Versatility of Heme Coordination Demonstrated in a Fungal Peroxidase. Absorption and Resonance Raman Studies of *Coprinus cinereus* Peroxidase and the Asp245→Asn Mutant at Various pH Values[†]

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ABSTRACT: The pH dependence of the electronic absorption and resonance Raman (RR) spectra of Fe^{III} and Fe^{II} forms of *Coprinus cinereus* peroxidase (CIP) and its Asp245→Asn (D245N) mutant has been examined in detail. The spectral data were obtained in the pH range 3.8−12.0. These spectra were used to assess the spin and ligation states of the heme via the porphyrin marker band frequencies and the wavelengths of the absorption maxima, especially that of the band (CT1) due to the charge transfer from the porphyrin to the heme iron via the $a'_{2u}(\pi) \rightarrow e_g(d_\pi)$ electronic transition. The RR spectra were obtained by using different excitation wavelengths and polarized light. The data obtained for ferric CIP show that two pH-induced structural transitions exist. At acid pH the Soret and the CT1 absorption maxima occur at 394 and 652 nm, respectively, compared with the values of 403 and 649 nm observed at neutral pH. The electronic data indicate that at acid pH the proximal Fe−Im bond might be weakened or ruptured, and the RR spectra show a new species (5-c HS*) different from the normal neutral 5-coordinate high-spin (5-c HS) heme. At pH 12.0, the protein converts to a 6-coordinate low-spin (6-c LS) heme with a hydroxyl ligand coordinated in the sixth position of the heme iron and strongly hydrogen-bonded with the positively charged guanidinium group of the distal Arg51 residue. Replacement of the aspartate carboxylate group of Asp245, which acts as hydrogen-bond acceptor to the proximal His183 ligand of the heme Fe, with a carboxamide group of an asparagine residue has a profound influence on the heme coordination. The RR spectra of the Fe^{II} form of this mutant at both neutral and alkaline pH values show a band at 204 cm^{-1} assigned to the Fe−His stretch associated with a fairly weak or non-hydrogen-bonded imidazole. The ferric form of the mutant shows a great variability in coordination and spin states upon pH titration. Between pH 8.8 and 3.8 the spectra are mainly characteristic of a 6-coordinate high-spin heme, presumably with a water molecule bound on the distal side of the Fe atom. The pK_a of the alkaline transition of the mutant is much lower than that of the wild-type protein. At pH 10.0 the D245N mutant is in its final alkaline form, which markedly differs from that of the parent enzyme. The spectral data indicate that the majority of the protein has 5-coordinate high-spin heme (5-c HS**), with the Fe−His183 bond broken and the distal axial coordination site of the heme iron occupied by a hydroxyl group, which is strongly hydrogen-bonded with distal Arg51. Therefore, the Asp245→Asn mutation on the proximal side results in the breakage of the Fe−His bond at alkaline pH.

A new fungal peroxidase, *Coprinus cinereus* peroxidase (CIP),[1] has received considerable attention in recent years. It can be obtained in high yields from fermentation of the parent organism (Shinmen et al., 1986; Morita et al., 1988) or by heterologous expression in *Aspergillus oryzae* (Dalbøge et al., 1992) and is commercially available under a confusing selection of names: *Coprinus cinereus* or *Coprinus macrorhizus* peroxidase (CMP) and *Arthromyces ramosus* peroxidase (ARP). These enzyme preparations, as well as recombinant CIP, were identical in their enzymatic and molecular properties, but all showed great carbohydrate heterogeneity (Kjalke et al., 1992; Limongi et al., 1995). The crystal structures of ARP (Kunishima et al., 1994) and recombinant CIP (Petersen et al., 1994) and NMR (Lukat et al., 1989; Veitch et al., 1994), electronic absorption, and resonance Raman (RR) spectra (Smulevich et al., 1994a) of fungal and recombinant CIP were also indistinguishable. Hence, we will refer to this fungal peroxidase by the name CIP or CIP/ARP when referring to information taken from the crystal structure of ARP (Kunishima et al., 1994, 1996).

Recombinant CIP and a great number of site-directed mutants, near the heme, at the presumed binding site of aromatic substrates, at sites of glycosylation, and at the N-terminus of the protein, have already been produced in quantity (Welinder & Andersen, 1993) and provide a great

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[1] Abbreviations: 5-c and 6-c, 5- and 6-coordinate; HS and LS, high- and low-spin; RR, resonance Raman; NMR, nuclear magnetic resonance; ARP, *Arthromyces ramosus* peroxidase; CIP, *Coprinus cinereus* peroxidase; CMP, *Coprinus macrorhizus* peroxidase; D245N, Asp245→Asn mutant of CIP; CCP, cytochrome *c* peroxidase; D235N, Asp235→Asn mutant of CCP; HRP-C, horseradish peroxidase isoenzyme C; Mb, myoglobin; wt, wild type.

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resource for the study of structure−function relationships of the heme-containing peroxidases. The heme structure of wild-type (wt) CIP at neutral pH studied previously by absorption and RR spectroscopy has been compared and contrasted with those of homologous peroxidases from yeast mitochondria (cytochrome *c* peroxidase, CCP) (Smulevich, 1993) and horseradish root (horseradish peroxidase, HRP-C) (Smulevich et al., 1994b).

In the present study, the electronic absorption and resonance Raman properties, over the entire pH range of protein stability, have been elucidated for wild-type CIP and for a CIP mutant, Asp245→Asn, at the site of the buried proximal aspartate Asp245, accepting a hydrogen bond from the proximal His183. The interpretation of these data allows us to assign the RR bands to specific features of the heme structure. A number of different, often coexistent, heme species have been detected. The different contributions of all of these heme species to the reaction with hydrogen peroxide are central to understanding the peroxidase catalytic mechanism.

MATERIALS AND METHODS

Wild-type (wt) CIP and the D245N CIP mutant were obtained by expression in transformed *A. oryzae* (Dalbøge et al., 1992). Wild-type CIP was purified as previously described (Smulevich et al., 1994a). The D245N mutant was precipitated by 2.5 M ammonium sulfate from the sterile-filtered fermentation broth and redissolved in 5 mM $CaCl_2$ before dialysis against 10 mM Bis-Tris buffer (pH 6.0) containing 1 mM $CaCl_2$. Ion exchange chromatography on HiLoad Q-Sepharose was performed as described for wt CIP. The fractions were analyzed by SDS−PAGE and absorption spectroscopy, and the pure fractions were pooled and stored at 4 °C in 3 M ammonium sulfate titrated to pH 7 with Tris base.

CIP and mutant preparations in the form of ammonium sulfate precipitates were centrifuged and the supernatant discarded. The precipitate was dissolved in Milli-Q water containing 10 mM $CaCl_2$, and the pH was adjusted to 7 with 2 M Tris base. Extensive dialysis was then carried out against Milli-Q water containing 2 mM $CaCl_2$. The following buffers were used: 10 mM citric acid−sodium citrate (pH 3.8−5.4), 10 mM sodium phosphate (pH 7), and 20 mM boric acid and 10 mM sodium phosphate (pH 8.8−10). All experiments were performed in the presence of 1 mM $CaCl_2$ and at a constant ionic strength of 0.1 M adjusted by the addition of potassium sulfate. The pH 11.8−12.1 experiments were performed in degassed NaOH solution added to a degassed solution of the dialyzed protein at very low ionic strength. The ferrous form was prepared by adding a minimum volume of fresh sodium dithionite solution to the degassed, buffered solution. Sample concentration was determined spectrophotometrically using an extinction coefficient of 109 cm^{-1} mM^{-1} at 405 nm for ferric CIP (Andersen et al., 1991) and was 0.1−0.4 mM enzyme for resonance Raman (RR) spectroscopy and 10 times more dilute for UV−visible absorption measurements.

