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Structural studies of cytochrome *c*-554 from *Chloroflexus aurantiacus* by resonance Raman spectroscopic techniques *,**

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The four-heme cytochrome *c*-554 of *Chloroflexus aurantiacus* was studied by resonance Raman and surface-enhanced resonance Raman spectroscopy. The resonance Raman spectra show the characteristic vibrational signature of *c*-type hemes as demonstrated by a comparison with those of horse-heart cytochrome *c*, which were obtained under identical conditions. Spectral differences between both cytochromes can be related to the porphyrin geometry and the interactions of the heme with the immediate protein environment. Asymmetry of bands of cytochrome *c*-554 in all spectral regions provides support for conformational heterogeneity among the four hemes. The core-size-sensitive marker bands are interpreted in terms of a slight core contraction of the tetrapyrrole macrocycle in the six-coordinated low-spin states with respect to horse-heart cytochrome *c*. Shoulders on the low-frequency side of these modes indicate that at least one of the cytochrome *c*-554 hemes partially exists in a five-coordinated high-spin configuration. The analysis of the low frequency region provides evidence that some of the hemes are located in a more open and flexible protein pocket than in cytochrome *c*, accounting for the low-lying redox potentials. These structural data are complemented by the surface-enhanced resonance Raman results of cytochrome *c*-554 adsorbed on colloidal silver. These spectra reveal a significantly larger fraction of the five-coordinated high-spin configuration than in the case of cytochrome *c*. This can be attributed to a higher susceptibility to conformational distortions of these heme pockets upon electrostatic interactions with the charged silver surface.

Introduction

Chloroflexus aurantiacus is the only member of a family of green gliding bacteria. Its structural similarities to both purple photosynthetic bacteria [1] and green sulfur bacteria [2] place it in a position crucial to our understanding of the development of various components of photosynthetic energy production. The four-heme cytochrome *c*-554 (C-554) is a membrane-bound cytochrome, the function of which is apparently to re-reduce the photooxidized special pair of bacteriochlorophylls in the reaction center of *C. aurantiacus* [3,4], analogous to the four heme cytochrome *c*-558-553 of *Rhodopseudomonas viridis* [5].

C-554 has recently been purified and partially characterized by Freeman and Blankenship [6], showing that the cytochrome has a molecular mass of 43 kDa and contains four hemes, the redox potentials of which vary relatively widely (0, +120, +220 and +300 mV, with respect to the normal hydrogen electrode), suggesting that the hemes are in quite different local environments. The protein is apparently not as intimately bound to the reaction center as is the cytochrome *c*-558-553 of *R. viridis*, based on the fact that it does not copurify with the reaction-center preparations [6,7].

It has long been a goal to uncover the parameters governing the midpoint redox potentials of cytochromes. So far, the factors which have emerged as being important are the heme ligands, the local dielectric constant, geometric distortions imposed upon the

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Abbreviations: C-554, cytochrome *c*-554 from *Chloroflexus aurantiacus*; HH, mitochondrial cytochrome *c* from horse heart; LDAO, lauryldimethylamine *N*-oxide; 6cLS, six-coordinated low-spin; 5cHS, five-coordinated high-spin; RR, resonance Raman; SERR, surface enhanced resonance Raman; Ag sol, suspensions of colloidal silver.

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heme by the surrounding protein, and hydrogen bonding networks involving the heme ligands and the heme itself [8]. Hence, structural information about the redox centers of C-554 is desired to elucidate the molecular basis for the different redox potentials and to contribute to a better understanding of the electron transfer mechanism.

In this respect, resonance Raman (RR) spectroscopic methods are a powerful tool, since they selectively probe the vibrational pattern of the heme and give insight into the structure of the heme and its interactions with the protein environment [9]. The sensitivity of the RR effect can be strongly improved by adsorbing the biomolecule on submicroscopically rough metal surfaces, such as colloidal silver (Ag sol). Under these conditions the RR signals exclusively of the adsorbed molecules can be enhanced by several orders of magnitude, depending on the proximity and orientation of the chromophores with respect to the surface (surface-enhanced resonance Raman (SERR) spectroscopy) [10]. This technique offers the additional advantage of studying conformational changes of the heme pocket induced by the electrostatic interactions with the electrical double layer of the charged metal surface [11,12].

