Communications to the Editor

Identification of Carbonyl Modes of P_{700} and P_{700}^+ by *in situ* Chlorophyll Labeling in Photosystem I†

Sunyoung Kim‡ and Bridgette A. Barry*

University of Minnesota
Department of Biochemistry, Molecular Biology, & Biophysics
St. Paul, Minnesota 55108-1022

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We have used isotopic labeling to identify vibrational stretching modes arising from ester carbonyl groups in P_{700} and P_{700}^+. P_{700} acts as the primary chlorophyll donor in the photosynthetic reaction center, photosystem I (PSI), which catalyzes photoinduced electron transfer from reduced plastocyanin to ferrodoxin.[1] The primary photochemical event in PSI results in the generation of the cation radical P_{700}^+. Our vibrational assignments for P_{700} and P_{700}^+ are based upon specific $^2H_3$-labeling of the chlorophyll (chl) 13^3 methoxy group (Figure 1A).

Chl serves several functional roles in photosynthesis. For example, chl can act as a primary electron donor in photoinduced charge separation or as an intermediate in energy transfer in the photosynthetic antenna complex. The structural factors leading to these differences in chl function have not as yet been elucidated. Of relevance is a complete description of chl conformation, of protein−chl interactions, and of local dielectric and polarizability.

X-ray diffraction analysis at 4.0 Å resolution[2,3] suggests that PSI contains a structural chl *a* dimer, which may play the role of the primary electron donor. The dimer is flanked by two additional chl molecules, A and A′, which may be analogous to the accessory pigments found in the bacterial reaction center.[4] Optical experiments have provided evidence that P_{700} is an exciton-coupled dimer (reviewed in ref 5). However, the correct description of the cation radical, P_{700}^+, has been controversial. Magnetic resonance experiments have been interpreted to give two results; some studies support a dimeric P_{700}^+, and some support a highly asymmetric charge/spin density distribution (reviewed in refs 5,6).

Vibrational spectroscopy can provide detailed structural information about the primary chl donor of PSI.[7−10] Isotope-based assignments are essential in the interpretation of complex vibrational spectra. In this report, chlorophyll has been labeled *in situ* by culturing a methionine-tolerant cyanobacterium, *Synechocystis sp*. PCC 6803, in the presence of $^2H_3$-methionine.[11,12] The 13^4 methyl group is the only methyl group of chl *a* that is derived from methionine.[13] Electrospray mass spectrometry demonstrates that 68% of chl *a* is $^2H_3$-labeled when the cyanobacterium is cultured in the presence of $^2H_3$-methionine (Figure 1B). Plasma desorption mass spectral analysis indicates that the phytol tail of chl is not labeled.[12] PSI was purified[14] from cyanobacterial cultures grown either on methionine or on $^2H_3$-methionine. EPR spectroscopy[6] at 264 K was used to show that the yield of P_{700}^+ in each preparation was indistinguishable under continuous illumination, that is, either 0.94 ± 0.09 ($^1H_3$) or 0.86 ± 0.06 ($^2H_3$) spin per 100 chl.[14]

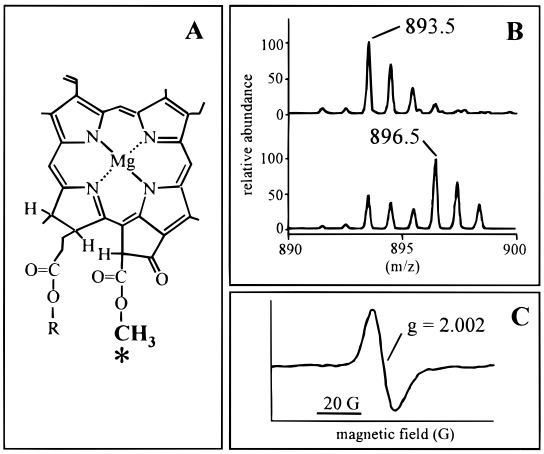

Figure 1. (A) Structure of chlorophyll *a*, with "R" representing the phytol tail and the asterisk denoting ^2H incorporation at position 13^4. (B) Electrospray mass spectra[12] of chlorophyll extracted from cyanobacteria, cultured[12] in the presence of $^1H_3$-methionine (top) or $^2H_3$-methionine (bottom). **(C)** Light-minus-dark EPR spectrum of P_{700}^+-minus-P_{700}, obtained from methionine-grown, cyanobacterial PSI samples[14] at pH 7.5. Data were recorded on a Bruker EMX 6/1 X-band spectrometer[17] with spectral conditions: microwave frequency, 9.1 GHz; power 0.2 mW; modulation amplitude, 3 G; scan time, 4 min; time constant, 2 s; and temperature, 264 K.

