Influence of Conserved Amino Acids on the Structure and Environment of the Heme of Cytochrome c_2. A Resonance Raman Study

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ABSTRACT: Resonance Raman spectra using Soret excitations of oxidized and reduced *Rhodobacter capsulatus* cytochrome c_2 at pH 7.5 were studied. The spectra of oxidized cytochrome c_2 show three components for the ν_{10} mode at 1638, 1633, and 1629 cm^{-1}. The intensities of these components are sensitive to the excitation wavelength. This effect is explained in the context of a conformational equilibrium of the ferriheme between a nearly planar structure and two ruffled structures. In the case of reduced cytochrome c_2, the absolute frequencies as well as the excitation-dependent frequency dispersion of the ν_{10} mode (1618−1621 cm^{-1}) indicate a displacement of the conformational equilibrium of heme toward the more planar structures. To measure the influence of some key amino acid residues on the heme−protein interaction of cytochrome c_2, four site-directed mutants of *Rb. capsulatus* cytochrome c_2 have been studied by resonance Raman spectroscopy and their spectra compared with the spectra obtained for the wild type cytochrome. The mutants studied are K14E/K32E, P35A, W67Y, and Y75F. The spectral changes induced by the mutations are interpreted in terms of alterations in the structure and/or environment of the cytochrome c_2 heme in the framework of the expected role of the different amino acid residues in the stability and redox potential.

The cytochromes c_2 (cyt[1] c_2) constitute a subclass of class I cytochromes *c* (cyt *c*) and function as soluble electron carriers in both respiration and photosynthetic systems (Bartsch, 1978; Meyer & Kamen, 1982; Moore & Pettigrew, 1990). In spite of structural similarities, cyt c_2 generally exhibit higher and more diverse redox potentials (+250−470 mV) than the mitochondrial cyt *c* (+265−275 mV) (Salemme et al., 1973a,b; Takano & Dickerson, 1981a,b; Bushnell et al., 1990; Benning et al., 1991). A number of residues near to or in contact with the heme of class I cytochromes *c* have been preserved during evolution (Moore & Pettigrew, 1990). The conservation of these residues suggests that they play an important role in the stability and/or the function of these hemoproteins.

The combination of high-resolution crystallography, resonance Raman (RR) spectroscopy, and site-directed mutagenesis has been particularly fruitful in the determination of structure−function relationships in various heme proteins (Morikis et al., 1989; Adachi et al., 1993; Smulevich, 1993; Shiro et al., 1994; Mitchell et al., 1996). A similar approach has been used to investigate the correlation between heme−protein interactions and cyt c_2 stability and function. The X-ray crystallographic structure of wild type *Rhodobacter capsulatus* cyt c_2 has been solved at a 2.5 Å resolution (Benning et al., 1991). The effects of mutations of some conserved amino acid residues on the stability and functional properties of this cyt c_2 have been characterized (Cusanovich & Caffrey, 1991; Caffrey & Cusanovich, 1991a,b, 1993; Caffrey et al., 1991, 1992). RR spectroscopy is a powerful tool in determining changes in heme structure and in interactions of the iron−porphyrin, its axial ligands, and its peripheral substituents with the surrounding protein residues or the solvent (Spiro, 1985; Kitagawa & Ozaki, 1987; Spiro & Li, 1988; Smulevich, 1993; Desbois, 1994). Therefore, this technique offers the opportunity to measure the alterations in heme structure and environment induced by the mutations of some of the highly conserved amino acids of *Rb. capsulatus* cyt c_2. Previous RR studies have investigated the sensitivities and assignments of most RR modes of the *c* type heme (Hu et al., 1993; Othman et al., 1993, 1994; Othman, 1994; Desbois, 1994). This vibrational information was used to interpret the RR data obtained for cyt c_2 and its mutants, in terms of the structure and environment of heme.

In this study, we report the RR spectra of wild type (wt) cyt c_2 and four of its mutants, in the oxidized and reduced states, at pH 7.5. The mutation sites studied include residues of the protein surface as well as those of buried positions in the vicinity of the heme (Figure 1). The double substitution of lysines 14 and 32 to glutamates (K14E/K32E) changes the net charge of the protein by −4 and strongly affects the redox potential of heme (Caffrey & Cusanovich, 1991a,b; Cusanovich & Caffrey, 1991; Caffrey et al., 1992). This double mutation, where sites are located at the cytochrome surface, was studied to determine if this net change in protein charge is able to affect the heme structure. Proline 35 (Figure 1) is an amino acid residue which is conserved in all the known sequences of bacterial cyt c_2 and mitochondrial cyt *c* (Moore & Pettigrew, 1990). The imine cycle of Pro is expected to play a role in determining the folding of the

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[1] Abbreviations: cyt, cytochrome; wt, wild type; MP8, microperoxidase-8; RR, resonance Raman; HP, heme propionate; HS, high-spin; LS, low-spin; 6c, six-coordinated; 5c, five-coordinated.

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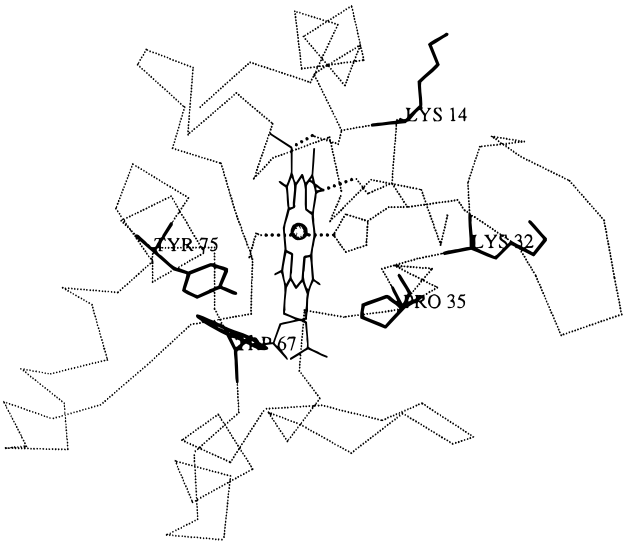

FIGURE 1: Heme binding pocket of cytochrome c_2 from *Rb. capsulatus* [from Benning et al. (1991)].

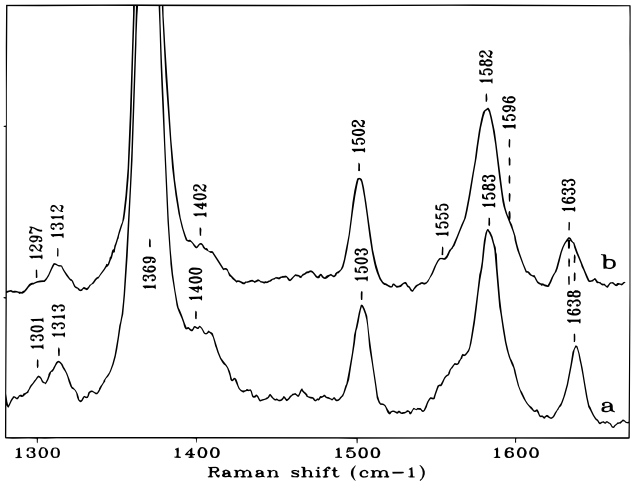

FIGURE 2: High-frequency regions (1300−1650 cm^{-1}) of resonance Raman spectra of wild type *Rb. capsulatus* cytochrome c_2 in its oxidized form at pH 7.5: (a) 406.7 nm excitation and (b) 413.1 nm excitation. Summation of six scans.