We report here the RR spectra of C-554 in both the oxidized and reduced states, along with the SERR spectra of C-554 on Ag colloids. These spectra are compared with those of horse-heart cytochrome *c* taken under identical conditions. The present work is directed to study the detailed interactions of the heme groups and the protein environment in an attempt to characterize the structural basis of the different redox potentials.

Materials and Methods

Chloroflexus aurantiacus was grown and membranes isolated as described previously [13]. Pelleted membranes were resuspended in 20 mM Tris-HCl (pH 9.0) buffer to an optical absorbance of 12–14 units at 865 nm and stored at −80°C until further isolation. Membrane proteins were solubilized by incubating the membranes with 0.7% lauryldimethylamine *N*-oxide (LDAO) for 1 h at 4°C under stirring. Incubated membranes were centrifuged at 43 000 rpm in a Beckmann Ti70 rotor for 1.5 h to remove chlorosomes. The supernatant was further purified on a DEAE-Sephacel (Pharmacia) column, equilibrated with 20 mM Tris-HCl (pH 9.0). Free bacteriochlorophyll and carotenoid pigments were removed by washing with 20 mM Tris-HCl (pH 8.0)/0.3% LDAO. Following the elution of the protein and reaction-center contaminations with 30 mM and 60 mM NaCl, respectively [7], the crude C-554 was obtained with 150 mM NaCl. From five different preparations, such fractions, which exhibit an absorbance ratio A_{280}/A_{413} of 1.25, were combined, diluted by a factor of 2 with 20 mM Tris-HCl (pH 9.0)/0.3% LDAO and purified on a TSK-DEAE-G50(S)-Fractogel (Merck) column, equilibrated with the same buffer solution. C-554 was eluted with a linear gradient of 20 mM Tris-HCl (pH 8.0)/50 mM NaCl/0.1% LDAO and 20 mM Tris-HCl (pH 6.0)/300 mM NaCl/0.1% LDAO. All cytochrome-containing fractions with an A_{413} of more than 2 were collected, yielding an A_{280}/A_{413} ratio of 0.8. The sample was concentrated by eluting with 300 mM NaCl from a DEAE-Sepharose CL-6B (Pharmacia) column, which was equilibrated with 20 mM Tris-HCl (pH 9.0). The A_{280}/A_{413} ratio was further decreased to 0.65 using a Pharmacia Sephacryl S100 HR column (elution with 20 mM Tris-HCl (pH 8.0)/0.1% LDAO). The final purification step was carried out with a DEAE-Sephacel column, equilibrated with 20 mM Tris-HCl (pH 8.0)/0.1% Deriphat-160 (Serva), to remove protein contaminations by washing with NaCl solutions of increasing concentrations up to 200 mM. The purified C-554, which was obtained with 250 mM, exhibited an A_{280}/A_{413} ratio of 0.35, which is in good agreement with the value reported by Freeman and Blankenship [6].

Polyacrylamide gel electrophoresis was performed as described elsewhere [13], using the Pharmacia PhastSystem and 10–15% PhastGels with SDS buffer strips. The samples were adjusted to an A_{280} of 0.05 for protein staining and of 0.8 for heme staining by diluting with the incubating buffer. Incubation was performed with buffer containing 5% SDS, 1% mercaptoethanol and a few drops of Bromophenol blue at 50 °C for 5 min. Sensitive silver staining and 3,3′,5,5′-tetramethylbenzidine staining were carried out according to Refs. 13–15. Molecular weights were determined according to Weber and Osborne [16] using the LKB molecular weight markers cytochrome *c* (equine), 12 300; myoglobin (equine), 17 200; carbonic anhydrase (bovine), 30 000 ; ovalbumin (hen egg), 43 000; albumin (bovine serum), 66 250; ovotransferrin (hen egg), 76 000–78 000. All these methods yield a molecular weight of 43 000, confirming results by Freeman and Blankenship [6]. Also, the absorption spectra are identical to those published by these authors.

