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Fourier Transform Infrared Difference Spectroscopy Shows No Evidence for an Enolization of Chlorophyll *a* upon Cation Formation either in Vitro or during P700 Photooxidation[†]

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ABSTRACT: Molecular changes associated with the photooxidation of the primary electron donor P700 in photosystem I from cyanobacteria have been investigated with Fourier transform infrared (FTIR) difference spectroscopy. Highly resolved signals are observed in the carbonyl stretching frequency region of the light-induced FTIR spectra. In order to assign and to interpret these signals, the FTIR spectra of isolated chlorophyll *a* and pyrochlorophyll *a* (lacking the 10a-ester carbonyl) in both their neutral and cation states were investigated. Comparison of the redox-induced FTIR difference spectra of these two model compounds demonstrates that upon chlorophyll *a* cation formation in tetrahydrofuran the 7c-ester carbonyl is essentially unperturbed while the 10a-ester carbonyl is upshifted from 1738 to 1751 cm^{-1}. For the 9-keto group, the shift is from 1693 to 1718 cm^{-1} in chlorophyll *a* and from 1686 to 1712 cm^{-1} in pyrochlorophyll *a*. The 1718-cm^{-1} band in the difference spectrum of chlorophyll *a* is thus unambiguously assigned to the 9-keto carbonyl of the cation. Comparison of the light-induced FTIR difference spectrum associated with the photooxidation of P700 in vivo with the difference FTIR spectrum of chlorophyll *a* cation formation leads to the assignment of the frequencies of the 9-keto carbonyl group(s) at 1700 cm^{-1} in P700 and at 1717 cm^{-1} in $P700^+$. These assignments are taken to indicate free keto carbonyl(s) in both P700 and $P700^+$ with no evidence for the participation either in chlorophyll a^+ or in $P700^+$ of the chlorophyll enol form previously suggested by Wasielewski et al. [Wasielewski, M. R., Norris, J. R., Shipman, L. L., Lin, C.-P., & Svec, W. A. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2957–2961] and more recently by Heald et al. [Heald, R. L., Callahan, P. M., & Cotton, T. M. (1988) *J. Phys. Chem. 92*, 4820–4824].

The primary photochemical act in photosynthesis leads to the generation of the radical cation of a specialized chlorophyll (Chl)[1] or bacteriochlorophyll (BChl) species. For purple bacteria, the primary electron donor is a pair of BChl whose molecular structure has been extensively investigated by various spectroscopic techniques and recently elucidated by X-ray crystallography of the bacterial reaction center (Deisenhofer et al., 1984; Allen et al., 1987). In the absence of high-resolution X-ray data for green plant photosystems I and II, proposals for the structure and bonding interactions of their primary donor rely only on spectroscopy. In particular, the primary donor of photosystem I, P700, is probably a Chl *a* dimer [for a review, see Golbeck (1987)], although several spectroscopic studies suggest that the positive charge in the $P700^+$ radical cation is localized on only one of the two Chl molecules that comprise P700 (Wasielewski et al., 1981a; O'Malley & Babcock, 1984; Ikegami & Itoh, 1988). Model studies also incorporate a possible keto–enol tautomerization of Chl *a* upon P700 photooxidation (Wasielewski et al., 1981b; Heald et al., 1988).

In previous work, we have shown that the nature of the bonding interactions of the primary reactants in purple bacteria and green plant photosynthesis can be investigated by Fourier transform infrared (FTIR) difference spectroscopy (Mäntele et al., 1985, 1988a; Nabedryk et al., 1986, 1987, 1988; Tavitian et al., 1986). In particular, the specific molecular changes associated with the photooxidation of P700 have been investigated by light-induced FTIR spectroscopy on particles enriched in photosystem I (Tavitian et al., 1986). Highly resolved FTIR difference spectra were obtained for these particles, showing features identical with those obtained for intact thylakoids. The most characteristic differential signals were observed in the carbonyl stretching frequency region (1620–1760 cm^{-1}), notably at 1700 cm^{-1} where a prominent band was assigned to a decrease in absorption of the 9-keto C═O of the Chl *a* molecule(s) constituting the primary donor (Tavitian et al., 1986). However, in the absence of IR model compound studies and especially of the in vitro Chl *a* cation IR spectrum, the assignments proposed for the differential IR bands observed in the $P700^+$ minus P700 spectrum were only tentative. In particular, the frequency of the 9-keto C═O in $P700^+$ could not be unambiguously determined.

