



Structural and functional roles of calcium ion in photosynthetic membrane protein complexes

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(Degree)

博士 (農学)

(Date of Degree)

2014-03-25

(Date of Publication)

2020-03-25

(Resource Type)

doctoral thesis

(Report Number)

甲第6024号

(URL)

<https://hdl.handle.net/20.500.14094/D1006024>

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Doctoral Dissertation

Structural and functional roles of calcium ion in photosynthetic membrane protein complexes

光合成膜蛋白質複合体におけるカルシウムイオンの
構造的および機能的役割

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January 2014

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Chapter 1 General introduction

1.1 Photosynthetic organisms

Photosynthesis is the fundamental chemical reaction that takes place in photosynthetic organisms to convert light energy into chemical energy through a series of photosynthetic redox events. Photoautotrophs can synthesize hydrocarbons directly from carbon dioxide using energy of light, whereas photoheterotrophs use organic compounds, rather than carbon dioxide, as a source of carbon. The photosynthetic organisms are divided into two categories depending on the kind of electron sources. When water molecules are utilized as electron donors, oxygen molecules are produced as a by-product. This type of photosynthesis is performed by cyanobacteria, algae and higher plants, and called as oxygenic photosynthesis which have maintained the atmospheric oxygen level and allowed the evolution of living organisms using aerobic respiration. On the other hand, no oxygen molecules are evolved when hydrogen, hydrogen sulfide or organic acids are used as the electron donors. This type of photosynthesis is called as anoxygenic photosynthesis, which occurs in primitive photosynthetic bacteria such as purple bacteria, green sulfur and non-sulfur bacteria, and heliobacteria, evolved early in the history of life.

Generally, a photosynthetic charge separation takes place at the reaction centers (RC) of photosynthetic organisms utilizing light energy collected by light-harvesting (LH) complexes, and the light-induced electrons are transferred through photosynthetic electron transport pathways mediated by various cofactors. The photosynthetic RCs are classified into two groups depending on the types of electron transport system; the type-I RC containing iron-sulfur complexes in photosystem I,

green sulfur bacteria and heliobacteria, and the type-II RC containing quinone complexes in photosystem II, purple bacteria, and green non-sulfur bacteria.

1.2 Photosynthesis in type-II reaction center

Photosynthetic organisms with type-II reaction centers are evolutionally related each other since they have commonly central domains of heterodimeric protein subunits and quinone-type electron transport system. These phototrophs are, however, largely different in the protein composition and several cofactors, which are closely related to the stability of photosystems. A number of biochemical and physicochemical studies have provided valuable information on the relation between structure and stability of type-II RCs, including LH1-RC complexes from purple bacteria, and PSII from cyanobacteria and higher plants.

1.2.1 Purple bacteria

In purple photosynthetic bacteria, light energy is captured by two types of light-harvesting (LH) pigment-protein complexes, i.e. peripheral antenna LH2 and core antenna LH1 complexes, and transferred efficiently to RCs where primary charge separation of *a*-type bacteriochlorophyll (BChl-*a*) special pairs occur across the membrane [1]. Two electrons from the RC are transferred to a ubiquinone to form a ubiquinol, which migrates to a quinone pool in the membrane. The photo-oxidized special pairs of the RCs are reduced by cytochrome bc_1 complexes through soluble heme proteins to form the cyclic electron flow. Electron transport pumps protons across the membrane and the resultant trans-membrane proton motive force is used by the ATP-synthase to generate ATP (Figure 1-1).

High resolution three dimensional crystallographic structures of isolated RC complexes have been reported for both mesophilic [2, 3] and thermophilic [4] purple bacteria. The results demonstrated that RCs are comprised of four intrinsic protein subunits (C, H, M and L), but the extrinsic proteins, ubiquitous for PS II, are not involved in the assembly of the bacterial RCs. In contrast, the X-ray structure of the RC associating with the LH1 has been limited to *Rhodospseudomonas (Rps.) palustris* at moderate resolution [5]. Generally, LH1 complexes are oligomers of minimal subunits composed of α - and β -polypeptides, BChl-*a* and carotenoid molecules. The RC is surrounded by the LH1 with a one-to-one stoichiometric ratio to form LH1-RC complexes (Figure 1-2). Recent electron microscopic (EM) and atomic force microscopic (AFM) studies showed two-dimensional projection maps at low resolution (8.5 to 26 Å), indicating a variety of LH1 complexes forming 12- to 16-meric assembly of $\alpha\beta$ -subunits depending on the species [6-11]. However, the LH1-RC structure at atomic resolution is absolutely required for elucidating details of the function and structure of LH1 pigment-protein complexes. Since the RC tightly associates with the LH1, interaction modes between both complexes, and the size and/or shape of LH1 rings are significant to understand the structural functional consequences, including structural stabilities, quinone transport mechanisms, and excited state dynamics of the LH1-RC complex.