Absorption spectra were measured with a Cary 5 spectrophotometer. These spectra were measured both prior to and after RR measurements to determine whether sample degradation had occurred. No degradation was detected under the experimental conditions applied in this study. At pH lower than 3.8, both CIP and the D245N mutant denatured.

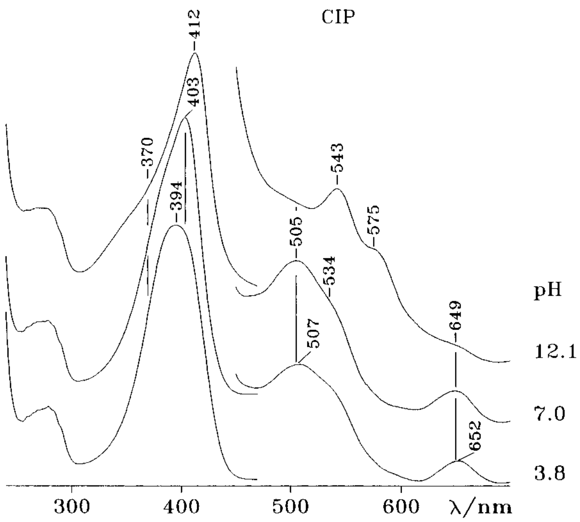

FIGURE 1: Electronic absorption spectra of ferric CIP at various pH values. The region between 450 and 700 nm has been expanded by 5 times.

The resonance Raman (RR) spectra were obtained by excitation with the 406.7, 413.1, and 568.2 nm lines of a Kr^+ laser (Coherent, Innova 90/K) and the 457.9 and 514.5 nm lines of an Ar^+ laser (Coherent, Innova 90/5). 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.

All of the electronic absorption spectra were collected at room temperature, i.e., about 23 °C. The resonance Raman spectra were collected at about 15 °C. To minimize the local heating of the protein induced by the laser beam, the rotating NMR tube was cooled by a gentle flow of N_2 gas passing through liquid N_2.

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-scattering geometry. The values obtained, 0.73 and 0.01, compared favorably with the theoretical values of 0.75 and 0.00, respectively.

RESULTS

Ferric Hemes

Heme Structure of CIP at Acid, Neutral, and Alkaline pH. Figure 1 compares the electronic absorption spectra of ferric CIP at various pH values. At pH 7.0 the spectrum shows a Soret band at 403 nm, α and β bands at 534 and 505 nm, and a charge transfer band (CT1) at 649 nm, indicating that the heme is mainly 5-coordinate high-spin (5-c HS) (Smulevich et al., 1994a).

Changes in the spectra are observed at both acid and alkaline pH. At pH 3.8 the Soret band broadens and shifts down to 394 nm, the β band red shifts to 507 nm, and the CT1 band shifts to 652 nm. The broadening of the Soret

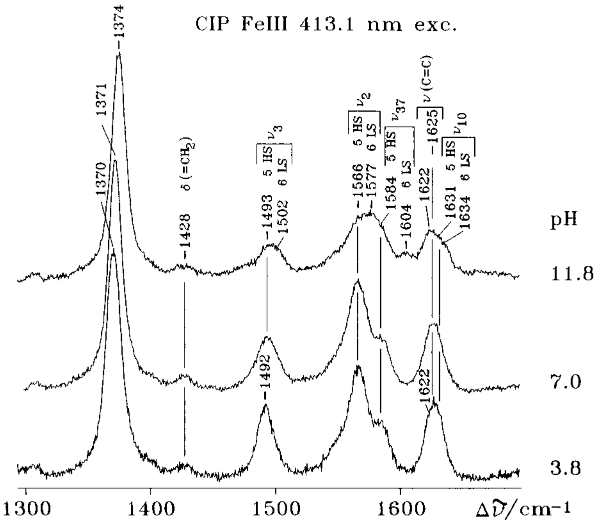

FIGURE 2: RR spectra of ferric CIP at various pH values, taken with 413.1 nm excitation. Experimental conditions: 5 cm^{-1} resolution; pH 3.8 and 11.8, 15 mW laser power at the sample, 3 s/0.5 cm^{-1} collection interval; pH 7.0, 10 mW laser power at the sample, 15 s/0.5 cm^{-1} collection interval.

band suggests the presence of overlapping bands. A blue shift of the Soret band is expected when the proximal histidine is replaced by a weaker ligand (Verma & Chaudhury, 1991). For metmyoglobin (met-Mb) at pH 4 (Sage et al., 1991; Palaniappan & Bocian, 1994) and horseradish peroxidase (HRP-C) at pH 3.1 (Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments), a broad band centered at 370 nm has been observed together with a shift of the bands in the visible region (510 and 640 nm for met-Mb and 519 and 652 nm for HRP-C). For these two proteins, the resonance Raman (RR) spectra indicated the presence of a 5-c HS heme with only a water molecule bound to the Fe atom. The broad band observed at 394 nm for CIP at pH 3.8 might result from the overlapping contributions of at least two bands: the Soret at 403 nm corresponding to the 5-c HS heme and another blue-shifted band corresponding to a new 5-c heme, hereafter indicated as 5-c HS* to distinguish it from a normal 5-c HS species, which has a histidine imidazole as the fifth ligand.

At pH 12.1 the Soret band of CIP upshifts to 412 nm with a clear shoulder at 370 nm. The visible region shows two maxima appearing at 543 (*β* band) and 575 (α band) nm, with a concomitant strong decrease in intensity of the three bands (at 403, 505, and 649 nm) arising from the 5-c HS heme. A very small amount of the 5-c HS heme is still present, indicating that the alkaline transition of CIP has a pK_a similar to or higher than that for HRP-C, which is around 11 (Harbury, 1957; Yamazaki & Nakajima, 1986). The spectrum resembles that previously observed for the alkaline form of ferric HRP-C (Feis et al., 1994), suggesting that a hydroxyl group is bound at the sixth coordination position of the Fe atom of CIP at pH 12.1 at low ionic strength. At higher ionic strength as well as at higher pH, unfolding CIP is observed (Tams & Welinder, 1996).

Figure 2 compares the RR spectra of ferric CIP obtained at different pH values. To assign the bands, we undertook a study using different excitation wavelengths and polarized light as shown in Figure 3. It can be seen that upon Soret excitation the spectra of CIP at pH 3.8 closely resemble those obtained previously at pH 7.0 (Smulevich et al., 1994a). The only difference is the sharpening and intensity increase of the band at 1492 cm^{-1} corresponding to the ν_3 mode. A

