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Effect of the His175 → Glu Mutation on the Heme Pocket Architecture of Cytochrome *c* Peroxidase[†]

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ABSTRACT: Resonance Raman (RR) and electronic absorption spectra of the ferric and ferrous forms of the His175Glu mutant of cytochrome *c* peroxidase are reported. At 296 K, the Fe^{III} form is five-coordinate high spin and the resonance Reman spectra are very similar to those obtained for the wild type enzyme, even though in the mutant the Fe atom is bound to an oxygen atom of the Glu residue. The only difference is that the bands due to the out-of-plane modes are very weak, indicating a less distorted heme plane compared to CCP. The absorption spectrum is similar to that of CCP, as far as the Soret and α,β bands are concerned, but the charge-transfer band due to the $a_{2u}(\pi) \rightarrow e_g(d_\pi)$ transition is 8 nm blue-shifted relative to that of the wild type enzyme, indicating that a more negative ligand is bound to the heme iron. As the temperature is lowered, the five-coordinate heme converts to a six-coordinate high-spin form. The conversion is readily reversible. A temperature effect on the protein structure is proposed that permits the Fe atom to approach the heme plane and to bind the distal water molecule. The results are discussed in terms of the X-ray structure, which shows a different disposition of the distal water molecules in the Glu175 mutant. The RR spectra also show that the heme is more contracted and distorted at 19 K than at room temperature. The RR spectra of the ferrous form are characterized by a slightly higher frequency of the core size marker bands and weaker out-of-plane modes for the mutant compared to wild type CCP, indicating a more planar heme. As expected, the bands due to the ν(Fe−Im) stretching modes are not present, and no new band due to the ν(Fe−ligand) is detected in the low-frequency region.

Peroxidases are heme-containing enzymes which catalyze the reduction of hydrogen peroxide by different substrates in the following multistep reaction

$$\mathrm{Fe(III)} + \mathrm{H_2O_2} \rightarrow \mathrm{Fe(IV){=}O\ R^{\bullet}} + \mathrm{H_2O}$$
$$\textbf{compound I}$$

$$\mathrm{Fe(IV){=}O\ R^{\bullet}} + \text{substrate} \rightarrow \mathrm{Fe(IV){=}O\ R} + \text{oxidized substrate}$$
$$\textbf{compound II}$$

$$\mathrm{Fe(IV){=}O\ R} + \text{substrate} \rightarrow \mathrm{Fe(III)} + \mathrm{H_2O} + \text{oxidized substrate}$$

The enzyme is first oxidized by a peroxide molecule to give compound **I**, in which one oxidizing equivalent resides on the iron as Fe(IV)=O and one resides as an organic radical, $R^{\bullet}$, which is either the porphyrin or a neighboring amino acid side chain. The majority of the heme peroxidases so far sequenced are characterized by the presence of a hydrogen bond between the N_δ atom of the histidine fifth ligand and the oxygen atom of a proximal aspartate side chain (Welinder, 1992) which gives the proximal ligand imidazolate character. To understand the role of the proximal ligand in the catalytic cycle of cytochrome *c* peroxidase (CCP),[1] different mutants containing Gln (H175Q), Glu (H175E), or Cys (H175C) in the fifth position have been studied (Choudhury et al., 1994). In steady state assays, the H175C mutant exhibited only about 7% wild type activity, the H175Q mutant was very nearly the same as the wild type CCP, and the H175E mutant was about seven times more active. In addition, stopped-flow experiments showed that the precise nature of the proximal ligand is not important for high rates of peroxide O−O bond cleavage but is important for the stability of compound **I**.

The X-ray structures showed that H175E contains a five-coordinated heme, while the H175Q has the distal water molecule coordinated to the Fe atom, although EPR data obtained at 4.2 K indicated the presence of six-coordinated hemes for both mutants. The apparent inconsistency between the X-ray structure and the EPR data observed for the H175E mutant has been attributed to the effect of methylpentanediol

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[1] Abbreviations: CCP, yeast cytochrome *c* peroxidase; CCPMI, cytochrome *c* peroxidase expressed in *E. coli* containing Met-Ile at the N-terminus; W51F, Trp51 → Phe CCPMI mutant; H175E, His175 → Glu CCP mutant; HRP-C, horseradish peroxidase, isoenzyme C; MetMb, MetMyoglobin; MetHb, MetHemoglobin; MPD, 2-methyl-2,4-pentanediol; EPR, electron paramagnetic resonance; RR, resonance Raman; 5-c and 6-c, five- and six-coordinate hemes, respectively; HS and LS, high spin and low spin.