In order to assign and to interpret the features observed in the light-induced spectra associated with the primary reactions in photosynthesis, the IR spectra of isolated chlorophyll model compounds in both their neutral and ionized states have to be investigated (Mäntele et al., 1988a,b; Tavitian et al., 1988; Leonhard et al., 1989). In the present work, the cation radicals of Chl *a* and of pyroChl *a* (lacking the 10a-ester carbonyl) were generated electrochemically in a special cell suited for the visible and IR spectral regions. Comparison of the

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[1] Abbreviations: (B)Chl, (bacterio)chlorophyll; FTIR, Fourier transform infrared; RR, resonance Raman; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane.

light-induced FTIR difference spectra associated with the photooxidation of P700 in vivo with difference FTIR spectra of the Chl *a* cation formation in vitro now leads to the assignment of the frequencies of the 9-keto and 10a-ester carbonyls in P700 and P700$^+$.

MATERIALS AND METHODS

The preparation of thylakoids of the cyanobacteria *Spirulina geitleri* is described elsewhere (Tavitian, 1987). Suspensions of these thylakoids were deposited on CaF_2 windows and air-dried. The absorption of these films ranged from 1 to 2 absorbance units at the red absorption maximum. The films were covered with a 5 mM, pH 7.0 Tris buffer (containing 10 mM sodium ascorbate) and sealed with another CaF_2 window, constituting microcells (≈10-μm optical path length) that were thermostated at 295 K. Some films were also prepared from thylakoids that have been incubated in ^2H_2O for 60 h at room temperature. Light minus dark FTIR spectra of these films, as well as the corresponding controls of P700 absorbance changes in the visible spectral range, were obtained as previously described (Tavitian et al., 1986). P700 absorption changes were measured by the bleaching at 706 nm and the absorption increase at 820 nm on a laboratory-built spectrophotometer [see Tavitian et al. (1986)]. Following saturating steady-state illumination (Flexilux, Schölly Fiberoptik, FRG; 400 nm < λ < 650 nm filter), the half-time of the decay was ≈9 s at 295 K. This demonstrates that the oxidized primary donor P700$^+$ can be accumulated in these hydrated films upon illumination with continuous actinic light. IR spectra were taken on a Nicolet 60 SX instrument equipped with an MCT-A detector. Interferograms were collected before and during saturating steady-state illumination of the sample with actinic light (Flexilux, Schölly Fiberoptik, FRG; 530 nm < λ < 1100 nm filter).

Chl *a* was isolated from spinach and purified by high-performance liquid chromatography to remove lipids (Berger et al., 1987). PyroChl *a* was obtained by a relatively mild pyrolysis method adapted from Pennington et al. (1964). PyroChl *a* was separated from Chl *a* and other impurities by high-performance liquid chromatography on reversed-phase columns (Leonhard et al., 1989). The purity, in terms of contamination by other pigments, was found to be better than 99% for both Chl *a* and pyroChl *a* (G. Berger, personal communication). Solutions of chlorophylls (1–2 mM) were prepared in deuterated solvents (tetrahydrofuran, dichloromethane, methanol). The techniques used for solvent drying, for purification of the supporting electrolyte (tetrabutylammonium hexafluorophosphate), and for electrochemistry (including cyclic voltammetry, controlled potential electrolysis, and coulometry) have been described elsewhere [Wollenweber, 1986; Mäntele et al. (1988b) and references cited therein]. With use of a transparent thin-layer electrochemical cell (Mäntele et al., 1988b), visible and IR spectra were recorded before and after cation formation.

RESULTS AND DISCUSSION

A spectrum of Chl *a* in fully deuterated tetrahydrofuran (THF), recorded in the visible spectral range with the spectroelectrochemical cell, is shown in Figure 1 (inset), together with the spectrum obtained after several minutes of electrolysis at $U = +0.8$ V. At this potential the π monocation radical is generated (Davis et al., 1979). The spectrum of the Chl *a* cation formation shows a decrease of the main absorbance band at 665 nm (Q_y transition of the neutral Chl *a*) and a slight increase of absorbance in the near-IR at ≈ 820 nm, which has been assigned to the Q_y transition of the cation.

