Structural and Conformational Stability of Horseradish Peroxidase: Effect of Temperature and pH

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Received March 30, 1999; Revised Manuscript Received August 23, 1999

ABSTRACT: Detailed circular dichroism and fluorescence studies at different pHs have been carried out to monitor thermal unfolding of horseradish peroxidase isoenzyme c (HRPc). The change in CD in the 222 nm region corresponds to changes in the overall secondary structure of the enzyme, while that in the 400 nm region (Soret region) corresponds to changes in the tertiary structure around the heme in the enzyme. The temperature dependence of the tertiary structure around the heme also affected the intrinsic tryptophan fluorescence emission spectrum of the enzyme. The results suggested that melting of the tertiary structure to a *pre-molten globule* form takes place at 45 °C, which is much lower than the temperature (T_m = 74 °C) at which depletion of heme from the heme cavity takes place. The melting of the tertiary structure was found to be associated with a pK_a of ∼5, indicating that this phase possibly involves breaking of the hydrogen-bonding network of the heme pocket, keeping the heme moiety still inside it. The stability of the secondary structure of the enzyme was also found to decrease at pH below 4.5. A 'high temperature' unfolding phase was observed which was, however, independent of pH. The stability of the secondary structure was found to drastically decrease in the presence of DTT (dithiothreitol), indicating that the 'high temperature' form is possibly stabilized due to interhelical disulfide bonds. Depletion of Ca^{2+} ions resulted in a marked decrease in the stability of the secondary structure of the enzyme.

Peroxidases catalyze oxidation of a large variety of substrates by hydrogen peroxide. This reaction gives an efficient way for removal of toxic hydrogen peroxide from the intracellular region. Horseradish peroxidase (HRP, EC 1.11.1.7) is the most widely studied member of this peroxidase family (*1*). HRP is a monomeric heme-containing plant enzyme (44 kDa) which has found enormous diagnostic, biosensing, and biotechnological applications (*2*−*4*) because of its unusually high stability in aqueous solution. Previous studies (*5*, *6*) on HRP and metmyoglobin showed that their reactivity to hydrogen peroxide are very much different albeit both of these heme proteins contain the same protoheme active site, indicating the importance of the amino acid residues in the heme cavity in regulating the function of the enzyme. Site-directed mutagenesis of amino acid residues around the heme indicated the existence of a hydrogen-bonding network around the active site. This extensive hydrogen-bonding network in the heme cavity of HRP has been proposed to enhance the stability of the heme cavity of this peroxidase enzyme.

The crystal structure of HRP (Figure 1) has been solved recently (*7*). The structural features of HRP include two Ca^{2+} binding sites proximal and distal to the heme, four disulfide bridges (Cys11−Cys91, Cys44−Cys49, Cys97−Cys301, and Cys177−Cys209), N-glycosylation, and an extensive hydrogen-bonding network (*8*, *9*). These may aid in stabilization of the secondary and/or tertiary structure of the enzyme. The iron of the heme prosthetic group of HRP was found to be pentacoordinated, the distal and proximal sites of the heme pocket were shown to have a hydrogen-bonding network, and the residues participating in this network were also identified in the enzyme from the crystal structure (*7*).

Several studies have earlier been carried out to understand the structural stability of this enzyme. Earlier studies on guanidinium chloride (Gdn·HCl)-induced unfolding of HRP and apo HRP (*10*, *11*) have shown the role of calcium on the stability of the enzyme. The structural stability of HRP was also compared with that of cytochrome *c* peroxidase (CCP) in temperature-dependent FT-IR (*12*) and Gdn·HCl (*13*) induced unfolding studies. These studies showed that the presence of two Ca^{2+} ions is important for the stability of this peroxidase enzyme. The hydrogen-bonding network is also expected to have a significant role in stabilizing the folded structure of the enzyme. However, any detailed study on the role of the hydrogen-bonding network in the stability of the enzyme has not yet been reported.

In the present study, we have carried out detailed circular dichroism and tryptophan fluorescence studies on the thermal unfolding of HRP at different pHs to understand the effect of the hydrogen-bonding network on the stability of the secondary and tertiary structures of the enzyme. The pH dependence of the tertiary and secondary structures has been used to determine the role of the hydrogen-bonding network in the stabilization of the heme cavity of the enzyme. The results have helped to identify various phases of thermal unfolding of the enzyme.

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10.1021/bi990729o CCC: $19.00
Published on Web 12/08/1999