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used for crystallization which may alter the disposition of the distal water molecule in the crystal (Choudhury et al., 1994).

In the present study we characterize the ferric form of the H175E mutant at room and low temperatures (19 K) by using electronic absorption and resonance Raman spectroscopy. The experiments were carried out on the proteins in buffer, without addition of any glassing agent. This has made it possible to clarify the effect of lowering the temperature on the heme cavity. The ferrous form also has been characterized by using resonance Raman spectroscopy. These results are compared with those previously obtained for CCP and recombinant CCPMI to determine the effect of replacing of the proximal His with Glu.

MATERIALS AND METHODS

The His175Glu mutant was prepared as described previusly (Choudhury et al., 1994). Crystals of the enzyme were dissolved in 0.1 M Tris[tris(hydroxymethyl)aminomethane] acetate/MES [2-(*N*-morpholino)ethanesulfonic acid] buffer at pH 5.5. The Fe(II) form was prepared by adding a minimum volume of fresh sodium dithionite solution in the deoxygenated buffered solution. The reduced form was very unstable and reverted to the Fe(III) form in about 20 min. Sample concentration was about 1×10^{-5} M for all of the experiments.

Absorption spectra were measured with a Cary 5 spectrophotometer.

The resonance Raman (RR) spectra were obtained by excitation with the 406.7 and 413.1 nm lines of a Kr^+ laser (Coherent, Innova 90/K) and the 457.9 nm line of a Ar^+ laser (Coherent, Innova 90/5). At room temperature the back-scattered light from a slowly rotating NMR tube was collected and focused into a computer-controlled double monochromator (Jobin-Yvon HG 2S) equipped with a cooled photomultiplier (RCA C31034 A) and photon-counting electronics. The RR spectra were calibrated with indene and CCl_4 as standards to an accuracy of 1 cm^{-1} for intense isolated bands.

Polarized spectra were obtained by inserting a Polaroid analyzer between the sample and the entrance slit of the spectrometer. The depolarization ratios, ρ, of the bands at 314 and 460 cm^{-1} of CCl_4 were measured to check the reliability of the polarization measurements using a rotating NMR tube with 180° back-scattered geometry. The values obtained, 0.73, and 0.01, compared favorably with the theoretical values of 0.75 and 0.0, respectively.

At low temperature both absorption and RR experiments were obtained on the same sample. Low-temperature spectra were obtained using a closed-cycle He cryotip with an automatic temperature controller, using a sealed quartz microcuvette (120 μL volume, 0.25 cm path length). The samples were transferred to the cuvette at room temperature, and the sealed cuvette was mounted on the cold finger of the cryotip. The temperature was then slowly decreased under vacuum.

RESULTS

Figure 1 shows the absorption spectra of the ferric H175E mutant in buffer at pH 5.5 taken at different temperatures. The wavelengths of the Soret and of the α,β bands correspond to a 5-c HS heme. With decreasing temperature the Soret band shifts from 407 to 406 nm. More dramatic are the changes observed in the visible region: the β,α bands shift from 506 to 497 nm and from 539 to 534 nm, respectively, and the band at 637 nm shifts down to 618 nm.

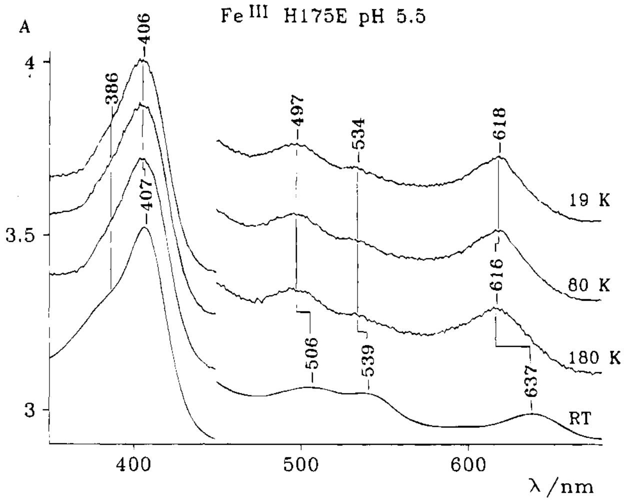


FIGURE 1: Absorption spectra of ferric H175E mutant at pH 5.5, taken at different temperatures. The region between 450 and 700 nm has been expanded 2.5 times.

