, since bands characteristic of protein aggregation (1616 and 1684 cm^{-1}) are apparent in the 55 °C spectrum. An abrupt loss in secondary structure

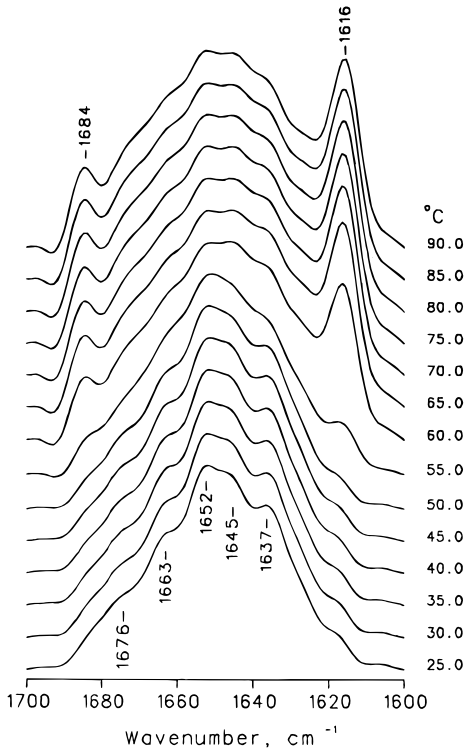

FIGURE 2: Deconvolved spectra in the amide I′ region of 2 mM Fe(III)CCP in 0.1 M phosphate buffer in D_2O at pD 7.0 vs temperature. The experimental conditions are given in the legend to Figure 1.

occurs above 55 °C, and aggregation appears to be complete at 60 °C since the aggregation bands have reached maximum intensity at this temperature. Kresheck and Erman (1988) studied the thermal denaturation of yeast Fe(III)CCP by calorimetry and found two transition midpoint temperatures at 44 and 63 °C at pH 7.0. They concluded that regions of domain I of the polypeptide, which are more flexible than the remainder of the molecule, unfold at 44 °C, and the low-temperature transition is complete before the rest of the polypeptide unfolds at 63 °C. A careful examination of the spectra in Figure 2 reveals essentially no alterations in the amide I′ bands between 25 and 50 °C; thus, the low-temperature transition of CCP at 44 °C does not involve secondary structure changes that lead to modified amide I absorption.

Comparison of Figures 1 and 2 underscores the significantly higher thermal stability of HRP relative to CCP. It has been reported that the stability of Fe(III)HRP to denaturants is greatly decreased when the tightly bound Ca^{2+} ions are removed (Ogawa, 1979; Morishima et al., 1986; Shiro et al., 1986; Papa & Cass, 1993) or in the presence of thiol-reducing agents such as dithiothreitol (G. Tsaprailis and A. English, unpublished observations). Hence, it is likely that the bound Ca^{2+} and disulfide linkages increase the thermal stability of HRP compared to CCP, which does not possess these stabilizing elements. Also evident from Figures 1 and 2 is the marked difference in the thermal denaturation mechanisms of the two Fe(III) peroxidases. In Fe(III)CCP, loss of secondary structure and aggregation occur simultaneously in the narrow temperature range between 50 and 55 °C. Fe(III)HRP, on the other hand, exhibits more gradual conformational changes over the entire temperature range examined, without any apparent aggregation even on complete unfolding of secondary structure at ∼90 °C.

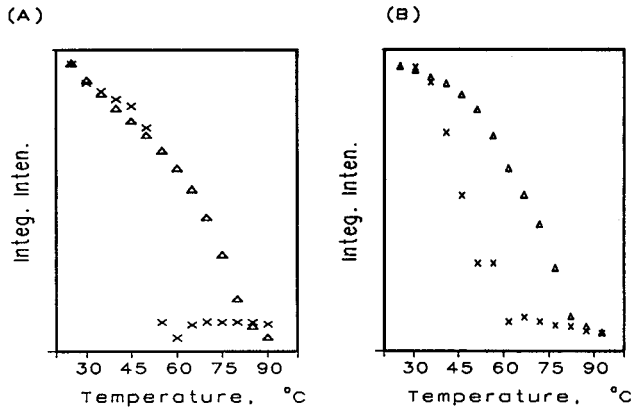

FIGURE 3: Plot of the integrated intensities of the amide II band (1560−1522 cm^{-1}) at pD 7.0 vs temperature for (A) Fe(III)HRP (△) and Fe(III)CCP (×), and (B) Fe(II)HRP (△) and Fe(II)CCP (×). The experimental conditions are given in the legend to Figure 1.