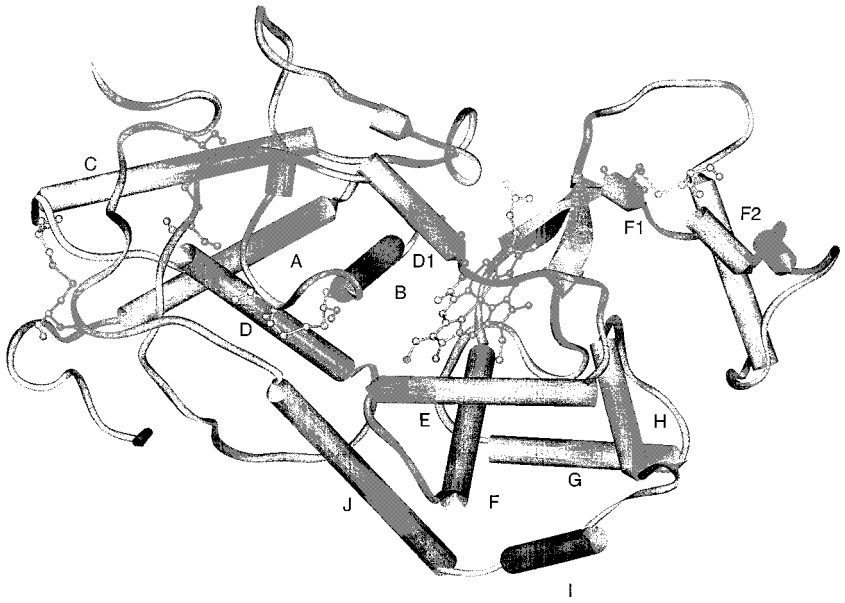

FIGURE 1: Structure of HRP obtained from the crystallographic results. All the helices are named according to nomenclature used by Gajhede et al. (1997) (*7*). The sketch was made by a MSI software, Insight II, v. 2.3, using coordinates downloaded from Protein Data Bank (PDB code: 1atj).

MATERIALS AND METHODS

Chemicals. Lyophilized powder of horseradish peroxidase isoenzyme *c* (HRPc) was a kind gift of Dr. Tony Cass, Imperial College of Science, Technology and Medicine, London, England. Before every experiment, protein solution was further purified in the following way (*14*):

The solid protein was dissolved in 5 mM acetate buffer at pH 4.4 and applied onto a CM52 column equilibrated with the same buffer. The bound protein was washed thoroughly with 5 mM acetate buffer at pH 4.4 and eluted by 100 mM acetate buffer at pH 4.4. The R_Z (ratio of A_{403}/A_{280}) of the protein solution used for the experiment was above 3.1. The concentration of HRPc was determined spectrophotometrically using the extinction coefficient of 102 mM^{-1} cm^{-1} at 403 nm (*15*) for the native enzyme. Guanidinium chloride (Gdn·HCl) was obtained from Sigma-Aldrich Co. All the other reagents used were of highest purity.

Preparation of Ca^{2+}-Depleted HRP. Ca^{2+}-depleted HRPc was prepared by a reported method (*16*). Purified HRPc was incubated with 6 M Gdn·HCl and 5 mM ethylenediaminetetraacetic acid (EDTA) at pH 7 for about 4 h at room temperature. The solution was then dialyzed overnight against 10 mM EDTA at pH 7. It was then followed by extensive dialysis against water.

Thermal Unfolding of HRP. Thermal denaturation of the enzyme was monitored both by tryptophan fluorescence and by circular dichroism techniques. Fluorescence measurements were carried out using a Shimadzu RF540 spectrofluorimeter. The excitation wavelength was chosen at 295 nm in order to avoid the contributions of the tyrosine residues present in HRPc (*17*). CD experiments were carried out using a Jasco J600 spectropolarimeter. CD in the UV region (250−200 nm, UV-CD) was monitored with a water-jacketed cylindrical cell of 1 mm path length with sample concentration of 6−7 μM. CD in the visible region (450−350 nm, Soret-CD) was monitored using a cuvette of 10 mm path length with a protein concentration of 29 μM. The CD data were expressed in terms of mean residue ellipticity, $[\theta]$, in deg·cm^2·$dmol^{-1}$. The temperature was raised from 20 to 95 °C in steps of 2 °C with an equilibration time of 2 min at each temperature. The pH of the solution was measured before every experiment, and care was taken to maintain constant ionic strength of the solutions during experiments. Loss of solvents due to evaporation at high temperature was less than 3%. Modification of the enzyme by dithiothreitol (DTT) was achieved by a reported method (*13*) after treatment with 30 mM DTT and incubation for 18 h at 4 °C. The disulfide bonds of HRP are reduced on treatment with DTT by this method; however, the number of free cysteines could not be estimated because of the presence of excess thiol. The reversibility of the unfolding transitions was always checked by measurement of the CD signals at room temperature upon cooling immediately after the conclusion of the transition (*18*). Reversibility of the transitions was also confirmed from the lack of hysteresis in the transition monitored by the tryptophan fluorescence at different temperatures.