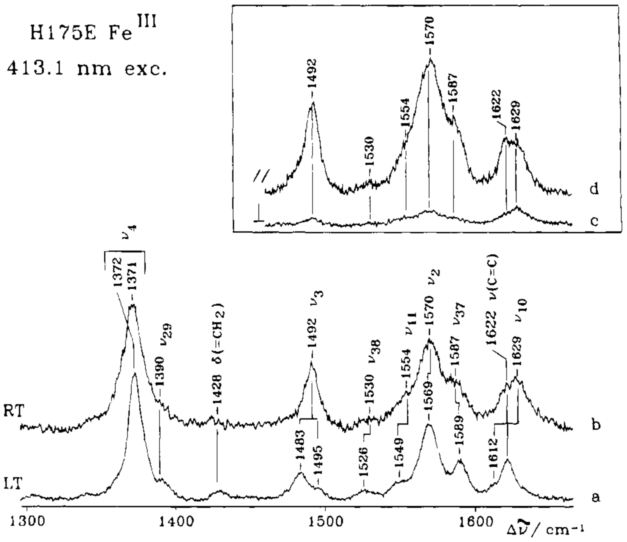


FIGURE 2: High-frequency region resonance Raman spectra of ferric H175E mutant obtained with 413.1 nm excitation wavelength at (a) 19 K and at (b) room temperature. Experimental conditions: 5 cm^{-1} resolution, 15 mW laser power at the sample; (a) 5 s/0.5 cm^{-1} and (b) 2 s/0.5 cm^{-1}, collection intervals. In the insert the resonance Raman spectra in perpendicular (⊥) (c) and parallel (||) (d) polarized light are shown. Experimental conditions are as given above, except 22 s/0.5 cm^{-1} (⊥) and 12 s/0.5 cm^{-1} (||) collection intervals were used.

Figure 2 compares the 1300–1700 cm^{-1} region RR spectra excited at 413.1 nm of ferric H175E mutant in buffer pH 5.5, obtained at (a) 19 K and at (b) room temperature. In order to assign the bands of the spectra at room temperature, we undertook a study in polarized light. The spectra obtained with the scattered light polarized perpendicular (⊥) and parallel (||) to the polarization of the exciting radiation are reported in the insert of Figure 2. On the basis of the depolarization ratios, $\rho = I_\perp/I_\parallel$, the bands were assigned as follows: ν_3 at 1492 cm^{-1} (A_{1g}), ν_2 at 1570 cm^{-1} (A_{1g}), ν_{38} at 1530 cm^{-1} (E_u), ν_{37} at 1587 cm^{-1} (E_u), ν_{11} at 1554 cm^{-1} (B_{1g}), ν_{10} at 1629 cm^{-1} (B_{1g}), and ν(C=C) at 1622 cm^{-1}.

Table 1: Skeletal and Vinyl Stretching Mode Frequenices (cm^{-1}) for Model Hemes and for Ferric and Ferrous H175E Mutant at Room Temperature and at 19 K

	FE^{III}			FE^{II}	
	H175E		(Me$_2$SO)$_2$PP[a]	H175E	(2-MeIm)PP[a]
mode	RT,[b] 5-c HS	LT,[b] 6-c HS	RT, 6-c HS	RT, 5-c HS	RT, 5-c HS
ν_4	1371	1372	1370	1359	1357
ν_3	1492	1483	1480	1474	1471
ν_{38}	1530	1526	1518		
ν_{11}	1554	1549	1545		
ν_2	1570	1569	1559	1566	1562
ν_{37}	1587	1589	1580	1584	1583
ν_{10}	1629	1612	1610	1607	1604
ν(C=C)	1622	1622	1621	1619	1622

[a] PP, Protoporphyrin IX, from Choi et al. (1982). [b] RT and LT, room and low (19 K) temperature.

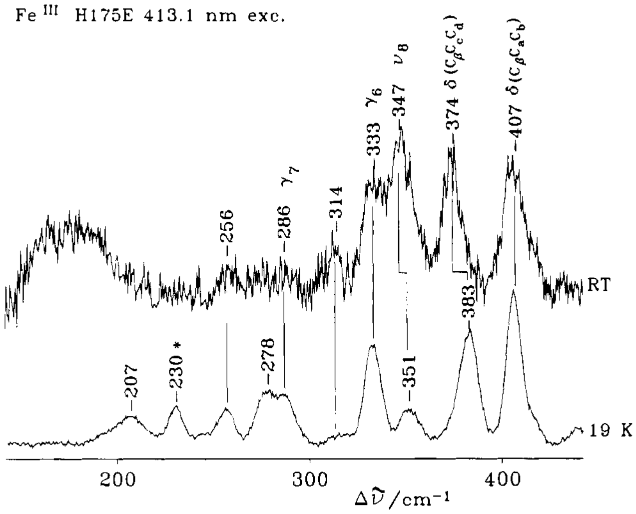


FIGURE 3: Low-frequency region resonance Raman spectra of ferric H175E mutant obtained with 413.1 nm excitation wavelength at room temperature (top) and 19 K (lower). Experimental conditions are as given in the caption to Figure 2, except a 5 s/0.5 cm^{-1} collection interval was used. The asterisk indicates the band of ice.