The LH1 complex shows characteristic electronic absorption properties due to BChl-*a* dimers and carotenoid molecules incorporated into the $\alpha\beta$ -subunits. These pigments are useful as internal probes for monitoring structural and functional properties of the LH1-RC complexes. In a typical mesophilic purple bacterium *Allochromatium (Alc.) vinosum*, the LH1 complex is suggested to have a macrocycle with a 16-fold symmetry [6]. The BChl-*a* – BChl-*a* interactions within the LH1 ring

resulted in the characteristic absorption property of the core complex as shown in Figure 1-3. The LH1-RC contains only *a*-type BChl molecule of which Q_y , Q_x and Soret bands appear at 880, 590 and 390 nm, respectively, as well as carotenoid bands around 450 – 550 nm and RC bands at 780 and 800 nm. Interestingly, thermophilic *Thermochromatium (Tch.) tepidum* showed an unusual red-shift of the LH1 Q_y peak to 915 nm along with slight red-shifts of Soret and Q_x bands although peak positions for carotenoid and RC bands remained to be unchanged [12]. The large red-shift of the Q_y band seems to be disadvantage in terms of the uphill energy transfer from the LH1 to the RC [13]. Taking into account that *Tch. tepidum* is a sole thermophile among purple bacteria, the relationship between thermostability and low-energy Q_y transition is a matter of interest.

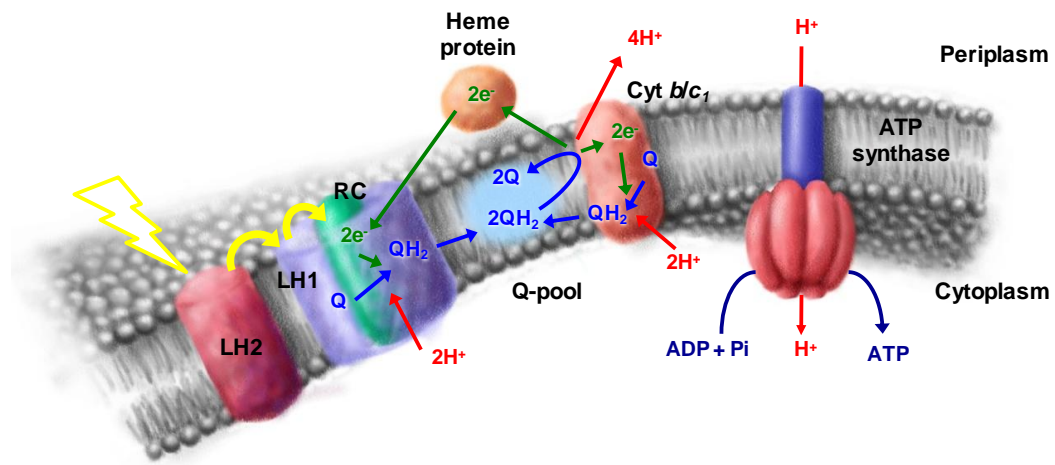


Figure 1-1. Photosynthetic apparatuses and the reaction scheme in an intracytoplasmic membrane of purple bacteria.

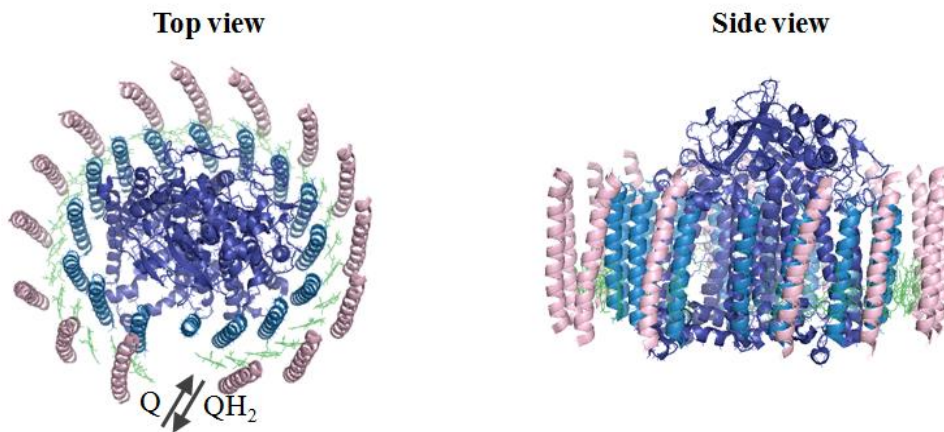


Figure 1-2. The crystallographic structure of an LH1-RC complex from *Rps. palustris* at 4.8 Å resolution [5]. The RC (purple) comprised of C, H, M, and L subunits is surrounded by the LH1 involving α - polypeptides (blue), β -polypeptides (magenta), BChl-*a* molecules (green).