In difference FT-IR spectroscopy, absorption spectra are obtained in the dark and under illumination, using a single protein sample.[15] This light-minus-dark spectrum reflects only signals from the fraction of the molecules that have undergone a structural change. Reversible photoinduced changes are advantageous for signal averaging. Figure 1C shows that P_{700}^+ can be generated reversibly at 264 K in the presence of an equimolar ratio of potassium ferricyanide and potassium ferrocyanide. The reversible yield of P_{700}^+ was indistinguishable in the two preparations, that is, either 0.18 ± 0.03 ($^1H_3$) or 0.15 ± 0.01 ($^2H_3$) spin per 100 chl.

In Figure 2A, we present difference FT-IR spectra obtained at 264 K from control PSI. In Figure 2A, positive lines at 1754, 1742, 1718, and 1688 cm^{-1} and negative lines at 1749, 1735,

* Author for correspondence. University of Minnesota, 1479 Gortner Avenue, St. Paul, MN 55108. Telephone: 612-624-6732. Fax: 612-625-5780. E-mail: barry@biosci.cbs.umn.edu.

‡ Present address: 124 Engel Hall, Department of Biochemistry, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

(1) Golbeck, J. H. In *The Molecular Biology of Cyanobacteria*; Bryant, D. A., Ed.; Kluwer Academic Publishers: Dordrecht, 1994; pp 319−360.
(2) Krauss, N.; Schubert, W. D.; Klukas, O.; Fromme, P.; Witt, H. T.; Saenger, W. *Nat. Struct. Biol.* **1996**, *3*, 965−973.
(3) Schubert, W. D.; Klukas, O.; Krauss, N.; Saenger, W.; Fromme, P.; Witt, H. *J. Mol. Biol.* **1997**, *272*, 741−769.
(4) Deisenhofer, J.; Michel, H. *Science* **1989**, *245*, 1463−1473.
(5) Brettel, K. *Biochim. Biophys. Acta* **1997**, *1318*, 322−373.
(6) Setif, P. In *The Photosystems: Structure, Function and Molecular Biology*; Barber, J., Ed.; Elsevier: Amsterdam, 1992; pp 471−499.
(7) Tavitian, B. A.; Nabedryk, E.; Mantele, W.; Breton, J. *FEBS Lett.* **1986**, *201*, 151−157.
(8) Moenne-Loccoz, P.; Robert, B.; Ikegami, I.; Lutz, M. *Biochemistry* **1990**, *29*, 4740−4746.
(9) Nabedryk, E.; Leonhard, M.; Mantele, W.; Breton, J. *Biochemistry* **1990**, *29*, 3242−3247.
(10) Breton, J.; Nabedryk, E.; Leibl, W. *Biochemistry* **1999**, *38*, 11585−11592.
(11) Barry, B. A.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7099−7103.
(12) Razeghifard, M. R.; Kim, S.; Patzlaff, J. S.; Hutchison, R. S.; Krick, T.; Ayala, I.; Steenhuis, J. J.; Boesch, S. E.; Wheeler, R. A.; Barry, B. A. *J. Phys. Chem. B* **1999**, *103*, 9790−9800.
(13) Porra, R. J.; Klein, O.; Wright, P. E. *Eur. J. Biochem.* **1983**, *130*, 509−516.
(14) Noren, G. H.; Boerner, R. J.; Barry, B. A. *Biochemistry* **1991**, *30*, 3943−3950; Noren, G. H.; Barry, B. A. *Biochemistry* **1992**, *31*, 3335−3342.
(15) Braiman, M. S.; Rothschild, K. J. *Annu. Rev. Biophys. Biophys. Chem.* **1988**, *17*, 541−570.