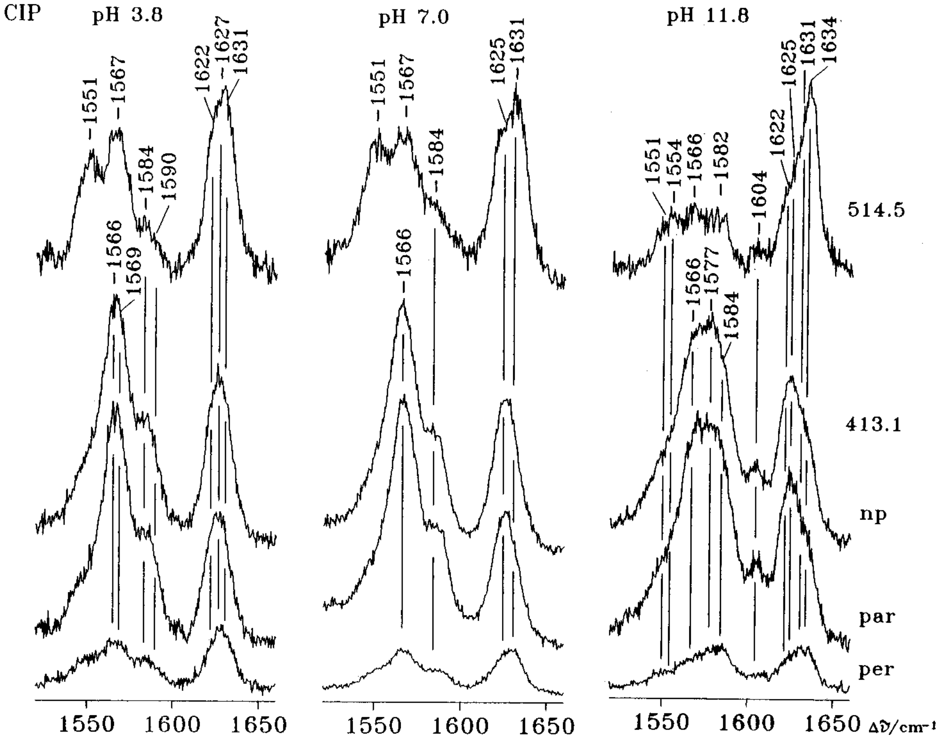

FIGURE 3: RR spectra of ferric CIP at various pH values, taken with different excitations and in polarized light. Experimental conditions: 5 cm^{-1} resolution; pH 3.8, 413.1 nm excitation, 15 mW laser power at the sample, 3 s/0.5 cm^{-1} (nonpolarized, np), 5 s/0.5 cm^{-1} (parallel polarization, par), and 8 s/0.5 cm^{-1} (perpendicular polarization, per) collection intervals; pH 3.8, 514.5 nm excitation, 50 mW laser power at the sample, 24 s/0.5 cm^{-1} collection interval; pH 7.0, 413.1 nm excitation, 10 mW laser power at the sample, 15 s/0.5 cm^{-1} (np), 12 s/0.5 cm^{-1} (par), and 24 s/0.5 cm^{-1} (per) collection intervals; pH 7.0, 514.5 nm excitation, 40 mW laser power at the sample, 30 s/0.5 cm^{-1} collection interval; pH 11.8, 413.1 nm excitation, 15 mW laser power at the sample, 7 s/0.5 cm^{-1} (np), 4 s/0.5 cm^{-1} (par), and 8 s/0.5 cm^{-1} (per), collection intervals; pH 11.8, 514.5 nm excitation, 60 mW laser power at the sample, 19 s/0.5 cm^{-1} collection interval.

careful analysis of the spectra at pH 3.8, obtained in polarized light, revealed that in the 1600−1650 cm^{-1} region, near the ν_{10} at 1631 cm^{-1}, a new depolarized band appeared at 1627 cm^{-1} that was enhanced upon excitation with the 514.5 nm line (Figure 3). Therefore, this new band is assigned as ν_{10} of the new acid 5-coordinate species, analogous with the species observed for acid met-Mb (Sage et al., 1991; Palaniappan & Bocian, 1994) and acid HRP-C (Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments). The other core size marker bands of this new form appear to overlap with those of the 5-c HS species still present at acid pH. The intensity increase in the ν_3 relative to the rest of the spectrum is due to a different cross section of the ν_3 mode of the 5-c HS* species. The RR spectra of met-Mb and HRP-C at acid pH showed that the 5-c HS* heme is characterized by a fairly strong ν_3 band compared to the rest of the spectrum. The ν(C═C) stretching modes are difficult to identify when two species are present. However, from the spectra in polarized light, it appears that at around 1622 cm^{-1} a new polarized band arises, which is therefore assigned to the ν(C═C) stretching mode of the 5-c HS* species. This change indicates that at least one vinyl group changes its conjugation with the heme chromophore upon acidification.

More dramatic changes are observed for CIP at alkaline pH. As observed from the absorption spectrum, the RR spectra indicate the presence of a small amount of the 5-c HS species, which dominates at neutral pH, together with a new form, 6-c LS, which indicates that a hydroxyl group could now be bound to the heme iron. The ν(Fe−OH) stretching mode of the LS species has been found for HRP-C at 503 cm^{-1} (Sitter et al., 1988; Feis et al., 1994). The 50 cm^{-1} lower energy together with the weaker intensity of this mode in HRP-C, as compared with the corresponding modes observed in globins, has been explained as a result of strong hydrogen bond(s) between the hydroxyl ligand and the distal Arg (and probably His) in HRP-C (Feis et al., 1994). In the low-frequency region of CIP at alkaline pH, a broad weak band around 505 cm^{-1} was observed (data not shown), which could be due to the ν(Fe−OH) stretching mode. However, experiments carried out in D_2O did not show any isotopic-sensitive band. The lack of a frequency-shifted band derives from the fact that the protein is not completely 6-c LS heme even at this extreme pH (12.1). In addition, the ν(Fe−OH) stretching mode for a 6-c LS is expected to be extremely weak and overlapped with a porphyrin mode, as observed for HRP-C (Feis et al., 1994). All the RR frequencies of the core size marker bands of CIP are lower than those previously observed for HRP-C at alkaline pH (Terner & Reed, 1984; Sitter et al., 1988; Feis et al., 1994). This indicates a slight expansion of the heme core of CIP as compared to HRP-C and can be ascribed to differences in both the proximal and the distal cavities between the two proteins. More detailed explanations are not possible at this stage, as the crystal structure of CIP only, and not HRP-C, is available at present. The alkaline-induced rearrangement should also be responsible for an orientation of the vinyl groups that is similar to that at acid pH, since a ν(C═C) stretch is observed at 1622 cm^{-1}.

Heme Structure of the D245N Mutant of CIP. Figure 4 compares the electronic absorption spectra of the ferric form of the D245N mutant of CIP from pH 3.8 to 10.0. The protein did not give rise to any further change in the absorption spectra from pH 10.0 to 11.3. At higher pH the mutant denatured. The protein was fairly stable at pH 10.0 and showed an identical absorption spectrum 24 h after preparation. In addition, the spectrum completely reversed to the form observed at neutral pH by readjusting the pH.

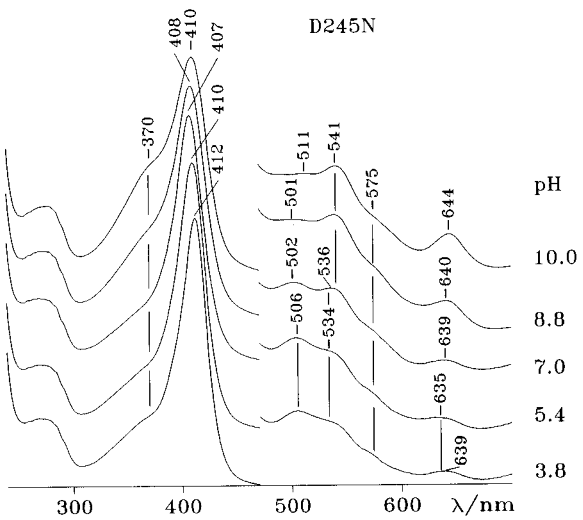

FIGURE 4: Electronic absorption spectra of ferric D245N mutant at various pH values. The region between 450 and 700 nm has been expanded by 6 times.