protein backbone and, in particular, in orienting the proline carbonyl group to form an H bond with the proximal histidine (Moore & Pettigrew, 1990). Tryptophan 67 is highly conserved in the cyt c_2 family and generally found among the class I *c* type cytochromes (Meyer & Kamen, 1982; Moore & Pettigrew, 1990). The crystal structures of a number class I *c* type cytochromes establish that the carboxylate group of one of the heme propionates is hydrogen bonded with the N_1 proton of the conserved Trp. Finally, the structural and electronic effects of the mutation of tyrosine 75 on the heme were investigated. This residue in *Rb. capsulatus* cyt c_2, or its equivalent in other *c* type cytochromes, is positioned adjacent to the Met ligand of the heme and is involved in a conserved H bond network (Figure 1) (Salemme et al., 1973; Takano & Dickerson, 1981a,b; Benning et al., 1991). Tyrosine 75 is believed to play an important role in maintaining both the redox potential and the stability of cytochromes *c*. It has been proposed that the oxidized state is partially stabilized by an electrostatic interaction between the Tyr hydroxyl group and the sulfur atom of the heme ligating Met (Salemme et al., 1973a,b). The stability and redox properties of the cytochromes c_2 mutants studied here have been extensively characterized (Caffrey & Cusanovich, 1991a,b, 1993; Cusanovich & Caffrey, 1991; Caffrey et al., 1991; Gooley et al., 1991) and provide a basis for analyzing the RR spectra described here.

EXPERIMENTAL PROCEDURES

Site-directed mutagenesis and genetic manipulations to produce mutant cyt c_2 were previously described (Caffrey et al., 1991; Gooley et al., 1991). The protein purifications were performed according to Bartsch (1971). The proteins were dissolved in 100 mM MOPS and 200 mM NaCl (pH 7.5) to give final concentrations of 25−100 μM. The fully oxidized forms were obtained by titration with potassium ferricyanide. The reduced samples were prepared under vacuum by addition of sodium dithionite (Othman et al., 1993, 1994).

The RR spectra were excited with the 406.7 and 413.1 nm lines of a Kr^+ laser (Coherent Innova) and the 441.6 nm line of a He/Cd laser (Liconix). They were recorded at 20 (± 1) °C using a Jobin-Yvon spectrometer (HG2S-UV). Radiant powers of 5−20 mW were used to avoid photoreduction of oxidized samples. Improvement of the signal-to-noise ratios of RR spectra was achieved by summations of two to six scans. For each excitation, the Raman spectrometer was calibrated with a 1:1 (v/v) mixture of benzene and dichloromethane. The RR spectra were analyzed with Spectra Calc software (Galactic Industries). Under these conditions, the frequency precision is 0.5−2 cm^{-1} depending on both the band intensity and the signal-to-noise ratio.

RESULTS

Wild Type Ferricytochrome c_2

The high-frequency regions (1300−1650 cm^{-1}) of RR spectra of wild type ferricyt c_2 excited at 406.7 and 413.1 nm are displayed in Figure 2. They show a clear dependence on the exciting wavelength used. The ν_2 (1582−1583 cm^{-1}), ν_3 (1502−1503 cm^{-1}), and ν_4 (1369 cm^{-1}) modes exhibit the same frequency to within 1 cm^{-1}, but the apparent frequency as well as the shape of the band corresponding to the ν_{10} mode varies when the excitation is changed (Figure 2). A strong band is observed at 1638 cm^{-1} with the 406.8 nm excitation, while a broader and relatively less intense band is seen at 1633 cm^{-1} with the 413.1 nm excitation (Figure 2). A close examination of the ν_{10} regions of RR spectra of wt cyt c_2 shows more precisely the spectral changes (Figure 3). In fact, the apparent excitation dependence of the ν_{10} frequency corresponds to a change in relative intensity of two main bands observed at 1633−1634 and 1637−1638 cm^{-1} (spectra a in Figure 3A,B). A third weaker ν_{10} component is observed at 1628−1629 cm^{-1} (Figure 3A,B). Other wavelength-dependent changes are seen in the 1550−1570 and 1300 cm^{-1} regions of the RR spectra (Figure 2). On one hand, a 1301 cm^{-1} band is downshifted to 1297 cm^{-1} when the excitation is red-shifted. On the other hand, the overall shape of the regions containing the ν_{11} and ν_{37} modes (1550−1570 cm^{-1}) appears to be modified, but both the weakness and the large overlapping of the corresponding bands do not allow us to determine precisely their frequency and/or intensity changes.

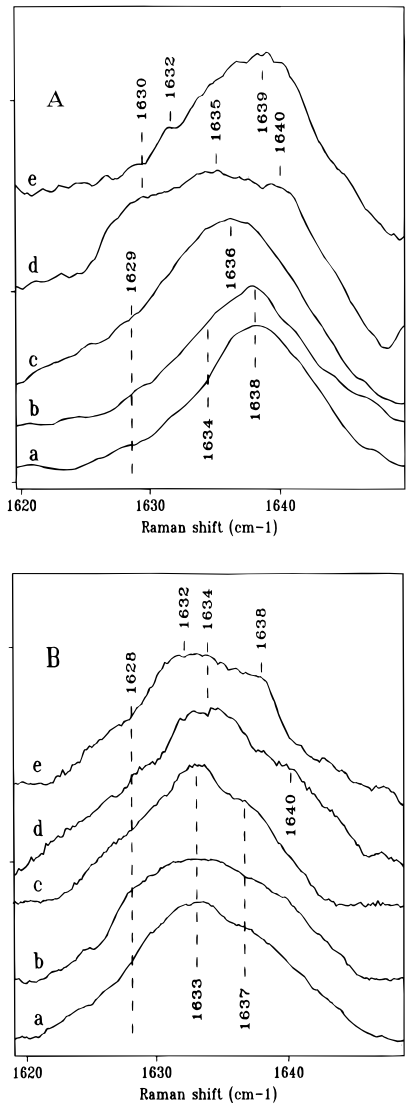

FIGURE 3: ν_{10} regions (1620−1650 cm^{-1}) of resonance Raman spectra of wild type oxidized cytochrome c_2 (a), K14E/K32E (b), P35A (c), W67Y (d), and Y75F (e) at pH 7.5: excitation at 406.7 (A) and 413.1 (B) nm. Summations of four to six scans.

In the low-frequency regions of RR spectra, a broad band detected at 445 cm^{-1} with the 413.1 nm excitation is apparently upshifted to 448 cm^{-1} with the 406.7 nm excitation (Figure 4A,B, spectra a).

Wild Type Ferrocytochrome c_2

In the high-frequency regions of RR spectra of reduced wt cyt c_2, the frequency of the ν_{10} mode is again sensitive to the exciting wavelength (Figure 5). However, the apparent frequency variation is weaker in the reduced state (3 cm^{-1}) than in the oxidized state (9 cm^{-1}). The maximum of the ν_{10} band of reduced cyt c_2 is observed at 1621, 1618, and 1621 cm^{-1} with the 406.7, 413.1, and 441.6 nm excitation, respectively (Figure 5 and spectra not shown). Given this small frequency variation, we cannot easily characterize the absolute frequency of each ν_{10} component.

