Heme–Protein Interactions in Cytochrome c Peroxidase Revealed by Site-Directed Mutagenesis and Resonance Raman Spectra of Isotopically Labeled Hemes

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SYNOPSIS

Isotope labeling has been used to assign the resonance Raman spectra of cytochrome c peroxidase, expressed in *Escherichia coli* [CCP(MKT)], and of the D235N site mutant. ^{54}Fe labeling establishes the coexistence of two separate bands (233 and 246 cm^{-1}), arising from the stretching of the bond between the Fe atom and the proximal histidine ligand, His175. These are assigned to tautomers of the H-bond between the His175 imidazole $N_\delta H$ proton and the Asp235 carboxylate side chain: In one tautomer the proton resides on the imidazole while in the other the proton is transferred to the carboxylate. When Asp235 is replaced by Asn, the H-bond is lost, and the Fe-His stretching frequency is markedly lowered. Two new RR bands are produced, at 205 and 185 cm^{-1}, as a result of coupling between the shifted Fe-His vibration and a nearby porphyrin mode; the two bands share the ^{54}Fe sensitivity expected for Fe-His stretching. C=C stretching and $C_\beta C=C$ bending vibrations have been separately assigned to the 2- and 4-vinyl groups of the protoheme prosthetic group via selective vinyl deuteration. In the acid form of the enzyme, the frequencies coincide for the two vinyl groups, at 1618 cm^{-1} for the C=C stretch, and at 406 cm^{-1} for the $C_\beta C=C$ bend. However, the 2-vinyl frequencies are elevated in the alkaline form of the enzyme, to 1628 cm^{-1} for C=C stretching, and to 418 cm^{-1} for $C_\beta C=C$ bending, while the 4-vinyl frequencies remain unshifted. Thus, the acid-alkaline transition involves a protein conformation change that specifically perturbs the 2-vinyl substituent. This perturbation might be a reorientation of the vinyl group, or an alteration of the porphyrin geometry that affects the porphyrin–vinyl coupling. The perturbation is attenuated when CO is bound to the enzyme; the C=C frequency is then unaffected in the alkaline form, while the $C_\beta C=C$ bending frequency is shifted to a smaller extent (412 cm^{-1}). This attenuation is probably linked to inhibition of distal histidine binding to the heme Fe in the alkaline form when the CO is bound.

INTRODUCTION

Cytochrome c peroxidase (CCP: ferrocytochrome c-hydrogen peroxide oxidoreductase, EC1.11.1.5) is a protoheme enzyme, found in yeast mitochondria, which catalyzes the oxidation of cytochrome c by hydroperoxides.[1,2] Because of the availability of high-resolution crystal structures of CCP[3–5] and of site-directed mutants of cloned protein,[6,7] it has been possible to probe the role of the heme pocket environment in directing the reactivity of the heme group in this enzyme. Resonance Raman spectroscopy has helped to elucidate this role by providing monitors of critical bonding interactions.[8–18] Among

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the insights provided by RR spectroscopy, two are of particular note:

1. The bond between the heme iron atom and the proximal histidine ligand His175 is unusually strong, as reflected in the high frequency of the Fe-His stretching RR band. This effect is due to the imidazolate character of the ligand, which is induced by the H-bond from His175 to the carboxylate side chain of the nearby Asp235 residue. The role of the proximal histidine ligand in peroxidase function has been recently studied by replacing the His side chain with Gln, Glu, or Cys[19] or by removing the side chain to allow nonnative ligands such as imidazoles.[20] In the former experiments, it has been found that the identity of the proximal ligand is not important for the high rates of peroxide O–O bond cleavage, but it is important for the stability of compound I.[19] However, replacement of His175 with imidazole reduces the enzyme activity by 95%, and also alters the stability of the Trp191 radical and its coupling to the heme center.[20] While the structure of the H175G/Im complex is very similar to the wild-type (wt) enzyme, a small tilt of the imidazole plane was proposed to diminish the strength of the Asp235 to His175 H-bond.[20] Thus, as previously proposed,[9,23] the strength of the H-bond between Asp235 and His175 is critical to the imidazolate character of His175.
2. The enzyme undergoes an acid-alkaline transition, with a pK_a between 7 and 9 (depending on the mutant and on the oxidation state), which involves a distinctive protein conformation change, as revealed by perturbations of RR bands associated with the heme vinyl substituents. This change permits binding of the distal histidine, His52, which is 5 Å distant from the Fe in the crystal structure of the acid form, although participation of His52 is not obligatory; the transition persists when His52 is replaced by leucine, or when its binding is blocked by the presence of bound CO. This transition may provide a mechanism for the coupling of peroxide reduction to cytochrome c binding.[20]

In this study, we clarify and extend the previous RR results with the aid of isotopic labels on the heme group. The variant CCP(MKT) has been expressed in *Escherichia coli* for this purpose, along with the D235N mutant, in which Asp235 (D) is replaced with the isosteric but uncharged asparagine residue (N). The suffix MKT stands for Met-Lys-Thr on the N-terminus.[22] These residue additions and substitutions have negligible effects on the enzyme structure or activity.[22] The expressed proteins were reconstituted with heme in which deuterium is incorporated in the 2- or 4-vinyl substituents (Fig. 1), or in the 1-methyl substituent, or at the meso-carbon atoms of the porphyrin ring. ^{54}Fe was also substituted for natural abundance iron (atm wt 55.85) in the heme. The isotope labeling has secured critical band assignments and has identified the 2-vinyl substituent as the site of the perturbing effect of the acid-alkaline protein conformation change.

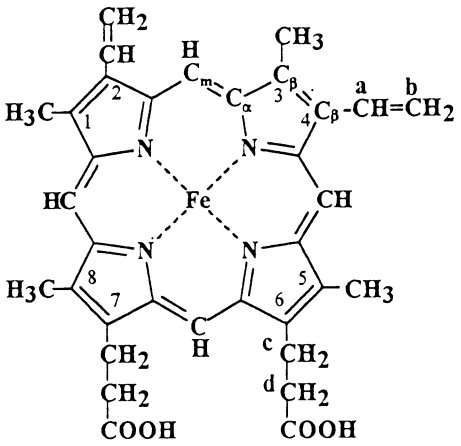

Figure 1. Structural and atom labeling of the heme group.