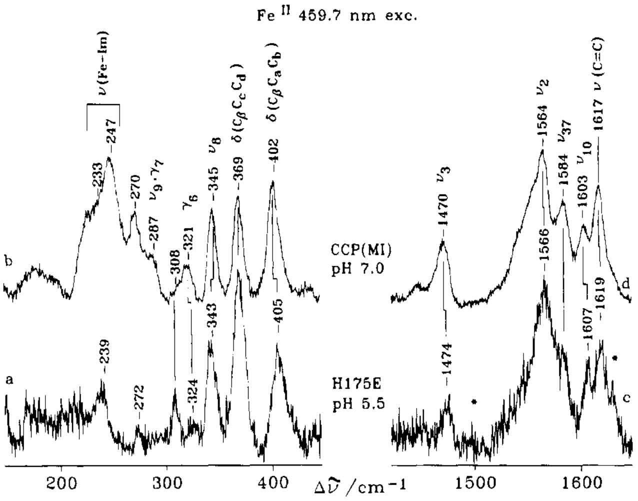


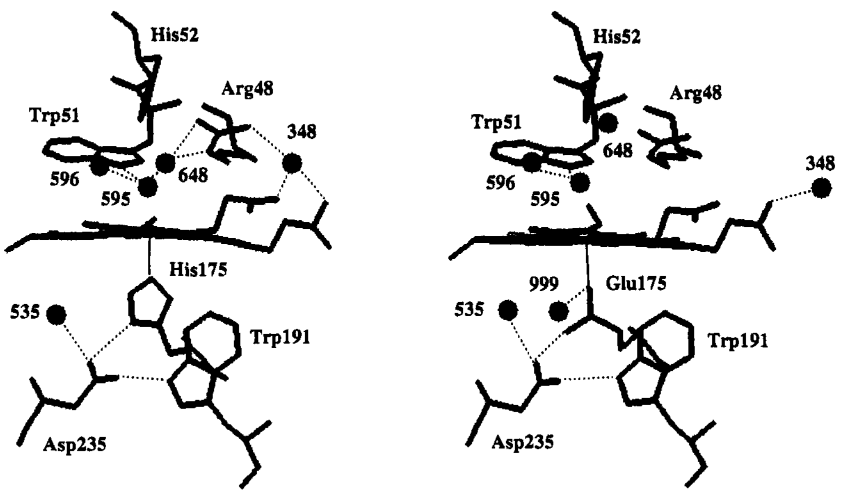
FIGURE 4: Low- (left) and high-frequency (right) region resonance Raman spectra of ferrous H175E mutant (a and c) and CCPMI [b and d, from Smulevich et al. (1994)] at room temperature obtained with 457.9 nm excitation wavelength. Experimental conditions: 5 cm^{-1} resolution, 25 mW laser power at the sample; (a) 9 s/0.5 cm^{-1}, (b) 6 s/0.5 cm^{-1}, (c) 2 s/0.5 cm^{-1}, and (d) 5 s/0.5 cm^{-1} collection intervals were used. The low-frequency region spectra have been expanded 2.5 times. The asterisks indicate the bands due to the oxidized form.

Therefore, the room temperature spectrum is characteristic of a pure five-coordinate high-spin (5-c HS) heme. Cooling gives rise to considerable changes in all the core size marker bands. In agreement with the changes observed in the absorption spectrum at 19 K, the corresponding RR spectrum shows the presence of a six-coordinate high-spin (6-c HS) heme. However, the porphyrin marker bands are at higher frequency than the corresponding bands observed for 6-c HS hemeproteins and model compounds at room temperature (Choi et al., 1982) (Table 1). A small amount of unchanged 5-c HS heme still can be observed in the RR spectrum at 19 K, as judged by the shoulder observed at 1495 cm^{-1} assigned to the ν_3 mode of a 5-c HS heme. The other corresponding modes are hidden by the bands due to the 6-c HS species. The changes induced by temperature are completely reversible, and the original absorption and RR spectra are reestablished when the sample is warmed to room temperature.