Among the other high-frequency bands, a downshift of the ν_{11} mode from 1547 to 1544 cm^{-1} parallels the downshift of ν_{10} when the exciting wavelength is blue-shifted from 441.6 to 413.1 nm (Figures 5 and 6A,B). The frequency of the δ(CH) mode of the C_β(pyrrole)−CH(CH_3)−S(Cys)

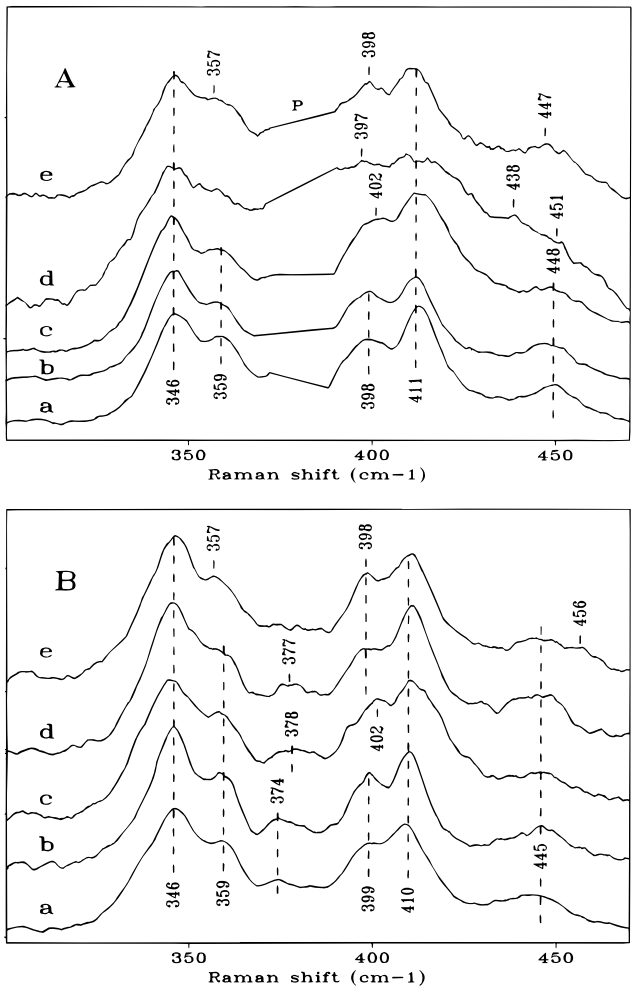

FIGURE 4: Low-frequency regions (300−470 cm^{-1}) of resonance Raman spectra of oxidized cytochrome c_2 (a), K14E/K32E (b), P35A (c), W67Y (d), and Y75F (e) at pH 7.5: excitation at 406.7 (A) and 413.1 (B) nm. Summations of four to six scans. P in panel A indicates a laser plasma line which has been removed.

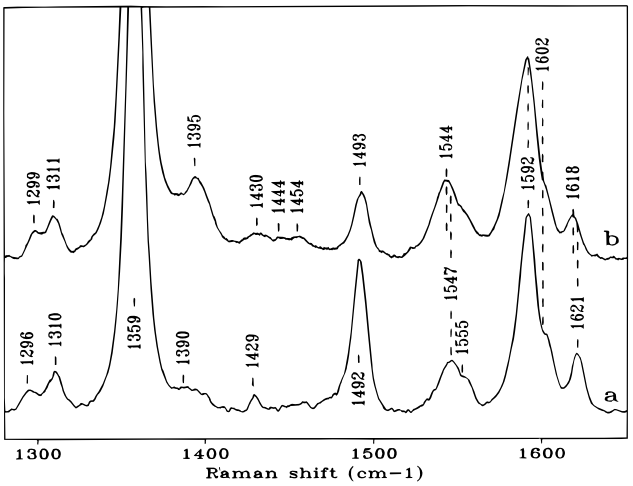

FIGURE 5: High-frequency regions (1300−1650 cm^{-1}) of resonance Raman spectra of wild type *Rb. capsulatus* cytochrome c_2 in its reduced form at pH 7.5: (a) 441.6 nm excitation and (b) 413.1 nm excitation. Summations of six scans.

bridges (1299 cm^{-1}) is apparently decreased to 1296 cm^{-1} upon the same shift in excitation (Figure 5).

In the low-frequency RR spectra, the relative intensities of two ν_{51} modes (302 and 307 cm^{-1}) and of two deformation modes of the thioether bridges (393 and 400 cm^{-1}) are

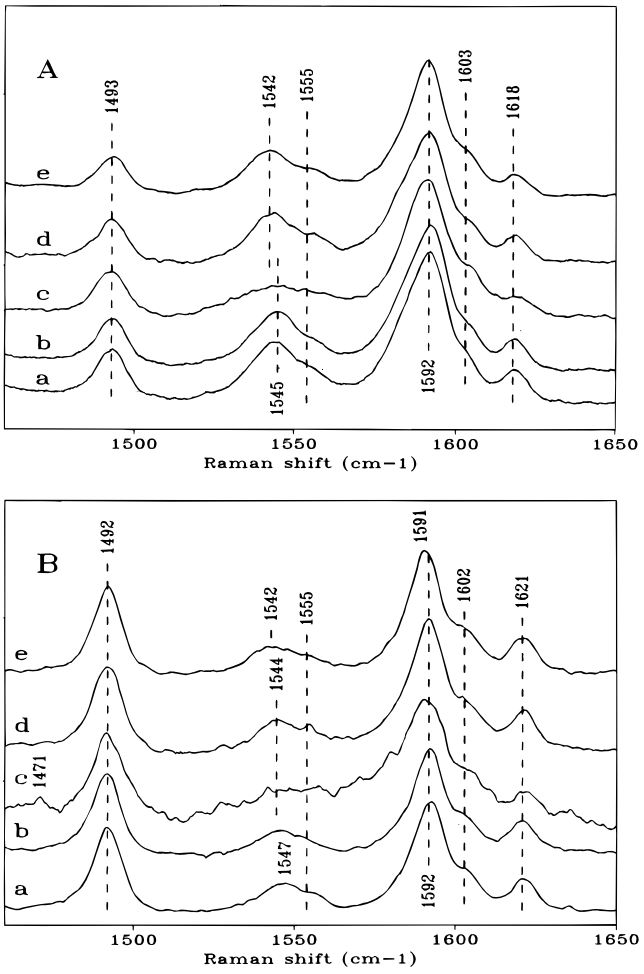

FIGURE 6: High-frequency regions (1470−1650 cm^{-1}) of resonance Raman spectra of wild type reduced cytochrome c_2 (a), K14E/K32E (b), P35A (c), W67Y (d), and Y75F (e): excitation at 413.1 (A) and 441.6 (B) nm. Summations of four to six scans.