MATERIAL AND METHODS

MKT and N235 CCP apoproteins were prepared as previously described.[23] All the heme isotopomers were synthesized according to published procedures.[24,25] The ^{54}Fe-heme was prepared as follows: $^{54}FeCl_2$ was added to a 1.1-fold excess of H_2-protoporphyrin IX-dimethylester (H_2PPIX-DME) in dimethylformamide (DMF) at 80°C under a nitrogen atmosphere. The nitrogen atmosphere was maintained and the solution was stirred at this temperature for 3 days to insure that all of the ^{54}Fe had been incorporated into the heme. Progress of the reaction was monitored by periodic acquisition of ultraviolet (UV)-visible spectra of the reaction mixture. After ^{54}Fe insertion was complete, the reaction was allowed to cool to room temperature and

a twofold excess of aqueous 2 *M* NaCl was added to DMF solution. The resulting precipitate was collected by vacuum filtration and dried under vacuum (10^{-3} torr) for 24 h. The dried crude hemin dimethyl ester fraction was purified by flash alumina chromatography. The pure hemin dimethyl ester fraction was evaporated to dryness, redissolved in a 1% methanolic KOH solution, and stirred overnight at room temperature to hydrolyze the ester group. The solution was then neutralized with a buffered aqueous acetate solution and the hemin was extracted into diethylether and crystallized by slow evaporation of the ether solution. The product thus obtained was shown to be pure and to have its vinyl groups intact via the pyridine hemechrome method. To reconstitute the apoproteins, an excess of each heme isotopomer was dissolved with a minimum amount of 0.1 N NaOH, diluted with chilled deionized water, and then mixed with apoprotein solution. To remove the excess heme, the mixture was passed over a Sephadex G-25 column, which had been equilibrated with 5 m*M* potassium phosphate buffer, pH 6.8. The CCP fractions were combined and concentrated with an Amicon Centricon-10 filter. The concentrated CCP solution was diluted with cold deionized water and concentrated several times until CCP crystals appeared. Typically, three cycles of concentration and dilution were enough to afford CCP crystals. The crystals and the mother liquor were kept on ice overnight to obtain complete crystallization of CCP. The CCP crystals were collected, washed three times with cold deionized water, suspended, and stored in liquid nitrogen until ready for use. Crystals were dissolved in 0.1 *M* phosphate (pH 6.0) and tris(hydroxymethyl)-aminomethane (Tris) (pH 8.5) buffers to give a protein concentration of 0.1 m*M*.

The Fe(II) forms were prepared by adding a minimum volume of dithionite to deoxygenated buffered solution of protein. The CO adducts were obtained by gently flowing CO (1 atm) (Matheson) from a gas cylinder over the surface of the reduced protein for 15 min.

The RR spectra were obtained with laser excitation at 413.1 nm [Kr^+ (Coherent)], 457.9 nm Ar^+ (Coherent) or 441.6 nm [He/Cd (Liconix)]. The back-scattered light from a slowly rotating NMR tube was dispersed with a Spex triplemate monochromator (2400 groove/mm grating) and collected with an optical multichannel analyzer (Princeton Instruments).

For the $^{54/56}$Fe(II) experiments as well as those in D_2O, the scattered light was collected and focused into a computer-controlled double monochromator (Spex 1404 or Jobin-Yvon HG 2S) equipped with a cooled photomultiplier (RCA) and photon-counting system. All the spectra were collected at room temperature and calibrated using indene and CCl_4 as standards. The frequencies are accurate to ± 1 cm^{-1} for isolated bands.

To minimize photolysis of the ligand, the spectra of the Fe(II) form at alkaline pH and those of the CO adducts were obtained using a cylindrical lens to focus the laser beam on the sample.

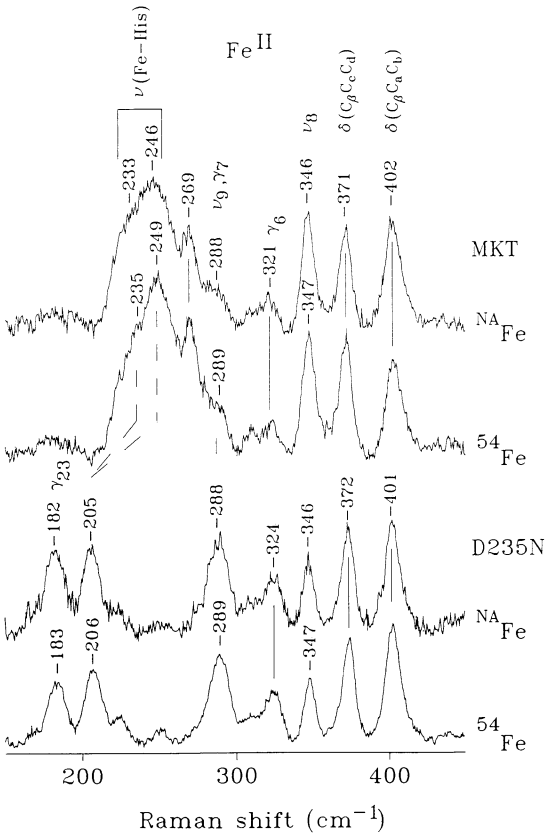

Figure 2. 441.6-nm-excited RR spectra of CCP(MKT) and the D235N mutant in the reduced (Fe(II) form, at pH 6.0 showing the effect of ^{54}Fe substitution on the Fe-His mode and on modes coupled to it. See discussion and Li et al.[25,26] for band labeling. Conditions: 5-mW laser power at the sample, 3-cm^{-1} resolution, 10-s/0.5 cm^{-1} collection interval.

RESULTS

Fe-Histidine Stretching Vibration

In common with other peroxidases, reduced CCP gives a broad band at ca. 240 cm^{-1} in the Soret-excited RR spectrum (Fig. 2), which is assigned to

the stretching vibration of the bond from the Fe atom to the proximal histidine ligand.[8] This band has two components, at 233 and 246 cm^{-1}, with frequencies and relative intensities that vary among different heme pocket mutants.[9] Upon ^{54}Fe substitution, both components are seen to shift up by ~ 3 cm^{-1} (Fig. 2). The shifts are within experimental error (~ 1 cm^{-1}) of those expected for isolated Fe-His oscillators (2.2 and 2.3 cm^{-1}, using 68.1 amu for the effective mass of the rigid imidazole ring). The two components therefore arise from separate Fe-His stretching vibrations.