RESULTS

Thermal Unfolding of the Secondary Structure of HRPc Monitored by UV-CD. The CD of HRPc in the UV region conforms to the helical structure of the enzyme with two minima at 209 and 222 nm (*19*). Computer simulation of the CD spectra using the program CONTIN (*20*) showed around 30% helical character, which agreed with the previous results (*21*). However, the helicity was decreased with increase in temperature as manifested by the decrease in ellipticity at 222 nm. The UV-CD spectra of HRPc at two different temperatures are shown in Figure 2a,b. The spectra of the enzyme obtained in the presence of 30 mM DTT (dithiothreitol) at 90 °C and that of completely unfolded enzyme obtained in 6 M Gdn·HCl (at room temperature) are shown respectively in Figures 2(c) and 2(d) for comparison. It is to be noted that the ellipticity of HRPc at 222 nm, even at 93 °C, was higher than that of the completely denatured enzyme (treated by 6 M Gdn·HCl) [Figure 2(d)], indicating that HRPc possibly does not lose its complete

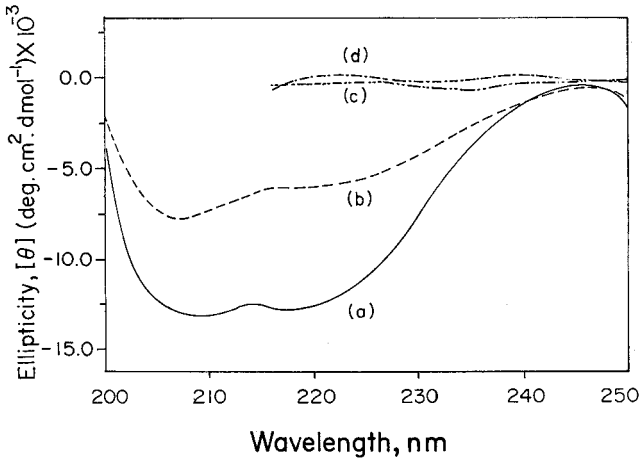

FIGURE 2: UV circular dichroism spectra of HRP at 25 °C (a), at 93 °C (b), in the presence of 30 mM DTT at 90 °C (c), and in the presence of 6 M GdN·HCl at 25 °C (d). The concentration of HRP is 7 μM. The path length is 1 mm.

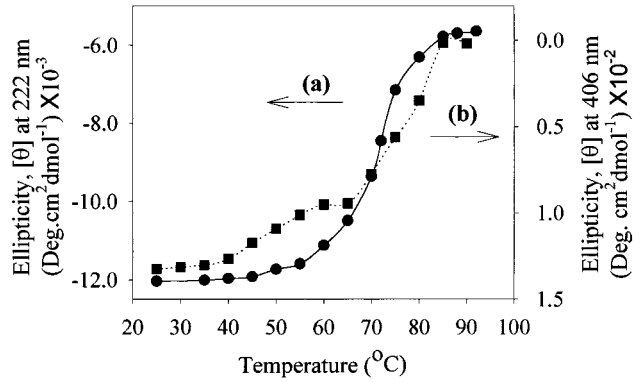

FIGURE 3: (a) Change in the ellipticity of HRP at 222 nm with temperature. The concentration of HRP is 7 μM. The path length is 1 mm. (b) Change in the ellipticity of HRP at 406 nm with temperature. The concentration of HRP is 20 μM. The path length is 10 mm.

secondary structure even at 93 °C. However, DTT-modified enzyme at 90 °C showed an almost similar CD spectrum [Figure 2(c)] as that of the completely denatured enzyme at room temperature [Figure 2(d)]. These results indicate that a second phase of backbone melting of HRPc possibly occurs at a temperature higher than 93 °C and the residual secondary structure at this temperature may arise due to stabilization by interhelical disulfide bonds in the enzyme (*13*).

The change in ellipticity of HRPc at 222 nm with temperature is plotted in Figure 3(a), which indicated a two-state melting of the secondary structure of the enzyme with an apparent T_m value (the midpoint of the transition) of 74 °C. The temperature dependence of UV-CD at 222 nm of the enzyme modified by DTT, however, showed a much lower and single transition temperature (T_m 65 °C), indicating that removal of interhelical disulfide bonding by DTT drastically decreases the stability of the enzyme.

Thermal Unfolding of the Tertiary Structure around Heme in HRPc Monitored by Soret-CD. CD at the Soret region was monitored to find out the effect of temperature on the heme active site. Hemin is CD-inactive in its free form while in an asymmetric environment of a protein it becomes CD-active. The CD activity of the heme transition in heme proteins results from the short- and long-range interactions of the heme with the protein matrix. A coupled oscillator

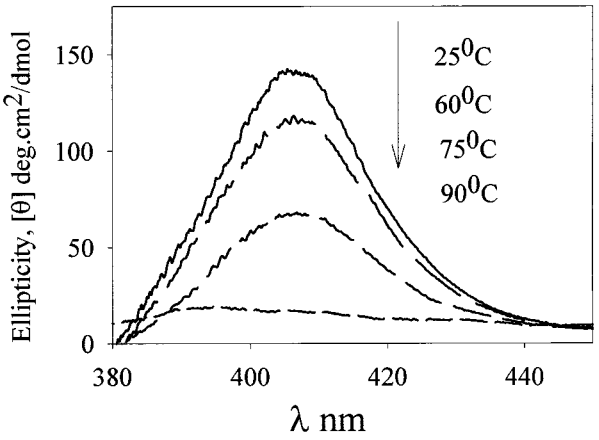

FIGURE 4: Soret circular dichroism spectra of HRP at different temperatures. The concentration of HRP is 20 μM. The temperatures are noted in the figure. The path length is 10 mm.

interaction between the transitions of the heme and those of the surrounding aromatic side chains gives rise to the observed CD bands in heme proteins (*22*, *23*). CD spectra of HRPc in the Soret region at different temperatures are shown in Figure 4, which shows that the CD intensity at 407 nm decreases with increase in temperature. The Soret-CD band vanishes at 93 °C, which conforms to the complete removal of heme from protein core at that high temperature. The variation in the CD intensity at 407 nm with temperature is plotted in Figure 3(b), which shows the presence of two distinct phases. The first phase of transition starts below 40 °C which is then followed by another phase of unfolding which reached saturation at 85 °C. Thus, unlike the melting of the secondary structure monitored by CD at 222 nm, the temperature-induced unfolding observed in the Soret-CD clearly showed the existence of one intermediate state.