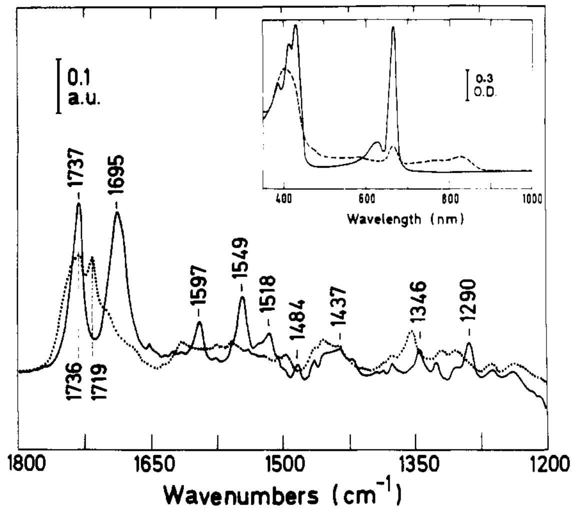

FIGURE 1: FTIR absorbance spectra of the neutral chlorophyll *a* in deuterated tetrahydrofuran in the electrochemical cell (250-μm optical path length), before electrolysis (full line) and after cation formation at $U = +0.8$ V (dashed line). $T = 295$ K; 4-cm^{-1} resolution. Inset: corresponding spectra in the visible spectral range.

Furthermore, changes in the other spectral regions are observed. This spectrum of Chl a^+ is in good agreement with the ones reported by Davis et al. (1979) and Heald et al. (1988). The reaction is reversible at $U = -0.5$ V, with a yield of at least 90%.

The corresponding IR absorption spectra of the neutral and radical cation of Chl *a* in THF are shown in Figure 1. The solvent and electrolyte bands have been subtracted by use of a blank spectrum taken in the same cell. The IR difference spectrum (Chl a^+ minus Chl *a*), hereafter referred as Chl a^+/Chl *a* spectrum, is shown in Figure 2a. Positive bands in this spectrum arise from the Chl *a* cation radical while the disappearing bands of the neutral Chl *a* are negative. The two bands at 1695 and 1737 cm^{-1} in the IR spectrum of neutral Chl *a* (Figure 1) have been assigned to the C═O vibration of the keto and ester groups (in this solvent, the two ester carbonyls at the 7c- and 10a-positions cannot be distinguished), respectively (Katz et al., 1966; Fujiwara & Tasumi, 1986). The frequencies are characteristic of noninteracting C═O groups as expected for a non-hydrogen-bonding solvent and unaggregated pigments (Ballschmiter & Katz, 1969). In the 1600–1400-cm^{-1} range, C═C and C–C modes of the porphyrin skeleton contribute to the spectrum, and the frequencies of the bands are in good agreement with the reported values (Katz et al., 1966; Fujiwara & Tasumi, 1986; Heald et al., 1988). Chl *a* cation formation results in a number of IR spectral changes, especially in the C═O frequency region (Figure 1). The 1737-cm^{-1} ester C═O band shows a significant decrease of its absorption while a shoulder on its high-frequency side appears. This change is reflected by the differential signal at 1751/1738 cm^{-1} in the Chl a^+/Chl *a* spectrum (Figure 2a). In addition, the 9-keto C═O band at 1695 cm^{-1} disappears, and a new band appears at 1719 cm^{-1} (Figure 1), giving rise to the differential signal at 1718/1693 cm^{-1} in the Chl a^+/Chl *a* spectrum (Figure 2a).

