Stability of Isolated Bacterial and Photosystem II Reaction Center Complexes on Ag Electrode Surfaces. A Surface-Enhanced Resonance Raman Study

Rafael Picorel,[†] Randall E. Holt,[‡,§] Randall Heald,[‡,∥] Therese M. Cotton,[‡] and Michael Seibert*[,⊥]

Contribution from the Estación Experimental de Aula Dei, CSIC, Apdo. 202, 50080 Zaragoza, Spain, the Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, Iowa 50011, and the Solar Energy Research Institute, 1617 Cole Boulevard, Golden, Colorado 80401. *Received September 28, 1990. Revised Manuscript Received December 14, 1990*

Abstract: Surface-enhanced resonance Raman scattering (SERRS) spectra from isolated *Rhodospirillum rubrum* bacterial reaction center complexes indicate that the carotenoid, spirilloxanthin, resides in the cis conformation, while SERRS spectra from isolated photosystem II (PSII) D1-D2-cytochrome *b*-559 reaction center complexes show that β-carotene is in the all-trans conformation. The fact that the cis conformation of the native bacterial reaction center carotenoid is maintained during SERRS experiments suggests that significant denaturation of the protein matrix does not occur as a result of contact with the anodized Ag metallic surface required for observation of SERRS spectra. Although a similar conclusion cannot be drawn from observations of β-carotene in PSII reaction centers, no new spectral bands previously ascribed to denatured cytochrome (Cyt) heme groups are observed with Cyt *b*-559 during SERRS experiments. Specific enhancement of SERRS signals from Cyt *b*-559 compared to the other chromophores of the PSII reaction center and in particular observation of a strong 1360-cm^{-1} band indicate that the heme group is located on or very close to the surface of the PSII reaction center complex and can be reduced by the Ag electrode.

Introduction

The unique characteristics of surface-enhanced resonance Raman scattering (SERRS) spectroscopy, including greatly enhanced signals compared to those of resonance Raman (RR) spectroscopy as well as extreme distance sensitivity, make this technique especially suitable for structural, functional, and topological studies of biological material.[1-4] However, before SERRS techniques can be applied to resolve surface or structural problems in biological systems, one must demonstrate that the native state of the sample material is preserved upon adsorption onto the Ag SERRS electrode surface. Close contact between the sample and electrode surface is a requisite for observing SERRS signals. There is growing concern that isolated proteins (or protein complexes) are denatured when they are adsorbed on metallic surfaces.[5-7] It is logical to suspect that purified proteins or protein complexes in solution are subject to greater potential surface damage effects than proteins embedded in the membranes. The following phenomena could be responsible for such damage: (a) direct surface contact between a purified protein and the metallic electrode results in protein denaturation; (b) membrane proteins may be more unstable in solution than when embedded in a membrane; and (c) chromophores associated with purified proteins or the active site of an enzyme might be directly exposed to the electrode surface, and the resulting chemical interaction with surface metallic ions could disrupt the chromophore or protein active site.

Knowledge of membrane-bound proteins has improved dramatically over the past few years as a result of X-ray crystallographic studies of the photosynthetic bacterial reaction center (RC) isolated from *Rhodopseudomonas (Rp.) viridis*.[8] Although similar studies have not as yet been possible for the photosystem II (PSII) D1-D2-cytochrome *b*-559 RC of oxygenic photosynthetic organisms, gene sequence homologies[9,10] and similarities in the chemical composition of the L-M heterodimer of the bacterial RC and the D1-D2 heterodimer of the PSII RC[11] suggest that the structures of the two reaction centers may be quite similar. The detection of the photoaccumulation of reduced pheophytin (Pheo) *a*,[11] the photoinduction of a spin-polarized triplet state,[12] and the determination of the charge separation rate[13] in the isolated PSII RC complex have led to the belief that the L-M and D1-D2 heterodimers of the two RCs are structural and functional analogues. Nevertheless, the two types of isolated RC complexes exhibit major structural differences in the absence of a counterpart to the bacterial H subunit in the PSII RC and the replacement of cytochrome (Cyt) *b*-559 for the *c*-type cytochrome found in the bacterial RC. More recent studies suggest differences in the structure of the primary donor associated with the two types of RCs,[14] and manganese-binding sites on the proteins of isolated PSII RCs,[15] presumably associated with O_2 evolution function,

* To whom correspondence should be addressed.
† Work performed at SERI while on leave from CSIC.
‡ Iowa State University.
§ Present address: UOP, Monirex Systems, 175 W. Oakton St., Des Plaines, IL 60018.
∥ Present address: Room 262PL, Phillips Petroleum Co., Bartlesville, OK 74004.
⊥ Solar Energy Research Institute.