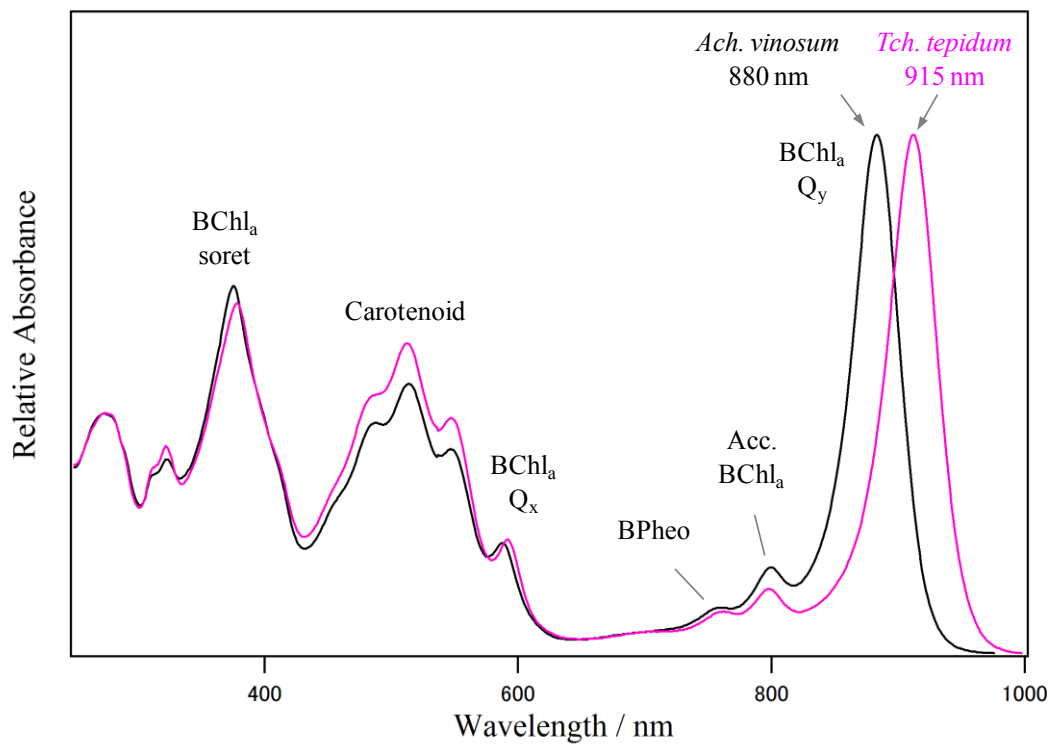


Figure 1-3. Absorption spectra of LH1-RC complexes from thermophilic *Tch. tepidum* (magenta) and mesophilic *Alc. vinosum* (black)(unpublished result).

1.2.2 Photosystem II in cyanobacteria and higher plants

Oxygenic phototrophs including cyanobacteria and higher plants convert photon energy into chemical energy through a series of light-induced electron transfer reactions initiated with the charge separation of chlorophyll special pairs located in the central part of photosystem I and II (PSI and PSII). The reducing power is transferred from PSII to PSI through cytochrome *b₆f*, and finally utilized for reduction of NADP⁺ to assimilate CO₂. The oxidized equivalents accumulated on the PSII donor side are neutralized by substrate waters to release protons for driving ATP synthase and O₂ molecules as a by-product.

The photosynthetic oxygen evolution occurs in the oxygen-evolving complex (OEC) of PSII. The OEC is composed of a heterodimer of D1 (psbA) and D2 (psbD) proteins associated with two chlorophyll proteins (CP), CP47 (psbB) and CP43 (psbC), and involves a catalytic Mn₄CaO₅ cluster located on the luminal side of PSII. These are highly conserved from cyanobacteria (Figure 1-4) to higher plants (Figure 1-5) to preserve the essential function of oxygenic phototrophs. In the past decade, X-ray crystallography has revealed the structures of cyanobacterial PSII at resolutions of 3.8 Å to 2.9 Å [14-18]. A very recent structural model at the atomic resolution level has revealed details of the ligation structure of the Mn₄CaO₅ cluster [19]. The Mn ions are bridged by several oxygen atoms and coordinated by water molecules as well as by Asp, Glu, Ala, and His residues in PsbA and/or PsbC proteins (Figure 1-6). The oxidized equivalents accumulated on the cluster and/or its ligands are reduced by electrons provided from a splitting reaction of substrate water molecules through a light-driven S-state cycle with five intermediate states S_n (n = 0 – 4), where n denotes the number of oxidizing equivalents stored (Figure 1-7). The OEC advances from the thermally stable S₁ state to the next oxidation state in a stepwise manner by absorbing

each photon and attains the highest oxidation state S_4 , followed by relaxation to the lowest oxidation state S_0 concurrent with a release of one oxygen molecule [20, 21]. Two water molecules are converted to one oxygen molecule by the OEC concurrent with release of four protons although details of the water oxidation mechanism are still a matter of debate [18, 22].