The differential signal at 1751/1738 cm^{-1} (Figure 2a) can be assigned to either one or both of the 7c- and 10a-ester C═O groups. For bonds not directly involved in conjugation such as these ester C═O groups, only inductive Coulombic effects from the π-electron system are expected [Mäntele et al. (1988a) and references cited therein]. From a comparison of the distances of these groups to the conjugated system, the 10a-ester should be predominantly affected. Indeed, this is

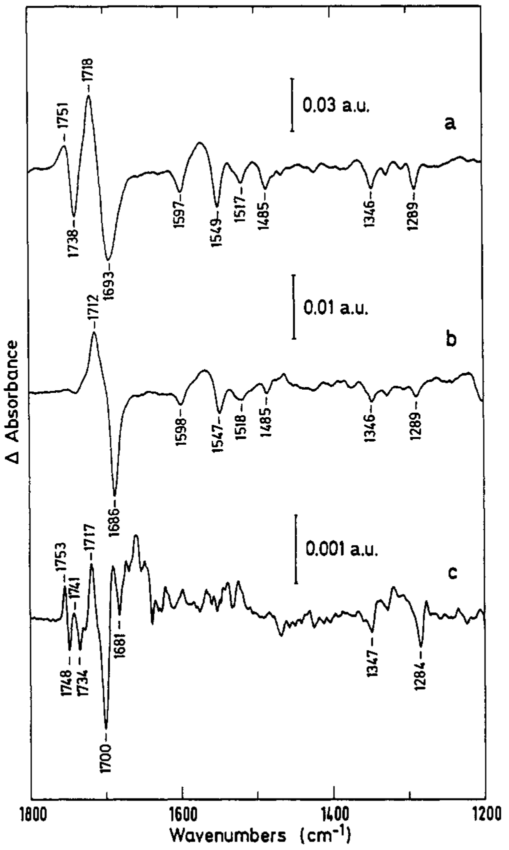

FIGURE 2: Redox-induced FTIR difference spectra of chlorophyll cation formation in deuterated tetrahydrofuran. (a) Chl a^+ minus Chl a at $U = +0.8$ V; (b) pyroChl a^+ minus pyroChl a at $U = +0.2$ V (64 interferograms coadded). In (a) the optical path length of the cell was 250 μm while in (b) it was 50 μm. (c) Light-induced FTIR difference spectrum of P700 photooxidation (P700$^+$ minus P700) in thylakoids from *S. geitleri* (excitation wavelength 530–1100 nm) (512 interferograms coadded). T = 295 K; 4-cm^{-1} resolution. a.u. is absorbance unit.

demonstrated by the absence of this differential signal in the pyroChl a^+/pyroChl a spectrum (Figure 2b). Since in pyroChl a the carbomethoxy group at C(10) is replaced by a hydrogen atom, this observation demonstrates that the IR absorption of the 7c-ester C═O is essentially unperturbed upon chlorophyll cation formation. The very small signal that can be detected at 1749/1737 cm^{-1} in the pyroChl a^+/pyroChl a spectrum (Figure 2b) is nevertheless attributed to a minor contribution (less than 5%) of the 7c-ester C═O. Therefore, the 1751/1738-cm^{-1} signal in the Chl a^+/Chl a spectrum is unambiguously assigned to the 10a-ester C═O. An almost identical feature at 1750/1737 cm^{-1} is also observed in the IR difference spectrum of the BChl a cation formation in THF (Mäntele et al., 1988a) but does not appear in its pyro derivative (Leonhard et al., 1989). When the Chl a cation is generated in dichloromethane or in methanol, a comparable shift at 1749/1735 or at 1750/1739 cm^{-1}, respectively, is observed (Tavitian, 1987). Indeed, the large perturbation observed on the 10a-ester C═O band upon cation formation reflects the direct effect on this group of the change in the charge density within the macrocycle. The partial conjugation of the 10a-ester C═O into the macrocycle has been already discussed for the neutral chlorophylls (Andersson et al., 1989). Upon cation formation, the observed frequency upshift of the 10a-ester can thus be related to the lowered electron density across the C(9)–C(10) bond, which tends to decrease the partial conjugation of the 10a-ester.