(1) Van Duyne, R. P. *Chemical and Biochemical Applications of Lasers*; Moore, C. B., Ed.; Academic Press: New York, 1979; Vol. 4, pp 101-185.
(2) Picorel, R.; Holt, R. E.; Cotton, T. M.; Seibert, M. *J. Biol. Chem.* **1988**, *263*, 4374-4380.
(3) Seibert, M.; Metz, J. G.; Cotton, T. M. *Biochim. Biophys. Acta* **1988**, *934*, 235-246.
(4) Picorel, R.; Lu, T.; Holt, R. E.; Cotton, T. M.; Seibert, M. *Biochemistry* **1990**, *29*, 707-712.
(5) Cotton, T. M. (1988) *Advances in Spectroscopy: Spectroscopy of Surfaces*; Clark, R. J. H., Hester, R. E., Eds.; Wiley and Sons: New York, 1988; pp 91-153.
(6) Holt, R. E.; Cotton, T. M. *J. Am. Chem. Soc.* **1987**, *109*, 1841-1845.
(7) Smulevich, G.; Spiro, T. G. *J. Phys. Chem.* **1985**, *89*, 5168-5173.
(8) Diesenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *Nature* **1985**, *318*, 618-624.
(9) Youvan, D. C.; Bylina, E. J.; Alberti, M.; Begush, H.; Hearst, J. E. *Cell.* **1984**, *37*, 949-957.
(10) Rochaix, J. D.; Dron, M.; Rahire, M.; Malnoe, P. *Plant Mol. Biol.* **1984**, *3*, 363-370.
(11) Nanba, O.; Satoh, K. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 109-112.
(12) Okamura, M. Y.; Satoh, K.; Isaacson, R. A.; Feher, G. *Progress in Photosynthesis Research*; Biggins, J., Ed.; Martinus Nijhoff: Dordrecht, 1987; Vol. 1, pp 379-383.
(13) Wasielewski, M. R.; Johnson, D. G.; Seibert, M.; Govindjee *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 524-528.
(14) Tetenkin, V. L.; Gulyaev, B. A.; Seibert, M.; Rubin, A. B. *FEBS Lett.* **1989**, *250*, 459-463.
(15) Seibert, M.; Tamura, N.; Inoue, Y. *Biochim. Biophys. Acta* **1989**, *974*, 185-191.

0002-7863/91/1513-2839$02.50/0

are not found on the bacterial RC.

Since the three-dimensional crystal structures of most membrane-bound protein complexes such as *Rhodospirillum* (*Rs.*) *rubrum* and PSII RCs are not known, new techniques must continue to be developed to ascertain both structural and functional information. SERRS approaches have been quite successful in ascertaining structural information about protein complexes embedded in biological membranes[2-4] and could be quite useful in examining isolated membrane-bound protein complexes. In this paper we present evidence that both isolated bacterial and PSII RC complexes are not denatured on the surface of a SERRS-active Ag electrode, opening up the possibility of employing SERRS spectroscopy for useful structural and functional studies of such protein complexes.

Materials and Methods

Rs. rubrum S1 was grown photosynthetically at 30 °C on the medium of Lascelle[16] supplemented with 2 g/L yeast extract. Cells were harvested during early stationary phase, pelleted at 4 °C, and stored at −20 °C until use. Chromatophores were released from cells and resuspended in 50 mM sodium phosphate buffer (pH 7.0) by two passages through a precooled French pressure cell at 20000 psi. The homogenate was centrifuged at 20000*g* for 20 min to remove cell debris, and the chromatophore supernatant fraction was purified by differential ultracentrifugation in 120000*g* for 90 min. The pellet obtained after centrifugation was suspended in 50 mM sodium phosphate buffer (pH 7.0) and kept overnight at 4 °C.

Bacterial RCs were purified by the procedure of Vadeboncoeur et al.[17] To a dense chromatophore suspension (A_{880nm} = 37.5) lauryl dimethylamine *N*-oxide (LDAO; Onyx Chemical Co.) was added dropwise to a final concentration of 0.3%. After a 1-h incubation, the concentration of LDAO was lowered to 0.1% by dilution with 50 mM sodium phosphate buffer (pH 7.0), and the suspension was centrifuged at 105000*g* for 90 min. The resultant supernatant was precipitated first with 35% (w/v) ammonium sulfate to remove contaminating material and then with 45% (w/v) ammonium sulfate to precipitate the RC fraction. RCs were further purified by two passages through a 15 by 1.5 cm diethylaminoethyl (DEAE)-Sephacel column. The column was eluted with 150 mM NaCl in the above-mentioned phosphate buffer, dialyzed against 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.5), and kept at 4 °C until use during the next 2 days.

Spirilloxanthin (Spx) was purified as in Boucher et al.[18] The carotenoid was extracted by using petroleum ether (bp 60–100 °C) and was further purified by silica gel thin-layer chromatography and developed in a petroleum ether/acetone (9:1) mixture at 4 °C in the dark. The major carotenoid band was scraped off the chromatographic plate and eluted with acetone. The sample was concentrated by evaporation under a nitrogen gas stream and stored in liquid nitrogen until use.