Exchange of the amide hydrogens of the polypeptide backbone is frequently monitored in studies of protein flexibility under different experimental conditions (Creighton, 1993). The -NH groups on the surface of a protein usually exchange rapidly, while buried -NH groups exchange more slowly, with rates that depend on the protein structure and environment (Creighton, 1993). The amide II bands, centered at ∼1540 cm^{-1}, rapidly lose intensity on dissolution in D_2O due to H−D exchange. The remaining amide II intensity can be attributed to slowly exchanging -NH groups that are protected from the solvent. As the temperature is increased, the amide II intensity further decreases due to greater solvent accessibility, which results in H−D exchange of buried -NH groups (Susi & Byler, 1986; van Stokkum et al., 1995; Haris & Chapman, 1995). van Stokkum et al. (1995) report enhanced H−D exchange at temperatures well below the unfolding of the secondary structure of a number of proteins in D_2O. They speculate that such intermediates with partially collapsed tertiary structure and intact secondary structure may be related to *pre-molten globule* folding intermediates.

Figure 3A compares the loss in integrated amide II intensity of Fe(III)HRP and Fe(III)CCP as a function of temperature. The amide II intensity of CCP slowly decreases between 30 and 50 °C; near 50 °C there is an abrupt loss of the remaining intensity. This indicates loosening of the tertiary structure *prior* to unfolding of the secondary structure and aggregation, which commence at ∼55 °C (Figure 2). In contrast to Fe(III)CCP, the amide II intensity of Fe(III)HRP exhibits a gradual decline over the 65-deg temperature range examined (Figure 3A), demonstrating the greater thermal stability of the tertiary structure of Fe(III)HRP relative to Fe(III)CCP. Nonetheless, extensive H−D exchange also occurs in Fe(III)HRP prior to loss of secondary structure at ∼80 °C (Figure 1), suggesting the formation of a partially unfolded state. Thus, above room temperature, both peroxidases form intermediate states with properties similar to pre-molten globules (van Stokkum et al., 1995).

Thermal Denaturation of the Fe(II) Peroxidases. Changes in the Fe(II) forms of both peroxidases were also examined by FTIR spectroscopy as a function of temperature. The amide I′ regions at pD 7.0 (data not shown) are similar to those of the Fe(III) proteins presented in Figures 1 and 2, except that aggregation bands appear in the Fe(II)HRP

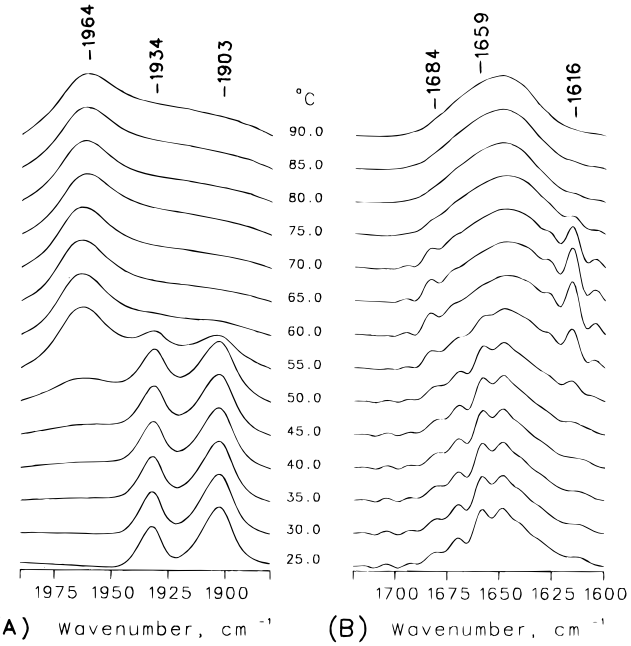

FIGURE 4: (A) Original spectra at pD 7.0 of the ν(CO) region of HRP-CO vs temperature from 25 to 90 °C. (B) Deconvolved spectra in the amide I′ region of 2 mM HRP-CO in 0.1 M phosphate buffer in D_2O at pD 7.0 vs temperature. The experimental conditions are given in the legend to Figure 1.

spectra around 55 °C, and the spectra are essentially identical to those shown in Figure 4B for HRP-CO. Thus, reduction of the iron in HRP promotes aggregation of the protein at a similar temperature to that observed for all forms of CCP at pD 7.0. As can be seen from Figure 3, there is little change in the amide II intensities of HRP vs temperature on reduction of the iron. The changes in CCP are more significant since the Fe(II) form does not exhibit the abrupt loss in amide II intensity at 50 °C like the Fe(III) form (Figure 3A). Nonetheless, in both redox states of CCP, the amide II intensities have reached the baseline at 60 °C, and are more sensitive to temperature than those of HRP.

