Conformational Changes in Cytochrome *c* and Cytochrome Oxidase upon Complex Formation: A Resonance Raman Study†

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ABSTRACT: The fully oxidized complex of cytochrome *c* and cytochrome oxidase formed at low ionic strength was studied by resonance Raman spectroscopy. The spectra of the complex and of the individual components were compared over a wide frequency range using Soret band excitation. In both partners of the complex, structural changes occur in the heme groups and in their immediate protein environment. The spectra of the complex in the 1600–1700 cm^{-1} frequency range were dominated by bands from the cytochrome oxidase component, whereas those in the 300–500 cm^{-1} range were dominated by bands from the cytochrome *c* component, hence allowing separation of the contributions from the two individual species. For cytochrome *c*, spectral changes were observed which correspond to the induction of the conformational state I and the six-coordinated low-spin configuration of state II on binding to cytochrome oxidase. While in state I the structure of cytochrome *c* is essentially the same as in solution, state II is characterized by a structural rearrangement of the heme pocket, leading to a weakening of the axial iron–methionine bond and an opening of the heme crevice which is situated in the center of the binding domain for cytochrome oxidase. The relative contributions of the two cytochrome *c* states were estimated to be approximately in the ratio 1:1 in the complex. It is proposed that the electron transfer from cytochrome *c* to cytochrome oxidase is controlled by the conformational equilibrium between the states I and II which exhibit different redox potentials. The spectral changes assignable to heme *a* are interpreted in terms of alterations in the interaction of the cytochrome oxidase protein matrix with the peripheral substituents of the heme group, on binding cytochrome *c*. It is suggested that, in particular, the mobility of the formyl group is reduced. These structural changes may reflect the establishment of the proper alignment of the heme *a* group for the electron transfer. Subtle effects on the resonance Raman bands of heme a_3 are attributed to modified interactions of the π-electron systems of the porphyrin with adjacent amino acid side chains, suggesting that there is a structural communication between the cytochrome *c* binding site and the heme a_3 pocket. Spectra of the complex between the reduced cytochrome *c* and the partially oxidized cyanide-ligated cytochrome oxidase also indicated that conformational changes take place in these redox partners.

The electron-transfer reaction from cytochrome *c* (cyt *c*)[1] to cytochrome oxidase (cyt ox; EC 1.9.3.1) constitutes the terminal step of the respiratory chain of aerobic organisms (Dickerson & Timkovich, 1975; Pettigrew & Moore, 1987). Cyt *c*, whose prosthetic group is a heme *c*, delivers the electrons required for the reduction of molecular oxygen to water. This process is coupled to the synthesis of ATP, the ultimate source of chemical energy for metabolic processes. Cyt ox contains four reducible sites, two hemes (a, a_3) and two copper centers (Cu_a, Cu_b) (Wikström et al., 1981). In the first step of the redox process, ferrocytochrome *c* binds tightly to the cytosolic face of the membrane-spanning cytochrome oxidase enzyme. This complex is held together via strong electrostatic interactions between the positively charged lysine-rich domain around the exposed heme crevice of cyt *c* and the negatively charged binding site of cyt ox, which is located in close vicinity to the heme *a* (Smith et al., 1977; Speck et al., 1979; Rieder & Bosshard, 1978). Heme *a*, which is regarded to be the primary electron acceptor, forms a rapid electron-transfer equilibrium with Cu_a. After complete reduction of these sites, the electrons are transferred to heme a_3 (and Cu_b) on the matrix side of the enzyme where the reduction of oxygen occurs.

The fundamental importance of the reaction between cyt *c* and cyt ox is in contrast to the still considerable lack of understanding of the underlying molecular mechanism (Pettigrew & Moore, 1987). Relatively little information is available concerning the structure and dynamics of cyt *c* and cyt ox during the redox process. Copeland et al. (1987, 1988) have demonstrated that the reduction of Cu_a is associated with a rapid conformational transition of cyt ox that is reflected in a modification of the tryptophan fluorescence. Similar observations were made by Malmström and co-workers, although the transition was assumed to be triggered by the reduction of heme *a* and Cu_a (Fabian et al., 1987; Thörnström et al., 1988). Furthermore, Musatov and Konstantinov (1988) showed that binding of cyt *c* to cyt ox can induce conformational changes in the environment of heme a_3. Of particular importance in this respect, are the spectroscopic investigations by Bosshard and co-workers (Weber et al., 1987; Michel et

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[1] Abbreviations: cyt *c*, cytochrome *c* (the indices I and II refer to the conformational states I and II); cyt ox, cytochrome oxidase; RR, resonance Raman; SERR, surface-enhanced RR; 6cLS, six-coordinated low spin; 6cHS, six-coordinated high spin; 5cHS, five-coordinated high spin.

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al., 1989), who provided evidence for structural modifications in the cyt *c*–cyt ox complex compared with the individual components. These studies, as well as the NMR work by Falk and Ångström (1983) and Satterlee et al. (1987), were the first to demonstrate that cyt *c* itself is also subject to conformational changes upon binding to cyt ox. These results suggest that the reduction of cyt ox by cyt *c* is a rather complex process including a variety of conformational transitions and electron-transfer steps. Hence, it is clear that a more adequate description of the physiological processes requires a detailed picture of the conformational changes which are involved.