The Soret band is observed at 412 nm at pH 3.8, shifts down to 410 nm at pH 5.4, and to 407 nm at pH 7.0. At alkaline pH it red shifts again and a shoulder develops at 370 nm. The weak α band at 575 nm, present at all pH, denotes the presence of a small amount of a 6-c LS heme. From pH 3.8 to 7.0 the dominant form is a 6-c HS heme, as revealed by the CT1 band in the range 639−635 nm and the band in the 506−501 nm range, assigned to the overlapping contribution of the β band with a charge transfer band (CT2). The similarity of the absorption spectrum to that of sperm whale met-Mb at neutral pH (Varadarajan et al., 1989) suggests that the sixth coordination position of the heme iron of D245N CIP is occupied by a water molecule. At alkaline pH, red-shifted bands are observed at 511 and 644 nm. These two latter bands, associated with the shoulder at 370 nm, indicate the presence of a new 5-c HS species, hereafter indicated as 5-c HS** to distinguish it from the species observed at neutral and acid pH for wt CIP.

The RR spectra of the D245N mutant taken at various pH values (Figures 5 and 6) are perfectly consistent with the electronic absorption spectra. Between pH 5.4 and 7 the core size marker bands are characteristic of a mixture of 6-c HS and LS hemes; at pH 3.8 a small amount of the 5-c HS* species is present, as judged from the appearance of a band at 1491 cm^{-1} (ν_3) with Soret excitation, but the RR spectrum obtained with visible excitation (Figure 6) shows that the core size marker bands of the 6-c HS form dominate. The 6-c HS heme is still present at pH 8.8, but disappears at pH 10.0. The 6-c LS form never disappeared, as confirmed by the RR spectrum at pH 10.0 obtained with the 568.2 nm excitation wavelength (Figure 6). At this wavelength the Raman spectrum of the 6-c LS species is in resonance with the α band, and the major contribution to the spectrum is from the bands of this low-spin form. At pH 7.0 a new heme form was detected, which becomes the major species at pH 10.0. This is characterized by an extremely intense band at 1491 cm^{-1} (ν_3) and bands at 1567 (ν_{19}), 1569 (ν_2), 1590

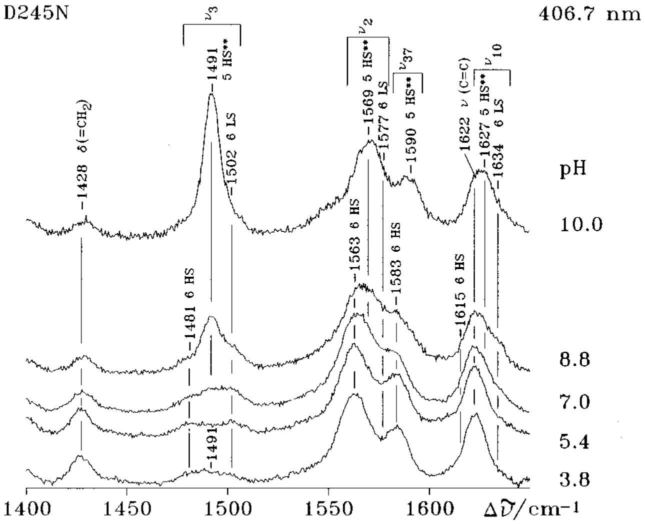

FIGURE 5: RR spectra of ferric D245N mutant at various pH values, taken with 406.7 nm excitation. Experimental conditions: 5 cm^{-1} resolution, 20 mW laser power at the sample; pH 3.8, 10 s/0.5 cm^{-1} collection interval; pH 5.4, 7 s/0.5 cm^{-1} collection interval; pH 7.0, 10 s/0.5 cm^{-1} collection interval; pH 8.8, 6 s/0.5 cm^{-1} collection interval; pH 10.0, 5 s/0.5 cm^{-1} collection interval.

(ν_{37}), and 1627 cm^{-1} (ν_{10}). At all of the pH values, the spectra taken in polarized light with Soret excitation (data not shown) revealed the presence of a polarized band at 1622 cm^{-1} (down-shifted by 3 cm^{-1} compared to wt CIP at neutral pH), assigned to the ν(C=C) stretching mode of the vinyl group, which appears oriented as for the acid and alkaline forms of wt CIP.

Table 1: Mode and Frequency (cm^{-1}) of the Principal Resonance Raman Bands Observed for the Major Species at Various pH of the D245N CIP Mutant together with Those Observed for CIP at pH 7.0 and 11.8

	CIP D245N mutant		CIP		
mode	pH 5.4 6-c HS	pH 10.0 5-c HS**	pH 3.8 5-c HS*	pH 7.0[a] 5-c HS	pH 11.8 6-c LS
ν_{10}	1615	1627	1627	1631	1634
ν(C=C)	1622	1622	1622	1625	1622
ν_{37}	1583	1590	1590	1584	1604
ν_{19}	1560	1567	1567	1567	1582
ν_2	1563	1569	1569	1566	1577
ν_{11}	1545	1551	1551	1551	1566[b]
ν_3	1481	1491	1492	1493	1502
δ(=CH_2)	1428	1428	1428	1428	1428
ν_4	1371	1373	1370	1371	1374
ν_8[c]	349	349		347	
γ_6[c]	323	323		323	
ν(Fe−Im)[c]	204	204		211, 230	

[a] Smulevich et al. (1994a). [b] Overlapped with the ν_2 mode at 1566 cm^{-1} of the 5-c HS species. [c] Frequencies observed in the ferrous forms.

Table 1 summarizes the frequencies of the most intense RR bands observed for the various species of CIP compared with the D245N mutant.

Ferrous Forms of wt CIP and the D245N CIP Mutant

Figure 7 shows the absorption spectra of the ferrous forms at pH 7.0 of CIP (Smulevich et al., 1994a) and the D245N mutant. They are characteristic of a 5-c HS species despite the fact that a 5 nm blue shift of the Soret band is observed

