Resonance Raman Study of the Interactions between Cytochrome *c* Variants and Cytochrome *c* Oxidase†

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ABSTRACT: The structural changes in oxidized yeast iso-1-cytochrome *c* and fully oxidized bovine cytochrome *c* oxidase that are induced upon complex formation have been analyzed by resonance Raman spectroscopy. The main spectral changes could be ascribed to cytochrome *c*, which in the case of the wild-type protein are essentially the same as previously observed in the complex of horse heart cytochrome *c* and bovine cytochrome *c* oxidase [Hildebrandt et al. (1990) *Biochemistry 29*, 1661–1668]. These spectral changes are attributed to the formation of the conformational state II (~45%) which exhibits an open heme pocket structure. The structural changes are assumed to be induced by the electrostatic interactions between the negatively charged binding domain on cytochrome *c* oxidase and the positively charged lysine residues on the front surface of cytochrome *c*. Substituting one of these lysine residues (i.e., Lys-72) by an alanine significantly lowers the state II content (<15%), implying that this lysine is essential for controlling the conformational equilibrium of the bound protein. On the other hand, the replacement of lysine-79 by alanine only slightly lowers the state II content (~35%). However, the analysis of the spectra suggests that lysine-79 may be involved in controlling conformational details within the heme pocket of the bound cytochrome *c*. Due to the underlying structural changes and the lowered redox potential, formation of state II may be of functional importance for the physiological electron-transfer process by lowering the reorganization energy and increasing the driving force. The spectral changes caused by complex formation that are attributable to cytochrome *c* oxidase indicate structural changes of the vinyl and formyl substituents while the ground-state conformations of the porphyrin macrocycles are preserved. This finding implies that the conformational changes in the heme pockets of cytochrome *c* oxidase are much smaller than those in cytochrome *c*. These changes refer not only to heme *a* but also to heme a_3, located remote from the cytochrome *c* binding site, pointing to a long-range structural communication between the binding domain and the oxygen reduction site. The possible functional implications of these structural changes are discussed.

The reduction of cytochrome *c* oxidase by cytochrome *c* constitutes the terminal process in the mitochondrial respiratory chain (Wikström et al., 1981; Pettigrew & Moore, 1987). In this process, four electrons are transferred to the redox centers (Cu_A, heme *a*, heme a_3–Cu_B) of the membrane-bound cytochrome *c* oxidase, where they are used to reduce molecular oxygen (Babcock & Wikström, 1992). While considerable progress has been made in recent years to elucidate the mechanism of the oxygen reduction at the heme a_3–Cu_B center (Babcock & Wikström, 1992; Rousseau et al., 1992), the electron-transfer step from ferrocytochrome *c* to cytochrome *c* oxidase is much less well understood. There is growing evidence that the Cu_A center serves as the entry for electrons delivered by ferrocytochrome *c* (Kobayashi et al., 1989; Pan et al., 1991). The binding of cytochrome *c* to cytochrome *c* oxidase which precedes the electron transfer occurs most likely in the vicinity of the Cu_A center (Bisson & Montecucco, 1982; Kadenbach & Stroh, 1984). The binding domain in subunit II is constituted by negatively charged amino acid side chains (Bisson et al., 1982) so that an electrostatic complex is formed with cytochrome *c* via Coulombic attractions with the positively charged lysine residues around the exposed heme crevice (Smith et al., 1977; Speck et al., 1979; Rieder & Bosshard, 1978). However, the molecular pathway and mechanism for the interprotein electron transfer are still unknown. A detailed knowledge of the architecture of the cytochrome *c*–cytochrome *c* oxidase complex for elucidation of this pathway is required, but currently only the structure for cytochrome *c* has been determined (Takano & Dickerson, 1981a,b; Bushnell et al., 1990; Louie & Brayer, 1990; Gao et al., 1990, 1991; Berghuis & Brayer, 1992). Thus, the cytochrome *c*–cytochrome *c* oxidase complex has been studied by various spectroscopic techniques that selectively probe the heme groups of both proteins (Falk & Angström, 1983; Michel & Bosshard, 1989; Michel et al., 1989b; Weber et al., 1987; Hildebrandt et al., 1990a; Lynch et al., 1992). In this way, strong evidence was provided for structural changes in the active sites, induced upon complex formation. Among these techniques, resonance Raman (RR)[1] spectroscopy is a particularly powerful tool since it provides information about the ground-state conformation of the heme groups and their interactions with the protein environment [for a review, see Spiro (1988)].

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[1] Abbreviations: Cyt, iso-1-cytochrome *c*; Cyt(WT), Cyt(A79), and Cyt(A72), the wild-type, the Ala-79, and the Ala-72 variant proteins, respectively (the amino acid sequence numbering scheme is based on the sequence alignment with tuna cytochrome *c*); CcO, cytochrome *c* oxidase; RR, resonance Raman; 6cLS, six-coordinated low spin; 6cHS, six-coordinated high spin; 5cHS, five-coordinated high spin.

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In a recent RR study of the fully oxidized complex between horse heart cytochrome c and beef heart cytochrome c oxidase, significant structural changes were found for the heme group of cytochrome c (heme c). These structural changes were attributed to the formation of the so-called conformational state II, which is the preferred state of both ferric and ferrous cytochrome c upon binding to negatively charged surfaces such as polyanions, phospholipid vesicles, or metal electrodes (Hildebrandt & Stockburger, 1989; Hildebrandt et al., 1990b; Hildebrandt, 1990, 1991). The structural properties of this state II in such model systems have been extensively characterized. It was shown that in this conformational state the heme pocket is open compared to the closed heme crevice structure in the unbound protein. The formation of state II is due to electrostatic interactions of the negative charges with the positively charged lysine residues around the exposed heme edge of cytochrome c. In general, state II constitutes a conformational equilibrium with the conformational state I. State I exhibits essentially the same structure as the unbound cytochrome c, but it can be distinguished from the latter by small changes of the frequencies (<1 cm^{-1}), half-widths (<2 cm^{-1}), and relative intensities (<10%) for some of the RR bands. The studies of model systems suggest that it is the spatial distribution of the negative charges on the binding surfaces that controls this conformational equilibrium (Heimburg et al., 1991; Hildebrandt, 1991).

In the present work, we have continued the studies of complex formation between ferricytochrome c and fully oxidized cytochrome c oxidase to elucidate details of these electrostatic interactions and their effect on the structure of the active sites of both partner proteins. In particular, we were interested to determine the role of individual lysine residues on the front surface of cytochrome c which may be involved in electrostatic binding. Thus, we have used iso-1-cytochrome c, for which genetically engineered mutants are available (Mauk, 1991). This offers the opportunity to probe the structural and functional importance of individual amino acids residues for the reaction with cytochrome c oxidase. The three-dimensional structure of the wild-type yeast protein is very similar to that of horse heart cytochrome c (Bushnell et al., 1990; Louie & Brayer, 1990; Berghuis & Brayer, 1992), and the reactivity toward bovine cytochrome c oxidase is essentially the same for both proteins (Michel et al., 1989a), justifying the combination of yeast cytochrome c and mammalian cytochrome c oxidase for the present structural investigations.

MATERIALS AND METHODS

Sample Preparation. Wild-type, Ala-72, and Ala-79 yeast iso-1-cytochromes c (Cyt) in which the cysteine at amino acid position 102 is replaced by a threonine were isolated and purified as described previously (Northrup et al., 1992; Ferrer et al., 1993). The proteins were exchanged into 10 mM phosphate buffer at pH 7.0. Cytochrome c oxidase (CcO) was isolated from beef heart as described in Heibel et al. (1993a). The enzyme was solubilized in 0.1% Triton X-100 at a pH of 7.6 (10 mM Tris-HCl buffer). For the RR experiments, the concentrations of the individual proteins were 15 μM. Stable complexes of the fully oxidized proteins were prepared by mixing equimolar (15 μM) solutions with a total ionic strength of about 12 mM and at pH 7.2. Under these conditions, the degree of complex formation is estimated to be about 95% and binding of Cyt occurs only at the high-affinity sites of CcO (Weber et al., 1987; Michel & Bosshard, 1989; Michel et al., 1989b). Physical mixtures of Cyt and CcO were obtained at high ionic strength where complex formation is negligibly small (Weber et al., 1987). As previously shown (Hildebrandt et al., 1990a), the RR spectra of such mixtures are identical to the sum of the spectra of the individual components.