When the carboxylate side-chain of Asp235 is replaced by the amide side chain of asparagine, in the D235N mutant, the low-frequency RR spectrum changes dramatically (Fig. 2). The 233- and 246-cm^{-1} components of the Fe-His band both disappear, along with the adjacent 269 cm^{-1} band, and two new bands appear, at 205 and 182 cm^{-1}. This alteration has been attributed[9] to loss of the His175-Asp235 H-bond. Thus, the 205-cm^{-1} band was assignable to the Fe-His stretch when the H-bond was lost, but the identity of the 182 cm^{-1} band was a puzzle.

Figure 2 shows that the 205- and 182-cm^{-1} bands are both sensitive to ^{54}Fe substitution, shifting up by ~ 1 cm^{-1}, each. Thus, the two bands share the 1.8-cm^{-1} shift expected for an isolated Fe-His oscillator at the average frequency, 194 cm^{-1}. It is evident that the Fe-His stretch becomes coupled to a nearby porphyrin mode when its frequency is shifted down to the vicinity of 200 cm^{-1}. We attribute the 182-cm^{-1} band to a pyrrole out-of-plane tilting mode, γ_{23}, which has been assigned at 254 cm^{-1} in NiOEP,[27] since pyrrole tilting is expected to couple mechanically with Fe-His stretching. (The frequency shift relative to NiOEP is attributable to the altered pyrrole substituents in protoporphyrin as well as the Fe-His coupling.) No meso-deuteration shift is observed (see below), or expected. The γ_{23} mode has little intrinsic RR enhancement, and it is undetectable in CCP itself (Fig. 2, top). In the D235N mutant, it shares the intensity of the Fe-His stretch, with which it becomes coupled because of the near coincidence in frequency.

The Fe-His bands are unaltered in frequency when the proteins are equilibrated in D_2O, as seen in Figure 3, but the 269-cm^{-1} band does shift in D_2O, by 3 cm^{-1}. We assign this band to an internal mode of the histidine ligand. The frequency is too low for any of the modes of the imidazole ring itself, but is reasonable for a bending mode centered at the histidine C_β atom to which the imidazole is attached. RR enhancement of this mode is attributed to vibrational coupling with the nearby Fe-His modes, because the 269-cm^{-1} band disappears when the Fe-His band is shifted to lower frequency, in the D235N mutant.

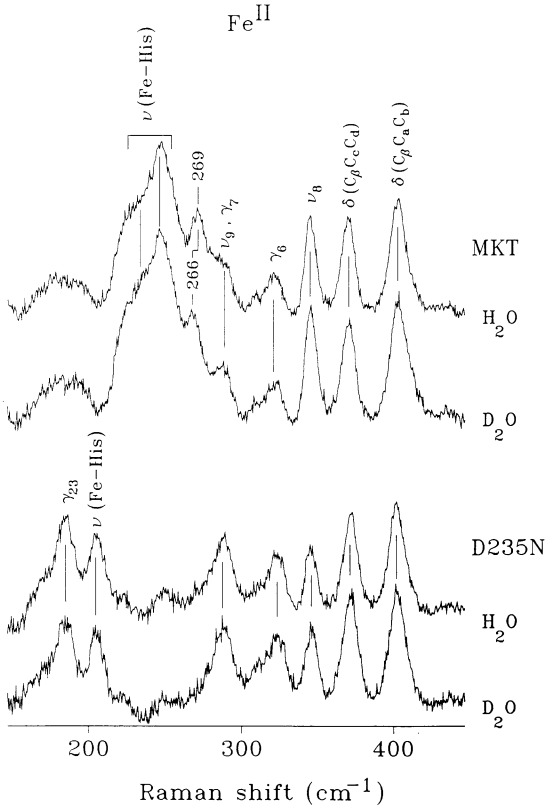

Figure 3. As in Figure 1, but showing the effects of replacing H_2O with D_2O. Conditions: 457.9-nm excitation, 50-mW laser power at the sample, 3-cm^{-1} resolution, 5-s/0.5 cm^{-1} collection interval.

Substituent Bending and Out-of-Plane Modes

Several other RR bands are seen in the 200–600-cm^{-1} region and are assigned as indicated by the labels in Figures 2–7. These assignments are made on the basis of the isotope shift patterns, with reference to the normal mode analysis of NiOEP,[26,27] and to recent assignments for cytochrome c[28] and myoglobin.[29]

Two in-plane porphyrin skeletal modes are observed in this region, ν_8 and ν_9. These are mixed Fe-pyrrole breathing and pyrrole-substituent bending modes, and are found at 346 and 288 cm^{-1}, independent of oxidation or ligation state. They couple slightly to the Fe-His vibration, as seen in the ~ 1-cm^{-1} ^{54}Fe shifts, and in their altered intensi-