Comparison of the temperature dependence of the UV-CD and Soret-CD of HRPc (Figure 3) provides information on the two phases of unfolding of the enzyme structure. The first phase is associated with a change in the local conformation of the heme active site monitored by CD at 407 nm. This phase is accompanied by only a very little or no change in the overall secondary structure of the enzyme (measured by CD at 222 nm). The second phase of the transition involves a large change in the secondary structure, which is a result of complete removal of heme from the heme active site indicated by the complete absence of the Soret-CD at 407 nm. The melting of the overall secondary structure of HRPc (observed in the temperature between 50 and 93 °C) and the first phase of the change in the tertiary structure near the heme region (monitored in the temperature range of 35−55 °C) form two distinct phases. Thus, in this temperature range, an intermediate structure of the enzyme is present where the secondary structure is intact while the tertiary structure near the heme center is changed. Such intermediate structure with partially collapsed tertiary structure and intact secondary structure, which can be related to *pre-molten globule* folding intermediates, was also predicted by earlier FT-IR studies on the thermal denaturation of HRP and cytochrome *c* peroxidase in D_2O (*12*). The unfolding of HRP thus involves a *pre-molten globule* intermediate where the secondary structure of the enzyme remains more or less unchanged while there is a substantial change in the conformation of the heme active site. Figure 3 shows that

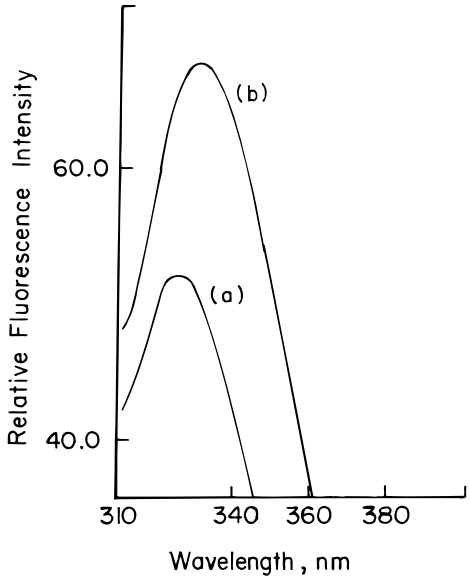

FIGURE 5: Fluorescence emission spectra of 5 μM HRP at 25 °C (a) and at 55 °C (b) at pH 7. Excitation wavelength is 295 nm.

the Soret-CD was decreased by ~30% at the end of the first phase (50 °C). The Soret-CD intensity further decreases drastically at temperatures above 50 °C and vanishes at 90 °C. The first phase thus possibly corresponds to a local conformational change in the heme pocket leading to intermediate *pre-molten globule* formation. This is then followed by release of heme from the protein cavity, indicated by a drastic decrease in the Soret-CD and subsequent change in the secondary structure of the enzyme (*13*) in the second phase.

Change in Tryptophan Fluorescence of HRPc with Temperature. The intrinsic fluorescence of the enzyme is highly dependent on the fluorescence energy transfer from tryptophan to heme (*24*−*27*). Thus, changes in the structure of the heme cavity affecting the distance/orientation between the heme and the tryptophan would affect the intrinsic fluorescence of the enzyme. On the other hand, a change in the microenvironment surrounding the tryptophan moiety changes the emission maximum of the tryptophan fluorescence (*28*). Denaturation of heme proteins is generally accompanied by an increase in the fluorescence intensity due to the decrease in the quenching effect of heme in the unfolded protein (*10*, *29*−*31*). In most of the proteins, unfolding also leads to a red shift of the fluorescence maximum as a result of a change in the tryptophan microenvironment to a more exposed and a more polar one in the unfolded form (*32*). Temperature dependence of the intrinsic tryptophan fluorescence (Trp117) of the HRPc has been studied in order to corroborate the conformational change at the heme active site in the temperature range 25−60 °C observed from the variation of the Soret-CD [Figure 3(b)]. A well-resolved reversible phase transition corresponding to the change in the tertiary structure in the heme cavity region was observed when the tryptophan fluorescence of the enzyme was monitored.