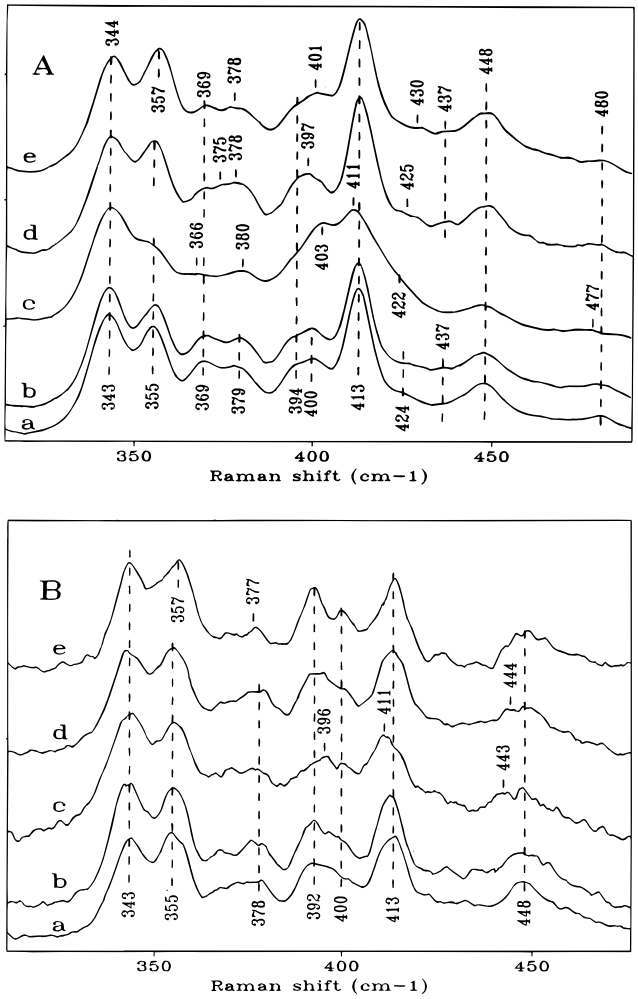

FIGURE 7: Low-frequency regions (310−490 cm^{-1}) of resonance Raman spectra of wild type reduced cytochrome c_2 (a), K14E/K32E (b), P35A (c), W67Y (d), and Y75F (e): excitation at 413.1 (A) and 441.6 (B) nm. Summations of six to eight scans.

sensitive to the excitation conditions (Figure 7A and spectra not shown).

K14E/K32E Double Mutant

The RR spectra of both redox states of the K14E/K32E mutant are presented in Figures 3, 4, 6, and 7. In comparison with the corresponding RR spectra of wt cyt c_2, they show very small differences for the oxidized state and no significant difference for the reduced form. In particular, a band shape analysis of the ν_{10} mode of the oxidized state shows slight increases in the relative contributions of two components at 1629 and 1639 cm^{-1} (Figure 3B).

P35A Mutant

Oxidized Form. In comparison with the RR spectra of wt cyt c_2(III), the ν_{10} region of RR spectra of the P35A mutant shows a change in shape (Figure 3A,B). In fact, this spectral modification corresponds to an increased contribution of the 1633−1634 cm^{-1} component relative to that of the 1638 cm^{-1} component. In the low-frequency regions, the most significant changes concern the broad bands observed at 374 and 399 cm^{-1} for wt cyt c_2(III) which are upshifted to 378 and 402 cm^{-1}, respectively, in the spectra of oxidized P35A (Figure 4A,B).

Reduced Form. The RR spectra of reduced P35A show important differences when compared to those of wt cyt c_2(II). In the high-frequency regions, the band corresponding to the ν_{11} mode peaks at 1545 cm^{-1} but loses intensity and becomes asymmetric because of a broadening toward its low-frequency side (Figure 6A). As compared to wt cyt c_2, the 406.7 nm-excited spectrum exhibits two clear ν_{11} components at 1547 and 1533 cm^{-1} (spectrum not shown). To a lesser extent, a band broadening is also detected for the ν_3 and ν_{10} modes. The band corresponding to the latter mode is upshifted by 2 cm^{-1} (Figure 6A,B). The 441.6 nm-excited spectrum shows a small contribution from a five-coordinated high-spin (5cHS) component since a weak ν_3 mode is observed at 1471 cm^{-1} (Figure 6B).

In the low-frequency RR spectra of reduced P35A, the relative intensity, the width, and/or the frequency of several bands are modified. The cluster formed in the ν_9 region by three bands at 250, 263, and 271 cm^{-1} is altered so that two broad bands are observed at 254 and 267 cm^{-1}. The two components of the ν_{51} mode of wt cyt c_2 (302 and 307 cm^{-1}) are shifted to 308 and 317 cm^{-1}, respectively, in the spectra of P35A (spectra not shown). The two $C_\beta CS$ deformation modes observed at 393 and 400 cm^{-1} in the spectra of wt cyt c_2 are broadened and shifted to 396 and 403 cm^{-1}, respectively, in P35A (Figure 7A,B). The splitting of the propionate deformation modes at 369 and 379 cm^{-1} is increased to 366 and 380 cm^{-1}, respectively. In the middle-frequency regions, bands observed at 693, 970, 1020, 1040,

1090, and 1216 cm^{-1} in the RR spectra of wt cyt c_2(II) are shifted to 692, 974, 1026, 1044, 1092, and 1218 cm^{-1}, respectively, in the spectra of reduced P35A (spectra not shown).

W67Y Mutant

Oxidized Form. With respect to the cluster of ν_{10} modes observed in the RR spectra of oxidized wt cyt c_2, that of oxidized W67Y, excited at either 406.7 or 413.1 nm, shows a broadening corresponding to a frequency shift of its high-frequency component from 1638 to 1640 cm^{-1} (Figure 3A,B). Moreover, the spectra excited at 406.7 nm exhibit an increased intensity of the 1630 cm^{-1} component (Figure 3B). A very small proportion of ferriheme *c* in a 5cHS coordination is detected since the 406.7 nm-excited spectrum exhibits a weak ν_3 component at 1488 cm^{-1} in addition to the major ν_3 mode at 1504 cm^{-1} (figure not shown).

In the low-frequency regions, a new band is detected at 200 cm^{-1} while the intensity of the 186 cm^{-1} band is decreased. The ν_9 mode of wt cyt c_2(III) (273 cm^{-1}) is downshifted for oxidized W67Y (268 cm^{-1}) (spectra not shown). On the contrary, the propionate deformation mode seen at 374 cm^{-1} in the spectra of wt cyt c_2 is upshifted at 378 cm^{-1} in that of the mutant (Figure 4B).

Reduced Form. The ν_{10} regions of RR spectra of reduced W67Y exhibit no significant difference with respect to those of wt cyt c_2(II), but the ν_{11} mode is systematically downshifted by 2−3 cm^{-1} (Figure 6A,B). Significant spectral changes are also seen in the low-frequency regions. (i) Three bands, observed at 250, 263, and 271 cm^{-1} in the spectra of wt cyt c_2(II), are all shifted in the spectra of the reduced W67Y mutant (245, 260, and 270 cm^{-1}, respectively). (ii) A new component of ν_{51} is found at 313 cm^{-1}. (iii) An additional component of the 369 cm^{-1} band is observed at 375 cm^{-1}. (iv) The 400 cm^{-1} band is downshifted at 397 cm^{-1} (Figure 7A and spectra not shown). In the medium-frequency regions, the frequency of the ν(CC) mode of heme propionate (HP) groups is broadened and upshifted from 970 to 973 cm^{-1} (spectra not shown).