Horse-heart cytochrome *c* was purchased from Sigma (type VI) and used without further purification. Reduced and oxidized cytochromes *c* and *c*-554 were obtained upon titrating the samples with freshly prepared solutions of sodium dithionite and $K_3[Fe(CN)_6]$, respectively, monitoring the relative RR intensities of the oxidation marker bands, ν_4.

RR spectra, excited with the 413 nm line of a Kr^+ laser (model 171, Spectra Physics), were measured using an optical multichannel detection system (Triplemate 1877, Spex Instruments, equipped with an

O-SMA intensified diode array, Spectroscopy Instruments). The spectral band width was about 5 cm^{-1} and the resolution per diode about 1 cm^{-1}. The samples were deposited in a rotating cuvette. The power of the exciting laser beam, which was focused by an 8 cm lens, was about 25 mW at the cell. The total accumulation time was between 15 and 30 min.

For RR experiments, the protein was concentrated to an optical absorbance of 2.0 at 413 nm in 20 mM Tris-HCl/0.3% LDAO at pH 8.0.

SERR spectra of the cytochromes were measured from colloidal silver suspensions (Ag sol) which were prepared as described previously [17]. In these experiments, the protein concentrations were about 10 nM.

Results and Discussion

The RR spectra of the fully oxidized and fully reduced C-554 were measured in the frequency range between 200 and 1700 cm^{-1}. In general, the spectra show far-reaching similarities with those of other *c*-type heme proteins, such as cytochrome *c* [12,18]. There is no evidence for bands which are characteristic for *b*-type hemes, such as bands originating from the vinyl vibrations. This confirms the assignment of C-554 as a *c*-type heme protein [6].

Several spectral regions are of particular interest, since they include RR bands which are well correlated with structural parameters of the heme and its immediate protein environment [9]. In previous RR studies on mammalian and bacterial cytochrome *c*, a vibrational assignment was suggested based on a comparison with experimental and theoretical data of model compounds [18]. This assignment is also adopted in this work.

Marker band region

Most of the bands above 1300 cm^{-1} are characteristic marker bands for the electronic configuration and the ligation state of the heme iron [19,20]. Among them is the mode ν_4, which is known to be a particularily sensitive marker for the oxidation state of the heme iron. In C-554, this band shifts down from 1374 cm^{-1} in the oxidized to 1361 cm^{-1} in the reduced state, showing the same behavior as in HH (spectra not shown here). It should be mentioned that for those hemes in which the iron is coordinated by potent electron donors such as thiolate or tyrosinate, frequency shifts greater than 20 cm^{-1} are observed upon reduction [21]. Thus, it is reasonable to assume that, in analogy to HH and other *c*-type heme proteins, a histidine and a methionine serve as axial ligands in C-554 [6].

Other marker bands, such as ν_3, ν_2 or ν_{10} are, in addition, indicators for the core size of porphyrins, which in turn depends on the spin- and coordination state of the heme iron [19,20]. This spectral region of both C-554 and HH is displayed on an extended view in Figs. 1 and 2. For the oxidized HH, this part of the spectrum was analyzed in great detail [18] and the bands at 1464, 1501, 1561, 1583 and 1634 cm^{-1} (Fig. 1B) could readily be assigned to the modes ν_{28}, ν_3, ν_{11}, ν_2 and ν_{10}. Furthermore, it was found that the asymmetric lineshape of the 1583 cm^{-1} peak is due to a contribution of a weak band at 1595 cm^{-1}, attributable to the E_u-mode, ν_{37} [18]. The RR-activity of this mode, which is forbidden within the D_{4h} point group, was taken as an indication for a deviation from the planar porphyrin geometry. The frequencies of these modes correspond well to the values expected for a six-coordinated low-spin (6cLS) heme [19,20]. This is also the case for the frequencies of the main peaks in the RR spectrum of C-554, although the values are distinctly higher than in HH. Furthermore, we note asymmetric bandshapes in particular for the modes ν_3 and ν_{10}, the latter revealing a shoulder at 1627 cm^{-1} (Fig. 1A*), which is not seen for HH.