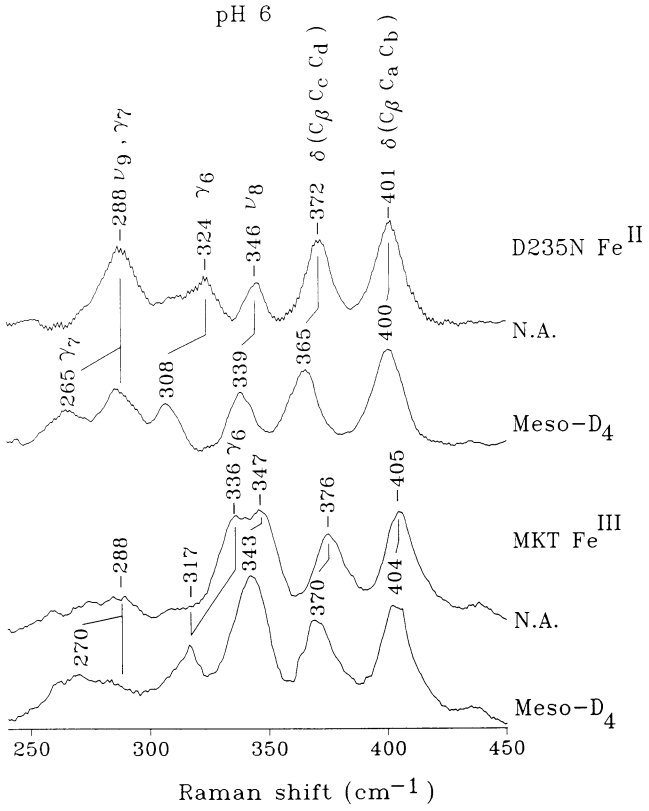

Figure 4. As in Figure 1, but showing the effects of meso-d_4 substitution on the porphyrin ring. The Fe(III) form of CCP(MKT) is shown because it avoids interference by the strong broad Fe-His band in the Fe(II) form. Conditions: the Fe(II) spectra were obtained with 441.6-nm and Fe(III) with 413.1-nm excitations, respectively; 5- and 50-mW laser power at the sample, for Fe(II) and Fe(III), respectively; 5-cm^{-1} resolution, 15-min collection.

ties when the Fe-His mode moves to lower frequency in the D235N mutant (Fig. 2). Figure 4 shows that ν_8 but not ν_9 is sensitive to deuteration of the methine bridges.

Two internal bending modes of the peripheral substituents, $\delta(C_\beta C_b{=}C_a)$ of the vinyl groups and $\delta(C_\beta C_c C_d)$ of the propionate groups, are strongly enhanced, at 402 and 371 cm^{-1} (Fig. 2). The propionate assignment is made by analogy with myoglobin, for which propionate-labeled heme was available.[29] This band is also sensitive to meso-deuteration (Fig. 4), reflecting a coupling of propionate bending with porphyrin out-of-plane modes. The propionate bending frequency varies somewhat, from 372 cm^{-1} in the Fe(II) form of the D235N mutant to 379 cm^{-1} in the Fe (III) form (376 cm^{-1} in the wt protein) to 389 cm^{-1} in the Fe(II)CO adduct at pH 6 (383 cm^{-1} at alkaline pH). This variability may reflect the out-of-plane coupling (see below), and/or alterations in the propionate orientation among the different forms.

The vinyl assignment is revealed in sensitivity to deuteration of the vinyl C_b atoms (Figs. 5 and 6). (The frequency is not altered by C_a deuteration, because the C_a atom is nearly stationary during the bending motion.) Sensitivity is also observed to deuteration of the 1-methyl substituent; the main part of the band shifts down 10–11 cm^{-1}, but a shoulder remains at the unshifted position, in both the Fe(III) and the Fe(II)CO forms at pH 6 (Figs. 5 and 6). The 1-methyl substituent is on the same pyrrole ring as the 2-vinyl substituent, and we therefore assign the shifted band to the 2-vinyl bending mode, coupled with deformation modes of the 1-methyl group, and the unshifted band to the 4-vinyl group. The two modes are coincident at pH 6 in the absence of 1-methyl deuteration.

At alkaline pH, there is a distinct upshift and

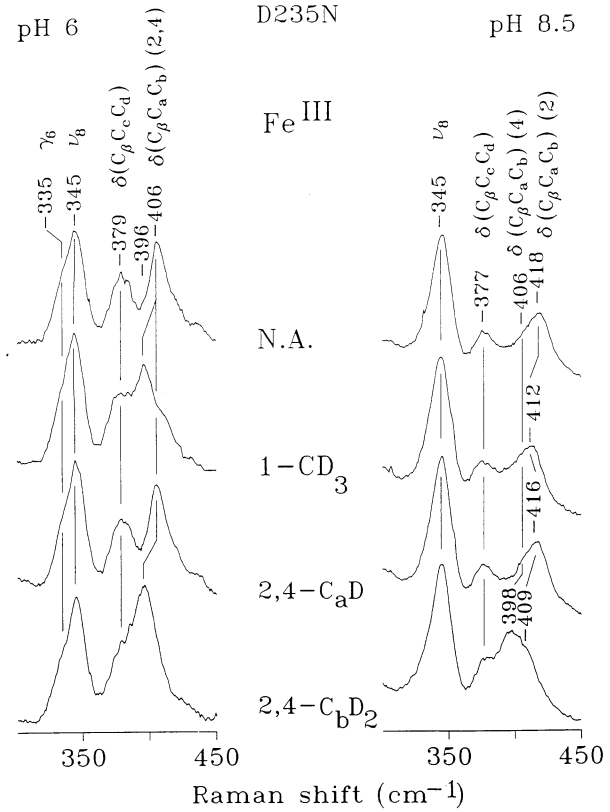

Figure 5. 413.1-nm-excited RR spectra of Fe(III) D235N mutant in acid and alkaline form, showing the effects of deuteration at the 1-methyl and the 2- and 4-vinyl substituents on the bands assigned to δ $C_\beta C{=}C$ bending. Conditions are as given in Figure 4.

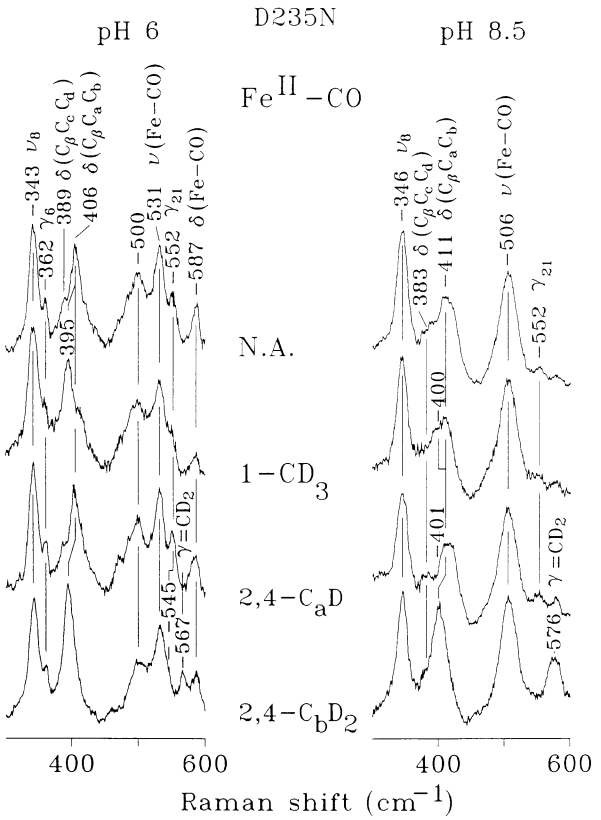

Figure 6. As in Figure 5, but the CO adduct of the Fe(II) form.