The large differential signal at 1718/1693 cm^{-1} in the Chl a^+/Chl a spectrum (Figure 2a) is closely analogous to the ones observed at 1712/1686 cm^{-1} in the pyroChl a^+/Chl a spectrum (Figure 2b). For pyroChl a, the absence of the 10a-ester C═O allows the positive band at 1712 cm^{-1} to be unambiguously identified to the 9-keto C═O of the pyroChl a cation radical. Thus, we assign the corresponding positive band at 1718 cm^{-1} in the Chl a^+/Chl a spectrum (Figure 2a) to the 9-keto C═O vibration of the Chl a radical cation. As will be discussed below, this assignment is at variance with the one recently proposed by Heald et al. (1988). When the Chl a cation is generated in dichloromethane or in methanol, a large differential signal at 1720/1689 or at 1720/1683 cm^{-1}, respectively, is observed for the 9-keto C═O (Tavitian, 1987). The sensitivity to solvent polarity of the 9-keto C═O frequency of Chl a (1693 cm^{-1} in THF, 1689 cm^{-1} in dichloromethane, and 1683 cm^{-1} in methanol) is in good agreement with the literature (Ballschmiter & Katz, 1969; Fujiwara & Tasumi, 1986; Koyama et al., 1986). Furthermore, as shown by Koyama et al. (1986), the resonance Raman (RR) stretching frequency of the 9-keto C═O of Chl a in the absence of hydrogen-bonding interactions is directly related to the dielectric constant of the solvent. Upon cation formation, we observed that this group is upshifted by 25–37 cm^{-1} (depending on the solvent). This upshift appears to be a general property as it has been previously observed for the cation of BChl a and b (Mäntele et al., 1988a). In contrast, a downshift of the 9-keto C═O has been documented upon generation of (bacterio)pheophytin (Mäntele et al., 1988a; Breton et al., 1990) and bacteriochlorophyll anions (Mäntele et al., 1988b). These effects are assigned to a partial change of bond order of the conjugated 9-keto C═O upon charge redistribution following addition or subtraction of one electron. In this regard, it is interesting to note that the effect of the solvent polarity on the frequency of the 9-keto C═O appears much smaller in the cation than in the neutral species. This suggests that for the cation the effect of bond-order change dominates over the influence of the dielectric constant of the solvent. On the other hand, the 9-keto C═O frequencies of the neutral and cation radicals are both downshifted by 6–7 cm^{-1} in pyroChl a with respect to the parent compound. A similar observation has been made for BChl a and pyroBChl a (Leonhard et al., 1989). As previously discussed for neutral chlorophylls by Andersson et al. (1989), the presence of the 10a-ester C═O induces molecular strain in the macrocycle and thus influences the vibrational mode of the 9-keto group. Loss of the 10a-ester C═O releases some strain and increases the conjugation of the 9-keto C═O, thus lowering its frequency (Andersson et al., 1989). The 26-cm^{-1} upshift of the 9-keto C═O vibration in the pyroChl a^+/pyroChl a spectrum (Figure 2b) indicates a decreased conjugation of this group with the macrocycle when the cation is formed. In addition, the observation of a 25-cm^{-1} upshift in the Chl a^+/Chl a spectrum (Figure 2a) shows that the decreased conjugation of this group upon cation formation is remarkably independent of the presence of the 10a-ester C═O.