Y75F Mutant

Oxidized Form. In the high-frequency RR spectra of oxidized Y75F, the band shape of the ν_{10} modes is very slightly modified with an apparent downshift of the 1633−1634 cm^{-1} components to 1632 cm^{-1} and a slight increase in the intensity of the 1638−1639 cm^{-1} component (Figure 3A,B). A small contribution of a 5cHS form is detected through the observation of a weak ν_3 mode at 1488 cm^{-1} (spectra not shown). In the low-frequency regions, the Y75 → F mutation downshifts the ν_{50} mode from 359 to 357 cm^{-1} and the ν_9 mode from 272 to 266 cm^{-1} (Figure 4A,B and spectra not shown).

Reduced Form. The ν_{11} mode of reduced Y75F is observed at a lower frequency (1542 cm^{-1}) than that of wt cyt c_2 (1545−1547 cm^{-1}) (Figure 6A,B). For this variant, the frequency of the ν_{11} mode is not clearly excitation-dependent. The low-frequency regions of RR spectra are also affected by the Y75 → F mutation. (i) The ν_9 region (250−280 cm^{-1}) contains two bands only in the 413.1 nm-excited spectra (250 and 268 cm^{-1}). (ii) A new band is seen at 228 cm^{-1} in the 441.6 nm-excited spectra. (iii) The ν_{51} mode has two components shifted at 307 and 311 cm^{-1}. (iv) The ν_8 and ν_{50} modes are shifted by 1 and 2 cm^{-1}, respectively (Figure 7A and spectra not shown). In the middle-frequency regions, the ν(CS) mode (693 cm^{-1} for wt cyt c_2) is downshifted by 1 cm^{-1} while a C_mH deformation mode is upshifted from 825 to 831 cm^{-1} (spectra not shown).

DISCUSSION

Heme Structure in Wild Type Ferricytochrome c_2

The high-frequency RR spectra of wt ferricyt c_2 confirm the six-coordinated low-spin (6cLS) coordination of heme (Kitagawa et al., 1977), but the frequency variation of the ν_{10} mode reveals some flexibility in its structure. A recent RR study on 6cLS ferriheme *c* complexes showed a frequency variation of the ν_{10} mode with the experimental conditions (Othman, 1994; Othman et al., 1994). From structural and RR studies of metalloporphyrin model compounds, a decrease in the ν_{10} frequency was correlated with an increase in out-of-plane distortion of the heme macrocycle (Alden et al., 1989; Anderson et al., 1993; Hobbs et al., 1994). Considering all these data, the 1633 cm^{-1} component of cyt c_2 is assigned to a heme tetrapyrrole exhibiting some ruffling (Othman et al., 1994). On the other hand, the 1638 cm^{-1} component can be associated with a more planar heme *c* structure such as that described in the crystal of wt cyt c_2 (Benning et al., 1991). The presence of a weak ν_{10} component at 1629 cm^{-1} suggests that a small amount of the cyt c_2 heme adopts a strongly distorted conformation, similar to that found for the heme of the imidazolate complex of ferric microperoxidase-8 (MP8) (Othman et al., 1994). The frequency variation of the 445−448 cm^{-1} band (γ_{22}) which was assigned to a pyrrole tilt (Hu et al., 1993) may have the same structural origin as that of ν_{10}, i.e. an equilibrium between different porphyrin conformers.

The crystal structure of cyt c_2 shows one of the most planar hemes of all cytochromes (Benning et al., 1991). For cyt c_2 in solution, the cluster of ν_{10} bands indicates some variation of the heme structure. This heme flexibility is also obvious for the mutants examined in this study (Figure 3). This feature was not detected for mitochondrial cyt *c*. On one hand, their tridimensional structures exhibit a strong heme ruffling (Takano & Dickerson, 1981b; Berghuis & Brayer, 1992). On the other hand, no frequency dispersion has been reported for the ν_{10} mode of ferricyt *c*. All these observations suggest that a large variability in the degree of nonplanarity of heme constitutes a particular property of ferricyt c_2.

The Soret band of oxidized cyt c_2 exhibits a maximum at 412 nm. The ν_{10} mode of the nearly planar form (1638 cm^{-1}) is more intense with the 406.7 nm excitation, i.e. shifted to the blue of the Soret maximum. Thus, it is probable that the nearly planar heme structure in cyt c_2 has a blue-shifted Soret maximum with respect to that of the ruffled form. A similar conclusion was drawn for Ni(II)−porphyrins (Shelnutt et al., 1991).

The apparent shift of the 1297−1300 cm^{-1} band assigned to a CH deformation mode in the thioether bridges of heme *c* (Hu et al., 1993) suggests that the conformational flexibility of the porphyrin is accompanied by small changes in peripheral interactions of heme. Alternatively, different Raman activities of the δ(CH) mode of the two thioether bridges, and thus different excitation profiles, could explain the apparent band shift. In this case, the thioether links would adopt different conformations.

Heme Structure in Wild Type Ferrocytochrome c_2

Like in the case of the oxidized form of cyt c_2, the 1621 and 1618 cm^{-1} frequencies are respectively associated with a nearly planar and slightly ruffled heme structure (Alden et al., 1989; Othman et al., 1994). Considering that the frequency dispersion of ν_{10} is larger for the oxidized form of wt cyt c_2 (1629−1638 cm^{-1}) than for its reduced form (1618−1621 cm^{-1}), it appears that the degree of porphyrin deformation is the highest for the oxidized heme. The crystal structures of *c* type cytochromes do not clearly characterize differences in heme distortion related to a change in the iron oxidation (Takano & Dickerson, 1981a,b; Louie & Brayer, 1990; Berghuis & Brayer, 1992). Model compounds however show that the shortest core size of oxidized hemes allows porphyrin ruffling while the core of reduced hemes is close to the optimum size of Fe−N(pyrrole) bonds of planar porphyrins (2.0 Å) (Scheidt & Gouterman, 1983). Thus, the large frequency dispersion of the ν_{10} mode of oxidized cyt c_2 in solution is the consequence of the coexistence of near planar and nonplanar heme conformers. When inducing a natural expansion of the porphyrin core, the heme reduction in fact shifts the conformational equilibrium toward the more planar conformers.

The frequency variation of ν_{11} cannot be associated with the conformational flexibility of heme since, in contrast to the ν_{10} frequency, that of ν_{11} is weakly altered by the porphyrin ruffling of Ni−porphyrins (Alden et al., 1989; Czernuszewicz et al., 1989; Shelnutt et al., 1991). For 6cLS ferroheme complexes, the frequency of the ν_{11} mode is in fact largely influenced by the nature and the orientation of the axial ligands through back-bonding effects (Spiro, 1985; Kitagawa & Ozaki, 1987; Desbois, 1994; Othman et al., 1994). Considering this latter sensitivity, the excitation-dependent frequency of the ν_{11} mode of cyt c_2 indicates a flexibility of the heme ligation. This ligand fluctuation may be either the origin or the consequence of the conformational equilibrium of heme in cyt c_2.

Both the frequency shift of the 1296−1299 cm^{-1} band and the changes in relative intensity of the two ν_{51} modes at 302 and 307 cm^{-1} and of the two thioether modes at 393 and 400 cm^{-1} could be due to either differences in the structure of the two thioether bridges or differences in their conformational mobility.