Resonance Raman Measurements. The RR spectra were measured with 413-nm excitation using a scanning double-monochromator with a spectral resolution of 2.8 cm^{-1} and a step width of 0.2 cm^{-1}. Details of the equipment are described elsewhere (Heibel et al., 1993a). All spectra were recorded at room temperature with repetitive scanning. The total accumulation time per 0.2-cm^{-1} increment was between 20 and 40 s. The samples were measured in a rotating cell to minimize laser-induced damage and photoreduction (Heibel et al., 1993a). In the case of the unbound Cyt, a catalytic amount of CcO (0.5%) had to be added to keep the protein in the oxidized state. Under these conditions Cyt was stable in the RR experiment. When the individual scans were compared, spectral changes were noted for CcO and the Cyt–CcO complex after 2–4 h of exposure to the laser beam so that such samples were replaced by fresh ones [cf. Hildebrandt et al. (1990a) and Heibel et al. (1993a)]. The spectra of individual experiments were combined only if the difference between them yielded a straight line of noise. The principles of the RR experiments using the dual-channel detection technique for quasi-simultaneous measurements have been described in detail elsewhere (Hildebrandt et al., 1990a). The raw spectra of CcO and of the Cyt–CcO complexes exhibit a relatively high scattering background which was removed by polynomial subtraction. Thus, the S/N ratio of these spectra is poorer than that of the unbound Cyt despite the considerably longer accumulation time.

Band Fitting. The RR spectra were analyzed with an interactive band fitting program utilizing Lorentzian line shapes. Details of the band-fitting procedure are discussed elsewhere (Hildebrandt et al., 1990a; Heibel et al., 1993a). The standard deviations for the calculated spectral parameters strongly depended on the quality of the spectra and are much better for strong and isolated bands than for weak and hidden shoulders. For example, typical values for the frequencies, half-widths, and intensities of the unbound Cyt (cf. Figures 2A, 3A, and 4A) were about 0.1 cm^{-1}, 0.4 cm^{-1}, and 3%, respectively. The corresponding values for the bound Cyt (cf. Figures 2B, 3B, and 4B) were ~2.5-fold greater.

RESULTS AND DISCUSSION

The RR spectra of the Cyt–CcO complexes as well as of their individual components were measured in three spectral regions which have been shown to provide complementary information about the structural changes in Cyt (280–480 and 1450–1530 cm^{-1}) and CcO (1600–1700 cm^{-1}) (Hildebrandt et al., 1990a).

Analysis of the Low-Frequency Region. Figure 1 shows the RR spectra of the unbound Cyt(WT) (A) and CcO (B) in the frequency region between 280 and 480 cm^{-1}. At 413-nm excitation, the RR spectrum of CcO predominantly exhibits RR bands of heme a_3 (Heibel et al., 1993a). The most prominent band of this heme group in this region (ν_8 at 332.3 cm^{-1}) is about one-third the intensity of the strongest band of Cyt(WT) at 348.8 cm^{-1} (ν_8). Thus, the RR spectrum of the fully oxidized complex is clearly dominated by the RR bands of the bound Cyt(WT) (Figure 1C). Comparing this spectrum with that of a mixture of both proteins at high ionic strength or with an appropriately weighted sum of the individual spectra reveals distinct spectral differences. This comparison is shown in the difference spectrum "complex

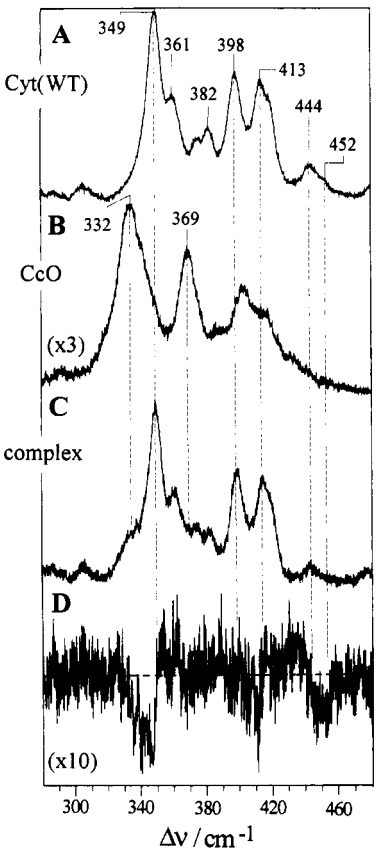

FIGURE 1: RR spectra of Cyt(WT) (A), CcO (B), and the Cyt(WT)–CcO complex (C) in the fingerprint region between 280 and 480 cm^{-1}, excited at 413 nm. The intensity scale of spectrum B is expanded by a factor of 3 compared to spectrum A. Spectrum D is the difference spectrum "C – [A + B]". The intensity scale of spectrum D is expanded by a factor of 10 compared to spectrum C.

minus mixture" depicted in Figure 1D. In particular, we note pronounced negative peaks at ~345, ~410, and ~445 cm^{-1}. The latter feature can unambiguously be related to a spectral change of bound Cyt(WT) since this heme protein exhibits two closely spaced bands at 443.8 and 452.4 cm^{-1}; CcO has no RR band in this region. Also, the negative peaks in the difference spectrum at ~350 and ~410 cm^{-1} most likely refer to Cyt(WT) rather than to CcO since they coincide with two of the most prominent bands of Cyt(WT) at 348.8 and 413.3 cm^{-1}. This difference spectrum is very similar to that obtained previously from the fully oxidized complex of horse heart cytochrome *c* and CcO (Hildebrandt et al., 1990a).

In the same way we have measured the RR spectra of the Ala-79 and Ala-72 variants and the corresponding complexes of these proteins formed with CcO. In the case of Ala-79 the difference spectrum (not shown) reveals some similarities with that of the wild-type protein such as the pronounced negative peak at ~440 cm^{-1} and the broad positive peaks at ~425 cm^{-1}, indicating that there are comparable structural changes of the bound Cyt in both cases. On the other hand, there are some additional features in the Cyt(A79) difference spectrum which are not seen in that of the wild-type protein, in particularly distinct positive peaks at ~408 and 342 cm^{-1}. The Ala-72 mutant, however, reveals quite a different behavior since there is no negative peak at ~440 cm^{-1}. Moreover, except for a dip at ~370 cm^{-1}, this difference spectrum is nearly a straight line.

To analyze the spectral changes of the bound Cyt in more detail, we have subtracted the contributions of CcO from the spectra of the complexes. The weighting factor for the

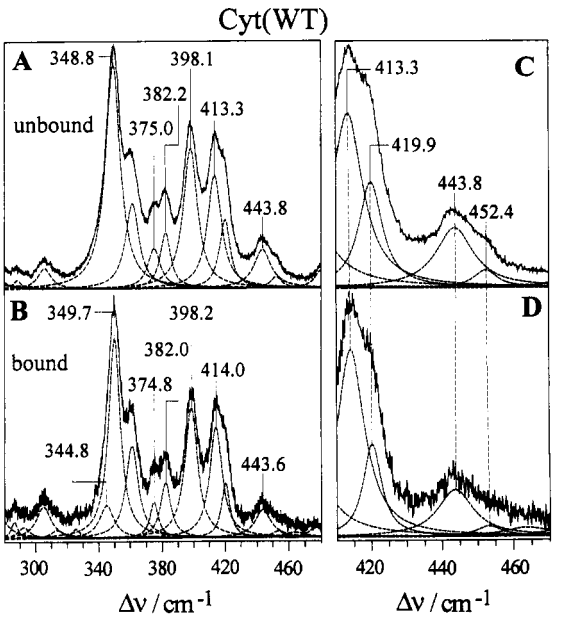

FIGURE 2: RR spectra in the fingerprint region of the Cyt(WT) in the unbound state (A) and in the complex with CcO (B). The RR contributions of CcO were subtracted from spectrum B as described in the text. Spectra C and D are expanded views of spectra A and B, respectively. The dotted lines represent the fitted Lorentzian components.

subtraction was determined by a band-fitting analysis of the complex spectrum using the same spectral parameters for CcO as previously determined for the unbound enzyme (Heibel et al., 1993a). The underlying assumption of this approach is that complex formation does not perturb the RR spectrum of CcO. This assumption is reasonable because the most pronounced effects in the difference spectra are related to Cyt unambiguously taking into account that the contributions of CcO to this region of the spectrum are minimal. Nonetheless, we show later that some of the RR bands of CcO in the high-frequency region are affected upon complex formation, so we cannot rule out subtle effects on the RR bands of CcO in the low-frequency range, that are beyond the limit of detection.

The RR spectra of the bound Cyt(WT), Cyt(A79), and Cyt(A72) obtained in this manner are displayed in Figures 2–4 along with the corresponding RR spectra of the unbound cytochromes. All spectra were analyzed by a band-fitting procedure. Such an analysis was straightforward in the case of the unbound Cyt species because the number of contributing bands and their approximate positions could readily be determined by careful visual inspection of the spectra. The assignment of the bands of Cyt(WT) is discussed elsewhere (Hildebrandt et al., 1991, 1992). All of these bands include relatively high contributions of vibrations involving the peripheral substituents and, hence, respond most sensitively to structural changes of the protein environment (fingerprint region). This is particularly true for the two bands at 443.8 and 452.4 cm^{-1} which can be assigned to porphyrin modes including bending vibrations of the propionate side chains of pyrrole rings D and A, respectively (Hildebrandt et al., 1992).[2,3]

For the Ala-79 and Ala-72 mutants, no data for the three-dimensional structure are available. In the case of Cyt(A72)

[2] For the numbering of the pyrrole rings, see Louie and Brayer (1989).