Figure 5 shows the fluorescence spectra of HRPc at room temperature and at 55 °C with excitation at 295 nm. Excitation at 295 nm ascertained that the observed emission arises solely from the tryptophan residue of the enzyme (*13*). The fluorescence emission of the native enzyme has a very low quantum yield (0.001) (*33*), which is a direct consequence of efficient energy transfer between tryptophan and the heme group. The fluorescence emission of HRP was at 326 nm at room temperature, which slowly shifted to 330 nm at high temperature. The intensity of the fluorescence emission increases with increase in temperature because of a change in the relative orientation or distance between the heme and the tryptophan residue leading to a decrease in the efficiency of energy transfer. The temperature dependence of the fluorescence intensity at 330 nm is shown in Figure 6. A typical two-state transition was observed with an apparent T_m of 42 °C. This T_m value agrees with the T_m obtained from temperature dependence of the Soret-CD signal (Figure 3b), indicating that it corresponds to the same tertiary structure change. On further increase in temperature, the second phase of unfolding, characterized by a much larger increase in fluorescence possibly because of complete release of the heme from the enzyme, was observed with a midpoint at ~72 °C (not shown in the figure). Release of heme from HRPc leading to a high increase in fluorescence intensity has also been reported earlier (*13*). The temperature-dependent fluorescence changes were always calibrated against a control of completely denatured enzyme to ensure that the observed temperature dependence arises primarily due to changes in the energy transfer between the heme and the tryptophan moiety.

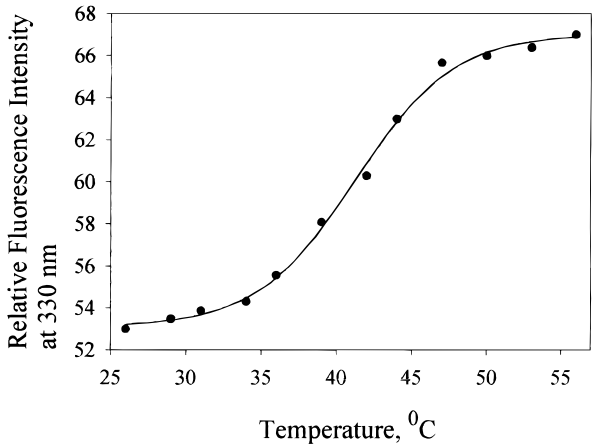

FIGURE 6: Change in the relative fluorescence intensity at 330 nm with temperature. HRP concentration is 5 μM. Excitation wavelength is 295 nm.

pH Dependence of the Melting of the Conformation of the Heme Cavity. HRP has a very extensive hydrogen-bonding network in the proximal and distal region of the heme (*7*−*9*). The proximal His (His170) is covalently bonded to the iron, and the existence of direct hydrogen bonds between the protein and the heme propionates through Gln176, Ser73, Ser35, and Arg31 was observed in the crystal structure of HRPc (*7*). The distal pocket is connected to the proximal side by a hydrogen-bonding network involving Arg38. Gln176 also has a role in connecting the two halves of the extended hydrogen-bonding network above and below the heme group. The role of the hydrogen-bonding network in the stability of the conformation of the heme cavity has been studied by monitoring the temperature dependence of the fluorescence intensity at 330 nm at different pHs. Figure 7-(a) shows the pH dependence of the T_m for melting of the tertiary structure around the heme center, obtained from the temperature dependence of the fluorescence emission at 330

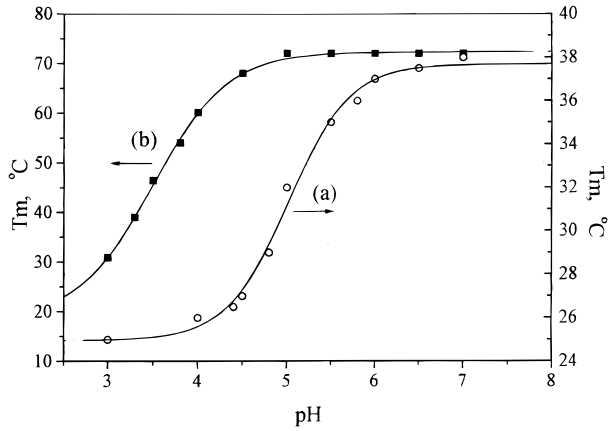

FIGURE 7: (a) Change in the value of T_m corresponding to the melting of the tertiary structure of the heme active site with pH. (b) Change in the value of T_m corresponding to the melting of the secondary structure of HRP with pH.

nm. Similar results were also observed from the variation of the Soret-CD. The magnitude of the T_m was found to increase with pH (from pH ~4.5 to pH ~7), and an apparent midpoint of the transition was observed at around pH 5, possibly indicating protonation of His42/His40 (distal histidine) which has a pK_a in this pH range. Some of the residues, which take part in the hydrogen-bonding network around the heme, are protonated with decrease in pH. This results in breaking up of the network, leading to the decreased stability of the heme pocket. The T_m for melting of the tertiary structure of the heme cavity of HRPc decreased to room temperature (298 K) at pH below ~4.5, indicating that the hydrogen-bonding network around the heme may be completely broken at this pH which follows release of heme from the protein cavity. The secondary structure of the enzyme monitored from the CD in the UV region (222 nm) was, however, found to be stable even at pH 4.5 [see below, Figure 7(b)].