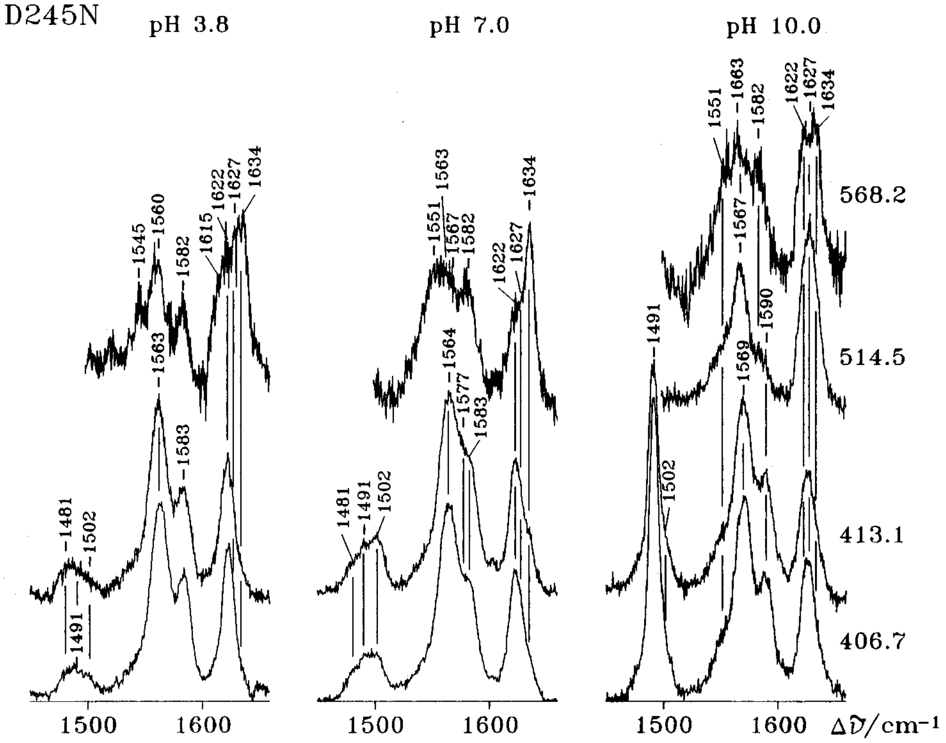

FIGURE 6: RR spectra of ferric D245N mutant at various pH values, taken with different excitations. Experimental conditions: 5 cm^{-1} resolution; pH 3.8, 406.7 nm excitation, 20 mW laser power at the sample, 10 s/0.5 cm^{-1} collection interval; pH 3.8, 413.1 nm excitation, 15 mW laser power at the sample, 5 s/0.5 cm^{-1} collection interval; pH 3.8, 514.5 nm excitation, 70 mW laser power at the sample, 61 s/0.5 cm^{-1} collection interval; pH 7.0, 406.7 nm excitation, 20 mW laser power at the sample, 10 s/0.5 cm^{-1} collection interval; pH 7.0, 413.1 nm excitation, 15 mW laser power at the sample, 10 s/0.5 cm^{-1} collection interval; pH 7.0 514.5 nm excitation, 60 mW laser power at the sample, 24 s/0.5 cm^{-1} collection interval; pH 10.0, 406.7 nm excitation, 20 mW laser power at the sample, 5 s/0.5 cm^{-1} collection interval; pH 10.0, 413.1 nm excitation, 20 mW laser power at the sample, 5 s/0.5 cm^{-1} collection interval; pH 10.0, 514.5 nm excitation, 80 mW laser power at the sample, 26 s/0.5 cm^{-1} collection interval; pH 10.0, 568.2 nm excitation, 80 mW laser power at the sample, 24 s/0.5 cm^{-1} collection interval.

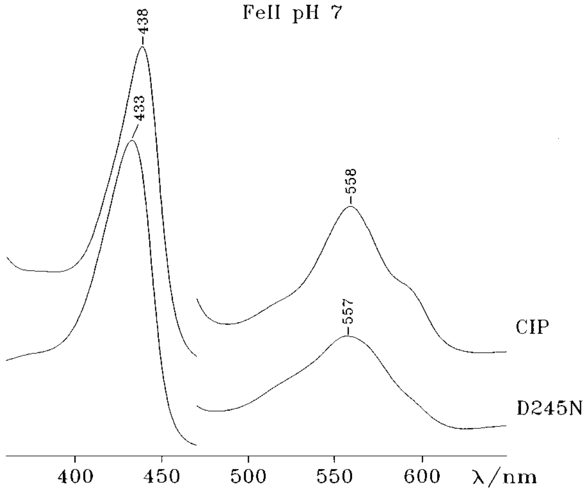

FIGURE 7: Electronic absorption spectra of ferrous CIP and D245N mutant at pH 7.0. The region between 450 and 700 nm has been expanded by 8 times.

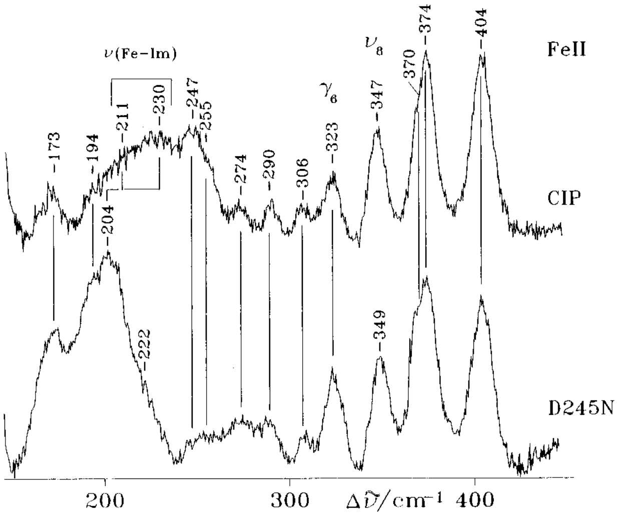

FIGURE 8: RR spectra in the low-frequency region of ferrous CIP and D245N mutant at pH 7.0, taken with 457.9 nm excitation. Experimental conditions: 5 cm^{-1} resolution; CIP, 15 mW laser power at the sample, 18 s/0.5 cm^{-1} collection interval; D245N 40 mW laser power at the sample, 47 s/0.5 cm^{-1} collection interval.

in the mutant, as previously reported for the corresponding D235N CCP mutant (Miller et al., 1990). Accordingly, the RR spectra in the high-frequency region of the two CIP proteins are almost identical, with core size marker bands characteristic of a 5-c HS heme (data not shown). The only difference is represented by the ν(C═C) stretching modes observed at 1619 cm^{-1} in the mutant and at 1621 cm^{-1} in the parent enzyme. Contrary to that previously observed for wt CIP (Smulevich et al., 1994a), increasing the pH to 10.0 did not give rise to any change in the absorption and RR spectra of ferrous D245N CIP.

Figure 8 compares the RR spectra taken at neutral pH of wt CIP and D245N CIP in the low-frequency region. The low-frequency region RR spectra of ferrous heme proteins are characterized by the presence of a strong band around 200−250 cm^{-1}, which is due to the ν(Fe−Im) stretching mode. Usually peroxidases show a ν(Fe−Im) stretching mode at higher frequency than other heme proteins, reflecting the imidazolate character of the proximal axial ligand induced by the polar $N_\delta^-\cdots HO$ hydrogen bond between the proximal histidine ligand of Fe and aspartate (Asp245 in CIP). For CIP, the ν(Fe−Im) stretching mode was assigned to the band at 230 cm^{-1} for the species at neutral pH and to the band at 211 cm^{-1} for the species at alkaline pH on the basis of the pH-dependent behavior of the bands (Smulevich et al., 1994a). The band at 230 cm^{-1} is replaced by a doublet at 194−204 cm^{-1} in the mutant. In addition, the band at 247 cm^{-1} of CIP markedly decreases in intensity in the mutant, and the band at 347 cm^{-1} upshifts to 349 cm^{-1}. Its intensity ratio to the 323 cm^{-1} band decreases.