Photosystem II D1-D2-Cyt *b*-559 RC complex was isolated from market spinach according to the Nanba–Satoh procedure[11] followed by poly(ethylene glycol) (PEG) precipitation as in Seibert and co-workers.[19,20] Reaction centers were extracted from PSII membrane fragments with 4% Triton X-100, loaded onto a DEAE-Toyopearl 650S ion-exchange column, washed overnight with 30 mM NaCl, and eluted with a 30–200 mM NaCl gradient. The PEG precipitation procedure allows for the removal of excess Triton X-100 and greatly stabilizes the material[19,20] compared to the Nanba–Satoh procedure.[11] All steps for the purification of bacterial and PSII RCs were performed at 4 °C in the dark.

Absorption spectra were recorded on a Cary 17D or a HP 8450A spectrophotometer in 1 cm path length cuvettes at room temperature. Resonance Raman and SERRS spectra were recorded on a multichannel instrument using the 457.9-, 488.0-, or 514.5-nm lines of a coherent INNOVA 90-5 Ar^+ laser or the 406.7-nm line of a coherent INNOVA 100-K3 Kr^+ laser. Laser power was kept below 20 mW for all measurements. The Raman scattered light was collected in a backscattering geometry and focused onto the entrance slit of a Spex Triplemate 1877 spectrometer. The light was dispersed across a PARC 1420 intensified silicon diode array detector, and the resulting signal was acquired with a PARC OMA II multichannel analyzer. Integration was 80 and 170 s for the SERR and RR measurements, respectively.

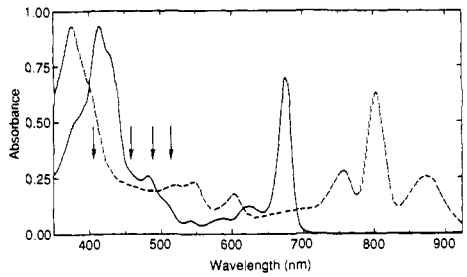

Figure 1. Absorption spectra of purified reaction center complex from *Rs. rubrum* (---) and PSII reaction center complex from spinach (—) at room temperature. Bacterial reaction center was suspended in 10 mM Tris-HCl, pH 7.5. PSII reaction center was suspended in 50 mM Tris-HCl, pH 7.2. The arrows indicate the wavelengths chosen to excite resonance Raman and SERRS spectra.

SERRS spectra were measured with the RC complexes adsorbed on a Ag electrode. The electrode was mechanically polished to a mirror finish by using three grades of alumina polishing compound (5.0, 3.0, and 0.5 μm). It was then anodized by stepping the potential of the electrode from −0.6 to +0.45 V and back to −0.6 V (all potentials vs SCE). The electrode was maintained at +0.45 V until 25 mC/cm² of charge had passed. After the anodization procedure, the electrode was dipped into a suspension of RC sample, removed and shaken to eliminate excess sample, and then cooled immediately to 77 K by submersion into liquid N_2 prior to laser exposure. Under these conditions the RC samples appeared quite stable since less than 10% of the SERRS signal was lost after the electrode had been exposed to the laser light for 30 min. RR control spectra were run with samples adsorbed to "smooth" (polished but unanodized) electrodes.

Results

The reaction center from *Rs. rubrum* exhibits three characteristic absorption bands in the near-infrared region centered at 865 and 803 nm due to the Q_y bands of the bacteriochlorophyll (BChl) *a* and at 755 nm due to the Q_y band of the bacteriopheophytin (BPhe) *a* (Figure 1). In the visible region, the spectrum shows bands at 600 and 540 nm due to the Q_x bands of the BChl *a* and BPheo *a*, respectively. Several Spx bands are observed in the 500-nm region. Purified Spx in acetone displayed peaks at 528, 494, and 465 nm (not shown). The absorption spectrum of purified PSII RC complex exhibits major peaks at around 675 and 435 nm due to both chlorophyll (Chl) *a* and Pheo *a* and at 415 nm due to Chl *a*, Pheo *a*, and the Soret band of the Cyt *b*-559. β-Carotene molecules present in the PSII RC absorb predominantly in the 490-nm region. Note that the electronic spectrum of Spx is red-shifted compared to that of β-carotene, resulting from a longer isoprenoic chain in the former.[21] The arrows in Figure 1 indicate the laser excitation wavelengths used to obtain resonance Raman and SERRS spectra in the RC materials.