Melting of the Conformation of the Heme Cavity by Gdn·HCl. The tertiary structure of the enzyme was found to be affected by even small amounts of guanidinium chloride (Gdn·HCl). The effect of small concentrations of the denaturant on the heme cavity tertiary structure has been studied by the temperature dependence of the intrinsic fluorescence at 330 nm at different Gdn·HCl concentrations. The values of T_m were found to be 40, 39, 37, 32, and 30 °C, respectively, at 200 μM, 1 mM, 10 mM, 100 mM, and 500 mM Gdn·HCl. A steady decrease in the T_m with increasing concentration of the denaturant indicates that the tertiary structure was more easily opened up with an increase in Gdn·HCl concentration, which agrees with earlier report (*13*). The tertiary structure was completely destroyed at the denaturing concentration (6 M Gdn·HCl).

Melting of Secondary Structure at Different pHs. The melting of the secondary structure of the enzyme was studied at different pHs by monitoring the change in the ellipticity at 222 nm (UV-CD) with temperature. Figure 7(b) shows the plot of the apparent T_m of melting of the secondary structure obtained from the temperature dependence of UV-CD of the enzyme, with pH. The apparent T_m for melting of the secondary structure increased with increase in pH in the range pH 3 to pH 5, and it remained almost constant (74 °C) above pH 5 to pH 7. The sharp decrease in the T_m below pH 4.5 indicates a decrease in the stability of the protein structure at low pH. However, comparison of the results shown in Figure 7(a) and Figure 7(b) shows that at pH ~4.5, although the T_m for melting of the tertiary structure around the heme is about room temperature, the T_m for the change in the secondary structure still remains quite high (63 °C). On the other hand, the T_m for the melting of the overall secondary structure of the enzyme decreased sharply below pH 4.5, where the T_m for the unfolding of the tertiary structure is already below room temperature. As mentioned above, variation of the T_m of heme active site melting with pH gives a pK_a of 5 corresponding to protonation of a distal histidine, indicating involvement of the hydrogen-bonding network in stabilizing the conformation of the heme active site. The overall stability of the protein also starts decreasing once the tertiary structure of the heme active site is melted, and a sharp decrease in the T_m corresponding to the unfolding of the overall secondary structure of the enzyme was observed at pH 4.5. The unfolding of the secondary structure becomes more feasible at pH below ~4, and the T_m decreased to room temperature at pH ≤3 which indicates that below pH 3 HRP starts responding to the acid-induced unfolding process at room temperature.

The secondary structure of HRPc, as mentioned before, was not completely unfolded even at 93 °C at ambient pH, indicating the possibility of the existence of yet another phase of unfolding of the enzyme at a temperature beyond 93 °C, which could not be determined because of experimental difficulty. Since the overall stability was found to decrease at lower pH (pH ≤4), we tried to check whether this 'high-temperature' phase could be detected at a temperature below 93 °C at a lower pH. But even at pH 3.5 the unfolding of the secondary structure was found to be incomplete at 93 °C, indicating that hydrogen bonding may not be responsible for the stability of the 'high temperature' phase of the secondary structure of the enzyme. Treatment with 30 mM DTT reduces the disulfide bonds (*13*) of HRPc, and thermal unfolding of the DTT-modified enzyme was found to be complete with no residual structure at 90 °C (Figure 2). This suggests that the partial stabilization of the helical structure of HRPc by the interhelical disulfide bonds may be responsible for the 'high temperature' form of the enzyme.

Unfolding of Ca^{2+}-Depleted HRP. The endogenous calcium ions (Ca^{2+}) present in HRPc were earlier shown to have important role in forming the heme pocket (*11*) as well as in maintaining the spin state of the heme iron which favors the enzyme activity of HRP (*34*). Previous studies on unfolding of this enzyme by Gdn·HCl have also shown the importance of the Ca^{2+} ions in the stability of this enzyme (*10*−*13*). In the present study, we monitored the thermal stability of the secondary structure from the CD at 222 nm at different temperatures to find out thermal unfolding of calcium-depleted HRPc. The depletion of Ca^{2+} ions was found to have a profound effect on the thermal stability of the secondary structure of HRPc, and the apparent T_m value obtained for melting of the secondary structure was decreased from 74 to 61 °C as a result of Ca^{2+} ion depletion.

Soret-CD and tryptophan fluorescence of the calcium-depleted enzyme were also studied at various temperatures to detect any intermediate form as observed for native HRP. However, no intermediate transition was observed in this temperature range which indicates that the conformation of

the heme cavity is already destroyed in calcium-depleted HRP.

DISCUSSION

Steady-state fluorescence as well as circular dichroism techniques have earlier been used to probe the structure and stability of proteins (*35*−*37*). Circular dichroism studies in the UV region (200−250 nm) have been very extensively used to understand the protein secondary structure. Ellipticity at 222 nm is used to monitor the unfolding of a helical protein. On the other hand, circular dichroism in the near-UV region, mostly in the Soret region, gives important information about the tertiary structure around the heme cavity of the heme protein. Unfolding of the heme protein generally disrupts the heme active sites, which can be probed by the change in CD at the Soret region. Very often, denaturation leads to the removal of heme from the cavity, which is characterized by complete loss of the heme CD. Taken together, UV-CD at 222 nm gives information on the global changes of the protein secondary structure whereas CD at the Soret region gives information about local changes in the heme cavity.