[3] This assignment is further supported by the RR spectra of the Gly-82 and Ser-82 mutants (unpublished results) for which X-ray data reveal structural changes at the propionate A but not at propionate D (Louie & Brayer, 1989; Louie et al., 1988).

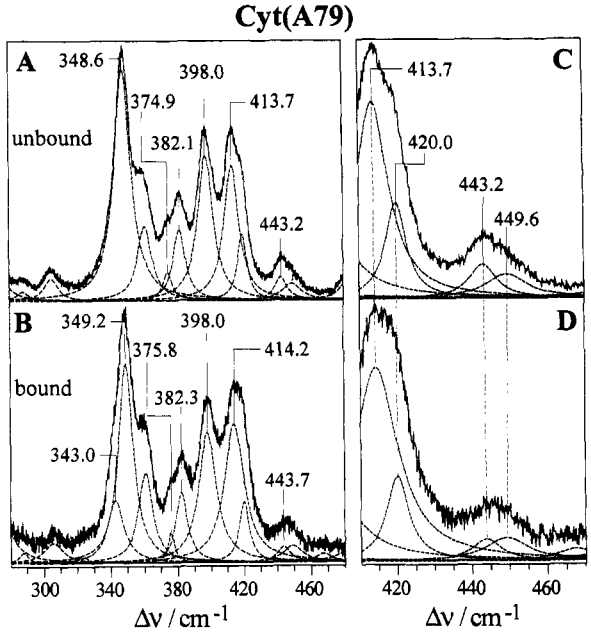

FIGURE 3: RR spectra in the fingerprint region of the Cyt(A79) in the unbound state (A) and in the complex with CcO (B). The RR contributions of CcO were subtracted from spectrum B as described in the text. Spectra C and D are expanded views of spectra A and B, respectively. The dotted lines represent the fitted Lorentzian components.

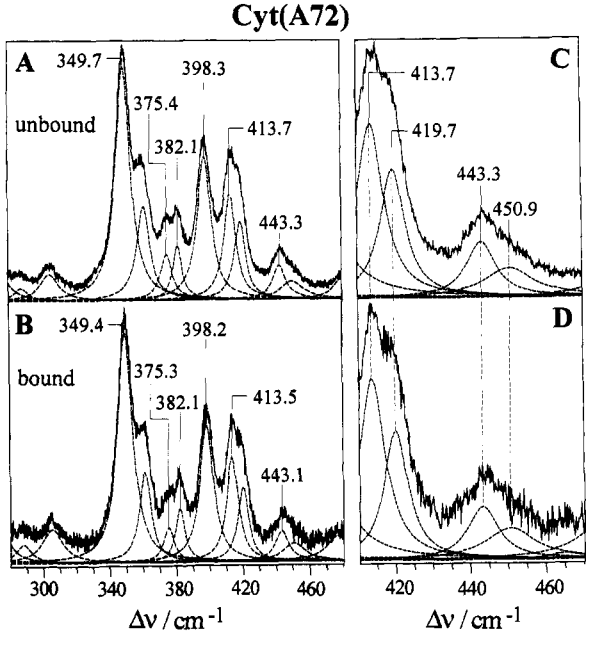

FIGURE 4: RR spectra in the fingerprint region of the Cyt(A72) in the unbound state (A) and in the complex with CcO (B). The RR contributions of CcO were subtracted from spectrum B as described in the text. Spectra C and D are expanded views of spectra A and B, respectively. The dotted lines represent the fitted Lorentzian components.

one would not expect significant structural differences compared to the wild-type protein because residue 72 is located on the surface of the protein. In fact, the only notable differences in Cyt(A72) are a 1-cm^{-1} upshift of ν_8 and a distinct narrowing of most of the bands in the fingerprint region (Table I).

In the Ala-79 mutant a more pronounced structural change in the heme pocket might be anticipated since the amide proton of Lys-79 is hydrogen-bonded to the propionate group of ring D and the charged side chain interacts with the carbonyl oxygen of Ser-47 (Figure 5; Louie & Brayer, 1990; Berghuis & Brayer, 1992). In particular, this latter hydrogen bond has been suggested to stabilize the heme crevice (Osheroff et al., 1980; Margoliash & Bosshard, 1983). However, the spectral differences, including those associated with the corresponding propionate bending, are comparable in magnitude to those in the Ala-72 mutant. This observation casts some doubt on the idea that Lys-79 plays a crucial role in stabilizing the closed heme pocket structure in the unbound protein.

The spectral parameters (number of components, frequencies, half-widths, and relative intensities) of the unbound Cyt were used as a starting point for the analysis of the RR spectra of the bound Cyt. For the wild-type protein and the Ala-79 mutant, however, a satisfactory fit was achieved only by increasing the number of bands (Figures 2 and 3). In particular, the RR spectrum of the bound Cyt(A79) required a band at 343.0 cm^{-1} adjacent to the dominant band at 349.2 cm^{-1}. The spectral parameters of the bound and unbound cytochromes obtained from the fitting procedure are listed in Table I.

The spectral changes in the Ala-72 variant induced upon binding to CcO are very small (Figure 4). Within the accuracy of the fitting procedure, there are essentially no frequency shifts, and a significant change of the relative intensity (>15%) is observed only for the 375.4-cm^{-1} band. However, some of the bands narrow by up to 1 cm^{-1}. These relatively small spectral changes might be attributed to less effective complex formation so that the spectrum in Figure 4B includes a significant contribution from the unbound Cyt(A72). In fact, the K_D value for complex formation between Cyt(A72) and CcO is not known. However, we have found that subtraction of the RR spectrum of the unbound Cyt(A72) (Figure 4A) from the spectrum in Figure 4B with weighting factors of more than ~25% produces artifacts such as an abnormally steep intensity decrease at the flanks of the 361- and 375-cm^{-1} bands so that the resulting spectrum cannot be properly fitted with Lorentzian line shapes. Thus, we conclude that in the case of Cyt(A72) the degree of complex formation is at least 75%. Also for Cyt(A79) the K_D value is not available. However, in this case the spectral changes induced by interaction with CcO are even more pronounced than for the wild-type protein and rule out less efficient complex formation by the variant.

We now consider the spectral changes in the bound Cyt(WT) and Cyt(A79) in greater detail, focusing at first on the two propionate bending vibrations at 443 and 450 cm^{-1}. The intensities of these two bands decrease significantly in the wild-type protein upon binding to CcO (Figure 2C,D). Using the 414-cm^{-1} band as a reference, the relative intensities have dropped by 25% and 40% for the 443- and 453-cm^{-1} components, respectively. For the Ala-79 mutant, a comparable decrease is observed for the 443-cm^{-1} band (35%), while the high-frequency component remains constant (Figure 3C,D). In a previous study of complex formation between horse heart ferricytochrome c and CcO, it was shown that the intensity decrease of these modes can be attributed to the formation of the six-coordinated low-spin (6cLS) configuration of the conformational state II which is characterized by the disappearance of the ~445-cm^{-1} band (Hildebrandt et al., 1990a).[4,5] This state II can be formed when cytochrome c is bound to an array of negative charges as it is induced by, e.g., large polyanions, metal electrodes, phospholipid vesicles, or, in this case, the binding domain of CcO (Hildebrandt, 1991). Hence, it is concluded that both the wild-type and the Ala-79 protein are partially converted to the conformational state II

Table I: Spectral Parameters[a] of Ferricytochrome *c* in the Unbound State and in the Complex with Oxidized Cytochrome *c* Oxidase

		wild type		Ala-79		Ala-72	
mode[b]	spectral param	unbound	complexed	unbound	complexed	unbound	complexed
ν_8	ν		344.8		343.0		
state II	$\Delta\nu$		10.7		11.7		
	I_{rel}		0.30		0.45		
ν_8	ν	348.8	349.7	348.6	349.2	349.7	349.4
state I	$\Delta\nu$	11.2	8.9	12.0	10.5	10.4	10.5
	I_{rel}	1.98	1.81	1.71	1.44	2.22	2.21
	ν	361.2	360.9	361.5	360.8	361.5	361.1
	$\Delta\nu$	10.5	9.6	10.3	10.3	9.4	8.5
	I_{rel}	0.75	0.84	0.55	0.65	0.89	0.87
	ν	375.0	374.8	374.9	375.8	375.4	375.3
	$\Delta\nu$	9.7	6.4	6.7	4.7	8.6	7.7
	I_{rel}	0.35	0.33	0.21	0.22	0.43	0.33
	ν	382.2	382.0	382.1	382.3	382.1	382.1
	$\Delta\nu$	8.0	7.9	8.9	8.5	6.6	6.7
	I_{rel}	0.49	0.50	0.52	0.50	0.52	0.51
	ν	398.1	398.2	398.0	398.0	398.3	398.2
	$\Delta\nu$	10.8	9.8	11.2	12.7	9.8	9.9
	I_{rel}	1.24	1.18	1.07	0.94	1.37	1.31
	ν	413.3	414.0	413.7	414.2	413.7	413.5
	$\Delta\nu$	10.6	10.0	10.7	14.3	8.7	8.5
	I_{rel}	1.00	1.00	1.00	1.00	1.00	1.00
	ν	419.9	420.0	420.0	420.0	419.7	419.9
	$\Delta\nu$	8.2	6.4	6.9	7.4	8.2	7.8
	I_{rel}	0.61	0.49	0.49	0.44	0.74	0.71
prop[c]	ν	443.8	443.6	443.2	443.7	443.3	443.1
ring D	$\Delta\nu$	13.0	12.3	8.9	9.4	9.4	9.9
	I_{rel}	0.34	0.25	0.17	0.11	0.32	0.29
prop[c]	ν	452.4	453.1	449.6	449.2	450.9	450.5
ring A	$\Delta\nu$	8.1	8.8	13.2	13.7	15.7	16.0
	I_{rel}	0.10	0.06	0.12	0.12	0.17	0.17