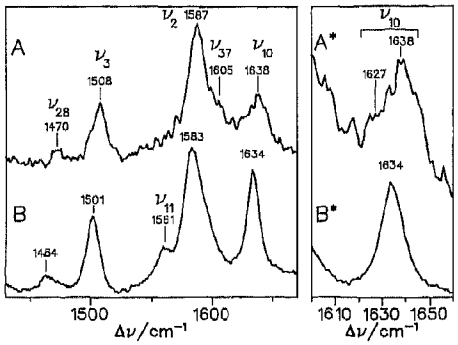

Fig. 1. RR spectra of the fully oxidized cytochrome *c*-554 (A) and the oxidized cytochrome *c* (B) in the marker band region excited at 413 nm. The spectra A* and B* display the ν_{10} band region on an extended scale.

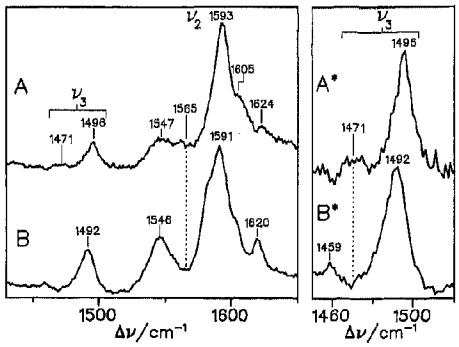

Fig. 2. RR spectra of the fully reduced cytochrome *c*-554 (A) and the reduced cytochrome *c* (B) in the marker band region excited at 413 nm. The spectra A* and B* display the ν_3 band region on an extended scale.

Comparable differences in the marker band region between C-554 and HH are also noted for the reduced forms (Fig. 2). Again, the main peaks of C-554 are in line with a 6cLS configuration, but they are upshifted compared to the corresponding modes of HH. Furthermore, a weak but unambiguously detectable band at 1471 cm^{-1} appears in C-554 which is missing in HH (Fig. 2A*, B*). Since no other fundamentals are expected in this region, the band at 1471 cm^{-1} should be assigned to ν_3 of a high-spin (HS) ferrous heme [19], although an additional contribution from the ν_{28} mode cannot be ruled out. A transition to the HS configuration is accompanied by an expansion of the porphyrin core, which in turn, is reflected by a frequency downshift of the marker bands. Correspondingly, the same interpretation holds for the increased RR intensity at about 1565 cm^{-1} in C-554 which has to be compared with the trough between the bands at 1546 (ν_{11}) and 1591 cm^{-1} (ν_2) in HH. This points to a new band at about 1565 cm^{-1} which is readily ascribed to ν_2 of a HS configuration. Furthermore, it may be that the broad shoulder at 1605 cm^{-1} not only results from the mode ν_{37} (as in HH) but also includes a contribution from the ν_{10} mode of a HS configuration. In a similar way, the 1627 cm^{-1} shoulder in the RR spectrum of the oxidized C-554 (Fig. 1A) may be interpreted in terms of the ν_{10} mode of an HS configuration. While for reduced hemes, the RR frequencies of a marker band of a five-coordinated high-spin (5cHS) and 6cHS configuration are very similar, in the oxidized state a frequency of 1627 cm^{-1} for ν_{10} clearly indicates a 5cHS species [19].

Since the RR cross-sections at 413 nm excitation can be substantially different for the marker bands of the HS and LS configurations [12], the comparison of the relative RR band intensities does not permit a precise quantification of the fractions of the two spin configurations. However, based on the relative RR cross-sections previously determined for the ν_3 mode of different spin and coordination states of HH [12], a crude estimation yields an upper limit of one out of four hemes of C-554 being in the HS configuration. Moreover, we assume that one heme exists in an equilibrium between a 6cLS and a 5cHS configuration so that the total fraction of HS hemes is even lower than 25%. This would explain why in the UV-vis absorption spectrum no characteristic features of a HS heme, such as a blue-shifted shoulder of the Soret band, can be detected.