A similar pattern of behavior was observed previously for the D235N CCP mutant (Smulevich et al., 1988). The strong bands at 233 and 246 cm^{-1} of CCP, assigned to two ν(Fe−Im) stretching modes, disappear in this mutant and are replaced by a new band at 205 cm^{-1}, and a concomitant increase in the intensity of the band at 182 cm^{-1} occurs. From experiments carried out on ^{54}Fe/^{56}Fe isotopic-substituted wild-type and D235N mutant of CCP, the band at 205 cm^{-1} was assigned to the ν(Fe−Im) stretching mode. The band at 182 cm^{-1} was assigned to the pyrrole tilting mode γ_{23}, coupled with the ν(Fe−Im) at 205 cm^{-1} from which it gained intensity (Smulevich, Hu, Rodgers, Goodin, Smith, and Spiro, unpublished experiments). Therefore, the replacement of Asp235 with Asn in CCP destroyed the strong hydrogen bond between the proximal aspartate and histidine side chains. From this experiment on D235N CCP, evidence that the band at 346 cm^{-1}, assigned to the ν_8 mode, is coupled to the ν(Fe−Im) stretching mode was obtained from the ^{54}Fe isotope shift and from its enhancement in the wild-type protein where the ν(Fe−Im) stretching mode is closer in frequency to the ν_8. On the basis of the similarity of the RR spectra of the D245N mutant of CIP and the D235N mutant of CCP, it also appears that in the CIP mutant the substitution of the proximal aspartate carboxylate group with the asparagine carboxamide group weakens the proximal hydrogen bond. The ν(Fe−Im) stretching mode of D245N CIP is therefore assigned to the band at 204 cm^{-1}, coupled to the mode giving rise to the band at 194 cm^{-1}. As a consequence, the band at 247 cm^{-1}, assigned to a heme mode strongly coupled to the ν(Fe−Im) stretching mode in CIP (Smulevich et al., 1994a), becomes a very weak band in the mutant. This is the same result that was found for the ferric form of the mutant (data not shown) and CIP (Smulevich et al., 1994a) where, in the absence of the ν(Fe−Im) stretching mode, the 247 cm^{-1} band cannot gain intensity through coupling. The band at 349 cm^{-1} is assigned to the ν_8 mode.

DISCUSSION

The characterization of the structural properties of the acid−alkaline equilibrium of CIP and its Asn245 mutant is important because the reactions of the enzymes are dictated by the heme pocket structure, in terms of the nature of the amino acid residues and the hydrogen-bonded network. These structural elements determine the strength of the axial ligands to the heme iron and the catalytic mechanism of these proteins.

To gain detailed information on the ligand structure of wt CIP and D245N CIP at the various pH values, we performed a careful analysis of the absorption and RR spectra and considered in particular the wavelengths of the Soret and CT1 charge transfer bands (Tables 2 and 3) and the intensity of the core size marker bands of the HS species. In fact, it has recently been shown for the His175→Glu (H175E)

Table 2: Soret and CT1 Bands (nm) for Different 5-c HS Heme Proteins

protein	Soret	CT1	axial ligand
HRP-C, pH 3.1[a]	<400	652	$H_2O\cdots R38^+$
CIP, pH 7.0[b]	403	649	Im^-
CCP, pH 7[c]	408	646	Im^-
D245N (CIP), pH 10.0[d]	410	644	$HO^-\cdots R51^+$
Mb, pH 3.0[e]	<400	640	H_2O
H175E (CCP), pH 5.5[f]	407	637	COO^-
H93C (Mb), pH 7.0[g]	391	629	S^-
H93Y (Mb), pH 7.0[g]	402	598	phenyl$-O^-$

[a] Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments. [b] Smulevich et al. (1994a). [c] Vitello et al. (1992). [d] A 6-c LS species is also observed. [e] Palaniappan and Bocian (1994). [f] Smulevich et al. (1995). [g] Adachi et al. (1993).

Table 3: Soret and CT1 Bands (nm) for Different 6-c HS Species Observed for Various Proteins

protein	Soret	CT1	proximal ligand	distal ligand
H64Q (Mb), pH 6.0[a]	409	637	Im	H_2O
D245N (CIP), pH 5.4[b]	410	635	Im	H_2O
Mb, pH 7.0[c]	409	632	Im	$H_2O\cdots H64$[g]
CCP-F, pH 5.0[d]	407	620	Im^-	$F^-\cdots R48^+$ [h]
Mb-F, pH 6.0[c]	406	606	Im	F^-
Mb, pH 10.4[e]	413	600	Im	OH^-
H175E (CCP), pH 7.0[f]	406	618	COO^-	H_2O

[a] Ikeda-Saito (1992). [b] A 6-c LS species is also observed. [c] Eaton and Hochstrasser (1968). [d] Yonetani and Anni (1987). [e] A 6-c LS species is also observed (Feis et al., 1994). [f] Experiment carried out at 19 K (Smulevich et al., 1995). [g] Takano (1977). [h] Edwards and Poulos (1990).

mutant of CCP (Smulevich et al., 1995) that the RR and electronic absorption spectra in the Soret and α and β regions furnished information on the heme coordination and spin states, but did not provide direct evidence that the heme iron is bound to the O_ϵ of the glutamate replacing the usual N_ϵ of the proximal histidine residue. Direct evidence for the presence of a ligand different from histidine at the fifth coordination position of the iron atom was revealed by the wavelength of the band resulting from the charge transfer (CT1) transition from the porphyrin to the iron $[a'_{2u}(\pi)\rightarrow e_g(d_\pi)]$. The higher π donor character of carboxylate compared to the imidazole raises the energy of the d_π orbitals of the iron atom and causes an 8 nm blue shift of the CT1 band in H175E CCP compared to wt CCP (Table 2). Likewise, the electronic absorption spectra of CIP and the D245N mutant at different pH values show great variability in the wavelength of the CT1 band (Tables 2 and 3). This indicates that both the π electrons and the p electrons of the ligand interact with the d_π electrons of the heme iron, as has also been observed for HRP-C at acid pH (Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments). Here we attempt to correlate the energy of the d_π orbitals of the heme iron to the nature of the axial ligands in terms of the p and π electrons. The wavelengths of the Soret and CT1 bands for different 5-c and 6-c HS proteins are compared in Tables 2 and 3, respectively. It can be seen that the CT1 band wavelength is extremely sensitive to both the proximal and distal ligands. For both the 5- and 6-c HS hemes, the maximum blue shifts when the p and π donor capabilities of the ligand(s) increase. In particular, hydrogen bonding affects the p donor capability. If the ligand acts as a hydrogen-bond donor, then the stronger the hydrogen bond, the lower the wavelength of CT1. This is the case of ferric met-Mb at neutral pH as compared to its distal mutant His64→Gln (H64Q), which has lost the hydrogen bond between the bound water molecule and the distal histidine (Quillin et al., 1993). If the ligand acts as a hydrogen-bond acceptor, the CT1 wavelength appears to increase with the strength of the hydrogen bond. This is the case for the fluoride complex of CCP, where the fluoride is hydrogen-bonded with the positively charged guanidinium group of Arg48 (Edwards & Poulos, 1990), in contrast to the Mb-F and Hb-F complexes, which exist as an equilibrium between a species with a hydrogen bond to water and a species free from hydrogen bonding (Deatherage et al., 1976; Asher et al., 1977; Asher & Schuster, 1981).

Wild-Type CIP. The fungal *Coprinus cinereus* peroxidase shows two pH-induced transitions. At pH 3.8, both the electronic absorption and RR data indicate the presence of two 5-coordinate species, which differ in the nature of the fifth ligand. At pH 12.1, CIP is predominantly 6-c LS due to a hydroxyl group bound at the sixth coordination position of the heme iron. The pK_a of the alkaline transition appears to be very high, as a small amount of the neutral 5-c HS species is still present at pH 12.1. For both of these pH-induced transitions, the conformational changes due to the extreme pH may also cause a change in the orientation of at least one vinyl group, as a new $\nu(C{=}C)$ stretching mode was observed at 1622 cm^{-1} instead of 1625 cm^{-1} at neutral pH. Therefore, it appears that CIP behaves similarly to HRP-C at extreme pH values.