In conclusion, the RR data obtained for reduced cyt c_2 establish that macrocycle flexibility occurs for both redox states. This structural variation could originate from conformational flexibility of at least one of the axial ligands since the ν_{11} mode of 6cLS ferroheme complexes is strongly sensitive to changes in axial coordination (Desbois & Lutz, 1992; Othman et al., 1994; Desbois, 1994). In addition, the low-frequency regions as well as the 1300 cm^{-1} regions of RR spectra suggest that the fluctuation in heme−ligand(s) interaction is accompanied by changes in peripheral heme−protein interaction.

Effects of the Amino Acid Mutations on the Heme Structure

K14E/K32E Double Mutation. The comparison of the RR data obtained for wt cyt c_2 and its K14E/K32E mutant is consistent with the fact that the structure of heme macrocycle as well as the axial and peripheral interactions of heme with the protein is not strongly affected by the alteration of the surface charge of the protein. A significant destabilization of the protein conformation has been characterized for the K14E/K32E mutant (Caffrey & Cusanovich, 1991b). RR spectroscopy shows that this destabilization cannot be associated with an important change in heme−protein interaction.

P35A Mutation. The spectral changes observed in the ν_{10} regions of RR spectra indicate that the heme structure of oxidized P35A is on average more distorted than that of wt cyt c_2(III). On the contrary, the broadening and the frequency upshift of the ν_{10} mode of reduced P35A are compatible with a trend toward a heme flattening. The RR bands observed at ca. 375 and 400 cm^{-1} were assigned to deformation modes of peripheral heme groups (i.e. the propionate and thioether groups, respectively) (Hu et al., 1993). Their shifts in the spectra of oxidized P35A are therefore markers of a change in conformation of at least one propionate and one thioether bridge when an alanine residue is substituted for the proline at position 35.

The shift as well as the broadening of the ν_{11} mode of reduced P35A is indicative of an alteration in the axial coordination of heme (Othman et al., 1994; Othman, 1994; Desbois, 1994). Since the near-infrared region of absorption spectra indicates that the Fe−S(Met) bond is not affected by the P35 → A mutation (Gooley et al., 1991), this spectral modification can be attributed to a change in the Fe−N(His 17) interaction. More precisely, the broadening of the ν_{11} mode of reduced P35A reflects the existence of several His−heme interactions. Moreover, the broadening of ν_{11} to its low-frequency side marks the trend of the Fe−N(His) bond being strengthened by protein and/or heme conformational fluctuations (Othman et al., 1994). This increased strength of the axial bond is consistent with a hydrogen bond between the N_1H(His 17) group and the carbonyl group of residue 35 which is slightly stronger in P35A (Gooley & MacKenzie, 1990). The slight formation of a 5cHS ferroheme in P35A could correspond to a partial loss of S(Met) axial ligation induced by an increased Fe−His bond strength and/or the conformational mobility of the His ligand in P35A.

In addition to the structural alterations of the heme structure and of the axial Fe−His bond, the RR spectra indicate modifications in peripheral heme−protein interaction. The changes observed in the low- and middle-frequency regions of RR spectra modify the frequencies of modes involving at least one propionate group and one thioether bridge (Hu et al., 1993). NMR investigations indicate that the rings of the Phe 51 and Tyr 53 residues flip faster in the P35A protein than in wt cyt c_2 (Gooley et al., 1991). In the crystal structure of wt cyt c_2, the OH group of Tyr 53 is hydrogen-bonded to the carboxyl group of the heme propionate-7 (HP-7) group and the phenyl ring of Phe 51 is in close proximity with this propionate group (Benning et al., 1991). Therefore, the shifts of propionate vibrations can plausibly be associated with a conformational change of the HP-7 group. Similarly, the alterations of the RR modes involving a thioether bridge can be assigned to the side chain of Cys 16. The X-ray structure of wt cyt c_2 suggests the presence of a hydrogen bond between Gly 34 and Cys 16 (Benning et al., 1991). Moreover, the change in the exchange rate of the NH group of Gly 34 in the P35A mutant suggests a destabilization of the H bond with the carbonyl group of Cys 16 (Gooley & MacKenzie, 1990).

The RR data show that this destabilization is propagated to the Cys 16 side chain that is bound to heme.

The NMR data obtained on wt cyt c_2 and its P35A variant have been interpreted in the context of two plausible models (Gooley & MacKenzie, 1990). On one hand, the P → A mutation creates a cavity allowing the entrance of a water molecule. On the other hand, the P → A substitution increases the local protein flexibility around the proximal His. The shifts as well as the enlargements of numerous RR modes of P35A are clearly compatible with the latter model involving a population of peripheral heme−protein interactions, and thus of protein conformers. From the RR data and the available structural information, one can conclude that the protein fluctuations could practically cover all the "right" side of heme with limits going from the Cys 16 side chain to the HP-7 propionate group (Figure 1).

W67Y Mutation. When the high-frequency RR spectra of oxidized wt cyt c_2 and W67Y are compared, a larger variation in the ν_{10} frequency (1630−1640 cm^{-1}) as well as an increased contribution of the 1630 and 1640 cm^{-1} components is observed. These changes reflect both a larger conformational flexibility and an access to a more planar structure of ferriheme in oxidized W67Y. In the RR spectra of the reduced forms, the low-frequency bands affected by the W67 → Y mutation involve deformation modes of peripheral substituents, i.e. propionate or thioether heme groups (Hu et al., 1993).

The three-dimensional structure of wt cyt c_2 indicates that the Trp 67 side chain interacts with at least one of the two HP groups (Benning et al., 1991). Indeed, the N_1(W67)−O(HP-6 carboxyl) distance was determined to be 2.9 Å. From molecular modeling of the W67Y structure, the distance between the hydroxyl group of Y67 and the same O(carboxyl propionate) atom would be 2.6 Å, suggesting that a stronger H bond could occur between the HP-6 group and the Y67 residue (Caffrey & Cusanovich, 1993). Therefore, the frequency changes of the propionate vibrations could correspond to a change in H bonding interaction of HP-6. In addition, the W → Y substitution modifies the volume of the amino acid side chain by 34 $Å^3$ (Zamyatnin, 1972) and thus could leave a cavity allowing a conformational change of the HP-7 propionate group. The shift of the ν_{11} mode observed upon the W67 → Y mutation in cyt c_2(II) can be viewed as an indirect probe for such a change. Considering that the ν_{11} mode is strongly sensitive to the axial ligation of heme (Othman et al., 1994; Othman, 1994; Desbois, 1994), the frequency shift of this mode can be interpreted as corresponding to an alteration of the axial ligation and thus indicative of interactions between W67 and at least one of the axial heme ligands (His 17 or Met 96). Both the red shift of the near-IR absorption band at ca. 700 nm and the decreased p*K* of the alkaline transition of oxidized W67Y (Caffrey & Cusanovich, 1993) provide evidence for concluding that this interaction involves the S(Met 96) heme ligand. Moreover, the crystal structure of wt cyt c_2 shows that the carboxyl group of HP-7 is connected to S(Met 96) via H bonds involving a water molecule and the hydroxyl group of Tyr 75 (Caffrey et al., 1991; Benning et al., 1991). On one hand, the downshift of the ν_{11} mode of W67F is indicative of a strengthening of the Fe−S(Met) bond and/or a change in heme−Met coordination geometry (Desbois, 1994). On the other hand, the decreased p*K* of the alkaline transition of oxidized W67F suggests that the heme−S(Met) interaction is weakened upon W → Y replacement in position 67 (Caffrey & Cusanovich, 1993). Therefore, the most probable effect of the W67 → Y substitution appears to be a stereochemical change of the Met coordination.