Resonance Raman (RR) spectroscopy is a highly specific technique since it selectively probes the vibrational pattern of chromophoric groups upon resonant excitation. It has successfully been employed for a variety of heme proteins [for reviews, see Spiro (1983) and Kitagawa and Ozaki (1987)]. In previous studies, this technique was used to analyze the structure of cyt *c* bound to negatively charged surfaces such as electrodes, phospholipid vesicles, and anionic macromolecules (Hildebrandt & Stockburger, 1989a,b). The underlying idea of this latter approach was to take these anionic systems as models which may mimic the negatively charged binding site on cyt ox. It turned out that, under the influence of strong electric fields, cyt *c* can exist in two thermodynamically stable conformational states (I and II). While in state I the native structure of the heme protein remains unaltered, a structural reorganization of the heme pocket was observed in state II. In this conformation, the native six-coordinated low-spin (6cLS) configuration is replaced by a temperature-dependent and electric field dependent equilibrium between a 6cLS and a five-coordinated high-spin (5cHS) configuration. These structural changes were found for both the reduced and the oxidized cyt *c*. In the case of the silver electrode system, it was possible to analyze the potential-dependent reactions of the adsorbed cyt *c* quantitatively by using surface-enhanced RR spectroscopy (SERR), whose extraordinarily high sensitivity permits the measurement of vibrational spectra even from submonolayer levels of coverage (Hildebrandt & Stockburger, 1989a). In this way, a complex reaction scheme was derived for the adsorbed cyt *c* that includes three electron-transfer pathways with different redox potentials corresponding to the conformational states I and II (5cHS and 6cLS). Since these conformational states were also detected in model systems other than the silver electrode, it was postulated that they may also be formed in the complex of cyt *c* with cyt ox (Hildebrandt & Stockburger, 1989b). This would imply that the physiological electron-transfer reactions of cyt *c* are also governed by electric field dependent conformational equilibria.

The present RR study is designed to examine this hypothesis. Using the RR spectra of the cyt *c*–cyt ox complex, we attempted to identify those bands which sensitively respond to structural alterations of the heme and its immediate protein environment. In particular, we have focused attention on those spectral regions which predominantly reflect the RR bands of either cyt *c* or cyt ox so that conformational changes of both heme proteins could be analyzed, separately.

Materials and Methods

Horse heart cytochrome *c* was obtained from Sigma (type VI) and purified according to the method described by Brautigan et al. (1978). Cytochrome oxidase was isolated from beef heart and purified following the procedure described by Yu et al. (1975) with the modifications given by Cable and Powell (1980). The molecular activity was 11 000 min^{-1} using the procedure of Yonetani (1966). The endogenous lipid was replaced by dimyristoylphosphatidylcholine as described in Powell et al. (1985, 1987). The enzyme was suspended in a solution containing 10 mM Tris-HCl, 0.15 mM EDTA, and 0.15% dodecyl maltoside at pH 7.4. This buffer was used in all spectroscopic experiments. The enzyme was stored in liquid nitrogen. Complexes of ferricytochrome *c* and the fully oxidized cytochrome oxidase were prepared by mixing the solutions of both components at a 1:1 molar ratio, taking M_r = 200 000 for cytochrome oxidase. In all RR experiments, the concentrations of cyt *c* or cyt ox were 21 μM. Under these conditions, the degree of complex formation was nearly 95% (Michel et al., 1989; Michel & Bosshard, 1989). The preparation of the complex between ferrocytochrome *c* and the semireduced, cyanide-ligated cytochrome oxidase followed the procedure described by Weber et al. (1987). All chemicals used in this study were of the highest purity grade available.

RR spectra were recorded at 20 °C using the divided-cell technique which permits the quasi-simultaneous measurement of two different samples (Rousseau, 1981). In addition, due to the continuous rotation of the cuvette through the laser beam, photoreduction of the oxidized enzyme was avoided. A detailed description of this apparatus is given elsewhere (Hildebrandt & Stockburger, 1989b). The spectra of the fully oxidized complex, as well as of the individual components, were excited with the 407-nm line of a Kr^+ laser which was attenuated to ~25 mW at the sample. For the reduced forms, the 413-nm line was used for excitation. The spectra were recorded by repetitive scanning. In general, the total dwell time per monochromator setting was 20–30 s. In all cases, the spectral resolution was 2.8 cm^{-1}. A calibration accuracy of ±0.1 cm^{-1} for all experiments was achieved by measuring the profile of the exciting laser line approximately each hour. Those spectra which were measured with a step width of 0.2 cm^{-1} were analyzed by a band fitting program which is described elsewhere (Hildebrandt & Stockburger, 1989a).

The integrity of the samples during the RR experiments was checked by monitoring characteristic RR bands. Only in the case of cyt ox were time-dependent spectral changes noted for the 335 cm^{-1} band (i.e., broadening of the band) after a period of about 10 h. Therefore, the samples were renewed after approximately 8 h. In separate experiments, it was found that the molecular activity of cyt ox did not decrease after incubating the sample in 0.15% dodecyl maltoside (with or without cyt *c*) at 16 °C for more than 24 h.

The study of the complexes of cyt *c* and cyt ox by RR spectroscopy encounters the difficulty that, with Soret band excitation, three different heme groups contribute to the measured spectrum. Thus, the analysis of such complex spectra required a specific strategy which was based on two steps. First, we carefully compared the RR spectra of the complex and the mixture of cyt *c* and cyt ox in order to verify if there were spectral changes upon complex formation. Then selected spectral ranges were analyzed in more detail for an assignment of these changes to the individual heme groups.

