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Possible ligation of the Mn cluster in photosystem II by the carboxyl-terminus of the D1 polypeptide: An FTIR study.

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Introduction

The catalytic site of photosynthetic oxygen evolution contains a $(Mn)_4$-Ca cluster that interacts closely with a redox-active tyrosine residue known as Y_Z [for review see (Britt, 1996; Hoganson & Babcock, 2000; Debus, 2000; Pecoraro & Hsieh, 2000; Tommos & Babcock, 2000; Renger, 2001)]. The $(Mn)_4$ cluster accumulates oxidizing equivalents in response to photoinduced electron transfer reactions within PSII, then catalyzes the oxidation of two molecules of water, releasing one molecule of O_2 as a by-product. Understanding the mechanism of water oxidation requires understanding the ligation environment of the $(Mn)_4$-Ca cluster and how this environment changes as oxidizing equivalents are accumulated. To provide this information, we are combining spectroscopic characterizations involving EPR, FTIR, and time-resolved optical absorption and fluorescence spectroscopies with site-directed mutagenesis and isotopic labeling. Our early studies, conducted with intact cells, suggested that Asp170, His332, Glu333, His337, and Asp342 of the D1 polypeptide are potential ligands of Mn and that Asp59, Asp61, and Asp342 of the D1 polypeptide are potential ligands of Ca (Chu *et al.*, 1994a,b; 1995a,b). Our recent efforts, conducted with purified PSII particles, have shown that D1-His332 almost certainly ligates the assembled $(Mn)_4$ cluster (Debus *et al.*, 2001) and that D1-Asp170 either ligates Mn directly or participates in a hydrogen bond to the $(Mn)_4$-Ca cluster (Chu *et al.*, 2001).

The free carboxylate group (α-COO^-) of Ala344 at the *C*-terminus of the D1 protein has been proposed to ligate the assembled $(Mn)_4$ cluster (Nixon *et al.*, 1992). To identify vibrational modes potentially associated with the α-COO^- moiety of Ala344, we have incorporated L-[1-^{13}C]alanine into wild-type PSII particles and recorded mid-frequency (1800-1200 cm^{-1}) S_2/S_1 FTIR difference spectra. Our preliminary results are consistent with ligation of the $(Mn)_4$-Ca cluster by the *C*-terminal α-COO^- carboxylate of Ala344 either directly or via a hydrogen bond.

Materials and Methods

Strains. For this study, a hexahistine-tag (His-tag) was fused to the *C*-terminus of CP47 in the glucose-tolerant wild-type strain (Williams, 1988) of *Synechocystis* sp. PCC 6803. Wild-type cells were first transformed with a construct that replaced the 3' 163 bp of *psbB* (the gene encoding CP47) plus 29 bp of downstream DNA (*i.e.*, the 192 bp *MscI*/*AflII* fragment) with DNA encoding resistance to kanamycin. The resulting km^r strain cannot assemble PSII and is unable to grow photoautotrophically.

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[3]*This contribution is dedicated to the memory of Gerald T. Babcock.*

Next, a plasmid containing a 1034 bp fragment of *Synechocystis* DNA containing the 3' 341 bp of *psbB* plus downstream DNA with six histidine codons inserted before the TAG stop codon (Debus *et al.*, 2001) was transformed into the non-photoautotrophic km^r strain and single colony survivors were selected for ability to grow photoautotrophically. The presence of the His-tag was verified as described previously (Debus *et al.*, 2001).

Incorporation of L-[1-^{13}C]alanine. Cultures, each consisting of 15 L of cells, were propagated photoautotrophically in 15 modified 1 L Erlenmeyer flasks (Chu *et al.*, 1994b) in the presence of 0.5 mM unlabeled (^{12}C) L-alanine or 0.5 mM L-[1-^{13}C]alanine (Peloquin *et al.*, 1999). Under these conditions, approximately 70% of the alanine incorporated into the thylakoid membranes of wild-type cells is supplied from the alanine in the growth medium, with little equilibration into pyruvate or the amino acids derived from pyruvate (*i.e.*, leucine, isoleucine, and valine) (Peloquin *et al.*, 1999). Three separate unlabeled and three separate L-[1-^{13}C]alanine cultures were employed for these studies.