Thermal Denaturation of Fe(II)−CO Peroxidases. Fe(II) heme peroxidases are known to bind CO with high affinity (Coletta et al., 1986; Miller et al., 1990). Figure 4 shows the IR spectra in the amide I′ and ν(CO) regions of HRP-CO at pD 7.0 as a function of temperature. A close examination of Figures 1 and 4B reveals that binding of CO results in little or no alteration in the amide I′ absorption of HRP at low temperature. Very weak features were observed in the amide I′ region of the FTIR difference spectrum of Mb-CO and Fe(II)Mb (Causgrove & Dyer, 1993), which indicates that only small absorption changes also occur on binding of CO to Fe(II)Mb.

Thermal denaturation of HRP-CO at pD 7.0 is accompanied by aggregation as evidenced by the appearance of bands at 1684 and 1616 cm^{-1} between 55 and 80 °C in Figure 4B. The disappearance of these bands around 80 °C reveals that aggregation is reversible, and the spectrum of nonaggregated, denatured HRP-CO at 90 °C is essentially the same as that of Fe(III)HRP (Figure 1). The reversibility of the aggregation at high temperature is further demonstrated in Figure 5, which shows the effect of subsequent cooling on the amide I′ absorption of HRP-CO. On reducing the temperature from 95 to 65 °C, the aggregation bands reappear and reach maximum intensity at ∼55 °C. Below 55 °C there

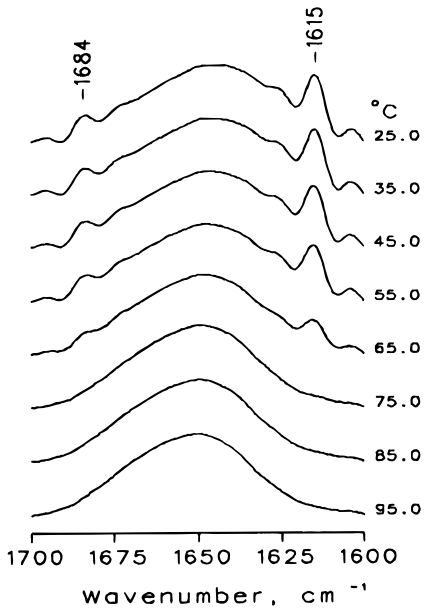

FIGURE 5: Deconvolved spectra in the amide I′ region on cooling the HRP-CO sample in Figure 4B.

is little further change in the spectra, and a comparison of the 25−°C spectra in Figures 4B and 5 clearly reveals that heating to 95 °C results in irreversible denaturation of HRP-CO.

Unligated Fe(II)HRP exhibits almost identical temperature-induced aggregation behavior (data not shown) to HRP-CO. The significantly lower denaturation temperature for the Fe(II) forms of HRP compared to Fe(III)HRP (∼55 vs ∼85 °C) raises the question of whether the disulfide linkages in HRP are cleaved on addition of dithionite, which was used to reduce the Fe(III) ion (see Experimental Procedures). Removal of stabilizing elements such as disulfide linkages should increase the polypeptide flexibility, and hence the H−D exchange. However, the temperature sensitivity of the amide II intensities is essentially identical for the three forms of HRP studied here [Fe(III)HRP (Figure 3A), Fe(II)HRP (Figure 3B), and HRP-CO (data not shown)], which is inconsistent with reduction of disulfide bridges in the Fe(II) forms only. Furthermore, no ν(SD) modes were observed around 1850 cm^{-1} in either Fe(II)HRP or HRP-CO, although these modes might be too weak to be observed under the present conditions (Bare et al., 1975).

The amide I′ regions of the FTIR spectra of Fe(III)CCP (Figure 2), Fe(II)CCP, and CCP-CO (data not shown) are all very similar at 25 °C. Furthermore, little difference between the spectra of the three forms is observed at higher temperatures. This indicates that, unlike HRP, reduction and ligation of CCP has little effect on its thermal stability. In each state examined, the CCP polypeptide undergoes aggregation between 55 and 60 °C, despite the dissimilar amide II intensity vs temperature profiles observed for the Fe(III) and Fe(II) forms (Figure 3).