Light minus dark FTIR spectra of the photooxidation of P700 (referred as P700$^+$/P700 spectra) obtained from hydrated films of thylakoids of *S. geitleri* at 295 K (Figure 2c) are very close to the ones previously reported for pea and spinach thylakoids and for photosystem I particles obtained from green plants and cyanobacteria at both room temperature (Tavitian et al., 1986; Tavitian, 1987) and low temperature (E. Nabedryk, C. Berthomieu and J. Breton, unpublished results). The largest FTIR differential signals (especially the pronounced negative signal at 1700 cm^{-1}) are observed in the C═O stretching frequency region (1620–1760 cm^{-1}), where contributions from protein, chlorophyll, and lipid carbonyls as well as the OH bending vibration of water might also arise. A 44 ± 1 cm^{-1} frequency downshift upon ^{13}C substitution together with an absence of shift (±1 cm^{-1}) upon ^{15}N substitution has been reported for all the bands in the 1620–1760-cm^{-1} region in both P700$^+$/P700 spectra and Chl a^+/Chl *a* spectra obtained with thylakoids and pigments extracted from cyanobacteria fed with these isotopes (Tavitian, 1987; Tavitian et al., 1988). This observation unambiguously demonstrates that the 1700-cm^{-1} signal of P700 is indeed a pure C=O mode. It is well established (Byler & Susi, 1986) that peptide C=O bonds absorb in the 1620–1690-cm^{-1} IR domain (the so-called amide I absorption band), depending on the peptide backbone conformation. In addition, a band around 1735 cm^{-1} with a higher and lower frequency component at 1744 and 1716 cm^{-1}, respectively, has been shown to be associated with the two ester C=O stretching vibrations of the lipid head groups (Mushayakarara & Levin, 1982). On the other hand, the 9-keto carbonyl of Chl *a* in vitro covers the 1705–1650-cm^{-1} frequency range (Ballschmiter & Katz, 1969; Fujiwara & Tasumi, 1986; Koyama et al., 1986). Thus, the frequency (1700 cm^{-1}) of the C=O group which disappears upon P700$^+$ formation lies within the range of vibration of the 9-keto C=O of Chl *a* but is outside the usual frequency range of both peptide C=O bonds and ester carbonyls from lipids and chlorophylls. The direct involvement of the 9-keto C=O of Chl *a* in the 1700-cm^{-1} signal appears therefore as a likely possibility.[2] This hypothesis is further substantiated by the striking similarities found between the in vivo and in vitro data upon comparison of the P700$^+$/P700 and Chl a^+/Chl *a* spectra (Figure 2). In particular, the largest differential signal at 1717/1700 cm^{-1} in the P700$^+$/P700 spectrum (Figure 2c) bears a close analogy with the largest differential signal observed at 1718/1693 cm^{-1} in the Chl a^+/Chl *a* spectrum (Figure 2a). This striking similarity actually appears to be a general observation when the light-induced difference FTIR spectra are compared with the relevant electrochemically induced difference spectra corresponding to the photooxidation (photoreduction) of a number of primary electron donors (acceptors) in both purple bacteria and photosystem II reaction centers (Mäntele et al., 1985, 1988a; Nabedryk et al., 1986, 1987, 1988; Tavitian et al., 1986; Breton et al., 1990). The negative component of the largest differential signal is always observed in the 1705–1675-cm^{-1} region and thus has been assigned to the 9-keto C=O group of the pigments in their neutral state. It therefore appears that the generation of chlorophyll radicals both in vitro and in vivo always leads to the detection of a large signal in the 9-keto C=O stretching region. Such large signals are not limited to free keto groups. The study of the BChl *a* cation generated in methanol indicates that, even when extensive hydrogen bonding of carbonyls induces a considerable downshift in frequency of the 9-keto C=O of the neutral species, a large differential signal at 1721/1652 cm^{-1} is observed upon cation formation (Mäntele et al., 1988a). Taking into account these IR data both in vivo and in vitro, the observation that the largest differential signal in the P700$^+$/P700 spectrum is at 1717/1700 cm^{-1}, and the fact that it is well established that photooxidation of P700 generates a Chl *a* cation, we thus favor the assignment of the 1717/1700-cm^{-1} signal observed in the P700$^+$/P700 spectrum to a frequency upshift upon primary donor photooxidation of the 9-keto C=O group(s) of the Chl *a* molecule(s) constituting P700. The value of 1700 cm^{-1} for the frequency of the 9-keto C=O in P700 is relatively high compared to the 1693-cm^{-1} value observed for Chl *a* in THF. However, it is close to the value measured for Chl *a* in solvents of very low dielectric constant. For example, frequencies of 1706 cm^{-1} (Ballschmiter & Katz, 1969) and 1702 cm^{-1} (Koyama et al., 1986) have been reported for Chl *a* in *n*-butylcyclohexane and diethyl ether, respectively. It therefore appears that the 9-keto C=O group(s) of P700 absorbing at 1700 cm^{-1} is (are) not only free of interaction in the neutral state of the primary donor but also immersed in a medium of quite low dielectric constant.

As discussed above, the high-frequency region of the Chl a^+/Chl *a* spectrum is understood in terms of a shift of the 10a-ester C=O from 1738 to 1751 cm^{-1}, with essentially no participation of the 7c-ester. In contrast, two differential signals are present in the P700$^+$/P700 spectra at 1753/1748 and 1741/1734 cm^{-1} (Figure 2c). Both signals might have several origins. A reasonable hypothesis for the signal at 1741/1734 cm^{-1} is that it corresponds to the 10a-ester C=O of the Chl molecule(s) constituting P700. Compared to the negative 1738-cm^{-1} signal detected in the Chl a^+/Chl *a* spectrum (Figure 2a), the negative 1734-cm^{-1} signal in the P700$^+$/P700 spectrum might be assigned to a weakly interacting 10a-ester C=O in the neutral state of P700. A less likely hypothesis for this signal is the involvement of the 7c-ester C=O provided it is bound to the protein in the neutral P700 state, and this bonding is affected by charge separation. In this respect, X-ray analysis of bacterial reaction centers indicates hydrogen bonds involving either the 10a-ester C=O (in *Rhodopseudomonas viridis*) or the 7c-ester C=O (in *Rhodobacter sphaeroides*) of the special pair BChls (Michel et al., 1986; Yeates et al., 1988).