[a] Frequencies (ν) and half-widths ($\Delta\nu$) are given in cm^{-1}. The relative intensities (I_{ref}) refer to the 413-cm^{-1} band. [b] The assignment is discussed in detail elsewhere (Hildebrandt et al., 1991, 1992). [c] "Prop" refers to a porphyrin mode including high contributions from the propionate bending vibrations of the rings A and D.

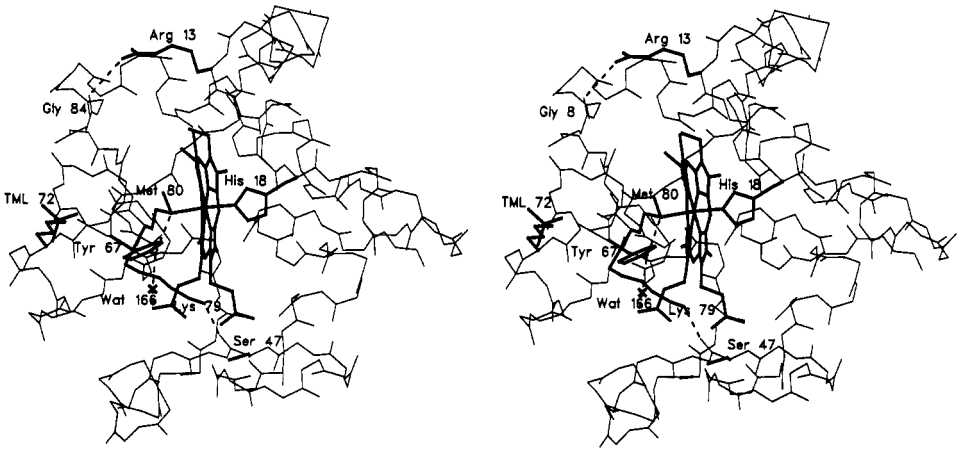

FIGURE 5: Stereo drawing showing the conformation of the main chain of yeast iso-1-ferricytochrome *c* according to the convention of Berghuis and Brayer (1992). The heme group and those amino acids which are referred to in the text are drawn in thick lines. TML-72 denotes the trimethyllysine residue 72.

upon binding to CcO. Further support for this conclusion comes from the analysis of the ν_8 band region between 340 and 350 cm^{-1}. The low-frequency component at 343.0 cm^{-1} in the complex of the Ala-79 variant with CcO agrees nicely with the position of the ν_8 mode found for state II of Cyt(WT) (Hildebrandt et al., 1991). Two ν_8 components were also included for fitting the RR spectrum of the bound Cyt(WT), although we note that in this specific case a fit with only one component was also satisfactory.

Analysis of the ν_3 Band Region. In other frequency regions, the RR spectrum of conformational state II also reveals characteristic differences compared to the unbound Cyt. In particular, the spectral parameters of the mode ν_3 have been determined precisely, both in state I and in state II (Hildebrandt et al., 1990b; Hildebrandt, 1990, 1991). This mode belongs to a set of bands that are referred to as marker bands

[4] State II of horse heart ferricytochrome *c* exhibits a temperature-dependent coordination equilibrium between a 6cLS and a five-coordinated high-spin (5cHS) configuration with a significant population of the 5cHS form at room temperature in some model systems (Hildebrandt, 1990). In the complex with CcO only the 6cLS configuration of state II was detected (Hildebrandt et al., 1990a). Similarly, yeast cytochrome *c* (WT, A79, A72) exhibits no indication of the 5cHS form of state II upon binding to CcO.

[5] The two components of the 445-cm^{-1} peak in the horse heart protein are at very similar frequencies. They can only safely be resolved in D_2O (unpublished data).

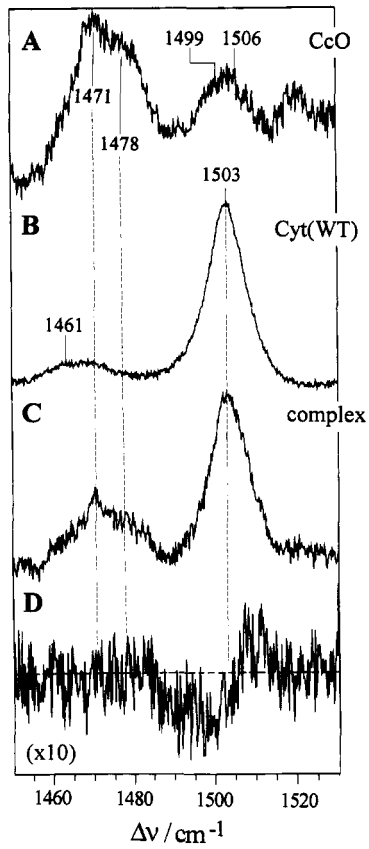

FIGURE 6: RR spectra of CcO (A), Cyt(WT) (B), and the Cyt-(WT)–CcO complex (C) in the ν_3 band region between 1450 and 1530 cm^{-1}, excited at 413 nm. The intensity scale of spectrum A is expanded by a factor of 3 compared to spectrum B. Spectrum D is the difference spectrum "C – [A + B]". The intensity scale of spectrum D is expanded by a factor of 10 compared to spectrum C.

and are located between 1450 and 1650 cm^{-1}. The frequencies of these bands exhibit a sensitive dependence on the geometry of and the electron density distribution within the porphyrin and, hence, on the spin, coordination, and oxidation state of the heme iron (Partharasathi et al., 1987; Kitagawa & Ozaki, 1987). Unfortunately, for CcO the resonance enhancement of these marker bands is much stronger than that of the low-frequency bands, so that their intensities are comparable in magnitude to those of Cyt. This similarity in marker band intensity for the two proteins complicates the analysis of the RR spectra of the protein complex in this region considerably. The most favorable situation is found in the region of the mode ν_3 which is expected at ~1480, ~1492, and 1503 cm^{-1} in ferric six-coordinated high-spin (6cHS), 5cHS, and 6cLS iron porphyrins, respectively. This correlation also holds for Cyt and CcO as can be seen in the RR spectra of Figure 6A,B. For the 6cLS hemes of Cyt(WT) and CcO (heme *a*), this mode is at 1503.0 or 1499.0 cm^{-1}, respectively, while in the 6cHS heme a_3 it is downshifted to 1479.6 cm^{-1} [for a vibrational assignment of the RR spectrum of CcO, see Heibel et al. (1993a) and Babcock (1988)]. In the RR spectrum of the fully oxidized complex of Cyt(WT) and CcO (Figure 6C), the Cyt(WT) mode ν_3 strongly overlaps with the corresponding mode of heme *a* and the heme a_3 mode ν_{38y} at 1505.6 cm^{-1}. These two bands of CcO, however, are one-fifth as intense as the ν_3 mode of Cyt(WT). On the other hand, the RR bands of CcO at 1470.1 and 1479.6 cm^{-1} (ν_{28} of heme *a* and ν_3 of heme a_3, respectively) are significantly stronger than the weak bands of Cyt(WT) in this region (1461.4 and 1471.2 cm^{-1}). Figure 6D shows the difference spectrum "complex minus

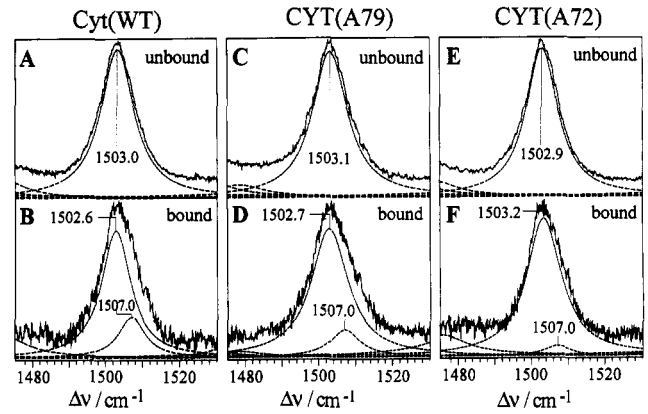

FIGURE 7: RR spectra in the ν_3 band region of the various Cyt variant proteins in the unbound state and in the complex with CcO: (A) Cyt(WT), unbound; (B) Cyt(WT), bound; (C) Cyt(A79), unbound; (D) Cyt(A79), bound; (E) Cyt(A72), unbound; (F) Cyt(A72), bound. The dotted lines represent the fitted Lorentzian components.