Following this interpretation, the remaining three heme groups are in a pure 6cLS configuration. Notwithstanding the far-reaching similarities with the RR spectra of HH, it is obvious that there are also some structural differences between the 6cLS hemes of both proteins. The notably higher frequencies of the marker bands in C-554 may reflect a core contraction compared to HH [19,20]. A possible explanation may be that the axially bound histidine assumes an orientation with respect to the porphyrin plane, in which the hydrogen atoms of the imidazole rings approach more closely the non-bonding d_{xz}- and d_{yz}-orbitals of the iron [12]. As was inferred from the crystal structures of model compounds in relation to mitochondrial cytochrome *c* [22,23], such a conformational difference can cause a core contraction by about 0.01 Å. This in turn would correspond to a frequency upshift of the marker bands by about 4 cm^{-1} [19], which compares well with the experimental data for both the oxidized and the reduced C-554. Alternatively or additionally, electronic effects may contribute to the frequency shifts [19]. It may be that the electron donating (accepting) properties of the axial ligands are different than in HH, for example, if these ligands are involved in different hydrogen-bonding interactions with nearby amino-acid residues or water molecules.

In this context, it is necessary to remember that there are, at least, three heme groups contributing to the 6cLS marker bands in C-554. It may be that due to structural differences among these hemes, the observed frequencies actually reflect the maxima of band envelopes including three slightly separated components for each mode. However, the resolution of the present RR spectra is not sufficient to resolve such envelopes adequately.

Fingerprint region

The bands below 500 cm^{-1} originate from modes involving significant contributions of the peripheral substituents of the heme [18]. Since these side-chains are in intimate contact with those amino-acid residues constituting the heme pocket, even subtle conformational differences in the protein environment are sensitively reflected by these bands so that they can be regarded as the characteristic fingerprint for the specific heme–protein interactions. These spectra of both C-554 and HH are shown in Figs. 3 and 4 on an extended scale. As expected, the spectral differences between these proteins are much more pronounced than in the marker band region. As was shown in previous, more detailed studies [11,18], in the region between 300 and 450 cm^{-1}, HH exhibits 8 and 10 bands in the oxidized and reduced form, respectively. The RR spectra of C-554 reveal an even more complex vibrational structure in this region which is only poorly resolved, indicating that the number of contributing bands is even larger than in HH. For example, the troughs between the relatively sharp peaks in the reduced HH appear to be partly filled up in C-554 (Fig. 4). This suggests that the vibrational modes of the individual hemes of C-554 are not located at the same position but are slightly separated. Taking into account that the 5cHS content is very small and, at least in the

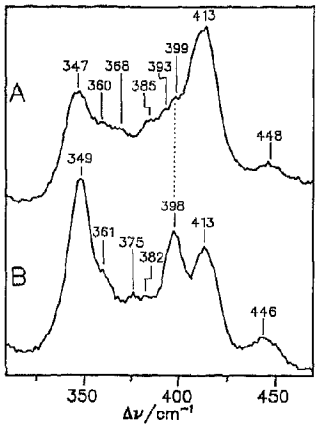

Fig. 3. RR spectra of the fully oxidized cytochrome *c*-554 (A) and the oxidized cytochrome *c* (B) in the fingerprint region excited at 413 nm.

reduced form, the RR bands of 5cHS hemes are much less enhanced at 413 nm excitation than those of 6cLS hemes [24], one can safely conclude that these spectra exclusively reflect the bands of those hemes which are in the 6cLS configuration.

Since a detailed analysis of this region require better-resolved spectra, at present, we just want to focus on those modes which are subject to the most pronounced changes and which can be correlated with structural properties of certain parts of the heme pocket. In the oxidized HH, the intensity of the bands at 398 and 446 cm^{-1}, which are assigned to the combination mode $\nu_{34} + \nu_{35}$ (unpublished data) and the propionate bending vibration, respectively, is strongly reduced upon opening of the heme pocket [18]. It is now interesting to note that a similar effect is observed in the RR spectrum of C-554, where both bands are very weak, compared for example with the 413 cm^{-1} band (Fig. 3). Adopting the interpretation elaborated for HH, it is suggested that there are heme groups in C-554 which are located in a more flexible environment, i.e., their heme pockets assume a more open structure than in the native HH.