Figure 3 compares the 150−450 cm^{-1} region RR spectra excited at 413.1 nm of ferric H175E mutant obtained at room temperature (upper) and 19 K (lower). Upon cooling, both frequency and intensity changes are observed. In particular, the bands at 347 and 374 cm^{-1} at room temperature are detected at 351 and 383 cm^{-1}, respectively, at 19 K. In addition, whereas the bands at 278, 286, and 333 cm^{-1} increase in intensity, the band at 351 cm^{-1} becomes extremely weak in the spectrum at low temperature, as compared to the vinyl bending modes $\delta(C_\beta C_a C_b)$ observed at 407 cm^{-1}. The bands at 347 and 374 cm^{-1} observed at room temperature are assigned to the ν_8 and $\delta(C_\beta C_c C_d)$ propionate bending modes, respectively, in agreement with the corresponding bands observed in myoglobin (Mb) (S. Hu and T. G. Spiro, private communication, 1994).

At room temperature the sample is not very stable. Using either low laser power irradiation for 1 h or after being stored for 1 day in the refrigerator, the sample showed irreversible changes. In both cases new bands at 1482 cm^{-1} (ν_3) and 1561 cm^{-1} (ν_2) appeared, indicating that a 6-c HS species grows in at the expense of the 5-c HS heme. The low-frequency region changes accordingly, and a noticeable decrease in intensity of the band at 333 cm^{-1} is observed (data not shown).

Figure 4 compares the low- (left) and high-frequency (right) region RR spectra of ferrous H175E mutant (lower) with that of CCPMI (upper). The mutant was unstable in the reduced form. After about 20 min, the oxidized form started to grow in at the expense of the reduced species. As a consequence, the spectral quality is not very good. As CCPMI, the H175E spectrum is characteristic of a 5-c HS heme, but the core size marker bands are all upshifted with respect to the corresponding bands observed for CCPMI. The most striking difference observed in the low-frequency region between the two proteins is the absence of the bands at 247 and 233 cm^{-1} in the spectrum of the mutant, which were assigned to the ν(Fe−Im) stretching modes (Hashimoto et al., 1986; Smulevich et al., 1988; Dasgupta et al., 1989). Differences in the frequency and intensity of the other bands also are observed. In particular, the bands of CCPMI at 270, 321, 345, and 402 cm^{-1} shift to 272, 324, 343, and 405 cm^{-1}, respectively, with a concomitant decrease in intensity with respect to the band at 369 cm^{-1}. The band at 287 cm^{-1} of



CCP **H175E**

FIGURE 5: Structural diagrams of the heme pocket of CCP (Finzel et al., 1984) and H175E (Choudhury et al., 1994). Dotted lines indicate inferred hydrogen bonds, on the basis of distance criteria. The water molecules present in the cavity are also shown.

CCPMI is not clearly observed in the spectrum of the mutant because of the poor signal-to-noise ratio, due to the instability of the protein in the reduced state.

DISCUSSION

The X-ray structure of the H175E mutant (Choudhury et al., 1994) shows that, whereas the Fe–ligand [Fe–O(Glu)] distance is comparable with that of CCP [Fe–N(His)] (about 2.0 Å), the distal water molecule (Wat595) moves away from the iron atom and closer to the N_ϵ of the distal histidine (His52) as compared to CCP. The new distance between the Wat595 and the heme iron is 3.3 Å, about 0.9 and 0.6 Å longer than that observed in baker's yeast CCP (Finzel et al., 1984) and CCPMI (Wang et al., 1990), respectively. Therefore, it was concluded that the mutant is pentacoordinate, as in the wild type or recombinant proteins. Both the absorption and the RR spectra taken at room temperature show that in solution the protein maintains the same coordination as observed in the crystal. The high-frequency RR spectrum compares well with those previously obtained for both CCP (Hashimoto et al., 1986; Dasgupta et al., 1989; Smulevich et al., 1989; Hildebrandt et al., 1992) and CCPMI (Smulevich, G., 1993; Smulevich et al., 1988, 1989) in solution at pH 7, as well as with the CCP single crystal (Smulevich et al., 1990a). However, it is noted that both native and recombinant wild type proteins show a very small amount of 6-c high- and low-spin heme. The absence of these species in the mutant spectrum is clearly indicated by the narrowing of the ν_3 band as a result of the missing shoulders at about 1480 and 1500 cm^{-1} due to the 6-c HS and LS hemes, respectively.