broadening of the vinyl band, as has previously been reported.[20] The effect is particularly clear for the Fe(III) form of the D235N mutant (Fig. 5), where it can be seen that there are two components at pH 8.5, a dominant one at 418 cm^{-1}, and a shoulder at 406 cm^{-1}. In the 1-CD_3 isotopomer, the 418-cm^{-1} component shifts to 412-cm^{-1}, while the 406-cm^{-1} shoulder remains unshifted. Thus, it is the 2-vinyl bending mode that shifts up from 406 cm^{-1} at pH 6 to 418 cm^{-1} at pH 8.5, while the 4-vinyl bending mode remains at 406 cm^{-1}. The 2-vinyl bend has greater RR enhancement at both pH values. Interestingly, an intensity reversal occurs upon vinyl C_b deuteration, as can be seen in the pH 8.5 spectra (Fig. 5). Both bending modes shift down ca. 10 cm^{-1}, and it is now the lower-frequency 4-vinyl component that is stronger.

In the Fe(II)CO adduct (Fig. 6), the alkaline effect is somewhat different. Both the vinyl bends shift up roughly 5 cm^{-1}, and the 2-vinyl bend shifts down 11 cm^{-1} in the 1-CD_3 isotopomer. The pH-induced shifts in FeCO vibrational bands (Figs. 6 and 7) have been discussed in detail elsewhere.[10]

Three out-of-plane modes, γ_6, γ_7, and γ_{21}, are identified via their meso-deuteration shifts and their frequency correspondences with NiOEP[27] and cytochrome c.[28] The γ_7 mode is observed at 288 cm^{-1}; it overlaps the ν_9 in-plane mode, but is shifted ca. 20 cm^{-1} upon meso-deuteration (Fig. 4). γ_{21} is observed at 552 cm^{-1}, and shows a ca. 12-cm^{-1} meso-deuteration shift (Fig. 7). [This mode is also sensitive to vinyl C_b deuteration (Fig. 6), possibly because of a near-resonant interaction with a vinyl $\gamma=CD_2$ mode, which is assigned to the additional band appearing at 567 cm^{-1}]. γ_6, which has a ca. 15-cm^{-1} meso-deuteration shift, is near 350 cm^{-1}, but its frequency depends on the oxidation and ligation state. It is at 324 cm^{-1} in the Fe(II) form [for D235N as well as MKT (Figs. 2 and 4)], but at 336 cm^{-1} for the Fe(III) form (Fig. 4), and at 362 cm^{-1} for the Fe(II)CO adduct (Fig. 6). Significantly, γ_6 vanishes when the pH is raised from 6 to 8.5, for the Fe(III) form (Fig. 5) and for the Fe(II)CO adduct (Fig. 6).

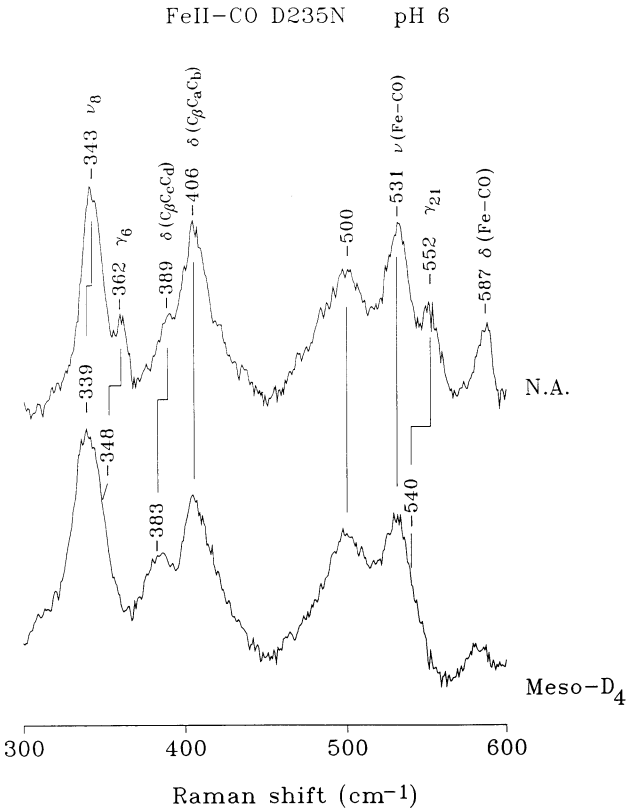

Figure 7. 413.1-nm-excited RR spectra of the CO adduct of the Fe(II) form of the D235N mutant, showing the effects of meso-d_4 substitution on substituted bending and out-of-plane modes. Conditions are as given in Figure 4.