A prerequisite for such a procedure is that all RR spectra were measured under exactly the same conditions which can be achieved by employing the divided-cell technique. In a series of measurements, the three different samples (cyt *c*, cyt ox, and the cyt *c* + cyt ox complex) were recorded in each possible pairwise combination so that two spectra of the same sample in different combinations were obtained. These conjugate spectra were only combined when the difference spectrum from the same species yielded a straight line. Thus, it was possible to avoid spectral shifts between the different experiments larger than ±0.1 cm^{-1} which corresponds to the calibration accuracy of the spectrometer. In order to improve

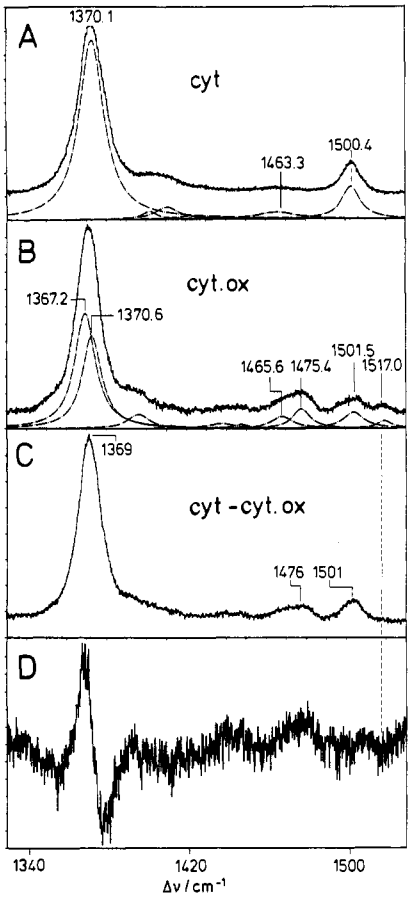

FIGURE 1: RR spectra of ferricytochrome c (A), fully oxidized cytochrome oxidase (B), and the 1:1 complex of both heme proteins (C), excited at 407 nm. (D) is the difference spectrum obtained by subtracting the sum of the spectra of the free heme proteins from that of the complex. The intensity scale of the difference spectrum is enlarged by a factor of 2.8 relative to spectrum C. The dotted lines represent the fitted Lorentzian profiles.

the S/N ratio, it was necessary to repeat this procedure several times using fresh samples. In general, the RR spectra of the mixture of cyt c and cyt ox were obtained by combining the spectra of the separate proteins. As checked by control experiments, these spectra were identical with those measured directly from a physical mixture of cyt c and cyt ox prepared at high ionic strength, for which complex formation does not take place (Weber et al., 1987).

Results and Discussion

Spectral Changes upon Complex Formation. Figure 1A,B shows the RR spectra of cyt c and cyt ox in the frequency range between 1330 and 1530 cm^{-1}. In the following, cyt c and cyt ox refer exclusively to the oxidized forms of both heme proteins. The most prominent bands in this region are the oxidation and spin-state marker bands, principally ν_4 and ν_3, whose frequencies are correlated with the electronic configuration and ligation state of the heme iron (Partharasathi et al., 1987). In the RR spectrum of cyt c (Figure 1A), band-fitting analysis reveals the exact positions of the ν_4 and ν_3 modes at 1370.1 and 1500.4 cm^{-1}, respectively, which agree well with the values expected for a ferric 6cLS heme. Other bands in this spectrum result from the modes ν_{28} (1463.3 cm^{-1}) and ν_{20} and ν_{29} (around 1400 cm^{-1}). In cyt ox, hemes a and a_3 are in different coordination states, hence accounting for the more complex vibrational structure of the RR spectrum in this region (Figure 1B). While the band at 1501.5 cm^{-1} can readily be assigned to the mode ν_3 of heme a (6cLS), in heme a_3 (6cHS) this mode is expected to shift down by approximately 20 cm^{-1}, so that it is assigned to the band at 1475.4 cm^{-1}. Then the overlapping band at 1465.6 cm^{-1} can be attributed to the mode ν_{28} of heme a. The expected downward shift in frequency of the mode ν_4 is significantly smaller (~5 cm^{-1}) than that for ν_3, and, in fact, visual inspection of the broad band at ~1370 cm^{-1} does not reveal any fine structure. From band-fitting analysis, one obtains two components at 1367.2 cm^{-1} (heme a_3) and 1370.6 cm^{-1} (heme a). Upon complex formation, we note distinct changes in the regions of both the ν_4 and ν_3 modes which appear very clearly in the difference spectrum (complex minus mixture; Figure 1D). These findings point to structural alterations in the coordination shell of (one of) the heme group(s).

An interesting feature in the RR spectrum of cyt ox is the weak band at 1517.0 cm^{-1}, which was not assigned in previous RR studies. There is no fundamental mode expected in this region, except for the E_u-mode ν_{38} of a 6cHS heme, so that this band is attributed to heme a_3 rather than to heme a. Most surprisingly, this band vanishes in the RR spectrum of the cyt c + cyt ox complex (Figure 1C).

Spectral changes upon complex formation are also observed in other frequency regions, in particular, between 1600 and 1700 cm^{-1} (Figure 2, below) and between 200 and 500 cm^{-1} (Figure 4, below), as can be seen from the corresponding difference spectra. Since the bands in these regions predominantly reflect the specific interactions of the peripheral substituents of the porphyrin groups with the protein matrix, a careful analysis holds the promise of obtaining more insight into the structural perturbations of the heme pocket(s). These spectral regions are discussed in more detail below.