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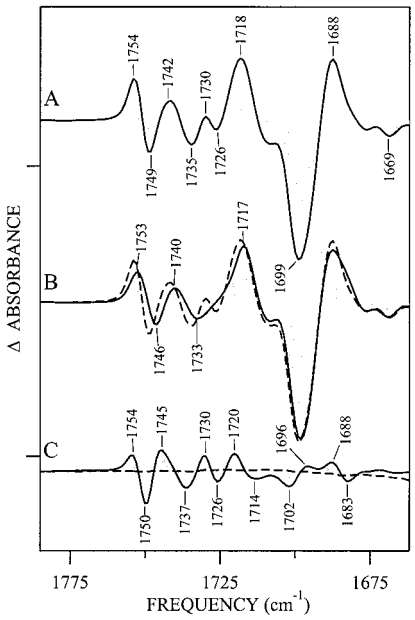

Figure 2. Light-minus-dark difference FT-IR spectra of P_{700}^{+}-minus-P_{700} obtained at pH 7.5. PSI was isolated from cyanobacteria grown on $^{1}H_3$-methionine (A and B, dashed line) or on $^{2}H_3$-methionine (B, solid line). (C, solid line) shows an isotope-edited spectrum, in which the data in (B, solid line) were subtracted from the data in (A). (C, dashed line) shows a subtraction control, in which half the data averaged to give (B, solid line) was subtracted from the other half of the data set. Data were recorded on a Nicolet Magna 550 II spectrometer with a MCT-A detector,[17] using spectral conditions: resolution, 4 cm^{-1}; mirror velocity, 2.5 cm/s; apodization function, Happ-Genzel; levels of zero filling, 1; data acquisition time, 4.0 min; and temperature, 264 K. The sample concentration was 4−5 mg chl/mL, the path length was less than 6 μm, and the sample area was held constant. Spectra in (A and B) were normalized to correct for the total amount of protein. Because the sample area was constant, this normalization procedure accounts for any differences in concentration and path length. Data are an average of 20 spectra; tick marks on the *y* axis represent $\Delta 2 \times 10^{-3}$ absorbance units.

1726, and 1699 cm^{-1} are observed. On the basis of the EPR experiment shown in Figure 1C, infrared modes associated with P_{700} oxidation are anticipated under these conditions. The terminal acceptor is expected to be potassium ferricyanide,[10,16] which has no fundamental vibrational modes in the 1800−1200 cm^{-1} region (see ref 17 and references therein). In agreement with these expectations, the spectrum obtained with an equimolar mixture of ferro/ferricyanide (Figure 2A) is similar to spectra previously assigned to P_{700}^{+}-minus-P_{700}.[7,9,10]

Oxidation of the chl macrocycle in vitro has been shown to cause an upshift of carbonyl stretching vibrations.[9,18] Only two derivative-shaped spectral features are observed between 1760 and 1650 cm^{-1} in the in vitro chl *a* oxidation spectrum. A feature at (+)1751/(−)1738 cm^{-1} was assigned to the ester carbonyl of chl *a*, on the basis of the absence of this spectral feature in data obtained from oxidation of 13^2-demethoxycarbonyl-chl *a*.[9,18] An intense spectral feature at (+)1718/(−)1693 cm^{-1} was assigned to the 13^1 keto carbonyl vibration of chl *a*.[9,18] Comparison to these in vitro data suggests assignment of the (+)1754/(−)1749 cm^{-1} feature in P_{700}^{+}-minus-P_{700} (Figure 2A) to an ester carbonyl vibration and of the intense (+)1718/(−)1699 cm^{-1} feature to the keto group.[9,10,18,19] However, the P_{700}^{+}-minus-P_{700} spectrum (Figure 2A) exhibits additional spectral lines that are not assignable based on comparison to chl *a* in vitro.