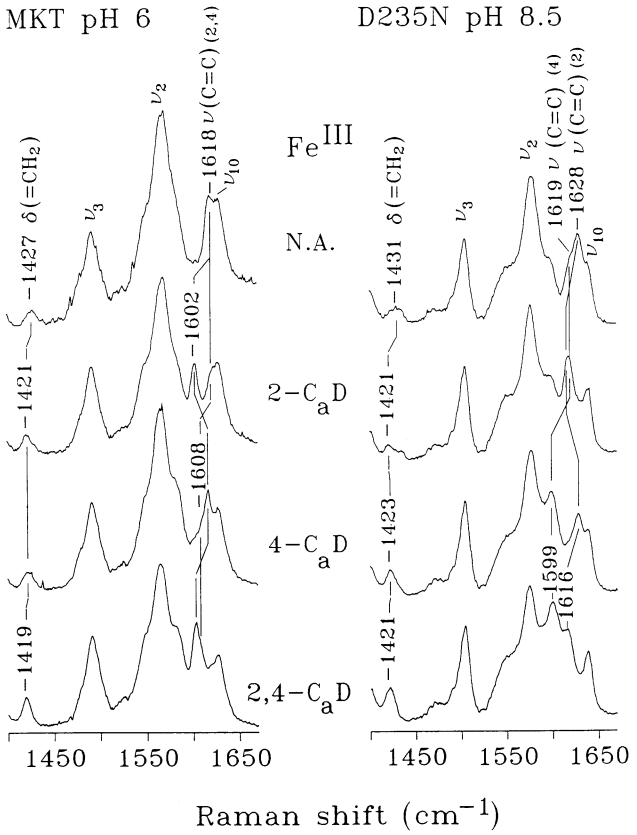

Figure 8. 413.1-nm-excited RR spectra of acid and alkaline Fe(III) forms, showing effects of selective vinyl C_a deuteration on the vinyl C=C stretches. CCP(MKT) and the D235N mutant show the shifts most clearly in the acid and alkaline forms, respectively. Conditions are as given in Figure 4.

Vinyl C=C Stretching Modes

Selective vinyl deuteration is also useful in assigning the vinyl C=C stretching modes, near 1620 cm^{-1}. As seen in Figure 8, the 1618-cm^{-1} band of the Fe(III) form at pH 6 splits into two bands if either the 2- or the 4-vinyl group is deuterated at the C_a atom. However, the deuteration shifts are not the same. Thus, the C=C stretches of both vinyl groups fall at 1618 cm^{-1}, but the 2-vinyl mode, which is the stronger component, shifts 16 cm^{-1} upon C_a deuteration, while the 4-vinyl mode shifts only 10 cm^{-1}. A similar pattern is shown by myoglobin[29]: the two C=C stretches coincide at 1620 cm^{-1}, but shift differentially upon C_a deuteration. In myoglobin, however, the sensitivities are reversed, with 9- and 15-cm^{-1} C_a deuteration shifts of the 2- and 4-vinyl modes, respectively.

At high pH, the C=C modes of CCP separate in frequency, giving rise to a doublet band, as has previously been reported.[20] This effect and the associated isotopic shifts are seen most clearly for the D235N mutant (Fig. 8), but the pattern is consistent with the data for CCP(MKT) as well. The selective deuteration shifts allow us to assign the higher-frequency component, at 1628 cm^{-1}, to the 2-vinyl mode, and the lower component, at 1619 cm^{-1}, to the 4-vinyl mode. Thus, the alkaline transition specifically shifts the 2-vinyl mode up 10 cm^{-1}. There is also a change in mode composition, since the C_a deuteration shift is lowered to 12 cm^{-1} at pH 8.5, while the 4-vinyl shift is increased to 20 cm^{-1}. Other bands in Figure 8 are associated with porphyrin skeletal modes, and with the vinyl=CH_2 scissors mode at ca. 1430 cm^{-1}. This modes shifts down ca. 10 cm^{-1} upon C_a deuteration.

The same pattern is seen for the Fe(II) form (Fig. 9). At pH 6 the vinyl C=C modes again coincide, and shift as expected upon selective deuteration, while at pH 8.5 the two vinyl modes again show a 10-cm^{-1} separation, with the 2-vinyl mode

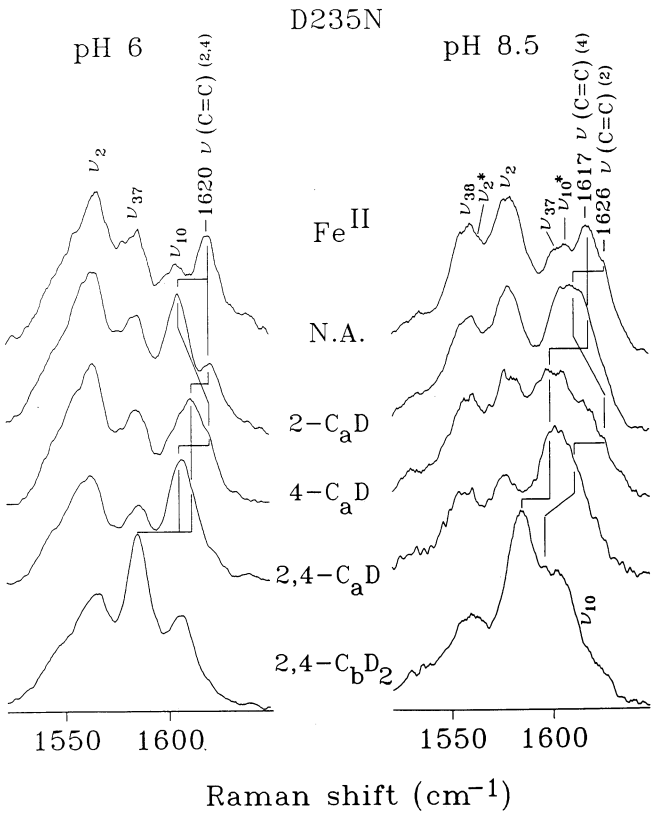

Figure 9. As in Figure 8, but for the Fe(II) form of the D235N mutant, obtained with 441.6 nm excitation. *Photolyzed 5-coordinate high-spin species induced by the laser beam. The intensities of these bands increase with increasing laser power.

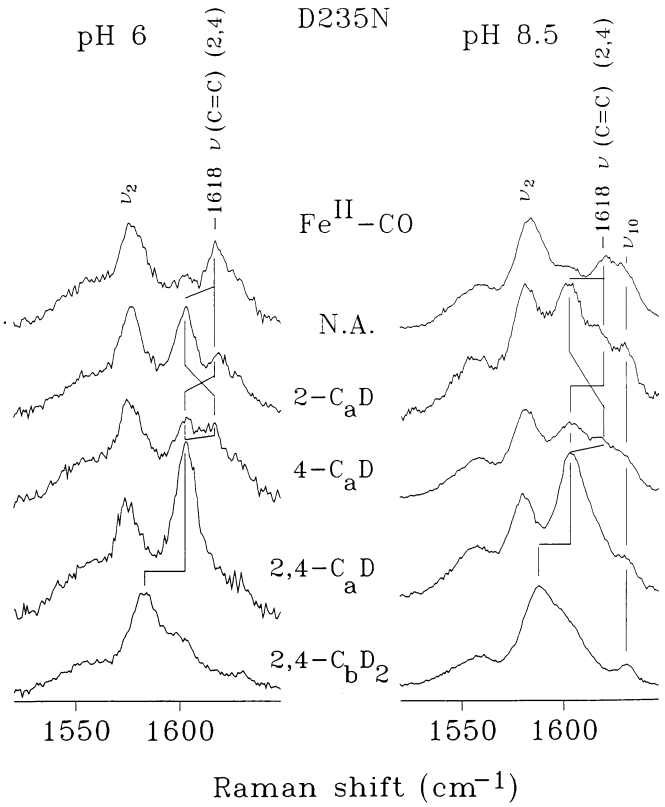

Figure 10. As in Figure 8, but for the Fe(II) CO adduct of the D235N mutant.