Assignment of the Spectral Changes to Individual Heme Groups. (A) High-Frequency Region. First, we consider the 1600–1700 cm^{-1} region in more detail (Figure 2). The RR spectrum of cyt c (Figure 2A) is dominated by the band at 1633.5 cm^{-1} which results from the mode ν_{10}. The weaker peak at 1669.0 cm^{-1} cannot be assigned with certainty, but presumably originates from a combination mode. The increasing intensity at the low-frequency extreme of this spectral region comes from the high-frequency wing of the 1584 cm^{-1} band (ν_2). Again, the RR spectrum of cyt ox (Figure 2B) is considerably more complex since, besides the modes ν_{10}, the vinyl and formyl stretching vibrations appear between 1620 and 1680 cm^{-1}. Furthermore, the RR intensity of the 1647 cm^{-1} band of cyt ox is higher by a factor of 2.9 than the strongest band of cyt c at 1633.5 cm^{-1}. This implies that the difference spectrum in Figure 2D predominantly reflects the spectral changes of cyt ox rather than of cyt c, particularly since the most pronounced effects are seen for the region above 1635 cm^{-1}. Thus, to a good approximation, a pure spectrum of cyt ox with cyt c bound can be obtained by subtracting the total contribution of cyt c (Figure 2A) from the spectrum of the complex (Figure 2C).[2] This difference spectrum was analyzed by a band-fitting program and compared with the RR spectrum of the solubilized cyt ox in Figure 3A,B.

The assignment of the bands in this region has been discussed elsewhere (Babcock et al., 1981; Woodruff et al., 1981; Choi et al., 1983; Babcock & Callahan, 1983; Ogura et al.,

[2] In the high-frequency region, the conformational changes of cyt c induced upon binding to cyt ox, i.e., the formation of the conformational state I and the 6cLS configuration of state II (see the following section), have only a small effect on the RR spectrum of cyt c. Subtracting a simulated spectrum of a 1:1 mixture of both states, which was constructed according to the spectral parameters determined in model systems (P. Hildebrandt, T. Heimburg, and D. Marsh, unpublished results), from the spectrum of the complex (Figure 2C) yielded essentially the same spectrum of the bound cyt ox as in Figure 3B.

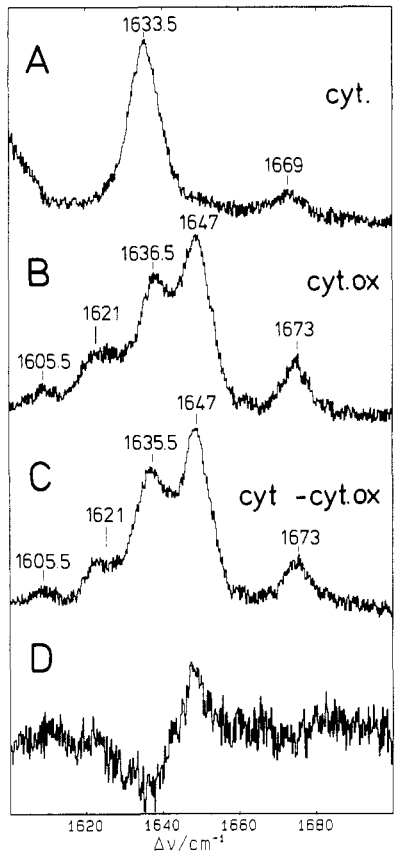

FIGURE 2: RR spectra of ferricytochrome *c* (A), fully oxidized cytochrome oxidase (B), and the 1:1 complex of both heme proteins (C) excited at 407 nm. (D) is the difference spectrum obtained by subtracting the sum of the spectra of the free heme proteins from that of the complex. The intensity scale of the difference spectrum is enlarged by a factor of 2.0 relative to spectrum C.

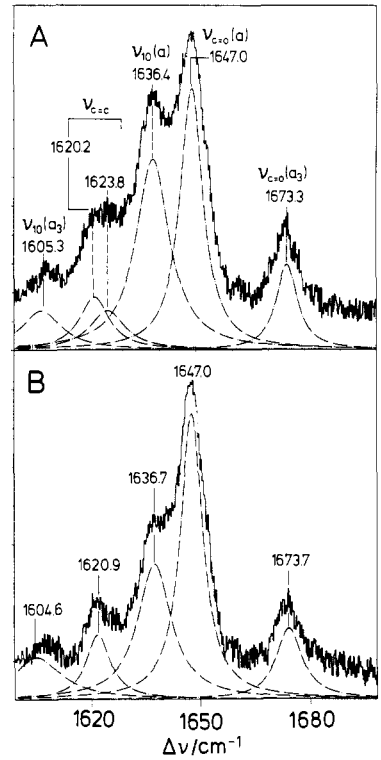

FIGURE 3: RR spectra of the free (A) and bound (B) fully oxidized cytochrome oxidase excited at 407 nm. Spectrum B was obtained by subtracting the total contribution of ferricytochrome *c* from the spectrum of the complex (details of the subtraction procedure are given in the text). The dotted lines represent the fitted Lorentzian profiles.

1984, 1985; Argade et al., 1986; Copeland & Spiro, 1986; Schoonover et al., 1988; Sassaroli et al., 1989). The formyl stretching of heme a_3 can readily be attributed to the peak at 1673.3 cm^{-1}, while the corresponding mode of heme *a* was assigned to the band at 1647.0 cm^{-1} (Figure 3A). The low frequency of this band was attributed to the formation of a hydrogen bond with an adjacent proton acceptor of the protein matrix (Babcock & Callahan, 1983), whereas the position of this band in heme a_3 is in agreement with a non-hydrogen-bonded carbonyl oxygen. For both bands, we note an unusually small half-width (7.6 and 7.1 cm^{-1}), suggesting that these substituents are enclosed in a rigid environment. The spectral parameters (frequencies and half-widths) remain essentially unchanged in the RR spectrum of the enzyme in the complex except for a slight broadening of the 1673 cm^{-1} band. This implies that the strong enhancement of the 1647 cm^{-1} band by a factor of 1.5, which is the most pronounced difference between the spectra in Figure 3A,B, cannot be associated with perturbation of the ground-state conformation of the formyl substituent.