Figure 2 shows 77 K SERRS spectra excited in *Rs. rubrum* RCs exposed to 457.9-, 488.0-, or 514.5-nm laser light. The spectra at 488.0 and 514.5 nm exhibit major peaks at 1526–1525 (ν_1), 1157–1154 (ν_2), and 1001 cm^{-1} (ν_3) arising from the single RC Spx molecule. The ν_1 mode is assigned to C=C symmetrical stretching of the isoprenoic chain, the ν_2 mode to C—C symmetrical stretching of the isoprenoic chain and C=C—C bending, and the ν_3 mode to CH_3 rocking. The weaker bands in the spectra, due to the other pigments in the RCs, are currently under study. No major resonance Raman signals were detected on the smooth electrode (the accompanying trace in the spectrum at 514.5 nm), providing clear evidence for the extreme sensitivity of the SERRS technique compared to bulk resonance Raman spectroscopy. Very small signals were detected after excitation at 457.9 nm, probably due to low absorption of RC Spx at this particular wavelength. The frequency of the ν_1 mode (1526–1525 cm^{-1}) is characteristic

(16) Lascelle, J. *Biochem. J.* **1956**, *62*, 78–93.
(17) Vadeboncoeur, C.; Noel, H.; Poirier, L.; Cloutier, Y.; Gingras, G. *Biochemistry* **1979**, *18*, 4301–4308.
(18) Boucher, F.; van der Rest, M.; Gingras, G. *Biochim. Biophys. Acta* **1977**, *461*, 339–357.
(19) Seibert, M.; Picorel, R.; Rubin, A. B.; Connolly, J. S. *Plant Physiol.* **1988**, *87*, 303–306.
(20) McTavish, H.; Picorel, R.; Seibert, M. *Plant Physiol.* **1989**, *89*, 452–456.
(21) Marlin, J. C. *Pure Appl. Chem.* **1985**, *57*, 785–792.

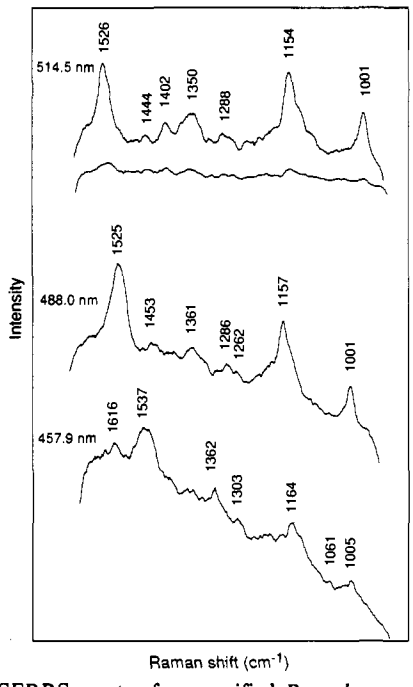

Figure 2. SERRS spectra from purified *Rs. rubrum* reaction center excited at 457.9, 488.0, and 514.5 nm (20 mW; 77 K). The frequencies of the peaks are reported in cm^{-1}. The major labeled peaks correspond to spirilloxanthin, and the accompanying trace in the spectrum at 514.5 nm is the bulk RR spectrum of the same sample material obtained prior to anodization of the Ag electrode.

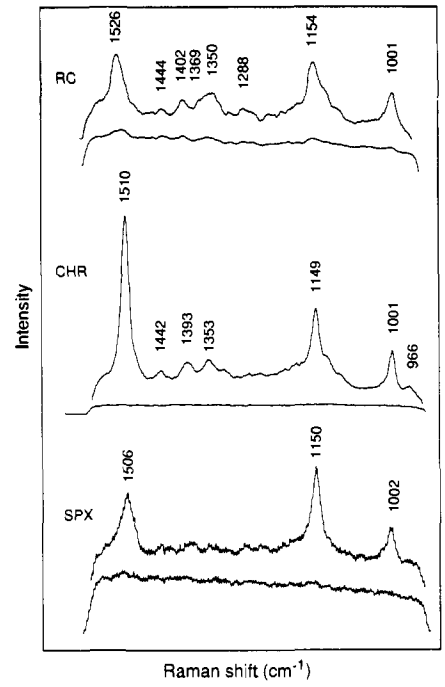

Figure 3. SERRS spectra of purified *Rs. rubrum* reaction center (RC), chromatophores (CHR), and spirilloxanthin in acetone (SPX) elicited with 514.5-nm laser light (20 mW) at 77 K. The frequencies are reported in cm^{-1}. The accompanying trace in each case is the spectrum of the samples prior to an anodization of the electrode. Note the ν_1 mode frequency shift in CHR and SPX samples compared to that in the RC sample.

of the cis isomer of Spx, as determined by resonance Raman spectroscopy.[22,23] Thus, the isomeric structure of the carotenoid molecule does not change when the RC is deposited on the anodized electrode surface (see below). Substantial physical and/or chemical interactions between the RC protein matrix and the electrode surface should induce structural changes on the protein surface that would affect the conformation of Spx. As a control experiment, we obtained SERRS spectra of purified Spx in acetone and chromatophores in addition to isolated RC complex (Figure 3). SERRS spectra arising from Spx in chromatophores and from extracted Spx are similar to that obtained for RC, but their peaks are shifted to lower frequencies. These lower frequencies correspond to the all-trans isomer of Spx normally found in antenna complexes.[22,23] The highest frequency peak shift (from 1526 to 1510–1506 cm^{-1}) corresponds to the ν_1 mode and has been correlated with the stereoisomerization of the carotenoid molecule.[22-24]