In contrast, structural analysis of PSII in higher plants has been delayed due to the instability of the membrane protein complex. The visualization of plant PSII structures has been limited to electron micrographs at low resolutions [23, 24]. Yet the findings to date strongly indicate that the structure and function of the PSII core assembly are almost identical to those of its prokaryotic counterparts, except for a critical difference in the composition of extrinsic proteins, which are lacking in purple bacteria and may provide valuable insights into the evolution of photosynthetic organisms [25]. Extrinsic proteins are thought to play essential roles for stabilizing and protecting PSII cores [26]. In cyanobacteria, PsbO, PsbV, and PsbU residing on the luminal side of PSII play significant roles in the regulation and stabilization of the water oxidation machinery. Higher plants possess major nuclear gene-encoded extrinsic proteins named PsbO, PsbP, and PsbQ. PsbO is a common extrinsic protein highly conserved among the oxygenic phototrophs. PsbP and PsbQ indigenous to plant PSII have been proposed as the functional equivalents of PsbV and PsbU, respectively, in bacterial PSII, having replaced them during the course of evolution from ancestral cyanobacteria to higher plants. These proteins play a key role for maintaining oxygen-evolving activity at physiological rates [26, 27]. PsbO independently associates with the PSII core [28, 29], whereas binding of PsbP occurred through electrostatic interactions with PsbO [30, 31]. PsbQ requires both PsbO and PsbP for its binding [29, 30]. PsbP protein is related to the stability of the

Mn₄CaO₅ cluster [32] as well as the binding affinity of functional Ca²⁺ and Cl⁻ ions [33, 34]. In addition, *psbP*-deletion mutants revealed that PsbP is indispensable for the normal PSII function in higher plants [35]. Another function of this protein was indicated that PsbP has a Mn²⁺-binding ability as a reservoir to keep or deliver manganese ions [36]. PsbQ protein is related to be binding of Cl ions [37]. Studies on transgenic tobacco [35] and Arabidopsis [38] revealed that PsbQ protein is not necessary under normal growth condition [35, 38] but is required for photoautotrophic growth under low-light condition [38].

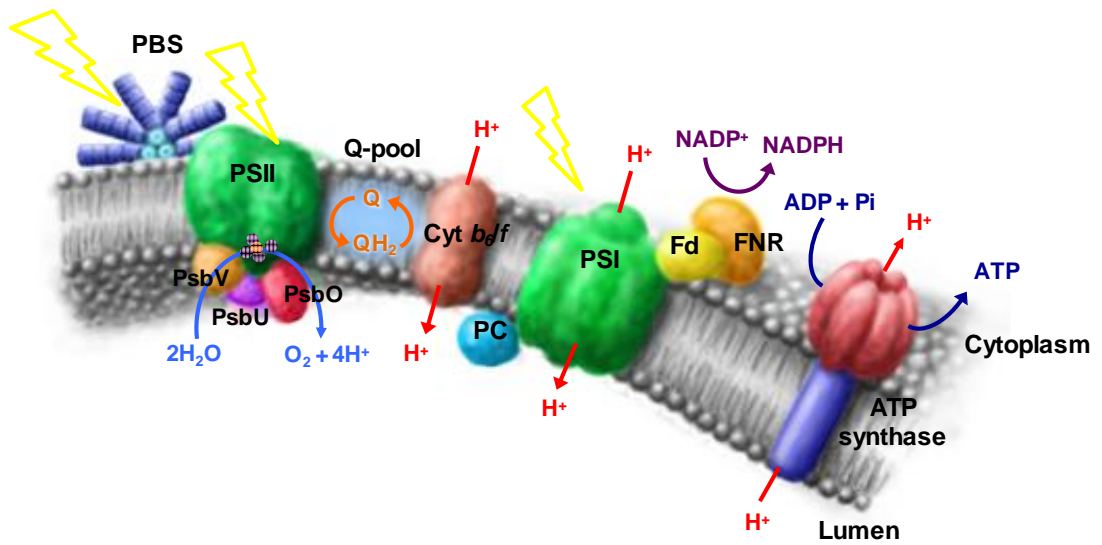


Figure 1-4. Photosynthetic apparatuses and the reaction scheme in a thylakoid membrane of cyanobacteria.