The situation is different for the vinyl stretching vibrations which give rise to the broad shoulder at ~1623 cm^{-1}. In the RR spectrum of the free cyt ox, band-fitting analysis reveals two components at 1620.2 and 1623.8 cm^{-1}. Since the spin and coordination state have no effect on the frequency of this mode, this splitting points to different conformations of these substituents in hemes *a* and a_3. Taking into account that the lowest stretching frequency should be observed for a coplanar geometry of the heme and the vinyl group, the high-frequency component is ascribed to a somewhat tilted substituent. This band shifts downward in the RR spectrum of the complexed cyt ox (Figure 3B) so that it overlaps with the low-frequency component. Attempts to resolve this peak into two components by band-fitting analysis failed, so that it is concluded that the conformations of the vinyl groups in both hemes have become very similar, i.e., coplanar with the hemes. Furthermore, it should be noted that the half-width of the single band is considerably smaller (8.0 cm^{-1}) in the complexed cyt ox than that of the high-frequency component in the RR spectrum of the free cyt ox (11.8 cm^{-1}), suggesting that the downward shift in frequency is accompanied by a narrowing of this band.

On the other hand, the porphyrin modes ν_{10} at 1636.4 cm^{-1} (heme *a*) and 1605.3 cm^{-1} (heme a_3) are largely unchanged on complexation of cyt ox by cyt *c*. Since this mode responds sensitively to changes of the heme core geometry (Kitagawa & Ozaki 1987), it can be concluded that binding of cyt *c* to cyt ox does not affect the coordination shells of hemes *a* and a_3.

(*B*) *Low-Frequency Region.* Now we turn to the low-frequency region (Figure 4), where the vibrational modes include large contributions from the peripheral substituents. Thus, the spectral parameters of these bands depend sensitively on the specific steric and electrostatic interactions of the porphyrin side chains with the immediate protein environment, so that this region can be regarded as a fingerprint of the structure of the heme pocket (Hildebrandt & Stockburger, 1989a). Consequently, it is not surprising that the vibrational patterns of cyt *c* and cyt ox are quite different in this region. This offers the advantage that the assignment of most of the RR bands in the spectrum of the complex (Figure 4C) to one of these heme proteins is straightforward.

The most prominent band of cyt ox at 335 cm^{-1} can be assigned to the ν_8 mode which includes considerable contributions from the bending vibrations of the formyl substituent (Copeland & Spiro, 1986). In the complex, this band sig-

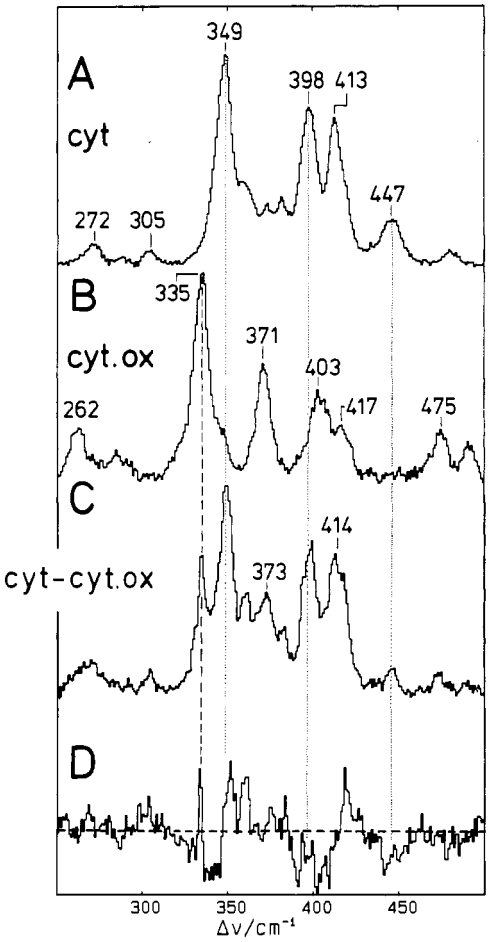

FIGURE 4: RR spectra of ferricytochrome *c* (A), fully oxidized cytochrome oxidase (B), and the 1:1 complex of both heme proteins (C) excited at 407 nm. (D) is the difference spectrum obtained by subtracting the sum of the spectra of the free heme proteins from that of the complex. The intensity scale of the difference spectrum is enlarged by a factor of 2.0 relative to spectrum C.