lying higher. For the Fe(II)CO adduct, however, the two modes do not separate at pH 8.5 (Fig. 10). Just as at pH 6, they coincide at 1618 cm^{-1}, and separately show selective deuteration shifts of the same amount. Upon deuteration at the C_b atom of both vinyl groups, the C=C stretches shift down further but remain coincident.

DISCUSSION

The heme isotope labeling of CCP(MKT) and its D235N mutant provides new insight into two issues of especial interest: 1) the nature of the proximal Fe-imidazole bond, and 2) the character of the protein conformational change in the acid–alkaline transition.

Fe–Histidine Bonding

Peroxidases all show elevated frequencies for the Fe(II)-His stretching vibration, as originally established for HRP by Kitagawa and co-workers,[30] who used ^{54}Fe substitution to assign this mode to an RR band at ca. 240 cm^{-1}. In myoglobin, the band is found at 220 cm^{-1}. This difference is attributable to the altered state of the proximal imidazole H-bond from the $N_\delta H$ to a protein acceptor group. In myoglobin, the acceptor is a backbone carbonyl group,[30] whereas in CCP it is the carboxylate side chain of Asp235.[4] This anionic acceptor forms a much stronger H-bond and increases the imidazolate character of the proximal ligand, thereby increasing the strength, and the stretching frequency, of the Fe(II)-His bond. The influence of the His175–Asp235 H-bond is dramatically illustrated by the altered RR spectrum of the D235N mutant (Fig. 2). Replacement of the Asp235 residue with Asn lowers the Fe-His frequency to 205 cm^{-1} and leads to coupling with a nearby porphyrin mode, γ_{23}, which shares intensity and ^{54}Fe sensitivity with the Fe-His stretch. In model systems, Fe(II)-imidazole frequencies are ca. 200 cm^{-1} when there is no opportunity for imidazole H-bonding.[31]

However, the Fe-His band is notably broad in native CCP, and separate components can be resolved, at 233 and 246 cm^{-1}. The latter frequency is as high as the Fe(II)-imidazolate frequency observed in a model system when the bound imidazole is fully deprotonated.[31] The two components have been suggested[9] to arise from tautomerism of the imidazole N_δ proton, with respect to the donor and acceptor atoms in the His175–Asp235 H-bond. In this view, the 233-cm^{-1} component arises from molecules in which the proton remains on the imidazole, while the 246-cm^{-1} component arises from molecules in which the proton is transferred to the carboxylate. The two structures are nearly equal in energy, and in fact, various heme pocket mutations produce variations in the frequencies and relative intensities of the two band components, consistent with slight perturbations of the H-bond and of the population of tautomers.[9]

This assignment of the two components is fully supported by the present observation that ^{54}Fe substitution shifts both frequencies by the full $\sim$ 3 cm^{-1} expected for an Fe-His oscillator, implying that the components arise from Fe-His stretches in separate molecules. The two components cannot be attributed to vibrational coupling of modes within a single molecule, because the isotope shift would then be shared, as is observed for the Fe-His and γ_{23} bands in the D235N mutant.

The apparent absence of a D_2O effect on the Fe-His modes is probably an artifact of band overlap. Recent experiments have revealed a D_2O effect when W191 mutants of CCP are examined.[32] The

Fe-His modes are better separated in the W191Q and W191G mutants, giving rise to bands at 233 and 250 cm^{-1}. The 233-cm^{-1} band shifts down by 4 cm^{-1} in D_2O, while the 250-cm^{-1} band remains unshifted. This behavior is consistent with the mode assignment: the Fe-His stretch shifts upon D_2O substitution when the proton is on the imidazole (233 in cm^{-1}) but not when it is on the aspartic acid (250 cm^{-1}). Thus, we would not expect the 246-cm^{-1} component of the CCP(MKT) Fe-His band to show D_2O sensitivity, and the expected shift of the 233-cm^{-1} component is masked by its overlap with the 246-cm^{-1} component, and also with a 220-cm^{-1} porphyrin band. The D_2O experiment did reveal that the nearby 269-cm^{-1} band is sensitive to H/D exchange, suggesting assignment to an internal histidine mode. Its intensification derives from coupling to the Fe-His stretch, as revealed by its disappearance when the latter is shifted out of the region in the D235N mutant.

Acid–Alkaline Conformation Change

CCP undergoes a well-defined acid-alkaline transition. The pK_a is between 7 and 9, depending on redox and ligation state, and on heme pocket mutations.[21] Two protons are lost in the transition, one of them from His181, which forms an H-bond to one of the heme propionate groups in the acid form. The other deprotonation site is presently unknown; it is not the distal His52, which remains unprotonated to much lower pH, and whose substitution by leucine does not alter the proton count in the acid–alkaline transition.[21] Replacement of His181 by glycine lowers the transition pK_a significantly. Because peroxide reduction is abolished in the alkaline form,[33] and because His181 is at the protein surface where cytochrome c is thought to dock,[34,35] it has been suggested[21] that the acid–alkaline transition might provide a regulatory mechanism for coupling peroxide reduction to cytochrome c oxidation.