mixture" which demonstrates that the spectral changes induced upon complex formation occur exclusively between 1490 and 1510 cm^{-1}, i.e., in that region which is dominated by the ν_3 band of Cyt(WT). On the other hand, there are no binding-induced differences below 1480 cm^{-1}. This implies that, as in the low-frequency region, there are no detectable spectral changes of the RR bands of CcO. Consequently, the spectral differences induced upon complex formation can be attributed to the structural changes of the bound Cyt(WT). Thus, in analogy to the fingerprint region, we have subtracted the content of CcO from the RR spectrum of the complex to obtain the RR spectrum of the bound Cyt(WT) which is shown in Figure 7. In principle, the RR spectrum of the complex could also be fitted by keeping the spectral parameters of Cyt(WT) constant and varying those of CcO. However, this would imply unusually large frequency shifts and intensity changes exclusively of the 1499.0- and 1505.6-cm^{-1} bands while all other modes of CcO remain unchanged.

The spectrum of the bound Cyt(WT) is fitted by including two ν_3 modes corresponding to the coexistence of the states I and II as inferred by the analysis of the low-frequency region. The high-frequency component is upshifted by about 4 cm^{-1} as it is usually found for state II in model systems (Hildebrandt et al., 1990b; Hildebrandt, 1991). The major component is at a slightly downshifted frequency, again in agreement with previous findings for state I.

In a similar manner we have measured and analyzed the corresponding spectra of the complexes formed by CcO with Cyt(A79) and Cyt(A72). The resultant spectra of the bound Cyt(A79) and Cyt(A72) are shown in Figure 7. In the case of the Ala-79 variant, the high-frequency component of the ν_3 envelope is slightly weaker than found with the wild-type protein, but it is still of considerable intensity. However, in the case of the Cyt(A72), this band is clearly at the limit of detection, and a fit without this component is of a comparable quality with the frequency and half-width of the major component being largely unchanged.

On the basis of the relative intensities of the two ν_3 bands in each spectrum, it is possible to estimate the relative concentrations of state I and state II by using the relative RR cross sections of ν_3 of both species determined from model systems (Hildebrandt & Stockburger, 1989). Thus, we obtain 45% and 35% state II for the wild-type and the Ala-79 variant proteins, respectively. In the case of Cyt(A72), we can only give an upper limit for the state II content of 15%, but the true value is probably lower.

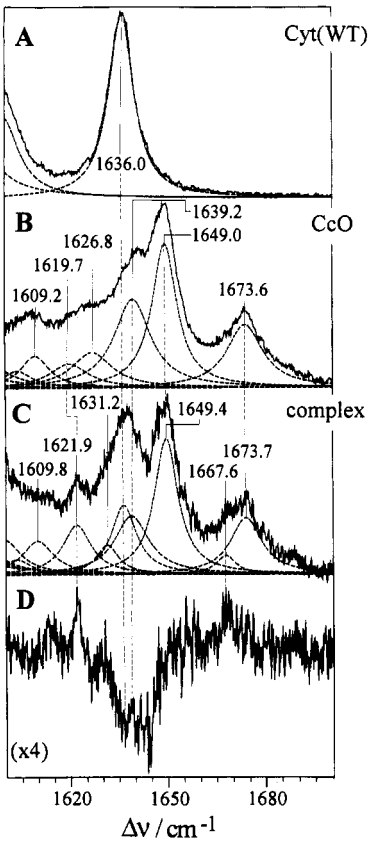

FIGURE 8: RR spectra of Cyt(WT) (A), CcO (B), and the Cyt-(WT)–CcO complex (C) in the high-frequency region between 1600 and 1700 cm^{-1}, excited at 413 nm. The intensity scale of spectrum A is expanded by a factor of 2 compared to spectrum B. Spectrum D is the difference spectrum "C – [A + B]". The intensity scale of spectrum D is expanded by a factor of 4 compared to spectrum C. The dotted lines represent the fitted Lorentzian components.

Analysis of the High-Frequency Region. In the region between 1600 and 1700 cm^{-1}, the RR spectrum of Cyt(WT) displays only one RR band, the mode ν_{10} at 1636.0 cm^{-1} (Figure 8A). In contrast, CcO shows a rich vibrational band pattern, and the RR cross section of the strongest band at 1649.0 cm^{-1} is about 50% greater than that of the ν_{10} mode of Cyt(WT) (Figure 8B). Most of the bands of CcO in this region originate from stretching vibrations of the vinyl (1626.8 and 1619.7 cm^{-1}) and formyl substituents (1649.0 and 1673.6 cm^{-1}) of heme *a* and heme a_3, respectively (Babcock, 1988). These modes have been shown to be extremely sensitive to structural changes of CcO, even on the level of quaternary structure (Heibel et al., 1993a). Hence, this region is of particular interest in detection of possible structural changes in CcO induced upon binding of Cyt(WT). The RR spectrum of the complex is displayed in Figure 8C. Visual inspection reveals an increase of the RR intensity at ~1620 cm^{-1}, i.e., in the vinyl stretching region. In addition, the difference spectrum (Figure 8D) shows a large negative feature with minima at ~1635 and ~1640 cm^{-1} and a small positive peak at ~1665 cm^{-1}. While the minima must be attributed to a spectral change of both Cyt (1635 cm^{-1}) and heme *a* (1640 cm^{-1}), the 1665-cm^{-1} peak is located in the region of the formyl stretching of heme a_3. Consequently, we must take spectral changes of both partner proteins into account.

The changes reflected in the RR spectrum of the bound Cyt(WT) are due to the coexistence of state I and state II. According to previous findings for horse heart ferricytochrome *c* bound to model systems, the ν_{10} mode of state I is characterized by the same spectral parameters as the unbound protein (Hildebrandt et al., 1990b). The corresponding mode of state II is upshifted by about 3–4 cm^{-1} but exhibits a much smaller RR cross section than in state I (Hildebrandt & Stockburger, 1989; Hildebrandt et al., 1990b). As the frequency of this mode would also coincide with the heme *a* mode ν_{10} at 1639 cm^{-1}, this band was neglected in the analysis of the spectrum. On the basis of these considerations, the RR spectrum of the complex (Figure 8C) was fitted by initially assuming a fixed frequency and half-width for the Cyt(WT) mode ν_{10} and allowing all other parameters to vary. Eventually, also the frequency and the half-width of this mode were also allowed to vary, though only minor deviations in this mode relative to the corresponding parameters of the unbound Cyt-(WT) resulted. The intensity of this mode obtained in this manner was ~30% lower than that observed for the unbound Cyt(WT). This result is in good agreement with the 45% content of state II inferred from the analysis of the ν_3 band region.

On the other hand, the spectral parameters of CcO were allowed to vary freely in the fitting procedure. The resulting values for the porphyrin skeleton modes of both heme *a* and heme a_3 were essentially identical to those observed for the unbound CcO (Table II). The only exception is the relative intensity of the heme *a* mode ν_{10}, which is ~25% weaker in the complex. However, there are significant changes in the internal stretching vibrations of the vinyl groups of heme *a* and heme a_3 as well as in the formyl stretching vibration of heme a_3. The vinyl stretching frequencies of heme *a* and heme a_3 shift up by 4.4 and 2.2 cm^{-1}, and the half-widths of these bands are narrowed by 6.7 and 4.1 cm^{-1}, respectively. Additionally, in the case of heme a_3, the intensity of this mode is increased by a factor of 2. Again, these results are similar to those obtained for the complex of horse heart cytochrome *c* and CcO, although in that study the vinyl stretching of heme *a* could not be resolved from the nearby ν_{10} modes (Hildebrandt et al., 1990a).

The most striking observation in the high-frequency region concerns the formyl stretching of heme a_3 (~1670 cm^{-1}). A satisfactory fit of this peak was not obtained by assuming the presence of just one component as was possible for the unbound enzyme (1673.6 cm^{-1}). As indicated by the difference spectrum (Figure 8D), there is a shoulder at the low-frequency side of this band (~1665 cm^{-1}) that generates a positive peak in the difference spectrum. A significant improvement of the fit was achieved by including a second component yielding a band at 1667.6 cm^{-1}. This finding suggests that there are two conformations of the formyl group of heme a_3 induced upon complexation of CcO with Cyt(WT).