In conclusion, the frequency shifts of the propionate vibrational modes as well as that of the ν_{11} mode can be plausibly interpreted as a conformational change of the HP-7 propionate induced by the different steric hindrance of the amino acid in position 67. As a consequence, this conformational change in W67Y could reorganize the network of H bonds between HP-7 and Met 96, to alter the interaction of the S(Met 96) ligating atom with the heme iron. It has been proposed that the primary role of the conserved Trp residue of cyt *c* and cyt c_2 is to provide a stabilizing hydrophobic core (Schweingurber et al., 1979; Caffrey & Cusanovich, 1993). Our RR data suggest that W67 could also play a steric role in controlling the conformation of HP-7 and subsequently in stabilizing the H bond networks going from this heme side group to the S(Met 96) heme ligand. As far as the ν_{10} modes of oxidized W67Y are concerned, a change in Fe−S(Met) interaction could influence the conformational equilibrium of ferriheme *c* in increasing the macrocycle flexibility.

Y75F Mutation. The RR spectra of wt cyt c_2 and its Y75F mutant exhibit spectral differences originating either from vibrations of peripheral heme groups [ν_9, ν_{51}, ν(CS), $\delta(C_mH)$, ...] or from modes influenced by the ligation of the heme iron (ν_8, ν_{11}, and ν_{50}) (Hu et al., 1993; Othman, 1994; Desbois, 1994). In the latter class of vibrations, the behavior of the ν_{50} mode is particularly interesting since, for the Y75F mutation, this mode decreases in vibrational frequency in the oxidized state (359 versus 357 cm^{-1}), while it increases in frequency in the reduced state (355 versus 357 cm^{-1}). In other words, the ν_{50} frequency is decreased from 359 to 355 cm^{-1} upon heme reduction of wt cyt c_2, while it remains constant for the oxidized and reduced forms of Y75F (357 cm^{-1}). Considering the *N*-acetylmethionine complex of MP8 as a model compound of the His/Met coordination of heme *c*, its ν_{50} frequency is decreased from 358 to 354 cm^{-1} when the heme iron is reduced (Othman, 1994). For yeast iso-1-cyt *c*, the heme reduction decreases the ν_{50} frequency from 361 to 356 cm^{-1} (Hildebrandt et al., 1991). Therefore, the normal trend of the ν_{50} mode is a decrease in its frequency by 4−5 cm^{-1} upon heme reduction. This is precisely what occurs for wt cyt c_2, but not for Y75F.

The normal mode composition of ν_{50} is likely complex. The RR spectra of 6cLS complexes of heme *c* showed that this mode is sensitive to both the nature of the axial ligands and the ionization state of the axial imidazole(s) (Othman, 1994; Othman et al., 1994). Considering all the sensitivities of ν_{50}, it was assigned to a coupled mode involving both an out-of-phase stretching mode of the Fe−N(pyrrole) bonds and a stretching or deformation mode of axial ligands (Hu et al., 1993; Othman et al., 1994). The different frequencies of the ν_{50} mode of oxidized and reduced wt cyt c_2 and Y75F can therefore be attributed to oxidation state-dependent alterations of the iron coordination. Taking into account the spatial proximity of the Tyr 75 and Met 96 residues in the crystal structure of cyt c_2 (Figure 1) (Benning et al., 1991), these alterations in the ν_{50} frequency most likely have an origin in a change in the electron density of the S(Met) ligand and/or in a change in heme−S(Met 96) interaction. Both types of changes could be controlled by the H bonding state

of the S(Met) atom. The presence or the absence of an H bond at the S(Met) site is expected to modulate the electron density of the Fe−S(Met) axial bond, an increased H bonding interaction at S(Met) site decreasing the electronegativity of the sulfur atom and thus the strength of the Fe−S(Met) bond. Moreover, the presence or the absence of an H bond at the S(Met) ligand can control the stereochemical orientation of the Met side chain and thus the geometry of the S(Met)−heme coordination (Senn & Wüthrich, 1985).

In the X-ray structure of *Rb. capsulatus* wt ferrocyt c_2, the OH group of Tyr 75 is apparently weakly H bonded with the S(Met 96) heme ligand (Benning et al., 1991). The crystal structure of the Y75F mutant is not available. However, that of the structurally equivalent Y67F mutant of yeast iso-1-cyt *c* is known. The crystal structures of wt iso-1-cyt *c* and the Y67F mutant have been determined both in the oxidized and in the reduced form (Berghuis & Brayer, 1992; Berghuis et al., 1994). In reduced wt iso-1-cyt *c*, the OH group of Tyr 67 is apparently involved in an H bond network and linked to both the S(Met 80) heme ligand and a bound water molecule (water 166). This network exhibits a clear similarity with that seen in the structure of reduced *Rb. capsulatus* cyt c_2, but the H bonding interaction between the OH group of the conserved Tyr and the S(Met) heme ligand appears to be stronger in iso-1-cyt *c* than in cyt c_2 (Benning et al., 1991). The distance between these H bond partners is 3.25 and 3.8 Å for yeast cyt *c* and *Rb. capsulatus* cyt c_2, respectively (Berghuis & Brayer, 1992; Berghuis et al., 1994; Caffrey et al., 1991). In the reduced state of the Y67F iso-1-cyt *c* mutant, an additional bound water molecule (water 300) replaces the missing hydroxyl group of residue 67 and this water molecule is able to form an H bond with the S(Met80) heme ligand (Berghuis et al., 1994). In the oxidized form of wt yeast iso-1-cyt *c*, the H bond between OH(Tyr 67) and S(Met 80) is apparently broken (Berghuis & Brayer, 1992; Berghuis et al., 1994). The structure of oxidized Y67F exhibits neither water 166 nor water 300 (Berghuis et al., 1994). The S(Met 80) atom of this mutant is apparently not H bonded. Thus, according to the available crystal structures (Berghuis & Brayer, 1992; Berghuis et al., 1994), the H bonding pattern of the S(Met 80) ligand of wt iso-1-cyt *c* would be S(Met)−HO(Tyr) and S(Met)−nothing for the reduced and oxidized state, respectively, and for the Y67F mutant S(Met)−H_2O and S(Met)−nothing, respectively. A similar oxidation state-dependent change in H bonding interaction of the S(Met 96) heme ligand of cyt c_2 can account for the observed ν_{50} frequency variations in the RR spectra of wt cyt c_2 and Y75F. The ν_{11} frequency downshift observed upon exchange of Y75 for F in reduced cyt c_2 (1545−1547 versus 1542 cm^{-1}) can be also interpreted in the context of an increase in the electron density of the Fe−S(Met) bond and/or of a change in the heme−S(Met) bonding geometry (Desbois, 1994).

Moreover, the RR data obtained on wt cyt c_2 and Y75F show the frequency shifts of several modes involving peripheral heme groups. These peripheral changes can be attributed to heme groups in proximity with the Tyr 75 residue, the structural water molecule, as well as the amino acid residues interacting with Tyr 75 (i.e. Val 76, Thr 94, and Met 96). The heme substituents perturbed upon mutation could be the 1- and 5-methyl groups, the HP-7 group, the proton of a methine bridge, and the heme side chain in position 2 (Benning et al., 1991).