It is also worth mentioning that the ν_1/ν_2 peak ratio in Figure 3 for chromatophores (2.2) is quite different from that of purified Spx (0.7). The ν_1/ν_2 peak ratio of the isolated RC (1.0) falls between the other two. It seems that ν_2 enhancement is particularly favored when a carotenoid molecule has free access to the electrode surface, as is the case for purified Spx in solution. In isolated RC, access of Spx to the Ag electrode surface is partially restricted (peak intensities are distance sensitive) by the protein matrix. Since the membrane matrix also would be expected to severly restrict direct contact between the electrode surface and most of the Spx molecule, the highest carotenoid ν_1/ν_2 ratio, observed in chromatophores, is expected. No signals were observed on the smooth electrode prior to anodization (Figure 3, accompanying traces), confirming that we are actually detecting SERRS signals.

Figure 4 shows 77 K SERRS spectra of isolated PSII RCs. Excitation at 406.7 nm is expected to enhance vibrational modes from all the PSII RC chromophores, because the Soret bands of

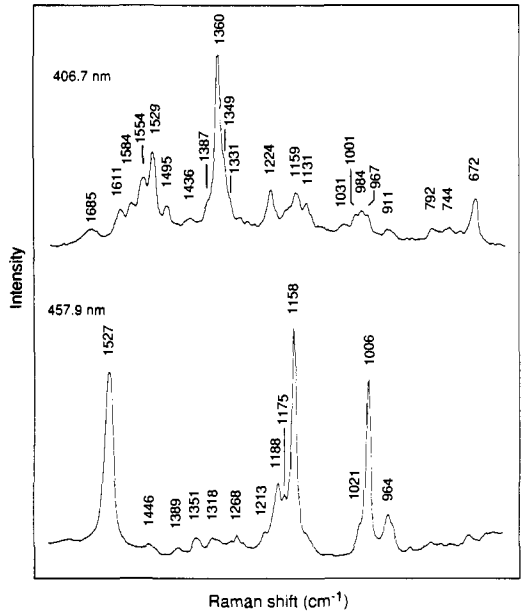

Figure 4. SERRS spectra excited in spinach PSII RC complex at 406.7 and 457.9 nm (10 mW; 77 K). The frequencies of the peaks are reported in cm^{-1}. The major labeled peaks of the spectrum at 457.9 nm are those of β-carotene, and the assignment for the labeled peaks of the spectrum at 406.7 nm is specified in Table I.

Chl *a*, Pheo *a*, and Cyt *b*-559 all lie within this region of the electronic absorption spectra. In addition, the strongly scattering β-carotene molecule also contributes to the observed SERRS spectrum. The spectrum excited by 457.9-nm (or 488.0-nm, not shown) laser light is much simpler than that elicited at 406.7 nm, and the former is completely dominated by β-carotene with main peaks at 1527 (ν_1), 1158 (ν_2), and 1006 (ν_3) cm^{-1}. These results demonstrate that the β-carotene of the PSII RC complex is in the all-trans conformation.[25,26] This represents an important

(22) Lutz, M.; Agalidis, I.; Hervo, G.; Cogdell, R. J.; Reiss-Husson, F. *Biochim. Biophys. Acta* **1978**, *503*, 287–303.
(23) Lutz, M.; Szponarski, W.; Berger, G.; Robert, B.; Newman, J.-M. *Biochim. Biophys. Acta* **1987**, *894*, 423–433.
(24) Koyama, Y.; Takii, T.; Saiki, K.; Tsukida, K. *Photobiochem. Photobiophys.* **1983**, *5*, 139–150.

Table I. Assignment of the Principal SERRS Peaks Observed for Isolated PSII Reaction Center Complex Excited with 406.7-nm Laser Light at 77 K

Raman shift, cm^{-1}	chromophore assignments
1685	Chl *a* and Pheo *a*
1611	Chl *a*
1584	Pheo *a* and Cyt *b*-559
1554	Chl *a*, Pheo *a*, and Cyt *b*-559
1529	β-carotene
1495	Chl *a*, Pheo *a*, and Cyt *b*-559
1436	Chl *a* and β-carotene
1387	Chl *a* and Cyt *b*-559
1360	Cyt *b*-559 (reduced form)
1349	Chl *a*
1331	Chl *a* and Pheo *a*
1224	Chl *a*, Pheo *a*, and Cyt *b*-559
1159	β-carotene
1001	β-carotene
984	Chl *a* and Pheo *a*
967	Chl *a*
911	Chl *a*
792	Chl *a* and Pheo *a*
744	Chl *a* and Cyt *b*-559
672	Chl *a*, Pheo *a*, and Cyt *b*-559

structural difference between the bacterial and PSII RC complexes and will be discussed later.