To directly probe changes in the heme pocket, the ν(CO) region (1980−1880 cm^{-1}) was examined as a function of temperature for both HRP-CO and CCP-CO. Figure 4A shows that, at pD 7.0, the CO ligand exists in two conformers in HRP-CO with bands at 1934 and 1903 cm^{-1}, in agreement with reported findings (Barlow et al., 1976; Smith et al., 1983; Evangelista-Kirkup et al., 1986; Uno et al., 1987, Holzbaur et al., 1996). The band at 1903 cm^{-1}, which

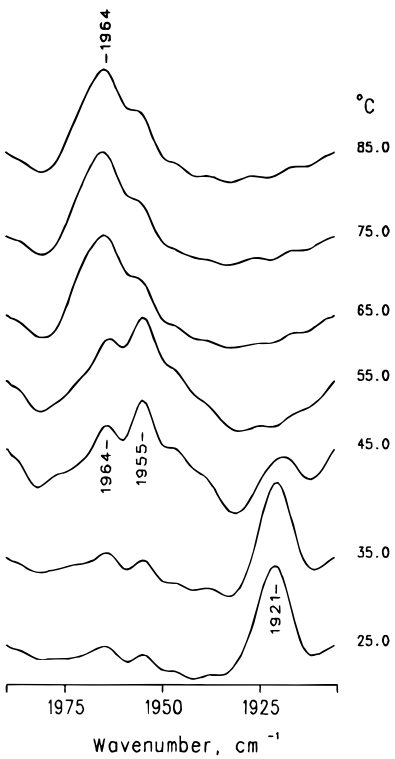

FIGURE 6: Original spectra in the ν(CO) region of 2 mM CCP-CO in 0.1 M phosphate buffer in D_2O at pD 7.0 vs temperature. The experimental conditions are given in the legend to Figure 1.

appears at 1905 cm^{-1} in H_2O, has been attributed to CO hydrogen-bonded to a distal group (Barlow et al., 1976; Smith et al., 1983; Uno et al., 1987, Holzbaur et al., 1996). When the temperature is increased to ∼50 °C, both ν(CO) bands decrease in intensity, and a new band is observed at 1964 cm^{-1} (Figure 4A). Since the appearance of the 1964-cm^{-1} band coincides with the onset of aggregation (Figure 4), this band is assigned to the ν(CO) vibration of *denatured* HRP-CO.

CCP-CO has been reported to exhibit a single CO stretching frequency at ∼1921 cm^{-1} in D_2O, which shifts to ∼1923 cm^{-1} in H_2O (Satterlee & Erman, 1984). A band at 1921 cm^{-1}, together with two weak bands at 1964 and 1955 cm^{-1}, are observed here at 25 °C (Figure 6); however, between 35 and 45 °C there is a dramatic drop in the 1921-cm^{-1} absorbance accompanied by an increase in the intensity of the bands at 1955 and 1964 cm^{-1}. On increasing the temperature to 55 °C, most of the ν(CO) absorbance shifts to the 1955- and 1964-cm^{-1} bands, and above 55 °C the 1964-cm^{-1} band is dominant. The temperature dependence of ν(CO) bands reveals that CCP-CO undergoes a conformational transition involving the heme pocket *prior* to the onset of global denaturation at ∼55 °C (Figure 2). This behavior is consistent with the calorimetric measurements of thermal denaturation of Fe(III)CCP reported by Kresheck and Erman (1988), who observed two transition midpoint temperatures at 44 and 63 °C, which they assigned to local and global unfolding of the polypeptide, respectively. Furthermore, a study of the temperature dependence of the visible absorption spectrum of Fe(III)CCP revealed a shift in the Soret maximum from 408 to 414 nm with a transition midpoint of 36 °C at pH 7.0, and a second shift from 414 to 395 nm between 45 and 63 °C (Gross & Erman, 1985). Thus, both Fe(III)CCP and CCP-CO (and presumably Fe(II)CCP also) exhibit biphasic thermal denaturation but, as discussed

above, the low-temperature transition does not involve secondary structure change that modifies the amide I′ absorption (Figure 2).