The presence of a higher frequency signal at 1753/1748 cm^{-1} in the P700$^+$/P700 spectrum can be interpreted in terms of a free 10a-ester C=O absorbing at 1748 cm^{-1} in the neutral state of P700. The frequency of this signal is too high to involve a 7c-ester C=O bonded to the protein. The observed shift for the 10a-ester C=O from 1738 cm^{-1} in vitro to 1748 cm^{-1} in vivo is comparable to the one found for the free 9-keto C=O (1693 cm^{-1} in vitro to 1700 cm^{-1} in vivo). Such an upshift by 7–10 cm^{-1} of the 9-keto and 10a-ester groups in vivo compared to Chl *a* in THF can be rationalized in terms of the difference in local environment and/or dielectric constant (Koyama et al., 1986).

Finally, the 1753/1748- and 1741/1734-cm^{-1} signals (Figure 2c) might also reflect the perturbation of side-chain groups from the protein as possible contributions from amino acid (Asp or Glu) carboxylic C=O groups are expected in the 1760–1720-cm^{-1} frequency region. However, the observed sharp differential signals exclude that carboxyl groups undergo protonation changes, which would shift the bands to a much lower frequency (≈1560 cm^{-1}). An absorption band arising from a C=O bond involved in a protonated carboxylic group can be distinguished from one of the pigment C=O by a 10–15-cm^{-1} downshift after ^{1}H–^{2}H exchange (Siebert et al., 1982). We have not observed such a deuterium isotopic effect on the signals in the 1760–1600-cm^{-1} frequency region when photooxidation of P700 was performed on films prepared from thylakoids that have been extensively exchanged with ^{2}H$_2$O buffer (Tavitian, 1987). Although it cannot definitely be excluded that the 1753/1748- and/or 1741/1734-cm^{-1} signals might arise from perturbation of the environment of a side-chain carboxylic group deeply buried in the protein, with no

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[2] An alternative assignment for the 1700-cm^{-1} band would be that of an unusually upshifted C=O amide I vibration from the protein backbone, the conformation of which is affected by charge separation. However, only signals of very low intensity are observed in the amide II region (≈1550 cm^{-1}), which makes this interpretation quite improbable.

accessibility to deuterium exchange, our results suggest that no carboxylic groups contribute to the spectrum. Furthermore, the relative amplitude of the 10a-ester differential signal with respect to the 9-keto signal in the Chl a^+/Chl *a* spectrum (Figure 2a), which is close to that observed in the P700$^+$/P700 spectrum (Figure 2c) for the corresponding spectral region, leads us to favor the hypothesis that both of the signals at 1753/1748 and 1741/1734 cm^{-1} originate from unequivalent 10a-ester carbonyls. In this hypothesis, which implies a dimeric model for P700, the 9-keto group of both Chl *a* molecules would absorb at 1700 cm^{-1}. However, it cannot be excluded that the negative 1681-cm^{-1} signal (Figure 2c) reflects a contribution from the 9-keto C=O of the second Chl *a*. Moënne-Loccoze et al. (1989) have recently reported differences between RR spectra measured at low and high laser light irradiation on detergent-treated PS1 particles. Bleaching at 1655 and 1675 cm^{-1} were assigned to 9-keto C=O of the dimeric P700. While those assignments appear difficult to reconcile with our FTIR data on P700 photooxidation, the lack of control experiments in the visible spectral range for the RR data leaves the possibility that chlorophylls which do not belong to P700 also contribute to the difference RR spectra.