CIP at neutral pH contains a 5-c HS heme with the proximal imidazole coordinated to the heme iron (Kunishima et al., 1996), as previously observed for CCP (Hashimoto et al., 1986). The wavelength of the CT1 band of CIP (649 nm) is, however, 3 nm higher than that for CCP (Vitello et al., 1992). Smulevich et al. (1994a) suggested that this red shift was due to the larger rotation angle, along the heme normal, of the imidazole plane with respect to the N(1)$-$Fe$-$N(3) axis (azimuthal angle, $\phi = 40°$) (Kunishima et al., 1994), as compared to CCP ($\phi = 7°$) (Finzel et al., 1984). The change in rotation decreases the π donation of the ligand and increases the wavelength of the CT1 band. Upon acidification, this neutral form partially converts to a species characterized by a CT1 at 652 nm, as previously observed for HRP-C at pH 3.1 (Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments), but very different from the CT1 reported at 640 nm for met-Mb at pH 3.0 (Palaniappan & Bocian, 1994). As discussed earlier, the fifth ligand of the heme iron might be a water molecule. A preliminary investigation of the Arg51→Leu CIP mutant at both pH 7.0 and 3.8 gave rise to electronic absorption spectra very similar to that observed for CIP at pH 3.8 (Neri, Indiani, Welinder, and Smulevich, unpublished experiments). In addition, a blue-shifted Soret band at 394 nm was also observed for the His64→Leu met-Mb. For this mutant the X-ray structure has been solved (Quillin et al., 1992) to show that the sixth coordination position of the iron atom was empty, but no changes were reported at the proximal side of the heme with respect to wt met-Mb. Therefore, the changes observed for CIP at pH 3.8 indicate a weakening of the proximal Fe$-$Im bond, but might also derive from other effects that are difficult to rationalize at the present stage.

At alkaline pH, in analogy with the findings for HRP-C (Feis et al., 1994), CIP should have a hydroxyl group bound to the heme iron and be hydrogen-bonded directly, or through

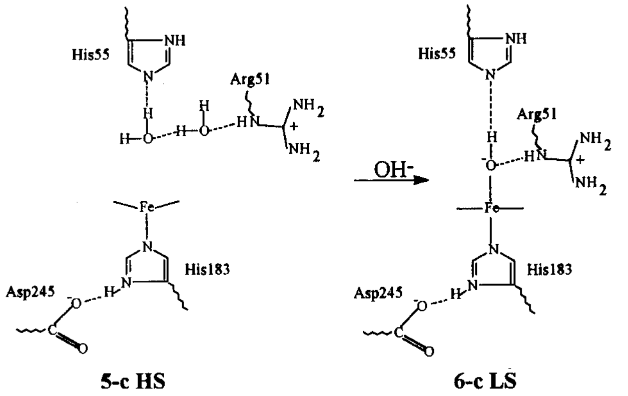

FIGURE 9: Schematic representation of the neutral−alkaline ligation equilibrium for CIP inferred from the RR and electronic absorption titration data.

a water molecule, to the distal Arg and also probably to the distal His, as indicated in Figure 9. In this figure, two distal water molecules observed in the CIP/ARP crystal structure at 1.8 Å resolution (Kunishima et al., 1996) are shown at pH 7.0. At pH 12.1, a hydrogen-bonding pattern similar to that of Cpd I of CCP (Fülöp et al., 1994) with one water molecule excluded and movement of the Arg51 side chain into the cavity is proposed.

The D245N CIP Mutant. Replacement of the proximal carboxylate group of Asp245 in CIP with an asparagine residue has a profound influence on the heme coordination. This mutant in the ferrous form has the Fe−Im stretching frequency at a low, non-hydrogen-bonded imidazole value, as was previously found for the CCP mutants at the corresponding position (D235N, D235A, D235E) (Smulevich et al., 1988; Goodin & McRee, 1993).

As far as the ferric form is concerned, a 6-c LS species is present at every pH under investigation. The fact that this species appears to be unaffected by the pH allows us to conclude that it derives from modified protein, and therefore, this species will not be discussed further. At pH 3.8 the ferric D245N CIP mutant is a mixture of a very small amount of a 5-c HS* and a 6-c HS heme. The presence of a 6-c instead of a 5-c heme, as found in wt CIP, can be explained as a consequence of the lack of the strong proximal hydrogen bond, which will allow the iron to move from the proximal side into the heme plane and to bind a distal water molecule. The CT1 observed at 639 nm, 7 nm higher than the corresponding band observed for met-Mb at neutral pH, is probably due to the dual contributions of the 6-c HS heme (see below) and the 5-c HS* heme, which supposedly corresponds to the form observed in wt CIP at the same acid pH (Figures 9 and 10). At pH 5.4 the CT1 band of the mutant shifts down to about 635 nm (Table 3), and the RR spectra indicate the presence of a 6-c HS species. Therefore, the CT1 band suggests the presence of a water molecule hydrogen-bonded to the distal His at the sixth coordination position of the iron atom, in analogy to met-Mb at neutral pH, which has CT1 at 632 nm. Many recombinant human myoglobin mutants in which the distal histidine was replaced have been studied to investigate how amino acid residues in the distal heme pocket control ligand binding to the iron atom. These studies clearly show that the wavelength of the CT1 is sensitive to the nature of the water molecule bound to the heme iron. In particular, the H64Q met-Mb appears to have a water molecule coordinated to the heme iron that is not hydrogen-bonded to glutamine, as shown by X-ray crystallography (Quillin et al., 1992). This mutant showed a CT1 band at 637 nm (Table 3) (Ikeda-Saito, 1992).

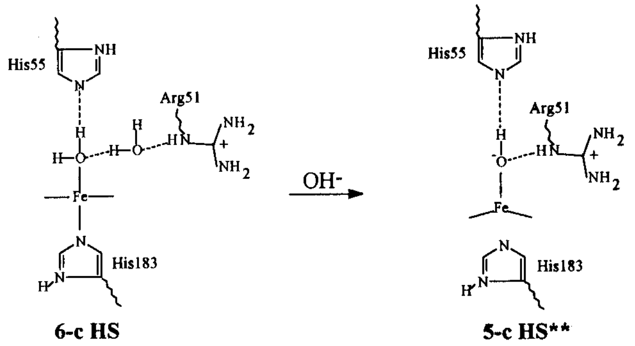

FIGURE 10: Schematic representation of the neutral−alkaline ligation equilibrium for the D245N CIP mutant inferred from the RR and electronic absorption titration data.

At pH 5.4 the D245N mutant of CIP behaves as previously reported for the corresponding D235N mutant of CCP. In fact, this CCP mutant was a mixture of a 6-c HS heme and a 6-c LS heme both in solution at pH 6 (Smulevich et al., 1988, 1991; Satterlee et al., 1990; Spiro et al., 1990; Vitello et al., 1992; Goodin & McRee, 1993; Smulevich, 1993; Ferrer et al., 1994) and as a single crystal (Smulevich et al., 1990). X-ray crystallography of D235N CCP (Wang et al., 1990) revealed that the distal Wat595 had moved toward the heme plane by ca. 0.6 Å compared to wt CCP, while the iron atom had shifted toward the distal side by ca. 0.2 Å. The distance of the iron to Wat595 decreased from 2.7 Å in CCP(MI) (2.4 Å in baker's yeast CCP; Finzel et al., 1984) to 1.9−2.0 Å in D235N CCP enzyme. Therefore, as the $Fe^{III}-O$ distance in D235N CCP was similar to that of 6-c sperm whale met-Mb (Takano, 1977), the 6-c HS species of this CCP mutant was also assigned to a water ligand bound to the Fe atom.