The frequencies of the bands in the 150–450 cm^{-1} region of the H175E mutant are very similar to those of CCP (Hildebrandt et al., 1992) and CCPMI (Smulevich et al., 1988), but the intensity of the bands at 286 and 333 cm^{-1} appears much weaker than the band at 347 cm^{-1}. These bands were assigned to out-of-plane modes, γ_7 and γ_6, respectively, from their large *meso*-d_4 shift observed in CCP (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results). Out-of-plane modes are not expected to be enhanced in the RR spectra of heme proteins, and their appearance is related to the distortion of the porphyrin skeleton, as shown by the study of the ruffled tetragonal crystal form of nickel octaethylporphyrin (Li et al., 1989). Therefore their decrease in intensity in the RR spectrum of the mutant could be due to a less-distorted heme with respect to that of the wild type. A comparison of the crystal structures (Figure 5) shows that the heme in the H175E mutant has a slightly less distorted heme than does wild type CCP.

The RR spectra do not give any direct evidence that the heme iron is bound to the O_ϵ of the glutamic acid residue instead of the usual N_ϵ of the histidine residue, in agreement with the unchanged Soret and α,β bands observed in the electronic absorption spectrum of the mutant (Figure 1) with respect to CCP (Yonetani & Anni, 1987). On the other hand, in the electronic absorption spectrum a strong band at 637 nm is observed. For CCP the corresponding band is at 645 nm (Yonetani & Anni, 1987). This probably results from the charge-transfer (CT) transition from the porphyrin to the iron [$a_{2u}(\pi) \rightarrow e_g(d_\pi)$], by analogy with the assignment of the band at 633 nm for (aquo)MetMb single crystal (Eaton & Hochstrasser, 1967). It increases in energy on going from the aquo-complex to formate- (624 nm) and to fluoride-MetMb (606 nm) due to the increase in the $e_g(d_\pi)$ orbital energy, as a result of the decreasing of the ligand field. The same results had also been observed in a series of ferrihemoglobins, ferricatalase, and ferriperoxidase complexes (Brill & Williams, 1961). Therefore, the 8 nm blue shift of the charge-transfer band of the 5-c mutant with respect to CCP can be explained as due to the different field of the proximal ligand bound to the heme iron. In fact, the Glu residue is expected to have a weaker ligand field with respect to the imidazole because Glu is also a better π donor, and the d_π orbitals are raised in energy. In accord with this view, the CT band of ferric *Coprinus cinereus* peroxidase (CIP) is at 649 nm (Smulevich et al., 1994), 4 nm higher than that observed for CCP. On the basis of X-ray structural data (Kunishima et al., 1994), it appears that the histidine plane of the fifth ligand is rotated about 40° with respect to the N(1)–Fe–N(3) axis (azimuthal angle, ϕ) (whereas in CCP the angle is about 7°). Such a rotation causes a weakening of the Fe−Im bond, as determined by the RR frequency of the ν(Fe−Im) stretching mode of the Fe^{II} form which appears about 15 cm^{-1} downshifted (Smulevich et al., 1994) with respect to the corresponding band observed for CCP (Hashimoto et al., 1986; Smulevich et al., 1988; Dasgupta et al., 1989). Therefore, the rotation of the imidazole plane decreases the π donation of the ligand, determining a red shift of the CT band with respect to that of CCP.

The EPR spectrum obtained at 4.2 K and pH 7 (Choudhury et al., 1994) showed that the mutant is 6-c HS. Since the crystal X-ray data at room temperature indicated a pentacoordinate heme, the authors ascribed the apparent inconsistency between the X-ray and EPR data as due to the presence of 30% methylpentanediol (MPD) necessary for crystallization. MPD has been found to affect the iron coordination state of W51F mutant of CCP. In this latter case, the X-ray structure (Wang et al., 1990) and the single-crystal RR spectra (Smulevich et al., 1990b) were characteristic of a 5-c HS heme, while the RR and absorption spectra (Smulevich et al., 1988, 1989) of the solutions between pH 5.7 and 7 gave rise to a mainly 6-c HS heme. The addition of 30% MPD to the buffered solution of the mutant resulted in a change in iron coordination state from hexa- to pentacoordinate (Smulevich et al., 1990b).