Table I identifies chromophores associated with the SERRS peaks identified in Figure 4. The assignments are based on this study and the RR studies of other groups.[27,28] The dominant peak at 1360 cm^{-1} arises from the ν_4 mode of Cyt *b*-559 (reduced form). Cyt *b*-559 also contributes to the bands at 1584, 1554, 1495, 1387, 1224, 744, and 672 cm^{-1}. Chl *a* accounts for the bands at 1611, 1349, 967, and 911 cm^{-1} and contributes to bands at 1685, 1554, 1495, 1436, 1387, 1331, 1224, 1131, 984, 792, 744, and 672 cm^{-1}. The peaks at 1611, 1554, 1495, and 1436 cm^{-1} indicate that the Chl *a* is five-coordinate. Pheo *a* partially accounts for bands at 1685, 1584, 1554, 1495, 1331, 1224, 1131, 984, 792, and 672 cm^{-1}. β-Carotene shows major peaks at 1529, 1159, and 1001 cm^{-1}.

Discussion

We have detected strong SERRS signals arising from the carotenoid molecule present in both purified bacterial and PSII RC complexes. X-ray analysis of the crystals from *Rp. viridis*[29] and *Rb. sphaeroides*[30] shows that the carotenoid molecule adopts a cis conformation and is located close to the surface of the protein complex near the monomeric BChl belonging to the M branch. Our SERRS data are consistent with a similar location for Spx found in the *Rs. rubrum* RC. Furthermore, our SERRS results confirm previous RR studies showing that the bacterial RC carotenoid is situated in the cis conformation[18,22,23] and that the PSII RC carotenoids are in the all-trans conformation.[26] This difference is surprising if we assume comparable structures and perhaps even similar functions (i.e., energy transfer and photoprotection) for the respective carotenoid molecules in the two different RCs. Thus, it is possible that the β-carotene of the PSII RC complex is not analogously located compared to Spx in the bacterial RC, and the former may exhibit additional functional properties compared to the bacterial RC carotenoid as suggested by others.[14]

SERRS spectra observed for the PSII RC complex at 406.7 nm are qualitatively similar to the bulk resonance Raman spectra obtained by others.[31,32] However, some peaks ascribed to Cyt *b*-559 are highly enhanced compared to that of normal resonance Raman peaks. This suggests that the heme moiety of the cytochrome is close to the surface of the PSII complex. Furthermore, the frequency of the major spectral peak (ν_4 = 1360 cm^{-1}) corresponds to the reduced form of the cytochrome; the oxidized cytochrome should exhibit a major peak around 1372 cm^{-1}.[27] Since most Cyt *b*-559 is observed in the oxidized form after the purification of PSII RCs from spinach,[11,31,33] the cytochrome must be reduced by the surface of the SERRS-active electrode. The open circuit potential of the electrode is determined by the solution environment (at the time the RC is adsorbed) and, as is the case with Ag sols,[34] is negative enough to reduce the cytochrome but apparently not Chl *a* or Pheo *a*.

The most important issue that we wish to address in this paper is that of protein stability on the SERRS-active anodized Ag electrode because this issue has elicited considerable controversy in the past.[5-7] As pointed out above, Spx is in the cis conformation in the bacterial RC, but it adopts an all-trans isomeric structure in the light-harvesting complexes (representing the majority of carotenoids in the bacterial chromatophore membrane) and after pigment extraction with organic solvents.[2,22] We would expect that Spx should also adopt an all-trans conformation if the RC structure were disturbed significantly and/or the carotenoid displaced from the apoprotein moiety due to electrode surface effects. However, this is not the case as seen in Figure 3. Since Spx is not covalently bound to the apoprotein moiety, it can be extracted easily from the RC complex and can be reconstituted back in a fully functional form.[18,35] Thus, both the SERRS (this work) and the biochemical data suggest a location on or near the surface of the protein matrix. Consequently, Spx in the bacterial RC can serve as a sensitive probe for potential surface damage in SERRS experiments. Significant distortion of the RC protein matrix should induce isomerization of the carotenoid from the cis to the all-trans conformation. Such an isomerization would lead to a shift of the ν_1 mode to lower frequency. Since the frequency of this mode is a very reliable parameter associated with the isomeric structure of carotenoids,[22-24,36] the lack of a ν_1 (1526 cm^{-1}) shift to lower frequency during SERRS analysis strongly suggests that the bacterial RC is stable on the Ag electrode surface. We should point out that SERRS spectra were recorded at low temperature and no stereoisomerization would be expected at this temperature. However, the adsorption of RCs onto the electrode surface was done at room temperature (see Materials and Methods), at which surface damage effects could occur. Indeed, control experiments (Figure 3) show that the Spx in chromatophores and in solution is in the all-trans conformation (ν_1 = 1510–1506 cm^{-1}).