Another remarkable spectral difference refers to the 265 cm^{-1} band of the reduced HH which is assigned to the normal mode ν_9. In C-554, this relatively strong band is replaced by a weak and very broad peak, centered at about 271 cm^{-1}, and, in addition, a new band at 287 cm^{-1} appears. A similar splitting of this mode was recently observed in a yeast iso-1-cytochrome *c* mutant, in which Phe-82, located at the exposed heme edge, was replaced by a serine (unpublished data). This replacement not only cause a structural change at the mutation site but also affects the interior of the heme pocket, in particular, the propionate side-chains of the heme and its immediate protein environment [25]. In conjunction with the intensity decrease and the frequency shifts of the propionate bending vibrations in the RR spectra of C-554, these findings suggest that hydrogen bonding interactions of these substituents are different compared with HH.

Also other regions in the RR spectra of C-554 indicate that the conformations of the heme pockets of the four hemes are slightly different. Fig. 5 compares the RR spectra of C-554 and HH in the range between 1060 cm^{-1} and 1260 cm^{-1}. One can immediately see that in C-554 most of the bands are considerably broader and exhibit quite asymmetric lineshapes. Typical examples are the peaks at 1229 and 1172 cm^{-1} of the reduced form and the band at 1129 cm^{-1} of the oxidized state. Even though the vibrational assignment of these bands is not fully settled, these observations can be taken as an evidence for different structural environments of the individual hemes.

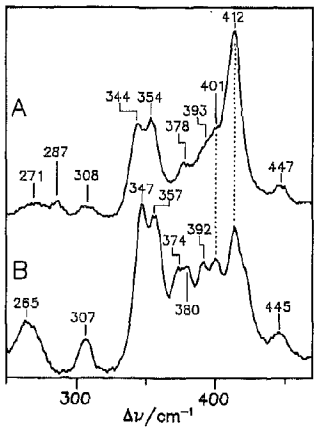

Fig. 4. RR spectra of the fully reduced cytochrome *c*-554 (A) and the reduced cytochrome *c* (B) in the fingerprint excited at 413 nm.

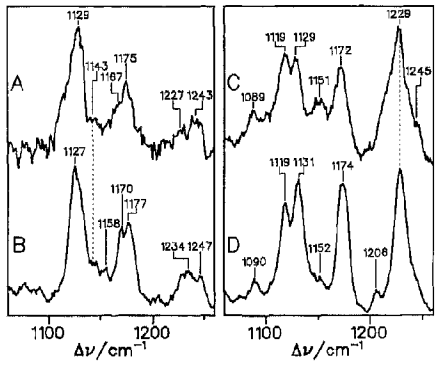

Fig. 5. RR spectra of the fully oxidized cytochrome c-554 (A), the oxidized cytochrome c (B), the fully reduced cytochrome c-554 (C) and the reduced cytochrome c (D) in the region between 1060 and 1260 cm^{-1}, excited at 413 nm.

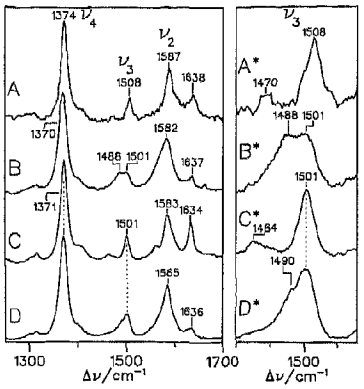

Fig. 6. RR and SERR spectra of the fully oxidized cytochrome *c*-554 and the oxidized cytochrome *c* the marker band region excited at 413 nm. (A) RR, cytochrome *c*-554, aqueous solution; (B) SERR, cytochrome *c*-554, adsorbed on Ag sol; (C) RR, cytochrome *c*, aqueous solution; (D) SERR, cytochrome *c*, adsorbed on Ag sol; The spectra labelled by * display the ν_3 band region on an extended scale.