As far as the structure of the heme macrocycle is concerned, the reduced form of wt iso-1-cyt *c* was observed to be less distorted than that of its Y67F mutant (Berghuis et al., 1994). This change in heme planarity is not found when the RR spectra of reduced cyt c_2 and its Y75F mutant are compared. Indeed, the frequencies of the ν_2, ν_3, and ν_{10} skeletal heme modes of both heme proteins are identical (Figure 6A,B). To explain these different results, we suggest that differences in macrocycle structure occur between the solution and crystal structures, as well as between cyt c_2 and mitochondrial cyt *c*. For the oxidized form of cyt c_2 and its Y75F variant, the slight increase in the contribution of the 1638−1639 cm^{-1} component of the ν_{10} cluster could reflect the increased stability of the nearly planar ferriheme structure in Y75F. In a way similar to that of the oxidized form of W67Y, the increased stability of this heme conformation appears to be related to a discrete change in heme−S(Met) interaction.

Structure−Function Relationships in Cytochrome c_2

The Soret excitations of *c* type cytochromes allow the RR enhancement of a large number of porphyrin modes (Cartling, 1983; Hu et al., 1993; Desbois, 1994). Considering the fact that each heme mode acts as a reporter for a given global or local heme structure, RR spectroscopy is able to precisely identify the effects of different amino acid mutations on the heme structure as well as the changes in protein−heme interaction. The preceding RR data clearly show that the Pro 35, Trp 67, and Tyr 75 residues play a role in the stabilization of the heme−protein interaction.

The redox potential of cytochromes is governed by a series of structural and environmental parameters affecting the heme, i.e. the number and type of the axial heme ligands, the H bonding state of these ligands, the electrostatic interactions arising from the protein charges and the propionate heme groups, the solvent exposure of heme, and the hydrophobicity of its environment (Harbury et al., 1965; Valentine et al., 1979; Moore et al., 1984; Churg & Warshel, 1986; Kassner, 1972; Stellwagen, 1976). Considering the Nernst equation, the parameters that stabilize the oxidized state of heme decrease its oxidation−reduction potential while those that stabilize the reduced state increase this potential (Churg & Warshel, 1986; Moore & Pettigrew, 1990). A number of these parameters is accessible for evaluation by RR spectroscopy (Desbois, 1994).

Heme Conformation. The crystalline structure of cyt c_2 shows that the heme macrocycle is nearly planar (Benning et al., 1991). The RR data on wt cyt c_2 in solution demonstrate the existence of a skeletal flexibility of the heme, both ferrous and ferric. An increased deformability has been found for the oxidized heme, indicating an oxidation state-dependent variation of the heme conformation. Such an effect could modulate the redox potential of heme, as macrocycle deformations can modify the energies of the highest occupied and lowest unoccupied molecular orbitals of the two oxidation states. A relationship between the heme ruffling and the redox potential is currently unknown. However, model studies on four-coordinated Zn−porphyrins suggest that a strong out-of-plane heme distortion is able to decrease the value of the redox potential by up to 200 mV (Barkigia et al., 1988).

The amino acid mutations we have investigated show a weak or no effect on the nature of porphyrin conformers as

well as their relative contributions, suggesting that point mutations even in the heme vicinity do not have large effects on the heme flexibility. In addition to the core dimension of heme, the protein could play a role in the heme deformability. For example, reduced wt cyt *c* and cyt c_2 are significantly more stable with respect to denaturation than the corresponding oxidized compounds (Takano & Dickerson, 1981b; Caffrey et al., 1991). This increased stability of the reduced state has been associated with an increased protein rigidity (Takano & Dickerson, 1981b). A higher mobility of the axial ligands in the oxidized state than in the reduced state could be the consequence of the weaker association of the S(Met) ligand with the ferric heme and thus could also participate in a greater deformation of the oxidized heme.

K12E/K32E Mutation. The K12 and K32 residues are located on the protein exterior near the exposed heme edge (Benning et al., 1991). The RR study on the K12E/K32E mutant allows us to conclude that the heme structure and environment are not strongly affected by a change of the net charge of the protein surface. The large change in the E_m value resulting from this double mutation is in good agreement with an alteration of the long range electrostatic effect between the positive charge of ferriheme and the surface charges (Moore & Pettigrew, 1990).

P35A Mutation. Changing the conserved Pro residue in cyt *c* and cyt c_2 produces no significant effect on the redox potential (Koshy et al., 1990; Gooley et al., 1991). This observation may appear strange since important changes are observed when the RR spectra of wt cyt c_2 and its P35A mutant are compared. In particular, the RR results show that this mutation induces an increase in the conformational mobility of the axial ligation and of some peripheral heme groups. The NMR data correlate these changes with an increased protein flexibility around the Pro 35 and His 17 residues. When forming a population of conformers around the wt structure, the nearly constant redox potential of P35A therefore resides in structural and environmental compensatory effects that affect both oxidation states. However, the increased protein mobility can have significant influences on the reaction mechanism of electron transfer with redox partners.

W67Y Mutation. The W → Y substitution in position 67 of cyt c_2 has no significant effect on the redox potential (Caffrey & Cusanovich, 1993). The comparison of RR spectra of wt cyt c_2 and W67Y indicates a conformational change of a propionate group affecting the H bond network between this heme group and the axial Met. In addition, some changes in heme−protein interaction have been detected. Because these effects were observed in both oxidation states, it seems likely that the W67 → Y mutation has no influence on the redox potential. Like in the case of the preceding mutant, some electron transfer properties could be however changed by this mutation.

Y75F Mutation. The redox potential of cyt c_2 is decreased by 59 mV in the Y75F mutant (308 versus 367 mV for wt cyt c_2) (Caffrey et al., 1991). The homologous mutation in cyt *c* produces a smaller negative shift (35 mV) (Luntz et al., 1989; Wallace et al., 1989). This decreased value in redox potential indicates that the mutation either stabilizes the oxidized form or destabilizes the reduced form. The RR spectra of wt cyt c_2 and Y75F are indicative of a change in either the electronegativity of the S(Met) ligand, the coordination geometry of the Met side chain, or both. Crystallographic data suggest that this effect is primarily due to oxidation state-dependent changes in H bonding of the S(Met 90) ligand with either the Tyr 75 residue in wt cyt c_2 or a water molecule in the Y75F mutant. A highly conserved water molecule near residues 90 and 75 and involved in an H bond network could be absent in the oxidized form of the Y75F mutant. Thus, the stabilization of the oxidized state of Y75F could be induced through the absence of an H bond at the S(Met) ligand, thereby increasing the electronegativity of the sulfur atom and/or altering the S(Met)−heme interaction. The RR data indicate an increase in electronegativity of the S(Met) ligand and/or a change in its coordination geometry when comparing the oxidized form of wt and Y75F. The comparison of RR data of the reduced forms is consistent with a decrease in the electronegativity of the S(Met) and/or a change in S(Met)−heme coordination geometry. The influence of the heme−Met coordination geometry on the stabilization of the reduced and oxidized heme is currently unknown. However, an increase in electronegativity of the Met sulfur is expected to stabilize the oxidized state of heme by increasing the stability of the Fe−S(Met) bond. The increased p*K* value of the alkaline transition of oxidized Y75F (10.7 versus 8.9 for wt cyt c_2) is also indicative of an increased heme−S(Met) interaction in ferric Y75F (Caffrey et al., 1991).

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