Purified PSII particles. Isolated PSII particles were purified as described previously (Debus *et al.*, 2001) except that the *n*-dodecyl β-D-maltoside-extracted thylakoid membranes were centrifuged for 10 min at 24,000 x g, the supernatant was pumped directly onto the 40 mL, 5-cm-diameter, Ni-NTA superflow affinity resin (Qiagen) at 3 mL/min, the column flow-through was saved for analysis by mass spectrometry, and the purified PSII particles were eluted with sample buffer [25% (w/v) glycerol, 50 mM MES-NaOH (pH 6.0), 20 mM $CaCl_2$, 5 mM $MgCl_2$, 0.3% (w/v) the *n*-dodecyl β-D-maltoside] containing 50 mM L-histidine. After the addition of EDTA to 1 mM, the eluted PSII particles were concentrated to ~ 0.5 mg of Chl/mL by ultrafiltration. Removal of histidine and transfer into sucrose buffer [sample buffer containing 0.4 M sucrose instead of glycerol (Chu *et al.*, 2001)] was accomplished by two cycles of concentration/dilution in Centricon-100 concentrators (Millipore Corp.) followed by concentration to ~ 5 mg of Chl/mL. Samples were then frozen in liquid nitrogen and stored at -80°C until use.

FTIR measurements. Final FTIR samples were prepared and FTIR spectra were recorded as described previously (Chu *et al.*, 2001).

Results and Discussion

The S_2/S_1 FTIR difference spectra of unlabeled and L-[1-^{13}C]alanine-labeled wild-type *Synechocystis* PSII particles are shown in Figure 1 [identical data were obtained with PSII particles isolated from the His-tagged wild-type* strain (Debus *et al.*, 2001) that we employ as a control for site-directed mutants]. The 1365/1400 cm^{-1} bands, previously assigned to carboxylate modes by Noguchi and coworkers (Noguchi *et al.*, 1995), were not affected by L-[1-^{13}C] labeling. Therefore, these modes do not originate from the *C*-terminal carboxylate (α-COO^-) of Ala344. However, several other modes are sensitive to L-[1-^{13}C]alanine-labeling. The most prominent of these are located in the symmetric $\nu_{sym}(COO^-)$ carboxylate stretching region (1450-1300 cm^{-1}). To display these modes more clearly, the ^{12}C-*minus*-^{13}C double difference spectrum is presented in Figure 2. This double-difference spectrum is compatible with S_1- and S_2-state modes at ~ 1356 and ~ 1320 cm^{-1}, respectively, in the unlabeled sample shifting to ~ 1338 and ~ 1304 cm^{-1}, respectively, in the L-[1-^{13}C]alanine-labeled sample. The magnitudes of these shifts, 16-18 cm^{-1}, are consistent with the ~ 17 cm^{-1} shift of the symmetric carboxylate mode observed in L-[1-^{13}C]alanine (data

not shown[4]). While we cannot exclude the possibility that these shifts correspond to altered amide III modes (predominantly C-N stretching and N-H bending) of peptide linkages involving alanine-derived carbonyl groups, we think that this possibility is

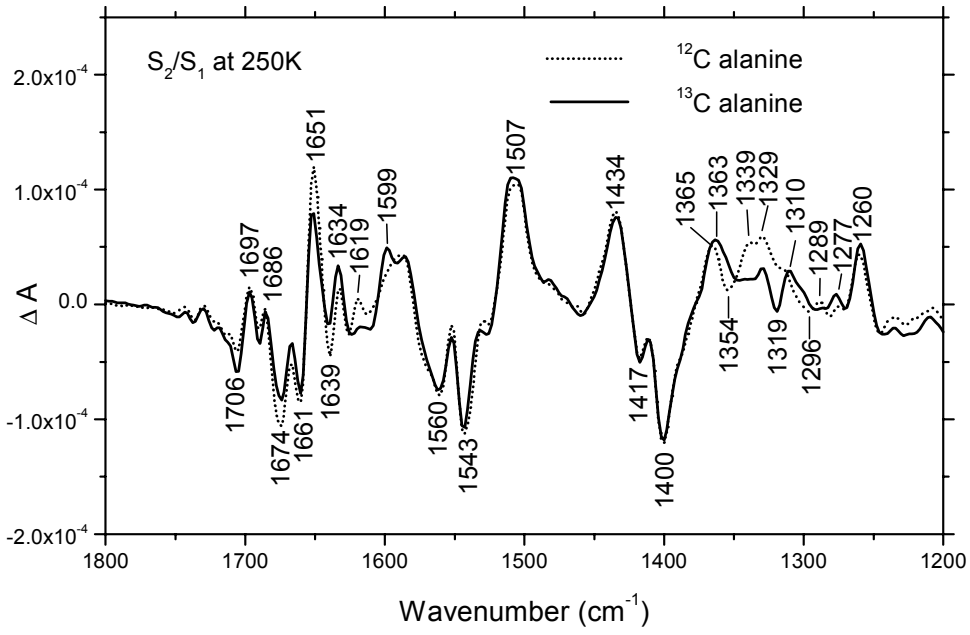

Fig. 1. Comparison of the mid-frequency S_2/S_1 FTIR difference spectra of wild-type *Synechocystis* PSII particles purified from cells propagated in the presence of unlabeled L-alanine (^{12}C, dashed line) and in the presence of L-[1-^{13}C]alanine (solid line). Both spectra were collected at 4 cm^{-1} resolution.

unlikely because global ^{15}N-labeling of spinach PSII preparations caused no shifts in this region of the spectrum (Noguchi *et al.*, 1995).