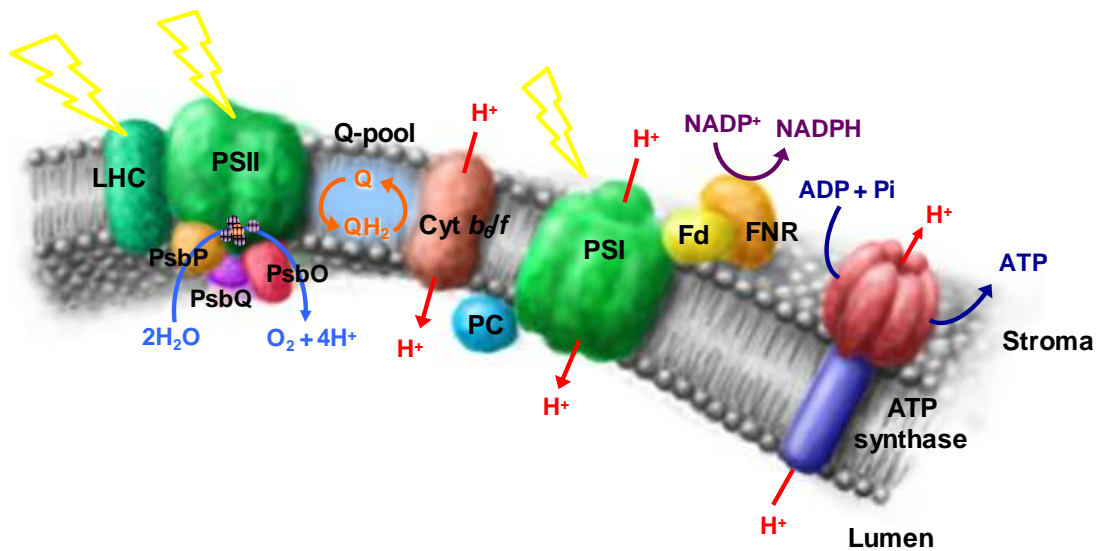


Figure 1-5. Photosynthetic apparatuses and the reaction scheme in a thylakoid membrane of higher plants.

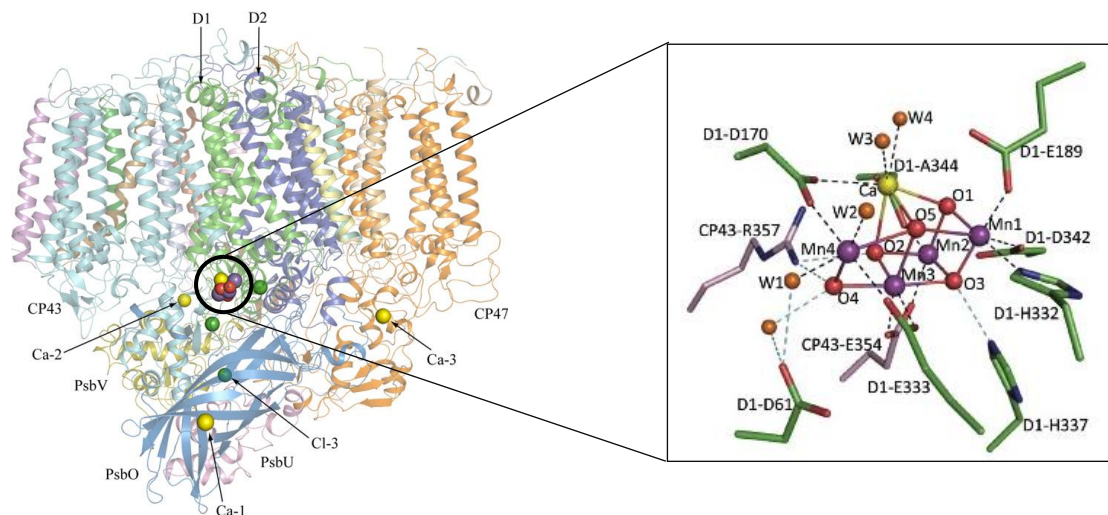


Figure 1-6. The crystallographic structure of PSII from *Thermosynechococcus vulcanus*. The inset shows an expanded view of the Mn_4CaO_5 cluster [19, 39].

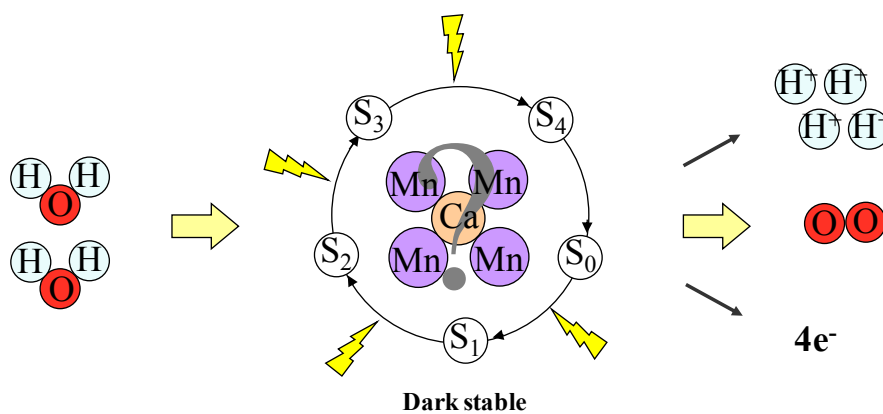


Figure 1-7. Photosynthetic water oxidation by the Kok cycle through five intermediate state (labeled S_0 to S_4) of the Mn_4CaO_5 cluster.