We cannot use Raman spectral changes from β-carotene to assess conformational modification of the PSII RC complex since the pigment is found in the all-trans conformation in the native purified complex. However, the heme moiety of Cyt *b*-559 can be used as a probe to detect protein denaturation on the Ag electrode surface during SERRS experiments. Strong SERRS signals from reduced Cyt *b*-559 (ν_4 = 1360 cm^{-1}) of the PSII reaction center complex are detected at 406.7 nm. As stated above, this suggests that the heme group is exposed on or near the surface of the protein. Because of the location of the heme group, any

(25) Note that the cis and all-trans conformations of β-carotene are at higher frequencies than the corresponding values of spirilloxanthin.[22-24] The expected frequency of the cis conformation of β-carotene is around 1540 cm^{-1}.

(26) Fujiwara, M.; Hayashi, H.; Tamusi, M.; Koyama, Y.; Satoh, K. *Chem. Lett.* **1987**, 2005–2008.

(27) Babcock, G. T.; Widger, W. R.; Cramer, W. A.; Oertling, W. A.; Metz, J. G. *Biochemistry* **1985**, *24*, 3638–3645.

(28) Tasumi, M.; Fujiwara, M. *Spectroscopy of Inorganic-Based Materials*; Clark, R. J. H., Hester, R. E., Eds.; Wiley and Sons: New York, 1987; pp 407–428.

(29) Deisenhofer, J.; Michel, H. *The Photosynthetic Bacterial Reaction Center, NATO ASI Series A: Life Sciences*; Breton, J., Vermeglio, A., Eds.; Plenum Press: New York, 1988; pp 1–4.

(30) Yeates, T. O.; Komiya, H.; Chirino, A.; Rees, D. C.; Allen, J. P.; Feher, G. *Proc. Natl. Acad. Sci. U.S.A.* **1988**, *85*, 7993–7997.

(31) Ghanotakis, D. F.; de Paula, J. C.; Demetriou, D. M.; Bowlby, N. R.; Petersen, J.; Babcock, G. T.; Yocum, C. Y. *Biochim. Biophys. Acta* **1989**, *974*, 44–53.

(32) Moënne-Loccoz, P.; Robert, B.; Lutz, M. *Biochemistry* **1989**, *28*, 3641–3645.

(33) Miyazaki, A.; Shina, T.; Toyoshima, Y.; Gounaris, K.; Barber, J. *Biochim. Biophys. Acta* **1989**, *975*, 142–147.

(34) Flemming, J.; Kneipp, K. *Stud. Biophys.* **1989**, *130*, 45–50.

(35) Chadwick, B. W.; Frank, H. A. *Biochim. Biophys. Acta* **1986**, *851*, 257–266.

(36) Koyama, Y.; Takatsuka, I.; Kanaji, M.; Tomimoto, K.; Kito, M.; Shimamura, T.; Yamashita, J.; Saiki, K.; Tsukida, K. *Photochem. Photobiol.* **1990**, *51*, 119–128.

protein denaturation resulting from binding of the RC complex to the Ag electrode surface during SERRS experiments should affect the structure of the heme group and hence its resonance Raman spectrum. Smulevich and Spiro claim that any major distortion of the heme active site gives rise to new resonance Raman spectral peaks at 1490 and 1570 cm^{-1} due to the formation of electrode-surface-bound hemin oxo dimers.[7] Also, it is important to note here that the results of Hildebrandt and Stockburger[37,38] have shown that a temperature-dependent, reversible spin-state change occurs in Cyt *c* when adsorbed on colloidal silver. However, even these reversible perturbations of the protein structure can be avoided, as we have succeeded in developing a Ag sol preparation that does not produce these spin-state changes.[39] Thus, in the results obtained here, no new peaks are observed at the positions indicative of oxo dimer formation or of spin-state changes, and we conclude that the PSII RC complex is also stable on the Ag electrode surface.

Since no evidence for structural protein damage is observed when bacterial or PSII RC complexes are adsorbed on Ag electrode surfaces,[40] SERRS spectroscopy appears to be a reliable technique for studying purified complex chromoproteins. Furthermore, the fact that the potential applied to SERRS-active electrodes can be varied makes this technique very promising for detecting intermediate redox species in redox-active chromoproteins, such as photochemically active RCs.

Acknowledgment. We thank Stephen P. Toon for preparing the PSII reaction centers and Jae-Ho Kim for assembling some of the data. M.S. thanks the Onyx Chemical Co. of Jersey City, NJ for its generous gift of LDAO used in this study. This work was supported by the Divisions of Chemical Sciences (M.S. and T.M.C.) and Energy Biosciences (M.S.), Office of Basic Energy Sciences, U.S. Department of Energy, and Consejo Superior de Investigaciones Cientificas, Spain (R.P.). Ames Laboratory is operated for the U.S. Department of Energy by Iowa State University under Contract No. W-7405-Eng-82. SERI is a division of the Midwest Research Institute and operated for the U.S. Department of Energy under Contract No. DE-AC02-83CH-10093.