Structural Changes upon Complex Formation: Cytochrome c. The RR spectra of the bound Cyt(WT) can be attributed readily to a mixture of the conformational states II and I. It is not surprising that these structural changes agree qualitatively and quantitatively (~45% state II) with the results obtained for the complex formed by horse heart cytochrome *c* and CcO (Hildebrandt et al., 1990a). This similarity implies that the mode of interaction with CcO is the same for both proteins independent of the replacement of Lys-13 by arginine and the (postsynthetical) modification of Lys-72 to trimethyllysine in the yeast protein (Figure 5). This conclusion is implicit upon comparison of the three-dimensional structures of the two proteins (Bushnell et al., 1990; Louie & Brayer, 1990; Berghuis & Brayer, 1992).

It was suggested that the opening of the heme crevice, the main structural change in state II, is due to the formation of "intermolecular salt bridges" at the expense of those internal

Table II: Spectral Parameters[a] of the Oxidized Cytochrome *c* Oxidase in the Unbound State and in the Complex with Wild-Type Ferricytochrome *c*

	unbound CcO			bound CcO		
mode[b]	ν	$\Delta\nu$	I_{rel}	ν	$\Delta\nu$	I_{rel}
ν_{37}, heme *a*	1603.7	11.0	0.48	1603.4	10.5	0.46
ν_{10}, heme a_3	1609.2	10.8	0.89	1609.8	11.7	1.03
$\nu_{C=C}$, heme a_3	1619.7	14.6	0.67	1621.9	10.5	1.52
$\nu_{C=C}$, heme *a*	1626.8	15.1	0.99	1631.2	8.4	0.90
ν_{10}, heme *a*	1639.2	13.4	2.47	1638.7	12.8	1.81
$\nu_{C=O}$, heme *a*	1649.0	9.2	4.02	1649.4	8.8	4.20
$\nu_{C=O}$, heme a_3				1667.6	7.3	0.57
$\nu_{C=O}$, heme a_3	1673.6	13.2	1.75	1673.7	11.5	1.74

[a] Frequencies (ν) and half-widths ($\Delta\nu$) are given in cm^{-1}. The relative intensities (I_{rel}) refer to the ν_{28} mode of heme *a* at 1470.1 cm^{-1}. [b] Assignment according to that given by Heibel et al. (1993a).

salt bridges and hydrogen bonds which stabilize the closed heme pocket (Weber et al., 1987; Hildebrandt et al., 1990a). Possible candidates for such "structurally relevant" heme crevice interactions may be the Lys-13–Glu-90 and Arg-13–Gly-84 couples in the horse heart and yeast protein, respectively [Figure 5; cf. Bushnell et al. (1990), Louie and Brayer (1990), Berghuis and Brayer (1992), and Margoliash and Bosshard (1983)].

In the Ala-72 mutant, there is, at most, a very small amount of state II formed in the complex with CcO. There is no evidence that Lys-72 is of importance for closing the heme cleft, but it has been argued that this lysine residue is crucial for a proper alignment of the partner proteins in the complex (Koppenol & Margoliash, 1982; Figure 9). The lack of Lys-72 may cause a reorientation of Cyt in the binding site of CcO so that structurally relevant internal salt bridges or hydrogen bonds of Cyt are not perturbed. Nevertheless, electrostatic binding of this variant protein to CcO still occurs since the RR spectra reveal small but distinct differences compared to the unbound protein. The subtle frequency shifts and the band narrowing of some of the modes are typical of state I (Hildebrandt, 1990, 1991).

The situation is more complicated in the case of the Ala-79 mutant. State II is only slightly less populated than in the wild-type protein complex, implying that Lys-79 is not involved in the intermolecular salt bridges that control the structures of the bound proteins. In fact, it has been suggested that Lys-79 is peripheral to the interaction domain (Rieder & Bosshard, 1980; Figure 9). However, the structural differences between the bound and the unbound Cyt(A79) are as pronounced as in the case of Cyt(WT) (Table I; Figures 2B, 3B, 7B, and D). This fact points to a stabilizing role of Lys-79 in maintaining the heme pocket structure of the bound Cyt. The most striking finding is that Cyt(A79) binding to CcO leads to an intensity reduction of the propionate bending of ring D only, while the corresponding mode of ring A remains unchanged. This observation is in contrast to that for Cyt(WT) and implies that states II of the wild-type protein and of the Ala-79 variant exhibit structural differences in the vicinity of ring A. Intuitively, one would expect the opposite behavior because Lys-79 interacts with the propionate group of ring D. Apparently, the conformational change caused by the substitution of this amino acid is not localized at the mutation site.

Furthermore, the 375-cm^{-1} band of Cyt(A79) narrows drastically upon binding to CcO to a value (4.7 cm^{-1}) that approaches the spectral resolution of the spectrometer. Both in the wild-type protein and in the Ala-72 mutant, this mode exhibits a much less pronounced narrowing. In general, the RR bands of biological chromophores are significantly broader, owing to conformational microheterogeneity of the chromophore and its protein environment (inhomogeneous broadening) or to efficient vibrational energy transfer to quasi-isoenergetic vibrational modes of nearby solvent molecules or protein groups (Hildebrandt & Stockburger, 1984; Hildebrandt, 1990, 1991; Sassaroli et al., 1989; Hashimoto et al., 1986). Evidently, such broadening mechanisms are largely missing for the 375-cm^{-1} band of the bound Cyt(A79), which is in sharp contrast to the remaining bands in the fingerprint region.

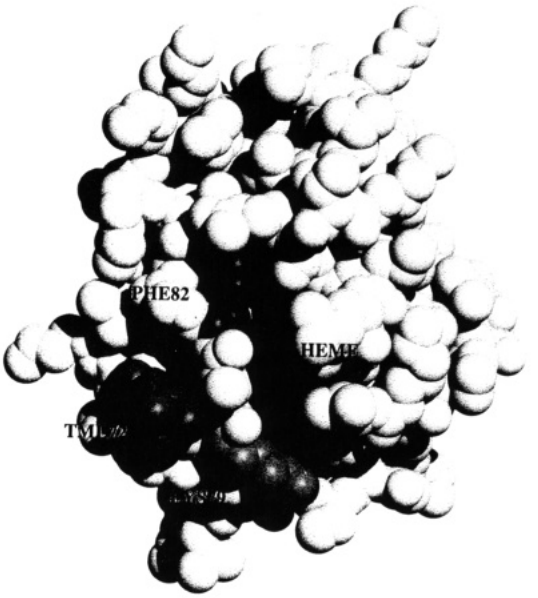

FIGURE 9: Space filling model of yeast iso-1-cytochrome *c*. The view presented here is directed onto the front surface of the protein including the CcO interaction domain. TML-72 denotes the trimethyllysine residue 72.

Structural Changes upon Complex Formation: Cytochrome c Oxidase. The RR spectra provide no evidence for changes of the ground-state conformations of the CcO porphyrin skeletons. This conclusion is in line with the results of a recent study by Lynch and Copeland (1992). The only effect noted for a pure porphyrin mode is the intensity decrease of the ν_{10} mode of heme *a*, which may reflect subtle perturbations of the excited state of heme *a*. Such changes in the electronic transition may be too small to be detected by absorption spectroscopy (Michel et al., 1989b). However, second-derivative absorption spectroscopy of the complex formed by ferrocytochrome *c* and cyanide-inhibited reduced CcO reveals a perturbation of the Soret transition of heme *a* (Lynch et al., 1992). It may be that the dielectric properties of the heme environment are modified (e.g., by the displacement of amino acid side chains or solvent molecules), which would affect the electronic transitions and the RR intensity

(Donovan, 1973; Hildebrandt et al., 1988; Larkin et al., 1991). Such an interpretation may in particular hold for the significant intensification of the vinyl stretching of heme a_3. A comparable increase of this mode is observed when (the unbound) CcO is solubilized in cholate instead of Triton X-100 (Heibel et al., 1993a). This increase has been attributed to a subtle change in the vicinity of the vinyl substituents which, in turn, may be due to the effect of the different detergents on the quaternary structure of CcO.

The main spectral changes in CcO caused by complex formation are associated with the internal stretching vibrations of the formyl and vinyl groups. This observation implies that the structural changes of CcO in the RR spectra are localized in these porphyrin substituents and their immediate protein environments. Most surprisingly, these changes involve not only heme *a* which is closer to the primary electron acceptor Cu_A near the Cyt binding site but also the remote heme a_3 (Saraste, 1990). The frequency upshifts of the vinyl stretching modes of both hemes indicate a conformational change of these substituents or its immediate protein environment (Debois et al., 1984). This structural change apparently includes a more rigid fixation of the vinyl as judged from the reduced bandwidths of the stretching modes (Table II).