Specific labeling of the 13^3 methoxy group is expected to have the most significant effect on the ester carbonyl stretching vibration (Figure 1A) and will identify vibrational modes arising from this normal coordinate. The difference FT-IR spectrum, obtained from labeled PSI, is presented in Figure 2B, solid line. $^{2}H_3$-labeling of chl causes 2−3 cm^{-1} downshifts of several infrared modes in the 1760−1710 cm^{-1} region. These isotope shifts cannot arise from labeling of carboxylic acids,[20] because GC−MS experiments[21] show that there is no significant deuterium incorporation (<2%) into aspartic or glutamic acid residues in PSI.

An isotope-edited spectrum (Figure 2C, solid line) was constructed by a 1:1 subtraction of the $^{2}H_3$-methionine spectrum (Figure 2B, solid line) from the control spectrum (Figure 2A). Spectral features, observed in the isotope-edited spectrum, were not observed in the subtraction control (Figure 2C, dashed line). The isotope-edited spectrum exhibits derivative-shaped spectral features at (+)1754/(−)1750 and (+)1745/(−)1737 cm^{-1} (Figure 2C, solid line). These data provide support for the assignment of both the (+)1754/(−)1749 and (+)1742/(−)1735 cm^{-1} lines in Figure 2A to ester carbonyl vibrations of (+)P_{700}^{+}/(−)P_{700}. Derivative-shaped features at (+)1730/(−)1726 and (+)1720/(−)1714 cm^{-1} are also observed in the isotope-edited spectrum (Figure 2C). Due to the observed similarities in intensities and in the oxidation-induced frequency shift, these additional isotope-sensitive infrared modes between 1730 and 1714 cm^{-1} are also assigned to ester carbonyl vibrations of P_{700}/P_{700}^{+}. The origin of weak isotope-sensitive features between 1702 and 1630 cm^{-1}, which apparently underlie the putative keto carbonyl stretching vibration, is under investigation.

This work has provided evidence that more than one chl ester carbonyl vibration is perturbed by the oxidation of P_{700}. The observation of more than one 13^3 C═O vibration demonstrates that the immediate environment of protein-bound chl has the potential to alter the frequencies of carbonyl vibrations in both the neutral and cationic states. Multiple frequencies could be the result of three different effects. First, a distribution of frequencies could be caused by local heterogeneity in the protein environment of P_{700}. Possible influences on frequency may originate from small alterations in hydrogen bonding, in other local nonbonding interactions, and in microscopic dielectric constants. Second, the primary donor may be more complex in structure than is commonly supposed. As one possible example, heterogeneous placement of an epimer of chl *a*, chl *a*′,[22] in the primary donor could give rise to multiple ester carbonyl frequencies. Third, a single chl molecule may contribute more than one ester carbonyl stretching mode to the spectrum because a dynamic or static process induces a non-homogeneous distribution of charge in P_{700}^{+},[23,24] and this charge heterogeneity alters the double bond character of the ester group. These possible explanations are under further investigation.

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(16) Lakshmi, K. V.; Jung, Y.-S.; Golbeck, J. H.; Brudvig, G. W. *Biochemistry* **1999**, *38*, 13210−13215.
(17) Kim, S.; Barry, B. A. *Biophys. J.* **1998**, *74*, 2588−2600.
(18) Heald, R. L.; Cotton, T. M. *J. Phys. Chem.* **1990**, *94*, 3968−3975.
(19) Boldt, N. J.; Donohoe, R. J.; Birge, R. R.; Bocian, D. F. *J. Am. Chem. Soc.* **1987**, *109*, 2284−2298.
(20) Flett, M. S. C. *Spectrochim. Acta* **1962**, *18*, 1537−1556.
(21) Hutchison, R. S.; Betts, S. D.; Yocum, C. F.; Barry, B. A. *Biochemistry* **1998**, *37*, 5643−5653.
(22) Kobayashi, M.; Watanabe, T.; Nakazato, M.; Ikegami, I.; Hiyama, T.; Matsunaga, T.; Murata, N. *Biochim. Biophys. Acta* **1988**, *936*, 81−89.
(23) Sieckmann, I.; Brettel, K.; Bock, C.; van der Est, A.; Stehlik, D. *Biochemistry* **1993**, *32*, 4842−4847.
(24) Kass, H.; Rautter, J.; Bonigk, B.; Hofer, P.; Lubitz, W. *J. Phys. Chem.* **1995**, *99*, 436−448.