1.3 Roles of calcium ions in type-II reaction centers

Calcium ion is an essential nutrient playing structural roles in the cell wall and membranes and functional roles as an intracellular messenger coordinating responses to developmental signals and environmental changes in living organisms. This is true for phototrophs with type-II reaction centers, however, other intriguing and important roles of calcium ions have been reported. In *Tch. tepidum*, Ca ions are closely related to the enhanced thermal stability and unusual spectroscopic properties of the LH1-RC complexes [12, 40]. Since *Tch. tepidum* was isolated from a living environment rich in the mineral calcium carbonate, it is presumed that this bacterium uses Ca ions to enhance its heat tolerance and thereby to survive in extreme environments. After the removal of Ca^{2+} from the *Tch. tepidum* LH1-RC, the thermal stability was significantly reduced, along with unusual Q_y blue-shifts from 915 nm to 880 nm that is comparable to those in the mesophilic counterparts. The modified properties are fully reversed upon the reconstitution of Ca^{2+} . Interestingly, only strontium ion is biosynthetically replaceable with Ca ion, resulting in different spectroscopic and thermodynamic properties of the LH1-RC complexes [41]. These results support that Ca ions play key roles in stabilizing the pigment-protein assembly and modulating the light-harvesting properties of the *Tch. tepidum* LH1-RC complex [42]. Based on the primary sequence of the *Tch. tepidum* LH1 complex [43], putative Ca^{2+} binding sites are the C-terminal region of α -polypeptide or the N-terminal region of β -polypeptide, where several neighboring acidic residues are located (Figure 1-8). A recent topological study strongly indicated the location of the binding site to be at the C-terminal region of the $\alpha\beta$ -subunit [44]. However, the structural changes of the LH1-RC proteins induced by the Ca^{2+} -binding are not detected and the molecular mechanism enhancing the thermal stability is largely unknown.

LH1 α -polypeptide

TTP MFTM^NANLYKI^WLILD^PRRV^LVS^IVAFQ^IVL^GLLI^HMIVL^ST-DLN^WL^{DD}NIPVSYQALGKK

AVN MSPDLWKI^WLLV^DPRRIL^IAVFA^FLTVL^GLAI^HMILL^STA^EFN^WL^{ED}GV^PAA

LH1 β -polypeptide

TTP AEQKSLTGL^T^{DD}EAK^EFHAI^FMQSMYAWFGLV^VIA^HLLAWLYRPWL

AVN NSSMTGL^T^{EQ}E^{AQ}E^FHGIFVQSM^TAFFGIV^VIA^HILAWLWRPWL

Figure 1-8. Amino acid sequences of LH1 α - and β -polypeptides from *Tch. tepidum* and *Alc. vinosum*. (Red: acidic residues forming putative metal binding sites. Green: BChl-*a* binding site.)

In the PSII of cyanobacteria and higher plants, calcium ion is an indispensable inorganic cofactor necessary for the photosynthetic water oxidation [45]. Previous studies demonstrated that O₂-evolving capabilities of the PSII were lost upon the depletion of Ca²⁺ ions, but restored by the replacement with Ca²⁺, or to a lesser extent, with Sr²⁺. In the absence of Ca²⁺, the OEC can be oxidized to the S₂-state but further oxidation steps beyond the S₂-state are interrupted [46, 47]. Therefore, Ca²⁺ ions are considered as an indispensable inorganic cofactor for O₂-evolution. It has been believed that Sr²⁺ is functionally replaceable with Ca²⁺ [48] although EPR studies for the Sr²⁺-substituted OEC revealed that magnetic properties of the Mn₄CaO₅ cluster was significantly altered [46, 47, 49].

A ¹¹³Cd-NMR study demonstrated the proximity of the Mn₄CaO₅ cluster to the ¹¹³Cd-bound Ca²⁺-site, in which ¹¹³Cd²⁺ is located in a symmetrical six-coordinate sphere of oxygen and nitrogen or chlorine [50]. In addition, EPR studies on the Mn²⁺-supplemented PS II membranes indicated that the Mn²⁺ ion that occupies the Ca²⁺-site is located outside the first coordination sphere of the Mn₄CaO₅ cluster [51]. Furthermore, site-directed mutagenesis studies of *Synechocystis* PCC6803 have demonstrated that mutants at Asp-59 and Asp-61 in the A-B loop of the D1 protein require higher Ca²⁺ concentrations in the culture medium for photoautotrophic growth, suggesting that these residues are involved in the Ca²⁺-binding [52, 53]. These results indicated that the Ca²⁺ ion is located at the periphery of the Mn₄CaO₅ cluster, rather than the inside of the cluster. However, the latest high-resolution X-ray crystallographic structure of the PSII from *T. vulcanus* demonstrated that one PSII involves 4 Ca²⁺ ions [19, 39], one of which is incorporated into the Mn₄CaO₅ cluster, and the other 3 Ca²⁺ ions are loosely bound to outside of the coordination sphere [39]. Findings to date strongly indicate that Ca²⁺ ions are bound to the extrinsic proteins,

PsbO [27, 33, 54-59], PsbP [33, 36], and PsbV [60-64]. Therefore, it is still a matter of debate which Ca^{2+} ion plays the key role in the reaction mechanism for the photosynthetic water oxidation.