In the asymmetric $\nu_{asym}(COO^-)$ carboxylate stretching region (1650-1550 cm^{-1}), several bands shifted in the L-[1-^{13}C]alanine-labeled sample (*e.g.*, positive bands near 1651 and 1619 cm^{-1} and the negative band near 1639 cm^{-1}). If some of these bands correspond to the asymmetric α-COO^- carboxylate stretching mode of Ala344 and the bands between 1360 and 1300 cm^{-1} correspond to the symmetric α-COO^- carboxylate stretching mode, then the difference in frequency between these bands ($\Delta\nu > 200$ cm^{-1}) would be consistent with unidentate coordination of Mn (Deacon & Phillips, 1980; Nakamoto, 1997) if Ala344 ligates the $(Mn)_4$-Ca cluster [although the accuracy of such an analysis for Mn ligation has been questioned (Smith *et al.*, 1997)]. However, another possibility is that the modes between 1650 and 1550 cm^{-1} correspond to one or more peptide linkages involving alanine-derived carbonyl groups that undergo structural alterations during the $S_1 \rightarrow S_2$ transition. For example, the amide I mode (predominantly C=O stretching) occurs at 1620-1690 cm^{-1} and the amide II mode (a mixture of N-H bending and C-N stretching) occurs around 1550 cm^{-1}.

To resolve the assignment ambiguities noted above, we are in the process of incorporating L-[1-^{13}C]alanine into cells of the *Synechocystis* mutants D1-Ala344Gly

[4]The symmetric stretching mode of the α-COO^- group of alanine appears at ~ 1411 cm^{-1} (Fukushima *et al.*, 1959). We observe that this band shifts from ~ 1413 cm^{-1} in unlabelled (^{12}C) L-alanine to ~ 1396 cm^{-1} in L-[1-^{13}C]alanine (not shown).

and D1-Ala344Ser. Incorporating L-[1-^{13}C]alanine will label the *C*-terminal α-COO^- carboxylate of the D1 polypeptide in neither mutant. Both mutants are photoautotrophic, assembling O_2-evolving $(Mn)_4$ clusters *in vivo* (Nixon *et al.*, 1992), and we have constructed both in a His-tag background.

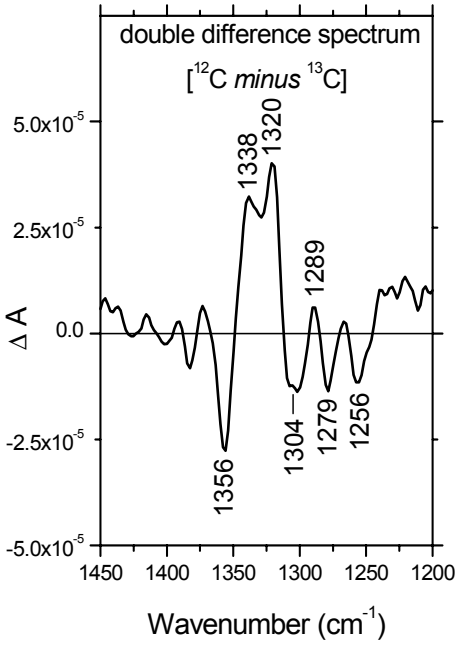

Fig. 2. Double difference spectrum, ^{12}C-*minus*-^{13}C, obtained by subtracting the S_2/S_1 difference spectrum of L-[1-^{13}C]labeled PSII particles from the S_2/S_1 difference spectrum of unlabeled PSII particles.

Concluding Remarks

The L-[1-^{13}C]alanine-induced shifts of putative carboxylate modes between 1360-1300 cm^{-1} are consistent with a structural coupling between the *C*-terminal α-COO^- carboxylate of Ala344 and the $(Mn)_4$-Ca cluster. If the carboxylate origin of these modes are confirmed by the incorporation of L-[1-^{13}C]alanine into the D1-Ala344Gly and D1-Ala344Ser mutants, they would show that the *C*-terminal α-COO^- carboxylate of Ala344 either directly ligates Mn or Ca or participates in a hydrogen bond to the $(Mn)_4$-Ca cluster.

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