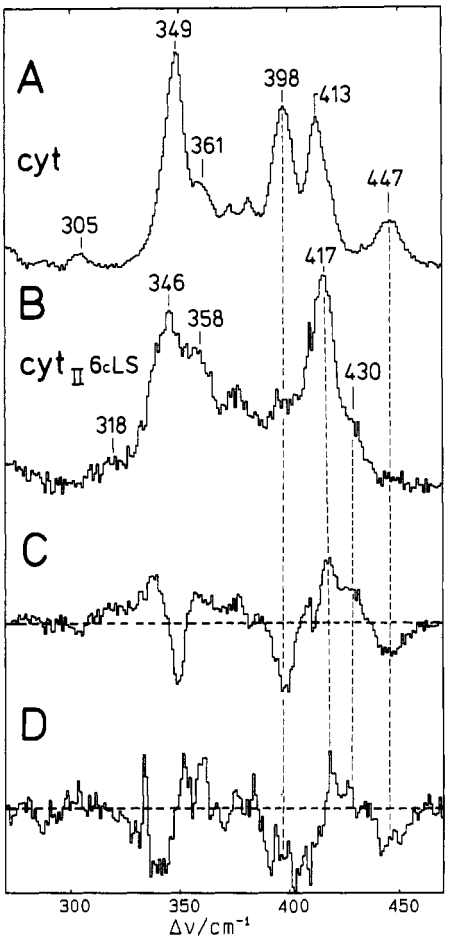

FIGURE 5: (A) RR spectrum of ferricytochrome *c* (as in Figure 4A); (B) SERR spectrum of the 6cLS configuration of state II of cyt *c* adsorbed on a Ag electrode, measured as described by Hildebrandt and Stockburger (1989a); (C) difference spectrum "state II(6cLS) minus free cyt *c*" obtained by subtracting spectrum A from spectrum B; (D) difference spectrum "complex minus free cyt *c*" (as in Figure 4D). The dotted vertical lines draw attention to the bands of greatest change at 447 and 398 cm^{-1}.

nificantly narrows to an approximate half-width of ~6 cm^{-1} (from ~12 cm^{-1} in the free cyt ox; Figure 4B,C), while the frequency remains constant. Evidently, this results from a reduction of the inhomogeneous broadening due to an increased rigidity of the protein matrix, in particular, in the vicinity of the C=O group. Since the C=O stretching vibration of heme a_3 is not subject to any spectral changes upon complex formation, this effect can be attributed to heme *a*.

Since the RR bands of cyt *c* in the low-frequency region are significantly more intense than those of cyt ox, only the strongest bands of cyt ox at 335 and 371 cm^{-1} can be identified unambiguously in the spectrum of the complex. Thus, most of the features in the difference spectrum over this frequency range should result from spectral changes of cyt *c* after binding to cyt ox. For example, the positive and negative peaks at ~350 and 400 cm^{-1} (Figure 4D) should be due mainly to changes in the relative intensities and/or frequencies of the cyt *c* bands at 349, 398, and 413 cm^{-1} (Figure 4A). The most pronounced difference is the decrease of the 447 cm^{-1} band in the complex (compare Figure 4A,C). Since the RR spectrum of cyt ox reveals no band close to this position (Figure 4B), this effect must exclusively be ascribed to cyt *c*. In previous studies, the RR bands in this region were used to monitor structural changes of cyt *c* bound to charged interfaces where cyt *c* exists in the conformational state I and in the 6cLS and 5cHS configurations of state II (Hildebrandt & Stockburger, 1989a,b). For state II, large spectral changes were noted in the low-frequency region when compared to state I or to the free cyt *c*. In Figure 5, such a spectrum of the 6cLS configuration of state II obtained from cyt *c* adsorbed on an Ag electrode is compared with the RR spectrum of the free cyt *c*. The most pronounced differences are the loss of the 447 cm^{-1} band and the decrease in intensity of the bands at 346 and 398 cm^{-1} (Figure 5B). These changes are more clearly evident in the difference spectrum "state II(6cLS) minus free cyt *c*" (Figure 5C). Striking similarities are found with the difference spectrum "complex minus mixture" of the cyt *c*–cyt ox system. (Figure 5D), indicating that the spectral changes of cyt *c* in the complex with cyt ox can be attributed to the (partial) transition to the 6cLS configuration of state II. On the other hand, there is no evidence for a contribution of the 5cHS configuration of state II, whose spectrum exhibits a strong band at 384 cm^{-1} (Hildebrandt & Stockburger, 1989a,b) which, if present, would produce a positive peak in the spectrum of the cyt *c*–cyt ox complex at this position. However, one has to take into account that the band intensities of this configuration under 407-nm excitation are much weaker than for the 6cLS form of state II, so that a small amount of cyt_{II}5cHS may be beyond the limit of detection. An approximate estimate of the cyt_{II}6cLS content, based on the decrease in intensity of the 447 cm^{-1} band in the spectra of Figure 4B,C, yields a value of ~45%. This implies that for the remaining fraction of the bound cyt *c*, the structure is very similar to that of the native cyt *c*. Thus, these molecules most

likely exist in the conformational state I whose low-frequency spectrum is nearly indistinguishable from that of the free species.

(C) Marker Band Region. The formation of state II is accompanied by structural changes of the coordination shell. This is reflected by frequency shifts of the oxidation and spin-state marker bands above 1350 cm^{-1} (Hildebrandt & Stockburger, 1989a). Furthermore, for the conformational state I, these bands also appear at different positions than for the free species. This suggests that the spectral changes observed in the ν_4/ν_3 band region (Figure 1D) arise from the structural modifications of cyt *c* in the complex, rather than of cyt ox. This idea is supported by the invariance of the sensitive marker bands ν_{10} of hemes *a* and a_3 which rules out structural changes of the coordination shells in cyt ox. The only spectral change in the marker band region which can be attributed to cyt ox is the reduction in intensity of the ν_{38} band which must be due to perturbations of the resonance enhancement rather than of the ground-state conformation, as will be described below.