Chapter 2 Analysis of molecular mechanism for the enhanced thermal stability of Light-harvesting 1 reaction center complexes from the thermophilic purple sulfur bacterium *Thermochromatium tepidum*

2.1 Introduction

Purple photosynthetic bacteria capture the light energy with antenna apparatuses (LH1 and LH2) complexes and transfer the energy into an RC to initiate a charge separation and the subsequent photosynthetic redox events [1]. An X-ray crystallographic study [5] and scanning probe microscopic analyses [6, 8-10, 65] on mesophilic purple bacteria have demonstrated that the RC is surrounded by a cylindrical LH1 to form an LH1-RC complex. However, the diffraction data for the LH1-RC complexes have been limited to moderate resolutions, and details of the interaction mode between RC and LH1 complexes and the roles of the LH1 complex are not fully understood.

Thermochromatium tepidum (*Tch. tepidum*) is a thermophilic purple sulfur bacterium [66, 67]. The growth temperature is the highest (up to 58°C) among purple bacteria, and the LH1 Q_y band appears at 915 nm, which is red-shifted unusually (~35 nm) compared to those of the mesophilic counterparts. Based on the primary sequence of the LH1 $\alpha\beta$ -subunit [43] and structural information of the RC [4], it was proposed that the electrostatic interactions between acidic residues at the C-terminal region of the LH1 α -polypeptides and four basic residues specific for the *Tch. tepidum* RC may be responsible for the unusual spectroscopic and thermodynamic properties of this thermophilic organism [4, 68, 69]. Recently, we demonstrated that Ca^{2+} ions play a key role in the unique properties of the *Tch. tepidum* LH1-RC complexes [12, 40]. Among all known purple bacteria, only the *Tch. tepidum* LH1-RC complex exhibits Ca^{2+} -dependent thermal stability and spectral changes of BChl-*a* molecules bound to

the LH1 complex. When Ca^{2+} ions were depleted from the native LH1-RC complex, the Q_y peak at 915 nm was blue-shifted to 880 nm with marked deterioration of the thermal stability. The modified properties of the *Tch. tepidum* LH1-RC complex were almost completely restored after reconstitution with Ca^{2+} or, to a lesser extent, with Sr^{2+} and Ba^{2+} , but the addition of Cd^{2+} scarcely changed the properties [12, 40]. Based on the topological analysis, a putative Ca^{2+} -binding site was expected to exist at the C-terminal region of the LH1 α -polypeptides [70]. However, these results were mostly obtained by monitoring the Q_y absorption of LH1 BChl-*a* molecules, and little information is available on the structural change of the LH1-RC protein upon the metal-substitution, which is essential to clarify the molecular mechanism responsible for how the *Tch. tepidum* LH1-RC complex enhances the thermal stability by utilizing Ca^{2+} .

Fourier transform infrared (FTIR) spectroscopy is a powerful tool to monitor subtle changes in molecular structures and chemical reactions that are essential to understand structure-function relationships of biomolecules [71]. In the recent years, perfusion-induced attenuated total reflection (ATR) FTIR spectroscopy has been used to monitor the fine structural changes of proteins upon the binding of ligands, substrates, and cofactors to nicotinic acetylcholine receptor [72, 73], transhydrogenase [74], halorhodopsin [71], cytochromes *c* [76] and *c* oxidase [77, 78], and V-ATPase [79], or upon the redox changes and/or state transitions between intermediates of cytochrome *c* oxidase [80-83], and photosystems I and II [84, 85]. As for purple bacteria, the redox-linked structural and/or conformational changes were reported for the cytochrome *bc*₁ complex of *Rhodobacter (Rba.) capsulatus* [86] and the RC complexes of *Rba. sphaeroides*, *Rps. viridis* [84] and *Tch. tepidum* [87]. Apart from the perfusion-induced ATR-FTIR investigations, a recent typical ATR-FTIR study

[88] reported reduced conformational flexibility of the *Tch. tepidum* LH1-RC complex upon the binding of Ca^{2+} . However, the spectra could only provide information concerning a macro change between the Ca^{2+} - and Mn^{2+} -bound forms, and the fine structural modifications of the LH1-RC proteins induced by metal exchanges have not been detected. Here, I report for the first time metal-sensitive fine structural changes of protein backbones and amino acid side chains in the highly purified LH1 and LH-RC complexes from *Tch. tepidum* by means of perfusion-induced ATR-FTIR spectroscopy. Possible assignments for the metal-sensitive ATR-FTIR signals and its relevance to the molecular mechanism enhancing the thermal stability of *Tch. tepidum* LH1-RC proteins are discussed based on the recent structural information on the Ca^{2+} -binding site.