Registry No. Pheo *a*, 603-17-8; Cyt *b*-559, 9044-61-5; Chl *a*, 479-61-8; Spx, 34255-08-8; Ag, 7440-22-4; β-carotene, 7235-40-7.

(37) Hildebrandt, P.; Stockburger, M. *J. Phys. Chem.* **1986**, *90*, 6017-6024.
(38) Hildebrandt, P.; Stockburger, M. *Biochemistry* **1989**, *28*, 6710-6721.
(39) Rospendowski, B. N.; Schlegel, V. L.; Holt, R. E.; Cotton, T. M. *Charge and Field Effects in Biosytems—2*; Allen, M. J., Cleary, S. F., Hawkridge, F. M., Eds.; Plenum Publishing: 1989; pp 43-58.

(40) It is likely that reaction centers adsorb to the Ag electrode in several different orientations. Since there is such an extreme distance effect in SERRS, only those orientations that bring either the spirilloxanthin or the Cyt *b*-559 in near proximity to the Ag electrode will contribute to our spectral observations.

Resonance Raman Characterization of Nitric Oxide Adducts of Cytochrome P450cam: The Effect of Substrate Structure on the Iron–Ligand Vibrations

Songzhou Hu and James R. Kincaid*

Contribution from the Chemistry Department, Marquette University, Milwaukee, Wisconsin 53233. *Received April 9, 1990*

Abstract: Resonance Raman spectra of the nitric oxide adducts of ferric cytochrome P450cam are acquired with Soret excitation. The frequencies of the heme skeletal vibrations are found 3–10 cm^{-1} lower than those of horseradish peroxidase and myoglobin. The axial vibrations are located and assigned by isotopic substitution. The stretching, ν(Fe–NO), and bending, δ(Fe–N–O), modes are detected at 522 and 546 cm^{-1}, respectively, for the title adduct in the presence of camphor. They shift to 520 and 533 cm^{-1} upon substitution by $^{15}N^{16}O$. The strong line at 522 cm^{-1} shifts to 528 cm^{-1} when the substrate is removed from the active site, whereas it appears at 524 cm^{-1} upon the addition of norcamphor. The isotopic sensitivity of these bands confirms their assignment as ν(Fe–NO). It is observed that only the larger substrates (camphor and adamantanone) give rise to the enhancement of the bending mode δ(Fe–N–O). As the substrate size increases, a band of medium intensity, which is tentatively assigned as ν(Fe–S), increases from 349 cm^{-1} (substrate free) to 359 cm^{-1} (adamantanone). On the basis of the consideration of both the electronic factors and the kinematic effect of distortion of the Fe–NO unit, it is suggested that the Fe–NO linkage adopts a linear structure in the absence of substrate but becomes slightly bent in the presence of substrates.

Introduction

The generic name, cytochrome P450, was given to a class of heme-containing enzymes that give rise to the electronic absorption maxima around 450 nm upon the formation of ferrous carbonyl complexes.[1] These b-type hemoproteins play a very important role in a wide variety of hydroxylation reactions, the oxidizing center being generated by two-electron reduction and cleavage of dioxygen.[2] Among the various cytochromes P450 that have been isolated and purified, that known as cytochrome P450cam, derived from *P. putida*, has been most thoroughly characterized, owing to its relative ease of isolation and aqueous solubility.[3]

(1) (a) Ortiz de Montellano, P. R., Ed. *Cytochrome P450 Structure, Mechanism, and Biochemistry*; Plenum Press: New York, 1986. (b) Schuster, I., Ed. *Cytochrome P450: Biochemistry and Biophysics*; Traylor & Francis, 1989. (c) Sato, R., Omura, T., Eds. *Cytochrome P450*; Academic Press: New York, 1978.

(2) (a) Black, S. D.; Coon, M. J. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1987**, *60*, 35-87. (b) Dawson, J. H. *Science* **1988**, *240*, 433-439. (c) Guengerich, F. P.; McDonald, T. L. *Acc. Chem. Res.* **1984**, *17*, 9-16. (d) Dawson, J. H.; Sono, M. *Chem. Rev.* **1987**, *87*, 1255-1276. (e) Sligar, S. G.; Gelb, M. H.; Heimbrook, D. C. *Xenobiotica* **1984**, *14*, 63-86.

(3) (a) Murray, R. I.; Fisher, M. T.; Debrunner, G.; Sligar, S. G. In *Metalloproteins Part I: Metal Proteins with Redox Roles*; Harrison, P. M., Ed.; Verlag Chemie: Weinheim, 1985; p 157. (b) Dawson, J. H.; Eble, K. S. *Adv. Inorg. Bioinorg. Mech.* **1986**, *4*, 1-64.

0002-7863/91/1513-2843$02.50/0