On the basis of these considerations, the spectral range between 1330 and 1530 cm^{-1} which includes the marker bands ν_4 and ν_3 (Figure 1C) was analyzed by a modified band-fitting procedure (Hildebrandt & Stockburger, 1989a). The starting point for this approach is the assumption that the spectral parameters of the marker bands (frequency, half-width, and intensity ratio of ν_4 and ν_3) of heme *a* and heme a_3 are the same as in the free cyt ox. For cyt *c*, two sets of parameters were adopted from a previous study corresponding to the conformational states I and II(6cLS).[3] These marker bands are at 1369.1 and 1499.4 cm^{-1} in state I and at 1374.0 and 1504.6 cm^{-1} in state II(6cLS). Then, in the first step of the fitting routine, the spectral parameters of all ν_4 and ν_3 bands were kept constant so that the spectrum of the complex (Figure 1C) was essentially simulated by an appropriate superposition of the RR spectra of the presumed components. In the second step, these spectral parameters were released in order to optimize the fit. It is interesting to note that this did not lead to variations of the spectral parameters greater than ± 0.4 cm^{-1} for the frequencies and half-widths and $\pm 10\%$ for the ν_4/ν_3 band intensity ratio, hence justifying the underlying assumptions of this approach. However, we noted a slight increase by nearly 10% in the intensities of the RR bands from heme a_3 compared to those from heme *a*, suggesting an increased resonance enhancement for heme a_3 in the complex.

The result of this procedure is shown in Figure 6. For the sake of clarity, we have subtracted the simulated bands of cyt ox from the spectrum so that it exclusively displays the RR bands of cyt *c*. The intensity distribution between the marker bands of state I and state II(6cLS) can then be used to calculate the fractions of both conformers, based on the relative Raman cross sections determined in a previous study.[3] Within experimental accuracy, the value of 55% obtained for the relative content of cyt_{II}6cLS is in good agreement with the estimate obtained from the low-frequency region.

Finally, we mention that spectral changes were also found for the complex between reduced cytochrome *c* and the partially reduced cyanide-ligated cyt ox (data not shown). However, these effects were significantly smaller than for the fully oxidized complex, so that the interpretation was extremely difficult. This may be due to the low resonance enhancement of the reduced state II compared to state IB with 413-nm excitation, so that even fractional populations of up to 50% are not adequately reflected in the RR spectrum.

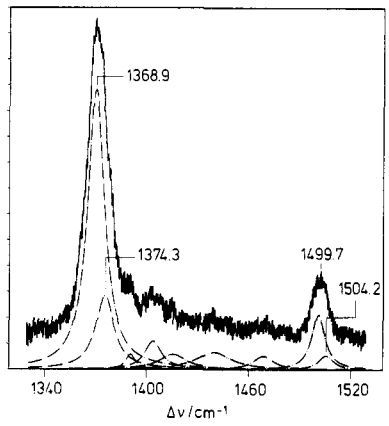

FIGURE 6: RR spectrum of ferricytochrome *c* bound to fully oxidized cytochrome oxidase. The excitation wavelength was 407 nm. The dotted lines represent the fitted Lorentzian profiles. The RR bands of cytochrome oxidase were subtracted (see text for further details).

Conformational Changes in Cytochrome c. Binding of cyt *c* to cyt ox induces the transition to the conformational states I and II. This process is a general effect for cyt *c* on binding to negatively charged interfaces (Hildebrandt & Stockburger, 1989b). It has been observed in a variety of systems, including metal electrodes covered with specifically adsorbed anions, phospholipid vesicles, and inverted micelles, large inorganic anions, and proteins. In all these systems, the interaction domain on cyt *c* is the same as for binding to cyt ox, i.e., the positively charged lysine-rich front surface around the exposed heme edge. The only common structural element of these model systems is the more or less regular array of negative charges which provide the binding site for cyt *c*. Although the dominant role of electrostatic interactions in the reaction with cyt ox has been demonstrated by a variety of experimental studies (Smith et al., 1977; Speck et al., 1979; Rieder & Bosshard, 1978; Koppenol & Margoliash, 1982), the similarity between the structural response of cyt *c* in the complexes with its physiological redox partner and with the model systems is surprising. It suggests that, in the cyt *c* + cyt ox complex, other types of interaction (e.g., hydrophobic, van der Waals, etc.), which may arise from the specific architecture of the binding site and hence can hardly be simulated by the model systems, are of minor importance.

On the basis of extensive studies of cyt *c* bound to these model systems (Hildebrandt & Stockburger, 1989a,b), a detailed picture is available of the molecular changes associated with the formation of states I and II. In state I, the entire protein and heme structure are the same as in solution, and the small frequency shifts of some of the porphyrin modes can satisfactorily be explained by an "electrochemical Stark effect". The changes in state II are restricted to the heme crevice, which assumes a more open structure than in state I. This is accompanied by a weakening of the iron–methionine bond which facilitates the thermal dissociation of this ligand. As a result, steric constraints on the heme, which, in state I, are imposed by the protein matrix, are largely removed in state II, and the mobility of the heme increases considerably. Furthermore, the heme environment becomes less hydrophobic, as is reflected by the large negative shift in redox potential.

[3] The spectral parameters of ν_4 and ν_3 for the states I and II(6cLS) differ slightly in the various model systems. This difference was attributed to the existence of conformational substates (Hildebrandt & Stockburger, 1989b). Here we have chosen the values determined from cyt *c* bound to phospholipid vesicles which appeared to be a more appropriate model system for the physiological complex than the Ag electrode (P. Hildebrandt, T. Heimburg, and D. Marsh, unpublished results). From that study, we also adopted the relative Raman cross sections for the 407-nm excitation line.

In this context, it is interesting to refer to the recent work by Bosshard and co-workers (Weber et al., 1987; Michel et al., 1989) whose conclusions from optical absorption, circular dichroism, and magnetic circular dichroism studies can be correlated rather well with the present RR data. The authors attributed the spectral changes, which were assignable to cyt *c*, to a localized structural modification of the protein surface around the exposed heme edge. Taking into account previous NMR results (Falk & Ångström, 1983; Satterlee et al., 1987), they suggested that binding to cyt ox induces the rupture of the Lys^{13}–Glu^{90} salt bridge so that loop 80–90 including Phe^{82} is pulled away from the heme edge and toward the cyt ox.