While the structure of the formyl group of heme *a* remains unaffected upon complex formation, the presence of two formyl stretching bands of heme a_3 indicates the formation of two conformers of this heme in the complex. It may be that both conformers differ exclusively in the structure of this substituent and its interactions in the heme pocket. While the stronger band reflects a conformer which apparently exhibits the same structure as in heme a_3 of the unbound CcO, the weaker band corresponds to a conformer with a modified formyl structure or environment. In each case, however, the frequency is in the range observed for non-hydrogen-bonded C=O groups (Babcock, 1988).

An alternative explanation is inspired by recent RR results on cytochrome aa_3 from *Sulfolobus acidocaldarius* (Heibel et al., 1993b). For this quinol oxidase, two formyl stretching vibrations of heme a_3 were detected at 1672.9 and 1665.7 cm^{-1} which are close to the values of the components in the bound CcO found in this work. In the *Sulfolobus* enzyme the high- and low-frequency components were assigned to a 6cHS- and a 5cHS heme a_3, respectively. If such an interpretation also holds for the two heme a_3 conformers of the bound CcO, there should be a splitting of the porphyrin modes of heme a_3, at least in the marker band region, which in fact could not be detected. However, taking the relative intensities of both formyl stretching bands as a measure of the population of both conformers, it is evident that those porphyrin modes associated with the minor fraction would be too weak to be detected in the more crowded regions of the RR spectra (e.g., 380–480, 1450–1530, 1600–1630 cm^{-1}; cf. Figures 2, 6, and 8). The sixth ligand of heme a_3, which must be a weak ligand, probably bridges the heme iron with the Cu_B (Saraste, 1990). In fact, there are indications that Cyt binding to CcO leads to a perturbation of the coordination shell of Cu_B (Musatov & Konstantinov, 1988), which in turn might promote the dissociation of the sixth ligand from the heme a_3 iron.

Functional Implications. In this work we have analyzed the structural changes in the fully oxidized complex of Cyt and CcO. On the other hand, the reactive complex is formed by ferrous Cyt and (partially) oxidized CcO (Babcock & Wikström, 1992). However, previous studies of Cyt bound to model systems have demonstrated that the formation of state II is largely independent of the oxidation state of Cyt (Hildebrandt et al., 1990b). In both the ferric and the ferrous forms, a conformationl equilibrium between states I and II is established which depends on the charge distribution at the interface of the complex partners. Hence, it is reasonable to assume that the structural changes found in the fully oxidized complex also occur in the reactive complex formed by ferrocytochrome *c* and oxidized CcO.

These electrostatic interactions involved in Cyt binding to a given surface are controlled by the ionic strength (Hildebrandt & Stockburger, 1989; Hildebrandt, 1990). Thus, it is possible to reconcile the present results with previous data for the ionic strength dependence of the electron-transfer process between Cyt and CcO by Hazzard et al. (1991). These authors concluded that there are structural differences between the Cyt–CcO complexes formed at low and high ionic strength. These data may reflect the ionic strength dependence of the conformational equilibrium of the bound Cyt. Conformational equilibria of the bound Cyt in the complex with CcO have also been invoked by other groups on the basis of a variety of experimental evidence (Brooks & Nicholls, 1982; Weber et al., 1987; Michel et al., 1989; Rush et al., 1988; Van Kuilenburg et al., 1992).

As suggested previously, state II formation may enhance the rate of CcO reduction due to a lowering of the reorganization energy associated with the electron-transfer step or by increasing the thermodynamic driving force for the reaction (Hildebrandt et al., 1990a; Hildebrandt, 1991). The reorganization energy involves those conformational changes of the redox center (heme) and the surrounding medium (protein) which are required to adapt to the electron density distribution in the heme after the electron-transfer step (Marcus & Sutin, 1985). A lowering of the reorganization energy occurs if state II assumes a structure which is intermediate between those of the reduced and the oxidized cytochromes.

The results of a wide range of spectroscopic studies (Chottard et al., 1987; Hildebrandt & Stockburger, 1989; Hildebrandt, 1990, 1991; Muga et al., 1991) indicate that the main structural change in state II is an opening of the heme crevice which is associated with a weakening of the iron–Met-80 ligand bond and an alteration of the hydrogen-bonding network in the interior of the heme pocket involving both heme propionates. On the other hand, the redox-linked conformational changes have been determined by an comparative X-ray diffraction analysis of yeast iso-1-ferri- and ferrocytochrome *c* (Berghuis & Brayer, 1992; Figure 5). The crucial changes occur on the Met-80 side of the heme and in the buried part of the heme pocket which becomes less rigidly closed in the ferricytochrome. In the heme pocket, the most remarkable effects are the modification of the hydrogen-bonding interactions of the heme propionate A and the rupture of the hydrogen bond between Tyr-67 and Met-80. As is well-known from various experimental studies [cf. Williams et al. (1985)], the coordinate bond of this amino acid to the heme iron becomes weaker upon oxidation. These alterations of the hydrogen-bonding interactions are linked through reorientation of the internal water molecule Wat-166, which is located between Tyr-67 and the propionate A (Berghuis & Brayer, 1992).

A comparison of these data demonstrates that the structural changes producing state II which are induced in Cyt upon binding to CcO exactly correspond to those regions of Cyt which undergo structural rearrangement with the transition from the reduced to the oxidized form. Furthermore, the opening of the heme pocket removes steric constraints from the heme so that it is less rigidly fixed in the protein matrix

(Hildebrandt, 1990, 1991). It may be that this more relaxed structure exhibits lower potential energy barriers for those atomic displacements which have to occur for the transition from the reduced to the oxidized form. Thus, the comparison of the binding-induced and the redox-linked conformational changes strongly supports the idea that electron transfer from state II to CcO occurs with a substantially lower reorganization energy than for state I.

In state II, the reduction potential exhibits a large negative shift by up to 400 mV (Hildebrandt & Stockburger, 1989; Chottard et al., 1987), similar to that found for the alkaline form of Cyt (Barker & Mauk, 1992). This shift is probably due to the opening of the heme crevice or modified hydrogen-bonding interaction of the heme propionates (Hildebrandt & Stockburger, 1989; Moore, 1983; Cutler et al., 1989; Davies et al., 1993). Such a negative shift of the potential would increase the driving force for the electron transfer to CcO. This suggestion is in agreement with the conclusion drawn from a recent kinetic study of the reaction between Cyt and human CcO (Van Kuilenburg et al., 1992).

Upon replacement of Lys-72 by Ala, there is little or no state II formed in the complex with CcO, which would imply significantly less effective electron transfer to CcO. In fact, this conclusion is supported by previous studies on the reaction of chemically modified Cyt with CcO (Rieder & Bosshard, 1978, 1980; Osheroff et al., 1980; Koppenol & Margoliash, 1982). Neutralization of the positive charge on the Lys-72 side chain leads to a drastic lowering of the effectiveness of Cyt as a CcO substrate by a factor of ~30 (Koppenol & Margoliash, 1982). These authors interpreted these results in terms of a poor alignment of the proteins in the Cyt–CcO complex caused by modification of the Cyt dipole moment. On the basis of the results presented in this work we suggest that the principal functional importance of Lys-72 in this reaction is its crucial role in inducing conformational state II, thereby significantly facilitating electron transfer to CcO. Our results with the Ala-79 mutant support this conclusion. For Cyt with chemically modified Lys-79, a smaller effect on the reaction with CcO was observed (Rieder & Bosshard, 1980) which is consistent with a slight decrease of the state II content compared to the wild-type protein.

Summarizing, we conclude that the reduction of CcO by Cyt is controlled by a conformational equilibrium that involves an activated form of Cyt which is more efficient in transferring electrons to CcO. Lys-72 but not Lys-79 is crucial to establishing a substantial equilibrium concentration of this activated form upon binding to CcO. For the native Cyt the conformational equilibrium depends exclusively on the charge distribution at the interaction site of CcO. This points to a possible mechanism for the regulation of electron flow and, hence, oxygen reduction.

The functional significance of the structural changes in CcO in the complex is more difficult to assess. These changes involve both the electron acceptor site (Cu_A, heme *a*) and the oxygen reduction site (heme a_3, Cu_B). It may be that the structural change associated with the vinyl group of heme *a* is relevant to reduction by ferrocytochrome *c*. Previous investigations have suggested that the conformation of the vinyl group and its interaction with the protein environment may influence the redox process by affecting the electron density distribution in the heme (Debois et al., 1984; Reid et al., 1986).

The fact that Cyt binding also induces structural changes in heme a_3 is *per se* of interest because it implies a long-range structural communication from the Cyt binding site to the oxygen reduction site. The structural changes of the heme a_3 include not only the vinyl but also the formyl group, which exists in two conformers. Whether or not these conformers represent 6cHS and 5cHS species of heme a_3 must be clarified by further experimental studies. Nevertheless, we mention that linkage of Cyt binding to the dissociation of the sixth ligand from the heme a_3 iron would be a reasonable event because this coordination site must be occupied by oxygen immediately after heme a_3 is reduced. If this mechanism is correct, it would imply that Cyt binding to CcO might support binding of oxygen.