The present results on H175E in solution clearly show that at room temperature the protein is pentacoordinate, and the heme iron becomes hexacoordinate at low temperature. These findings are in perfect agreement with the X-ray data taken at room temperature indicating a pentacoordinate iron and with the EPR data taken at 4.2 K indicating the presence of a 6-c HS heme (Choudhury et al., 1994). These results, together with the fact that the change of the coordination state is perfectly reversible upon warming the sample, rule out the hypothesis of an inconsistency between the X-ray and the EPR data as reflecting the known effect of MPD, added to induce crystallization, on the ligation geometry (Choudhury et al., 1994; Yonetani & Anni, 1987; Smulevich et al., 1989) and demonstrate that coordination change is due solely to a temperature-induced transition. Cooling causes a contraction of the heme pocket, and therefore the iron atom approaches the heme plane and can bind a distal ligand. A change of the coordination and/or the spin state upon freezing has been previously observed for HRP-C, CCP, and many of its site-directed mutants. HRP-C gives rise to a mixture of low- and intermediate-spin hemes, the latter being the prevalent species, as demonstrated by both EPR (Maltempo et al., 1979) and RR (Evangelista-Kirkup et al., 1985; Smulevich et al., 1991) data. On the other hand, CCP and recombinant CCPMI give rise to different mixtures of 5-c HS and LS hemes (Yonetani et al., 1966; Yonetani & Anni, 1987; Smulevich et al., 1989; Choudhury et al., 1994). A small amount of 6-c HS has also been detected at low temperature in both RR (Evangelista-Kirkup et al., 1985; Smulevich et al., 1989) and EPR (Yonetani & Anni, 1987). However, the latter experiment provided clear evidence that the 6-c HS EPR signal at low temperature is associated with aging of the CCP. Furthermore, aged CCP shows the presence of a 6-c HS heme also at room temperature, as, in fact, does the mutant under investigation in the present study.

RR experiments carried out on a fresh CCP single crystal showed that at 80 K the protein is a mixture of 5-c HS and 6-c LS hemes and that the conversion of the pentacoordinate species to the low-spin species is very gradual and completely reversible (Smulevich et al., 1990a). The only other CCP mutant so far studied showing HS heme at low temperature with a prevalent six-coordinated species was found to be the W51F, as observed by EPR (Fishel et al., 1987) and RR experiments on the solution (Smulevich et al., 1989) and by the RR study on the single crystal (Smulevich et al., 1990b).

Comparing the X-ray structures of CCP (Finzel et al., 1987) and H175E (Choudury et al., 1994) (Figure 5) it appears that the disposition of the water molecules on the distal side of the cavity is quite different in the two proteins. In CCP the distal water molecule, Wat595, which is 2.4 Å away from the heme iron, is H-bonded to Wat596 (2.6 Å), Wat648 (3.2 Å), and Trp51 (2.7 Å). Wat648 is also H-bonded to Arg48 (2.5 Å) which in turn is H-bonded to Wat348 (2.7 Å). This latter water molecule, situated between the two-propionate heme, is H-bonded to the propionate heme located on pyrrole IV (2.8 Å) (Finzel et al., 1987). Replacement of the proximal histidine with glutamic acid alters the disposition of these water molecules in such a way that the distal Wat595 moves away from the iron atom (3.3 Å) and the H-bonds with both Wat596 and Trp51 become longer (2.9 Å). But the most striking effect is shown by the Wat648 that moves very far from both Wat595 (8.1 Å) and Arg48 (5.0 Å from N_{h1} and 7.2 Å from N_ϵ). The Wat348 also moves away from Arg48 (6.6 Å). Therefore, the spin state difference observed in CCP and the H175E mutant at low temperature can be ascribed either to the different ligand field of the proximal residue, and to the different nature of the water molecules bound to the iron. In CCP the aquo-ligand, involved in three strong H-bonds, has an anionic character and, therefore, is expected to behave similarly to an OH^- group (Feis et al., 1994). In the mutant, the Wat595 can be considered a normal aquo-ligand, as in MetMb where the distal water molecule is H-bonded to the distal histidine (His 7E) (Takano, 1977). We noticed that the W51F mutant, which gives rise to a 6-c HS heme upon cooling, shows a disposition of water molecules on the distal side of the cavity similar to that of the H175E mutant. The W51F mutant crystal structure shows that Wat595 remains centered at 2.7 Å from the heme iron even though it no longer H-bonds with N_ϵ of Trp51. However, Wat596 has moved approximately 1 Å away from Wat595 (3.7 Å), and no water molecule is present in, or near, position 648.