SERR experiments

Previous studies have shown that adsorption of HH on negatively charged surfaces such as silver electrodes (colloids), induce the formation of a conformational equilibrium between two states (I, II) [12,18,26]. While in state I the structure of the heme pocket (and of the entire protein) is unchanged compared to the unbound HH, in state II the electrostatic interactions with the positively charged lysine residues around the exposed heme edge can cause an opening of the heme crevice [18]. This structural rearrangement of the heme pocket is accompanied by a weakening of the iron–methionine bond so that a thermal coordination equilibrium between a 5cHS and a 6cLS configuration is established, which is nicely detectable in the SERR experiment. Fig. 6D shows such a SERR spectrum of the oxidized HH adsorbed on colloidal Ag. Compared to the RR spectrum of the dissolved HH (Fig. 6C), the marker bands are considerably broadened reflecting the overlapping contributions of the various spin and coordination states. This is most clearly seen for the mode ν_3 (Fig. 6D*), which exhibits a distinct shoulder at 1490 cm^{-1} indicative of the 5cHS configuration of state II, while the modes of the 6cLS configurations of the states I and II (at 1499.5 and 1503.1 cm^{-1} [12]) constitute the broad peak at 1501 cm^{-1}. Most likely, a similar conformational change occurs when C-554 is bound to the metal surface, since the strong band at 1488 cm^{-1} indicates a significant fraction of the hemes being in the 5cHS configuration. A crude estimate yields a total amount of the 5cHS form of about 50%.

Since the three-dimensional structure of C-554 is not known, we have no information about the proximity and the orientation of the individual hemes with respect to the Ag surface. These parameters, however, determine the enhancement of the RR scattering in the SERR experiments [10]. Thus, it is possible that not all of the heme groups contribute to the SERR signals to the same extent. Moreover, it may be that only those porphyrin chromophores which are located close to that part of the protein surface which is in contact with the metal surface are selectively probed by this technique [27,28]. For these hemes, however, the transition from the 6cLS- to the 5cHS configuration is facilitated compared to HH, as can be inferred from the intensity distribution of the corresponding marker bands ν_3 (cf. Fig. 6B*, D*). This suggests that these heme pockets of C-554 are structurally more flexible than in the mitochondrial cytochrome *c*.

Heme structure and redox potentials

The RR and SERR spectra of C-554 were interpreted in terms of structurally different heme pockets. The partial conversion to the 5cHS state indicates that, at least for one heme, the interactions with the immediate protein environment are significantly different than for the others, facilitating the dissociation of one axial ligand. In addition, a differentiation of the heme pockets of the 6cLS states can be inferred from the intensity lowering of the $\nu_{34} + \nu_{35}$ combination (≈ 399 cm^{-1}) and the propionate bending mode (≈ 448 cm^{-1}; Fig. 3A; see above). In this context it is interesting to refer to a previous study of cytochrome c_3, another four-heme-containing cytochrome [29]. Verma et al. did not find any indication for a structural heterogeneity of the heme pockets. Instead, these authors discussed the effect of vibrational exciton coupling between the individual heme groups on the RR spectra. Although, at present, we cannot rule out such a mechanism being operative also in the case of C-554, it is evident that in this protein there are qualitatively different interactions of the four heme groups with the protein environment.

Based on these results, we would finally like to discuss the redox properties of C-554. In a recent study, Freeman and Blankenship [6] found quite different redox potentials for the individual hemes, i.e. +300, 220, 120 and 0.0 mV. Such a distribution in a range of 300 mV must be related to structural differences of the heme pockets, which is in fact supported by the present RR results. In particular, the RR spectra include the characteristic vibrational signature for an open heme pocket. Such a structural element implies solvent accessibility and a higher dielectric constant of the heme environment which, in turn, is accompanied by a negative shift of the redox potential [8,26]. Thus, it is very likely that the heme with the most positive redox potential is bound in a closed pocket of the protein. Consequently, it is reasonable that this heme gives rise to those RR bands which, in the case of HH, have

been shown to be characteristic marker bands for a closed heme crevice, e.g., the weak bands at 399 and 448 cm^{-1} of the oxidized C-554. The remaining three heme pockets must assume a more or less open structure accounting for low redox potentials and the low intensities of the 399 and 448 cm^{-1} bands. The lowest redox potential is assigned to that heme, for which a (partial) conversion to the 5cHS configuration was already noted in the RR experiment.

As pointed out by Freeman and Blankenship [6], this distribution of redox potentials is quite unusual compared to analogous cytochromes serving as electron donors for the reaction centers of other photosynthetic bacteria such as *R. viridis* [5]. This suggests that the route and the mechanism of the electron transfer may be different in *C. aurantiacus*. Further structural studies are required to elucidate the intramolecular redox processes in C-554.

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