The X-ray structure of wt CIP/ARP revealed the presence of hydrogen-bonded water molecules in the distal pocket (Kunishima et al., 1996). Despite the fact that the structure of the D245N mutant of CIP is not known, it is conceivable that the mutation causes structural effects similar to those observed for the D235N mutant of CCP and gives rise to an aquo ligand (6-c HS) (Table 3, Figure 10). At neutral pH, the most abundant species of the D245N mutant of CIP is the 6-c HS form, but a small amount of the 5-c HS** form is also observed.

For D245N CIP at pH 10.0, the 6-c HS disappears and the most abundant species is the 5-c HS** form, which had already appeared at pH 7.0. The RR core size marker bands, in particular ν_3, are more intense than the ν_4 at pH 10.0 than at pH 7.0 (Figure 5). This means that at pH 10.0 variation in the size of the core between the ground and the excited electronic states (along the normal coordinate corresponding to the ν_3 mode) is larger than at pH 7.0. We have noticed that an intense band corresponding to the ν_3 mode can be observed in the RR spectra of Mb mutants where the proximal imidazole ligand was substituted by tyrosine (Morikis et al., 1990; Adachi et al., 1993) or cysteine (Adachi et al., 1993). Therefore, the effect appears to be associated with the presence in the fifth position of a ligand different from histidine. From the electronic and RR spectra it is not possible to determine whether the water molecule binds on the proximal or the distal side of the heme. In any case, the

Table 4: Summary of Heme Coordination and Spin States in the Ferric Forms of CIP, D245N CIP, CCP, and D235N CCP Observed at Various pH Values

	acid				neutral[a]				alkaline[b]		
CIP	5-c HS*	5-c HS			5-c HS				5-c HS	6-c LS	
D245N(CIP)	5-c HS*		6-c HS		5-c HS**		6-c HS		5-c HS**		
CCP[c]		5-c HS	6-c HS			5-c HS					6-c LS$^{\#}$ [d]
D235N(CCP)[e]			6-c HS	6-c LS			6-c HS	6-c LS			6-c LS$^{\#}$
D235A(CCP)[f]			6-c HS	6-c LS			6-c HS	6-c LS			6-c LS$^{\#}$
D235E(CCP)[f]		5-c HS	6-c HS	6-c LS		5-c HS	6-c HS	6-c LS			6-c LS$^{\#}$

[a] pH 6 for the CCP mutants. [b] pH 7 for the CCP mutants (Smulevich et al., 1988). [c] The amount of the 6-c HS depends on the ionic strength, buffer, and purification procedure (Vitello et al., 1990). [d] 6-c LS$^{\#}$ indicates a bis(imidazole) 6-coordinate low-spin heme. [e] From Smulevich et al. (1988). [f] From Goodin and McRee (1993). Smulevich, G., unpublished experiments. The most abundant species is underlined.

D245N mutation weakens the Fe−Im bond and could explain the breakage of the bond. On the other hand, the CT1 occurs at 644 nm, which is 8 nm lower than for the corresponding band of HRP-C at pH 3.1 (Table 2) (Smulevich, Paoli, De Sanctis, Mantini, Ascoli, and Coletta, unpublished experiments). Hence, a ligand different from H_2O, and most likely a OH^- group strongly hydrogen-bonded to the distal arginine (and distal histidine), is the most likely candidate for coordination in the alkaline form of D245N CIP heme iron (Figure 10). Thus, this 5-c HS** species has the same ligand in the sixth position as CIP at alkaline pH. Most significantly, it differs from CIP as the proximal histidine is not coordinated to the Fe atom.

The alkaline forms of the D245N CIP and D235N CCP mutants are very different. CCP, as well as all of the mutants investigated so far (His52→Leu CCP being the only exception), exhibits an alkaline transition to a low-spin Fe^{III} form (Smulevich et al., 1988, 1991; Miller et al., 1988) characterized by α and β bands distinctly blue-shifted relative to those of the alkaline H52L CCP mutant, met-Mb (George & Hanania, 1952; Feis et al., 1994), and HRP-C (Keilin & Hartree, 1951; Feis et al., 1994) and very similar to those of ferric cytochrome b_5 (Strittmatter & Velick, 1956). Therefore, it was concluded that in CCP and its mutants at alkaline pH the imidazole side chain of distal His52 binds to Fe^{III} (Table 4, indicated by 6-c LS$^{\#}$). When histidine was replaced by leucine, a hydroxide ion was bound instead (Smulevich et al., 1991). The acid−alkaline transition of CCP and its mutants was also accompanied by a change in the orientation with respect to the heme plane of the vinyl group at position 2 of the heme (Smulevich et al., 1991; Smulevich, Hu, Rodgers, Goodin, Smith, and Spiro, unpublished experiments). This proposed conformational change is independent of the heme oxidation and was observed in both the ferric and ferrous forms. It is also independent of the ligation state, but controls the heme chemistry by permitting the His52 side chain to bind as a sixth ligand, despite its original distance of ca. 5 Å from the Fe^{III} at neutral pH.

An important difference between peroxidases of class I (like CCP) and those of class II (CIP) and class III (HRP-C) is that the peroxidases of class I lack the stabilizing disulfide bridges and structural Ca^{2+} (Welinder, 1992). This may explain the unique instability of CCP at alkaline pH.

The unique 5-c form (5-c HS**) present in the D245N mutant of CIP at alkaline pH, to our knowledge, has never been observed before in any other heme protein or mutant. As this form is absent from wt CIP, the proposed breaking of the Fe−Im bond appears to be due to the weakening or breaking of the proximal hydrogen bond between the His183 ligand and the Asp245 carboxylate group. The corresponding D235N CCP mutant does not show this 5-coordinate species at pH 7.0 or at alkaline pH, because a distal conformational change induced at these pH values brings the distal His52 into a position to displace the OH^- sixth ligand, giving rise to a bis(imidazole) 6-c LS species (Smulevich et al., 1991). The different heme cavity structure of CIP and CCP might account for this, or again the reduced internal mobility of apo-CIP resulting from disulfide bonds and two structural Ca^{2+} ions might prevent the structural adjustments and the formation of the 6-c LS heme with an internal ligand bound at the distal side.

CONCLUSIONS

The electronic absorption and RR spectra obtained for CIP and the D245N CIP mutant indicate that the structure of the heme group is dramatically influenced by pH changes. In wt CIP, the proximal histidine ligand is coordinated at both neutral and alkaline pH, but the iron−histidine bond might be modified and/or ruptured at acid pH and the Fe atom ligated with a partially protonated water molecule. At alkaline pH the heme is characteristic of a 6-c LS species. A hydroxyl group strongly hydrogen-bonded with the distal Arg51 is suggested to be bound in the sixth position of the heme iron, in analogy with alkaline HRP-C.

The replacement of Asp245 by Asn in CIP significantly weakens or breaks the hydrogen bond from His183 and, consequently, the Fe−Im bond, as concluded by the 25 cm^{-1} downshift of the ν(Fe−Im) stretching mode in the ferrous form of the mutant compared with wt CIP. The appearance of a 6-c high-spin (aquo) heme in the ferric form in the pH range 3.8−8.8 shows that the His183−Asp245 interaction restrains the Fe atom from entering the heme plane and from binding the distal water molecule, as observed previously for the corresponding D235N CCP mutant. A novel feature is the 5-c HS** species observed for the mutant at alkaline pH. This form is suggested to result from the breakage of the Fe−Im bond and the concomitant binding in the distal position of the iron atom of a hydroxyl group strongly hydrogen-bonded to the distal Arg51.

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