The participation of enol forms of chlorophylls in primary reactions of photosynthesis is still debated (Fetterman et al., 1977; Wasielewski et al., 1981b; Bocian et al., 1987; Hanson et al., 1988; Heald et al., 1988). On the basis of redox potential measurements and ENDOR spectroscopy of Chl *a* enol models, Wasielewski et al. (1981b) favored an oxidized Chl *a* enol form at the C(9) of ring V for P700$^+$, while no prediction could be made about the nature of the neutral P700 from the magnetic resonance studies. Recently, from RR spectroscopy of the neutral and electrochemically generated cation radical of Chl *a*, Heald et al. (1988) also raised the possibility that oxidation of Chl *a* might generate the enol form of the 9-keto group, both in vitro and in vivo. In agreement with our IR data, these authors observed a loss of intensity for the 9-keto C=O absorption at 1687 cm^{-1} when Chl *a* (in dichloromethane) is oxidized while a new peak appears at 1717 cm^{-1}. In general, the 10a-ester C=O cannot be observed in RR spectra of Chl *a* because this bond is not sufficiently in conjugation with the π-electron system to be resonance enhanced. However, Heald et al. (1988) are reluctant to attribute the large upshift from 1687 to 1717 cm^{-1} observed upon Chl *a* cation formation to the 9-keto C=O. Instead, they identify the appearing 1717-cm^{-1} band in the RR spectrum of the Chl *a* cation to a downshifted 10a-ester C=O vibration consequent to the enolization of the 9-keto C=O after oxidation of Chl *a*. In this scenario, the resulting formation of a double bond across C(9) and C(10) on ring V concomitant with the transfer of the hydrogen at C(10) to the 9-keto function leads to a redistribution of charge, which increases the partial conjugation of the 10a-ester C=O into the macrocycle. This partial conjugation of the 10a-ester C=O decreases its energy (frequency) and allows it to be detected in RR experiments. On the contrary, our FTIR data on Chl *a*, pyroChl *a*, BChl *a*, and pyroBChl *a* (Leonhard et al., 1989) demonstrate that upon cation formation (i) the 10a-ester C=O of Chl *a* is upshifted from 1738 to 1751 cm^{-1} and not downshifted to 1717 cm^{-1} as proposed in the enol model by Heald et al. (1988) and (ii) the 9-keto C=O is upshifted by 25–26 cm^{-1} in both Chl *a* and pyroChl *a*. From the nonexchangeability of the two C(10) protons in pyro derivatives, Katz et al. (1966) have concluded that these modified pigments essentially exist in the keto form. Our observation that cation formation induces the same frequency upshift (25–26 cm^{-1}) independently of the presence of the 10a-ester group provides compelling evidence for the absence of enolization in the Chl *a* cation.[3] Thus, we disagree both with the assignment of the 1717-cm^{-1} band in the RR spectrum of Chl a^+ to the 10a-ester C=O and with the proposal of the enolization of the 9-keto C=O after Chl *a* cation formation in vitro (Heald et al., 1988).

In conclusion, the present FTIR spectroscopy study provides a solid basis for the assignment of the vibrational frequencies of ester and keto carbonyl groups in Chl a^+, P700, and P700$^+$. In particular, studies of the molecular interactions in vivo reveal free keto C=O groups in both P700 and P700$^+$. Therefore, it appears that, for Chl *a* both in vitro and in P700, we find no evidence in favor of the keto–enol tautomerization consequent to cation formation previously suggested by Wasielewski et al. (1981b) and Heald et al. (1988).

ADDED IN PROOF

After submission of this paper, Cotton and Heald kindly made us aware of their recent RR investigation on the electrochemically generated cation radical of Chl *a* and Chl *a* derivatives, including pyroChl *a* and 10-OH-Chl *a*. These new RR data (Heald & Cotton, 1990) are in complete agreement with our conclusion regarding the Chl *a* cation radical FTIR spectrum, namely, that the enol form is not involved in the Chl *a* cation radical: the examination of RR spectra of Chl *a* derivatives clearly shows that the band at 1717 cm^{-1} in the Chl *a* cation RR spectrum is indeed due to the 9-keto C=O stretching mode.

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[3] Although the C(9)–O bond from a Chl enol model in its neutral state is expected to absorb at much lower frequency than the corresponding 9-keto C=O (Hynninen et al., 1979; Bellamy, 1975), we have no knowledge of experimental or theoretical data on the vibrational absorption frequency of the corresponding C–O bond in the cation of the Chl enol form.

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