Thermal denaturation of HRPc has been studied using CD both at 222 nm and at 407 nm. Changes in the CD at 222 nm at pH 7.0 with temperature gave rise to a two-state transition, whereas the Soret-CD (at 407 nm) clearly demonstrated the existence of an intermediate state between those two states. The CD spectrum of the enzyme in the 222 nm region shows that even at 93 °C denaturation was not complete and the 'high temperature' form was found to be independent of pH.

The CD in the 222 nm region is often attributed not only to the peptide bonds but also to aromatic residues (*19*, *38*). HRPc has 26 aromatic residues (5 Tyr, 1 Trp, and 20 Phe) out of 308 residues in the protein (*40*). The maximum ellipticity at 222 nm due to the aromatic residues in HRPc can be calculated to be ∼1013 deg.cm^2/dmol on a mean residue basis assuming that the contribution of each aromatic residue is 12 000 deg.cm^2/dmol (*19*). The observed ellipticity of HRPc at 222 nm (Figure 2) is several orders of magnitude larger than this; hence, the contributions of the aromatic residues were within the error limit of the experiment even in the fully denatured enzyme. This confirms that the observed CD of the enzyme at 93 °C originates solely from the residual helicity of the enzyme.

Dithiothreitol (DTT) is known to break cystine disulfide bonds in proteins. Earlier studies (*13*) have shown that 30 mM DTT can efficiently cleave the disulfides, causing large enhancement in the fluorescence intensity (∼10 times) of HRPc at ambient conditions. The CD spectrum of the DTT-modified enzyme at 90 °C (Figure 2) was very similar to that of the fully denatured enzyme, indicating that the interhelical disulfide bonds are responsible for stabilization of the residual helicity (in the 'high temperature' form) of the native enzyme at high temperature. The following unfolding pathway may thus be proposed based on the results of fluorescence emission and CD experiments in the UV and Soret regions of the enzyme:

$$N \rightarrow U' \rightarrow U'' \rightarrow U$$

The native state of HRPc (N), on thermal denaturation, goes toward the final unfolded state through at least two intermediate states, U′ and U″. The intermediate state U′ has almost intact secondary structure of native HRPc but with a difference in the tertiary structure due to the change in the heme cavity. The presence of a *pre-molten globule* intermediate analogous to U′ was also predicted by earlier FT-IR studies (*12*) on the thermal unfolding of HRPc.

The transition between the native state, N, and the first intermediate state, U′, was observed very clearly by the intrinsic tryptophan fluorescence of the enzyme. The intrinsic fluorescence probe tryptophan is very sensitive to the microenvironment surrounding the tryptophan residue in a protein (*39*). Nevertheless, the use of tryptophan fluorescence in heme proteins is somewhat restricted because of the low quantum yield due to very efficient energy transfer between the tryptophan and the heme residue (*26*). Isoenzyme *c* of HRP has a single tryptophan residue (Trp117) (*40*), and hence it is relatively simple to analyze the fluorescence data. Intrinsic fluorescence was earlier used to monitor the structural transitions of apo and holo HRP in the presence of Gdn·HCl (*10*). The technique was applied to study the effect of pH-induced conformational perturbation in the heme active site of HRPc (*17*). It was also used for the study of the stability of HRPc and CCP with reference to the stabilizing effects of the two calcium ions present in the former peroxidase (*13*).

The reversible increase in fluorescence with temperature observed in the present case might arise due to loosening of the heme cavity at a temperature of ∼50 °C. The tryptophan residue would then move away with respect to the heme, leading to a decrease in the energy transfer efficiency and thus an increase in the tryptophan fluorescence intensity. This change in the heme active site in the intermediate form (U′) resulted in a ∼2-fold increase in tryptophan fluorescence intensity. As a result of the conformational transition, the tryptophan residue becomes more exposed to the external solvent, leading to a red shift of the fluorescence emission maximum (*31*). The pH dependence of this transition revealed that the formation of the intermediate form U′ is associated with a pK_a of ∼5, which indicated protonation of a distal histidine residue. This suggests that the intermediate U′ might be formed by breaking up of the hydrogen-bonding network involving the distal histidine around the heme in HRPc.

The transition from U′ to U″ was characterized by a complete loss of CD at the Soret region, which can be explained by the removal of heme from the cavity. This is also associated with a large increase (>10-fold) in the fluorescence intensity and a significant loss of secondary structure as evident from the CD at 222 nm. The loss of secondary structure of HRPc with the release of heme from the active site was also reported earlier (*10*, *13*). pH-dependent studies indicated that complete depletion of heme from the enzyme leading to the intermediate U″ was associated with breaking up of the Fe−His (proximal) bond which takes place at pH <3 at room temperature.