Because no crystal structure is available for the alkaline form, the nature of the transition is uncertain, but spectroscopic evidence points to a pronounced structural change. Both Fe(III) and Fe(II) forms are 5-coordinate and high-spin in the acid form but 6-coordinate and low-spin in the alkaline form. The sixth ligand has been determined via spectroscopic indicators and mutagenesis to be the His52 residue.[21] Since the His52 imidazole is ca. 5 Å away from the Fe atom in the crystal structure of the acid form, its binding in the alkaline form requires a substantial change in protein conformation. This change generates tension in the protein, as evidenced by the unusual photolability of the His52-Fe bond in the alkaline Fe(II) form of the protein.[11]

However, the acid–alkaline transition induces a protein conformation change even in the absence of His52 binding, as evidenced by its persistence when CO is bound to the Fe(II) form of CCP, or when the His52 residue is replaced by leucine.[11] The RR indications for protein conformation change are the vinyl C=C stretching and $C_\beta C{=}C$ bending bands. In the previous study,[11] these bands were found to broaden and shift up slightly in the alkaline form, suggesting that the contributions from the individual vinyl substituents separate in frequency. The present results allow us to confirm this suggestion, and establish that the 2-vinyl group is specifically perturbed. The two C=C stretches and $C_\beta C{=}C$ bends are coincident, at 1618 and 406 cm^{-1}, respectively, in the acid form, but the 2-vinyl frequencies increase, to 1628 and 418 cm^{-1}, in the alkaline form, while the 4-vinyl frequencies are unshifted. Thus, a conformation change is indicated which specifically perturbs the heme 2-vinyl group.

The effect is attenuated, however, in the Fe(II)CO form of the enzyme. In the alkaline form of the Fe(II)CO adduct, the 2-vinyl $C_\beta C{=}C$ bending frequency shifts only to 411 cm^{-1}, while the C=C frequency is unaltered. Thus, the protein influence on the 2-vinyl group is diminished when His52 binding is blocked by CO. At the same time, the C—O and Fe—CO stretching frequencies reflect other consequences of the protein conformation change: both the proximal His175–Asp235 H-bond, and the distal H-bond from the Arg48 residue, via a water molecule, to the bound CO, are apparently eliminated in the alkaline form.[10] Thus, the blocking of His52 by CO alters the consequences of the protein conformation change on the heme. At the same time, the loss of intensity for porphyrin out-of-plane modes, especially γ_6, indicates that distortions of the porphyrin ring, which are evident in the acid-form crystal structures,[4] become relaxed in the alkaline form. This effect is seen whether or not CO is bound.

What is the nature of the protein perturbation of the 2-vinyl group? One possibility is rotation of the vinyl group with respect to the porphyrin plane. The $C_\beta C_\beta C{=}C$ dihedral angle is expected to influence both electronic and kinematic coupling between the vinyl and porphyrin bonds. Arguments for an orientational effect have been advanced by Kalsbeck et al.,[36] who found two C=C RR bands,

at 1620 and 1631 cm^{-1} in solutions containing hemes having one or two vinyl substituents. The relative intensities were temperature dependent, indicating contributions from different conformers. Density functional calculations on vinyl-substituted models gave two low-energy conformers with predicted C=C frequencies differing by 10–20 cm^{-1}. Both CCP in its acid form and Met-myoglobin have coincident 2- and 4-vinyl C=C stretches, at essentially the same frequency (1618 and 1621 cm^{-1}, respectively), and the vinyl $C_\beta C_\beta C{=}C$ dihedral angles, are essentially the same, 30–37°,[37] and are well away from being coplanar with the porphyrin ring, so that the coupling is minimized. The 10-cm^{-1} increase in the 2-vinyl frequency in alkaline form of CCP might then be attributed to a rotation of the 2-vinyl group into the porphyrin plane, where the coupling should be maximal. A similar explanation might apply to other reported instances of a second vinyl C=C stretch, ca. 10 cm^{-1} higher than the usual 1620 cm^{-1} frequency, in protoheme-containing proteins: insect hemoglobin,[38] horseradish peroxidase,[39,40] cytochromes $P450_{LM2}$, and $P450_{SCC}$,[41,42] and in homodimeric myoglobin from *Nassa* mutabilis[43]; in none of these cases is a crystal structure available. However, Kalsbeck et al.[36] calculated that the in-plane conformation of their vinyl-substituted model has a lower C=C frequency than the out-of-plane conformation, opposite to what the protein data suggest.

Alternatively, the differential responses of the vinyl modes might reflect out-of-plane distortions of the porphyrin, which affect the pyrrole rings differentially and modify the vinyl couplings to the porphyrin modes. This explanation is suggested by the vinyl C=C deuteration shifts, which are different for the 2- and 4-vinyl groups of both CCP and metMb; thus, the couplings of the two substituents are not the same, despite the frequencies being coincident. Interestingly MetMb has two well-separated $C_\beta C{=}C$ bending bands, at 440 and 409 cm^{-1} for the 2- and 4-vinyl groups, whereas in CCP both frequencies are 406 cm^{-1} in the acid form, and are at 418 and 406 cm^{-1} in the alkaline form. Differential couplings are likely to be more pronounced for $C_\beta C{=}C$ bending than for C=C stretching because of stronger kinematic interactions with low-frequency in- and out-of-plane porphyrin skeletal modes. Thus, alterations in the porphyrin geometry, which are indicated by the loss of the γ_6 band, might give rise to the observed vinyl mode alterations in the alkaline form of CCP.

CONCLUSIONS

The ^{54}Fe-labeling experiment allows us to conclude that the complex line shape of the Fe-His stretching RR band in CCP, and by extension in other peroxidases, is due to overlapping bands from separate molecules. These are proposed to reflect tautomerism in the His175–Asp235 H-bond. At the same time, the 205/182-cm^{-1} doublet in the D235N mutant is suggested to arise from vibrational coupling between the Fe-His mode, its frequency lowered by the loss of the proximal H-bond, and a nearby porphyrin mode, which is tentatively identified as a pyrrole tilting mode, γ_{23}.

The deuterium labeling of the vinyl groups establishes that the C=C stretching and $C_\beta C_a{=}C_b$ bending frequencies are coincident for the 2- and 4-vinyl groups in the acid form of CCP, but that the 2-vinyl frequencies are raised in the alkaline form. This experiment establishes that the protein conformation change in the acid–alkaline transition specifically perturbs the 2-vinyl group.

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