It is noteworthy that both HRP-CO and CCP-CO give rise to a ν(CO) band at 1964 cm^{-1} on denaturation. Similar ν(CO) frequencies were reported for acid denatured Mb-CO (Morikis et al., 1989), base denatured cytochrome P_{450}-CO (P_{420}) (O'Keefe et al., 1978), and for free heme-CO with imidazole as an axial ligand in methylene chloride (1960 cm^{-1}) (Evangelista-Kirkup et al., 1986). From their extensive vibrational studies on mutant forms of Mb-CO, Li et al. (1994) found that the ν(CO) frequencies blue-shift in nonpolar environments or when surrounded by negative charge. The inhomogeneous broadening of the ν(CO) bands of the denatured peroxidases (Figures 4A and 6) and the other denatured proteins strongly suggests that the CO ligand undergoes increased random polar interactions in the denatured proteins. Hence, the large blue-shifts in ν(CO) observed here for the denatured peroxidases (30−60 cm^{-1}) are attributed to heme exposure and interaction of the CO ligand with the negative end of the dipole of the aqueous solvent. The reported Soret absorption of denatured Fe(III)-CCP is also characteristic of heme in an aqueous environment (Gross & Erman, 1985).

From the spectral and thermal data discussed in the previous paragraphs, it appears that the low-temperature transition of CCP includes conformational change in the heme cavity. This is most likely to involve disruption of the well-characterized hydrogen-bonding network, Trp51−Arg48−heme propionate−His181, that connects the proximal and distal sides of the heme (Smulevich et al., 1991). This network is important in stabilizing the heme cavity of CCP, and more recent studies indicate that residues around the heme of HRP are also involved in a hydrogen-bonding network (Smulevich et al., 1994; Veitch et al., 1992). We speculate here that redox-linked changes in this network may give rise to the aggregation and lower denaturation temperatures of the Fe(II) forms of HRP, since, as Figure 4 indicates, denaturation of the heme cavity coincides with the global denaturation of HRP-CO. However, an examination of Figures 4A and 6 shows the greater thermal sensitivity of the ν(CO) bands of CCP-CO compared to those of HRP-CO, indicating greater stability of the structural features controlling the integrity of the heme cavity in the latter peroxidase.

Correlation between Crystal Structures and FTIR Data. The X-ray structures obtained to date on proteins of the plant peroxidase superfamily (Welinder, 1992) reveal that these enzymes are built largely from 10 α-helices connected by loop regions of various lengths (Patterson & Poulos, 1995; Kunishima et al., 1994; Petersen et al., 1994; Sundaramoorthy et al., 1994; Poulos et al., 1993; Finzel et al., 1984). In CCP, for example, 53% of the residues adopt an α-helical conformation, and less than 12% are involved in antiparallel β-sheet structure (Finzel et al., 1984). Therefore, the relatively high intensity of the 1637-cm^{-1} peak in the CCP spectra (Figure 2), which is within the consensus range for β-structure (Krimm & Bandekar, 1986; Susi & Byler, 1986), was somewhat surprising. CCP has 29 β-turns, largely of type I (Finzel et al., 1984), but in keeping with literature assignments (Susi & Byler, 1986; Krimm & Bandekar, 1986), the peaks attributed to turns are at 1663 cm^{-1} and above (Figure 2). More recently, absorbance around 1640 cm^{-1} has been assigned to 3_{10}-helical (Prestrelski et al., 1991) and α-helical absorption (Haris & Chapman, 1995; Martinez & Millhauser, 1995). Since only four -CO groups are involved in 3_{10}-helices in CCP (Finzel et al., 1984), the 1637-cm^{-1} absorbance is assigned to α-helical absorption. Instability (Chirgadze et al., 1976) and extent of hydration (Martinez & Millhauser, 1995) have been cited as significant factors in determining the frequency of α-helical absorption. However, the reasons for the low-frequency α-helical absorption are not apparent from an examination of the X-ray structure of CCP and require further investigation. Based on sequence alignment within the plant peroxidase superfamily (Welinder, 1992), approximately 45% of the residues in HRP are expected to be present in α-helices. The remaining residues are expected to be largely in loop regions that connect the α-helices. This is consistent with the domination of the amide I′ regions of the HRP spectra by peaks arising from turns, α-helices, and random structure (Figures 1 and 4B). The presence of two α-helical components in a single protein, as observed here for HRP and CCP, has been reported previously for lysozyme and α-lactalbumin (Prestrelski et al., 1991; van Stokkum et al., 1995).

Extension of this FTIR spectral survey to other members of the plant peroxidase superfamily is warranted to determine the nature of the subtle changes and differences in the α-helical components reported here for HRP and CCP. Also, the structural reasons for the different denaturation mechanisms of the peroxidases is of interest, including the sensitivity of HRP denaturation to the redox state of the heme iron. Now that X-ray structures are available for at least six members of the plant peroxidase family of homologous enzymes, such studies could shed new light on the more elusive details of structure−function relationships in heme proteins.

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