One might argue that the complex formation of cyt *c* at two different binding sites of cyt ox [high- and low-affinity binding sites; see Wikström et al. (1981)] stabilizes either of the conformational states I or II at the separate sites. However, this is unlikely since the approximate 1:1 mixture of both conformational states would imply that the binding constants are nearly the same for both sites which is in contradiction to the experimental findings [see, for example, Pettigrew & Moore (1987)]. Furthermore, Bosshard's group has provided strong evidence that the spectral changes of cyt *c* (and cyt ox) are exclusively associated with the occupation of the high-affinity binding site (Michel & Bosshard, 1989; Michel et al., 1989), which, in addition, appears to be the only functionally relevant interaction site (Michel & Bosshard, 1989). Thus, we conclude that the distribution between states I and II simply reflects the conformational equilibrium of cyt *c* bound to the high-affinity site of cyt ox. This is in agreement with the situation observed for cyt *c* bound to other negatively charged surfaces (Hildebrandt & Stockburger, 1989a,b).

Conformational Changes in Cytochrome Oxidase. The translation of the spectral changes associated with hemes *a* and a_3 to a molecular description is far more difficult than in the case of cyt *c*. Since there are no frequency shifts except for the vinyl stretching vibration, it must be concluded that the ground-state conformations of the porphyrin ring systems themselves are not affected. Hence, there must be other structural parameters which are modified upon complex formation.

Let us consider first the intensity changes of the porphyrin modes of heme a_3. The A_{1g} modes ν_4 (1367.2 cm^{-1}) and ν_3 (1475.4 cm^{-1}), whose intensities are governed by the oscillator strength of the Soret transition (Spiro, 1983; Remba et al., 1979), increase slightly in the complex, while the E_u mode ν_{38} (1517.0 cm^{-1}) shows a considerable lowering in intensity. A reasonable explanation is that the Soret transition is slightly blue-shifted so that it comes in closer resonance with the 407-nm excitation line. This blue-shift may be induced by the displacement of hydrophobic or polar amino acids, so that the effective dielectric constant is modified in the surroundings of the π-electron system (Hildebrandt et al., 1988). Similar intensity changes are induced in the RR spectrum of cytochrome P-450 by binding of aromatic substrates which are assumed to form a sandwich-type arrangement with the porphyrin ring system (Hildebrandt et al., 1989). The idea of structural changes at the binding site of heme a_3 is consistent with recent findings by Musatov and Konstantinov (1988) that cyanide binding to heme a_3 is strongly accelerated after the complex formation of cyt *c* with cyt ox. This was taken as evidence for a conformational change of the protein matrix around heme *a*.

Now we turn to the formyl stretching vibration of heme *a* at 1647 cm^{-1} (Figure 3). This band becomes RR-active due to the coupling of the C=O bond with the delocalized π-electron system of the porphyrin. Since for heme *a* no intensity changes of the porphyrin modes are observed, the strong enhancement of the C=O stretching band cannot be explained by a perturbation of the Soret transition. Furthermore, it is not very likely that an increase of the electronic coupling is induced by a change of the porphyrin–formyl bond angle, since this would lead to a frequency shift of the C=O stretching vibration. The position of this band, however, remains constant. Consequently, one has to conclude that the origin of the increased RR intensity lies in the Franck–Condon factor, i.e., in a structural parameter localized to the C=O group.

As seen from the decrease in half-width of the ν_8 band (335 cm^{-1}), the flexibility of the formyl substituent of heme *a* is strongly hindered in the complexed cyt ox. This may arise from a fixing of this group in a narrow pocket in the protein, formed by displacement of the amino acid side chains. Hence, it is possible that the underlying molecular changes are the same as those giving rise to the enhancement of the C=O stretching band.

Finally, we consider the downward shift in frequency and band narrowing of the vinyl stretching vibration (Figure 3). Although the assignment to heme *a* is not unequivocal, these spectral changes are consistent with the discussion of the heme *a* pocket outlined above. This would also imply that, in the opposite part of the heme periphery, cyt *c* binding induces structural changes which force the porphyrin substituents into a rigid conformation.

Conclusions

The physiologically more important complex is formed between ferrocytochrome *c* and cyt ox, while the present study was restricted mostly to the fully oxidized complex of cyt *c* and cyt ox. Nevertheless, it is very likely that the observed conformational changes also occur in the electron-transfer complex since, for cyt *c*, it was found that the conformational transition to state II occurs under essentially the same conditions in the reduced and in the oxidized form of the heme iron (Hildebrandt & Stockburger, 1989a,b). Thus, our findings suggest that the electron transfer from ferrocytochrome *c* to cyt ox is governed by the conformational equilibrium between states I and II. These states exhibit different redox potentials, i.e., +0.02 and −0.41 V (vs saturated calomel electrode), so that the conformational equilibrium of the bound cyt *c* can control the rate and the unidirectional flow of the electron transfer [for a detailed model, see Hildebrandt and Stockburger (1989b)]. Also, the conformational changes in cyt ox upon cyt *c* binding may be of physiological relevance. The proposed rigid fixation of heme *a* in the protein matrix may lead to the proper alignment required for the electron transfer.

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Registry No. Cyt *c*, 9007-43-6; cyt *c* ox, 9001-16-5.

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