The presence of calcium ions and interhelical disulfide linkages has earlier (*12*) been proposed to be responsible for the increased thermal stability of HRP compared to cytochrome *c* peroxidase (CCP). Significant α-helical absorption at 1659 cm^{-1} up to 80 °C in the FT-IR spectrum of HRPc (*12*) was consistent with high thermal stability of the

secondary structure of the enzyme. Aggregation of the enzyme was not detected by the FT-IR study even on further increase in temperature to 95 °C (*12*). However, the exact extent of unfolding of the enzyme at this temperature could not be inferred from the earlier report (*12*) as that would require comparison of the FT-IR results with those of fully denatured (by Gdn·HCl) enzyme. Our results on thermal unfolding of the enzyme suggest that modification by DTT drastically decreases the T_m (65 °C) of unfolding of the overall structure of the enzyme and no 'high temperature' form with residual helicity was observed in the modified enzyme. The intermediate U″ may thus have partially unfolded helical structure with part of the helicity still conserved due to interhelical disulfide bonds.

Earlier studies (*13*) showed complete loss of secondary structure of HRPc accompanied by depletion of heme from the protein cavity at room temperature in 2.5 M Gdn·HCl at ambient pH. Complete loss of secondary structure by Gdn·HCl possibly indicates that the interhelical disulfide bonding may not efficiently protect the helical structure of the enzyme in the presence of the chemical denaturant (*13*). Nevertheless, a steady decrease in the helicity with increase in Gdn·HCl concentration from 2.5 to 6 M [Figure 7B of (*13*)] and an increase in tryptophan fluorescence intensity of the enzyme [Figure 8 of (*13*)] noted in the earlier report (*13*) might accrue from partial stabilization of the helical structure by the disulfide bonds. It is, however, noteworthy that the *pre-molten globule* intermediate which was observed in earlier thermal unfolding studies (*12*) as well as in the present study was not formed in Gdn·HCl-induced unfolding of HRP.

The first step of thermal unfolding (N to U′) of HRPc thus leads to an intermediate of intact secondary structure but with a different conformation of the heme cavity. The second step (U′ to U″), is accompanied by complete loss of the heme prosthetic group from the enzyme leading to a substantial loss of the secondary structure. The presence of at least two intermediates was thus established in the unfolding pathway of HRPc. The presence of disulfide bridges, two endogenous Ca^{2+} ions, and a hydrogen-bonding network have important roles in the thermal stability of the enzyme. The depletion of Ca^{2+} from native HRP leads to a conformational change in the heme pocket, which leads to the shift of the emission maximum of the tryptophan residue to 337 nm with an increase in the fluorescence quantum yield to 0.030 (*11*). Removal of Ca^{2+} resulted in depletion of the heme residue from the enzyme at a much lower temperature (61 °C compared to 74 °C in the presence of Ca^{2+}) at ambient pH, indicating that Ca^{2+} ions play a significant role in stabilizing the enzyme.

Modification of the enzyme by DTT was shown to have a drastic effect on the thermal stability of the 'high temperature' species of the enzyme (U″) which was completely unfolded at a temperature of 90 °C. The presence of four disulfide bridges thus might be responsible for the incomplete unfolding of the enzyme. The structure of HRP given in Figure 1 shows the disulfide bridges (displayed using ball-and-stick representation) between the helices. It is clearly evident that these disulfide bridges may have significant effects on the stability of the neighborhood helices A, B, C, D, F1, and F2. The presence of intact or incompletely broken disulfide bridges should be able to maintain some residual structures in their vicinity even when the other sites of the enzyme are completely randomized. Thus, intact or partially broken disulfide bridges might be the reason behind the residual ellipticity observed even at very high temperature.

CONCLUSIONS

Thermal unfolding of HRPc has been carried out using circular dichroism and fluorescence techniques. The changes in the CD in the 220 nm region correspond to changes in the secondary structure of the enzyme while changes in the Soret-CD (407 nm) correspond to changes in the tertiary structure around the heme cavity of the enzyme. The results from CD studies indicated formation of an intermediate structure in the temperature range 40−50 °C at ambient pH, in which the tertiary structure around the heme cavity was melted while the overall secondary structure of the enzyme remained almost unchanged. This form has been identified as the *pre-molten globule* intermediate (*12*). This intermediate form (U′) was found to be highly pH-dependent with an apparent pK_a of ∼5, which corresponds to protonation of a distal histidine residue. This indicated that the intermediate (U′) may not have the hydrogen-bonding network of the heme cavity of HRPc, although the heme might still be coordinated to the proximal histidine and residing inside the cavity. The overall secondary structure of the enzyme was found to change drastically with an apparent T_m of 74 °C which is associated with complete release of the heme moiety from the enzyme. Depletion of the Ca^{2+} ion had a marked effect on the stability of the enzyme. The Ca^{2+}-depleted HRPc showed melting of the secondary structure at 61 °C compared to 74 °C observed in the native enzyme at ambient pH. The unfolding of the secondary structure was, however, found to be incomplete even at 93 °C, and a 'high temperature' unfolding phase (U″) was detected. Reduction of the disulfide bonds in the enzyme resulted in a drastic decrease in the T_m (65 °C) corresponding to complete unfolding of the secondary structure. The results indicated that these disulfide bonds might be responsible for stabilizing the partially unfolded intermediate (U″) at high temperature.

ACKNOWLEDGMENT

We thank Dr. Tony Cass for kindly donating samples of HRPc used in this work.

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BI990729O