The new disposition of the water molecules in the distal side of the cavity of the H175E mutant is not reflected in any major change in enzyme activity since the mutant is even more active than wild type CCP. This hyperactivity has been explained by an increase in thermodynamic driving force of electron transfer (Choudury et al., 1994). The more negative fifth ligand in the mutant with respect to CCP gives rise to a lower effective charge on the Fe atom, determining a weaker electrostatic attraction for the distal water molecule. Therefore, the Wat595 moves away from the Fe atom and the H-bonds with the Wat596 and the N atom of Trp51 are lengthened by about 0.2−0.3 Å. A lengthening of the H-bond between Wat595 and Wat648 leads to a breaking of this H-bond, which already is weak in CCP (3.2 Å). As a consequence, the Wat648 moves farther away.

The electronic absorption spectrum at 19 K shows the CT band at 618 nm, 15 nm lower than the corresponding band observed for the (aquo)MetMb-complex at room temperature. The blue shift is due to the replacement of the fifth His ligand with the weaker ligand field (Glu residue, as previously discussed for the 5-c HS form.

From a comparison of the RR spectra of the variant at 19 K and those obtained for other six-coordinated HS heme proteins and model compounds (Table 1) at room temperature, it appears that the core size marker bands are upshifted at low temperature, indicating that core contraction occurs, due the increase of the protein packing forces with decreasing temperature. This is in agreement with a similar effect previously observed for alkaline MetMb, MetHb, and HRP-C at 20 K (Feis et al., 1994). The intensity increase of the out-of-plane modes at 286 and 333 cm^{-1} suggests also that distortion of the heme plane occurs. In addition, cooling causes an upshift of the propionyl bending mode that can be due to a changed orientation of the propionyl side chains, as previously observed for alkaline MetMb, MetHb, and HRP-C (Feis et al., 1994). Less clear is the origin of the upshift and intensity decrease of the ν_8 mode.

Even though the H175E mutant was extremely unstable in its ferrous form and reverted to the ferric form in about 20 min, it was possible to obtain the RR spectra. Upon comparison of these results with those obtained for the parent enzyme, it appears that the variant exhibits a more planar heme, as judged from the slightly higher frequency of the core size marker bands and from the intensity decrease of the out-of-plane modes at 287 (γ_7) 324 (γ_6) cm^{-1} bands. The Fe^{II} RR spectrum lacks the bands at 233 and 247 cm^{-1}, previously assigned to the ν(Fe−Im) stretching modes (Hashimoto et al., 1986; Smulevich et al., 1988; Dasgupta et al., 1989). Accordingly, the ν_8 mode, which has been found to be coupled to the Fe−His stretching mode (G. Smulevich, S. Hu, K. R. Rodgers, D. B. Goodin, K. Smith, and T. G. Spiro, unpublished results), shifts down by 3 cm^{-1} with a concomitant decrease in intensity. No new band due to the ν[Fe−O(Glu)] has been observed.

CONCLUSIONS

The replacement of the imidazole fifth ligand with a Glu side chain does not alter the energy of the $\pi \rightarrow \pi^*$ transitions, giving rise at room temperature to electronic absorption and resonance Raman spectra about identical to those previously obtained for the wild type CCP, and therefore characteristic of a 5-c HS heme. On the other hand, the charge-transfer band [$a_{2u}(\pi) \rightarrow e_g(d_\pi)$] is 8 nm downshifted with respect to the parent enzyme, indicating the presence of a more polar fifth ligand.

Both the ferrous and ferric RR spectra are characterized by very weak bands due to the out-of-plane modes, indicating a less distorted heme plane with respect to CCP, in agreement with the X-ray structure. In the spectrum of the Fe^{II} form, the missing bands due to the ν(Fe−Im) stretching modes are not replaced by any other new band assignable to the ν[Fe−O(Glu)] stretching mode.

Upon cooling, a temperature-induced transition from 5-c to 6-c HS heme is observed. No low-spin heme is formed, in contrast with CCP and CCPMI. The different behavior of the variant at low temperature is a consequence of the replacement of the N with the more negative O atom as the fifth ligand, which results in a lower effective charge on the iron atom, causing a lower attraction for the distal water molecules. In fact, from the X-ray structure it appears that the Wat595 moves 0.8 Å away from the iron atom with a concomitant lengthening of the H-bonds with Trp51 and Wat596 and the breaking of the H-bond with Wat648, which moves about 8 Å away. Therefore, upon cooling, the protein packing forces increase, and the Fe atom moves into the heme plane and binds the distal water molecule which has lost its polar character, as compared with CCP. The increase of the packing forces also causes a contraction of the core and a distortion of the heme plane, as indicated by the RR spectra.

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