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REFERENCES

Babcock, G. T. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) pp 293–346, Wiley, New York.

Babcock, G. T., & Wikström, M. (1992) *Nature 356*, 301–309.

Barker, P. D., & Mauk, A. G. (1992) *J. Am. Chem. Soc. 114*, 3619–3624.

Berghuis, A. M., & Brayer, G. D. (1992) *J. Mol. Biol. 223*, 959–976.

Bisson, R., & Montecucco, C. (1982) *FEBS Lett. 150*, 49–53.

Bisson, R., Steffens, G. C. M., Capaldi, R. A., & Buse, G. (1982) *FEBS Lett. 144*, 359–363.

Brooks, S. P. J., & Nicholls, P. (1982) *Biochim. Biophys. Acta 680*, 33–43.

Bushnell, G. W., Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 585–595.

Chottard, G., Michelon, M.; Hervé, M., & Hervé, G. (1987) *Biochim. Biophys. Acta 916*, 402–410.

Cutler, R. L., Davies, A. M.; Creighton, S., Warshel, A., Moore, G. R., Smith, M., & Mauk, A. G. (1989) *Biochemistry 28*, 3188–3197.

Davies, A. M., Guillemette, J. G., Smith, M., Greenwood, C., Thurgood, A. G. P., Mauk, A. G., & Moore, G. R. (1993) *Biochemistry* (in press).

Debois, A., Mazza, G., Stetzkowski, F., & Lutz, M. (1984) *Biochim. Biophys. Acta 785*, 161–176.

Donovan, J. W. (1973) *Methods Enzymol. Part D 27*, 497–548.

Falk, K. E., & Angström, J. (1983) *Biochim. Biophys. Acta 722*, 291–296.

Ferrer, J. C., Guillemette, J. G., Bogumil, R., Inglis, S. V., Smith, M., & Mauk, A. G. (1993) *Nature* (submitted for publication).

Gao, Y., Boyd, J., Pielak, G. J., Williams, R. J. P., & Pielak, G. J. (1990) *Biochemistry 29*, 6944–7003.

Gao, Y.; Boyd, J., Pielak, G. J., & Williams, R. J. P. (1991) *Biochemistry 30*, 7033–7044.

Hashimoto, S., Teroaka, J., Inubushi, T., Yonetani, T., & Kitagawa, T. (1986) *J. Biol. Chem. 261*, 1110–1118.

Hazzard, J. T., Rong, S.-Y., & Tollin, G. (1991) *Biochemistry 30*, 213–222.

Heibel, G., Hildebrandt, P., Ludwig, B., Steinrücke, P., Soulimane, T., & Buse, G. (1993a) *Biochemistry* (in press).

Heibel, G., Anzenbacher, P., Hildebrandt, P., & Schäfer, G. (1993b) *Biochemistry* (in press).

Heimburg, T., Hildebrandt, P., & Marsh, D. (1991) *Biochemistry 30*, 9084–9089.

Hildebrandt, P. (1990) *Biochim. Biophys. Acta 1040*, 175–186.

Hildebrandt, P. (1991) *J. Mol. Struct. 242*, 379–395.
Hildebrandt, P., & Stockburger, M. (1984) *Biochemistry 23*, 5539–5548.
Hildebrandt, P., & Stockburger, M. (1989) *Biochemistry 28*, 6710–6721.
Hildebrandt, P., Copeland, R. A., Spiro, T. G., Otlewski, J., Laskowski, M., & Prendergast, F. G. (1988) *Biochemistry 27*, 5426–5433.
Hildebrandt, P., Heimburg, T., Marsh, D., & Powell, G. L. (1990a) *Biochemistry 29*, 1661–1668.
Hildebrandt, P., Heimburg, T., & Marsh, D. (1990b) *Eur. Biophys. J. 18*, 193–201.
Hildebrandt, P., Pielak, G. J., & Williams, R. J. P. (1991) *Eur. J. Biochem. 201*, 211–216.
Hildebrandt, P., English, A. M., & Smulevich, G. (1992) *Biochemistry 31*, 2384–2392.
Kadenbach, B., & Stroh, A. (1984) *FEBS Lett. 173*, 374–380.
Kitagawa, T., & Ozaki, Y. (1987) *Struct. Bonding 64*, 71–114.
Kobayashi, K., Une, H., & Hayashi, K. (1989) *J. Biol. Chem. 264*, 7976–7980.
Koppenol, W. H., & Margoliash, E. (1982) *J. Biol. Chem. 257*, 4426–4437.
Larkin, P. J., Gustafson, W. G., & Asher, S. A. (1991) *J. Chem. Phys. 94*, 5324–5330.
Louie, G. V., & Brayer, G. D. (1989) *J. Mol. Biol. 209*, 313–322.
Louie, G. V., & Brayer, G. D. (1990) *J. Mol. Biol. 214*, 527–555.
Louie, G. V., Pielak, G. J., Smith, M., & Brayer, G. D. (1988) *Biochemistry 27*, 7870–7876.
Lynch, S. R., & Copeland, R. A. (1992) *Protein Sci. 1*, 1428–1434.
Lynch, S. R., Sherman, D., & Copeland, R. A. (1992) *J. Biol. Chem. 267*, 298–302.
Marcus, R. A., & Sutin, N. (1985) *Biochim. Biophys. Acta 811*, 265–322.
Margoliash, E., & Bosshard, H. R. (1983) *Trends Biochem. Sci. 8*, 316–320.
Mauk, A. G. (1991) *Struct. Bonding 75*, 131–157.
Michel, B., & Bosshard, H. R. (1989) *Biochemistry 28*, 244–252.
Michel, B., Mauk, A. G., & Bosshard, H. R. (1989a) *FEBS Lett. 243*, 149–152.
Michel, B., Proudfoot, A. E. I., Wallace, C. J. A., & Bosshard, H. R. (1989b) *Biochemistry 28*, 456–462.
Moore, G. R. (1983) *FEBS Lett. 161*, 171–175.
Muga, A., Mantsch, H. H., & Surewicz, W. (1991) *Biochemistry 30*, 7219–7224.
Musatov, A., & Kostantinov, A. A. (1988) *FEBS Lett. 238*, 295–299.
Northrup, S. H., Thomasson, K. A., Miller, C. M., Barker, P. D., Eltis, E. D., Guillemette, J. G., Inglis, S. V., & Mauk, A. G. (1993) *Biochemistry* (submitted for publication).
Osheroff, N., Borden, D., Koppenol, W. H., & Margoliash, E. (1980) *J. Biol. Chem. 255*, 1689–1697.
Pan, L.-P., Hazzard, J. T., Lin, J., Tollin, G., & Chan, S. I. (1991) *J. Am. Chem. Soc. 113*, 5908–5910.
Parthasarathi, N., Hansen, C., Yamaguchi, S., & Spiro, T. G. (1987) *J. Am. Chem. Soc. 109*, 3865–3871.
Pettigrew, G. W., & Moore, G. R. (1987) *Cytochrome c—Biological Aspects*, Springer-Verlag, Berlin.
Reid, L. S., Lim, A. R., & Mauk, A. G. (1986) *J. Am. Chem. Soc. 108*, 8197–8201.
Rieder, R., & Bosshard, H. R. (1978) *J. Biol. Chem. 253*, 6045–6053.
Rieder, R., & Bosshard, H. R. (1980) *J. Biol. Chem. 255*, 4732–4739.
Rousseau, D. L., Han, S., Song, S., & Ching, Y.-C. (1992) *J. Raman Spectrosc. 23*, 551–556.
Rush, J. D., Koppenol, W. H., Garber, E. A. E., & Margoliash, E. (1988) *J. Biol. Chem. 263*, 7514–7520.
Saraste, M. (1990) *Q. Rev. Biophys. 23*, 331–366.
Sassaroli, M., Ching, Y.-C., Dasgupta, S., & Rousseau, D. L. (1989) *Biochemistry 28*, 3128–3132.
Smith, H. T., Staudenmayer, N., & Millet, F. (1977) *Biochemistry 16*, 4971–4974.
Speck, S. H., Ferguson-Miller, S., Osheroff, N., & Margoliash, E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 155–159.
Spiro, T. G. (1988) *Biological Applications of Raman Spectroscopy*, Vol. III, Wiley, New York.
Takano, T., & Dickerson, R. E.(1981a) *J. Mol. Biol. 153*, 79–94.
Takano, T., & Dickerson, R. E. (1981b) *J. Mol. Biol. 153*, 95–115.
Van Kuilenburg, A. B. P., Gorren, A. C. F., Dekker, H. L., Nieboer, P., Van Gelder, B. F., & Muijsers, A. O. (1992) *Eur. J. Biochem. 205*, 1145–1154.
Weber, C., Michel, B., & Bosshard, H. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6687–6691.
Wikström, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase, A Synthesis*, Academic Press, London.
Williams, G., Moore, G. R., & Williams, R. J. P. (1985) *Comments Inorg. Chem. 4*, 55–98.