2.2 Materials and methods

2.2.1 Sample preparation

LH1-RC complexes from *Tch. tepidum* were purified as described previously [41] with slight modifications. Briefly, *Tch. tepidum* cells cultured at 48–50°C for 7–10 days were disrupted in 20 mM Tris-HCl buffer (pH 8.5) at 0°C by sonication (Sonopuls HD3200, Bandelin). The resulting chromatophores were treated with 0.32–0.35% (w/v) lauryldimethylamine *N*-oxide (Anatrace) at 25°C for 60 min followed by ultracentrifugation to remove a large portion of the LH2 complexes. The pellets were further treated with 0.9% (w/v) *n*-octyl- β -D-glucopyranoside (Anatrace) at 25°C for 60 min to extract the LH1-RC crudes. After ultracentrifugation, the supernatant was loaded onto a DEAE anion-exchange column (Toyopearl 650S, TOSOH) equilibrated at 4°C with 20 mM Tris-HCl buffer (pH 7.5) containing 0.08% (w/v) dodecylphosphocoline (DDPC, Anatrace). Upon a linear gradient of CaCl₂ concentration from 0 mM to 25 mM, I isolated for the first time the LH1 complex lacking the RC at 7~10 mM, and then the typical LH1-RC complex at ~20 mM. Sample fractions with a ratio of A₉₁₅/A₂₈₀ over 3.0 for LH1 complexes and A₉₁₅/A₂₈₀ over 2.1 for LH1-RC complexes were collected. The purity of the LH1 and LH1-RC complexes were confirmed by SDS-PAGE on a 12-22% gradient gel stained with CBB and sucrose density gradient centrifugation under a 10-40% (w/v) continuous gradient of sucrose concentration in a buffer containing 20 mM Tris-HCl (pH 7.5) and 0.08% (w/v) DDPC. Purification of *Allochromatium (Alc.) vinosum* LH1-RC complexes was conducted as described previously[42], and fractions with a ratio of A₈₈₄/A₂₈₀ over 2.0 were collected.

The LH1 or LH1-RC fractions were concentrated with Amicon Ultra 100K filters (Millipore), and diluted 10-fold with buffer A (20 mM Tris-HCl, 20 mM CaCl₂,

pH7.5), and ultracentrifuged at 195,000×g for 10 min. The resulting pellet was suspended in buffer A containing 0.008% DDPC to be ~100 μM of LH1-RC complex, and used in the ATR-FTIR measurements.

Uniform ¹⁵N- or ¹³C-isotope labeling of the *Tch. tepidum* cells was performed in a medium containing ¹⁵NH₄Cl (Masstrace Inc., 99 atom% ¹⁵N) or ¹³CH₃¹³COOH and NaH¹³CO₃ (Masstrace Inc., 99 atom% ¹³C) as the nitrogen or carbon source.

2.2.2 ATR-FTIR Measurements

The ATR-FTIR measurement system was constructed as described in the literature by Rich and Breton [72] with modifications (Figure 2-1). FTIR spectra were recorded on a Prestige-21 spectrophotometer (Shimadzu) equipped with a mercury-cadmium-telluride (MCT) detector (Shimadzu) and a DuraSamplIR II ATR accessory with a three-bounce silicon microprism (Smiths Detection). A silicon long-pass filter was placed in front of the detector to improve the S/N ratio. An aliquot of the LH1-RC sample solution (5 μl) was deposited on the Si/ZnSe ATR prism and dehydrated for 5 min under a stream of dry nitrogen gas. Upon this dehydration, no spectral change of the LH1-RC complex was confirmed. Then, a flow-attachment consisting of a transparent acrylic plate and a rubber O-ring was laid over the sample film and connected to a peristaltic pump (MP-1000-H EYELA) via silicon tubing. Background spectra were measured after a perfusion of Ca²⁺-buffer (20 mM Tris-HCl, 25 mM NaCl, 20 mM CaCl₂, pH 7.5) at a flow rate of 1 ml/min for 30 min. The buffer was switched to Sr²⁺-buffer (20 mM Tris-HCl, 25 mM NaCl, 20 mM SrCl₂, pH 7.5) at a flow rate of 1 ml/min for 20 min, and the sample spectra were recorded to yield a perfusion-induced Sr²⁺/Ca²⁺ ATR-FTIR difference spectrum. In a similar manner, Ca²⁺/Sr²⁺ difference spectra were obtained by switching back from

Sr^{2+} -buffer to Ca^{2+} -buffer. In some cases, BaCl_2 or CdCl_2 was used instead of SrCl_2 . Each spectrum was accumulated at 25°C for 1.5 min (150 scans), and 30–40 spectra (4500–6000 scans) from different samples were averaged to improve the S/N ratio.

For deuterium substitution, LH1-RC samples were suspended in D_2O -buffer (20 mM Tris-DCI, 20 mM CaCl_2 , 0.008% DDPC, pD 7.5) and incubated at 4°C for 12 hr. Then, spectral changes upon metal-exchanges in D_2O -buffer were examined. ^{44}Ca -isotope labeling was performed using $^{44}\text{Ca}^{2+}$ -buffer including 20 mM Tris-HCl, 25 mM NaCl, 20 mM $^{44}\text{CaCl}_2$ (98.7 atom% ^{44}Ca , Oak Ridge National Laboratory) at pH 7.5.

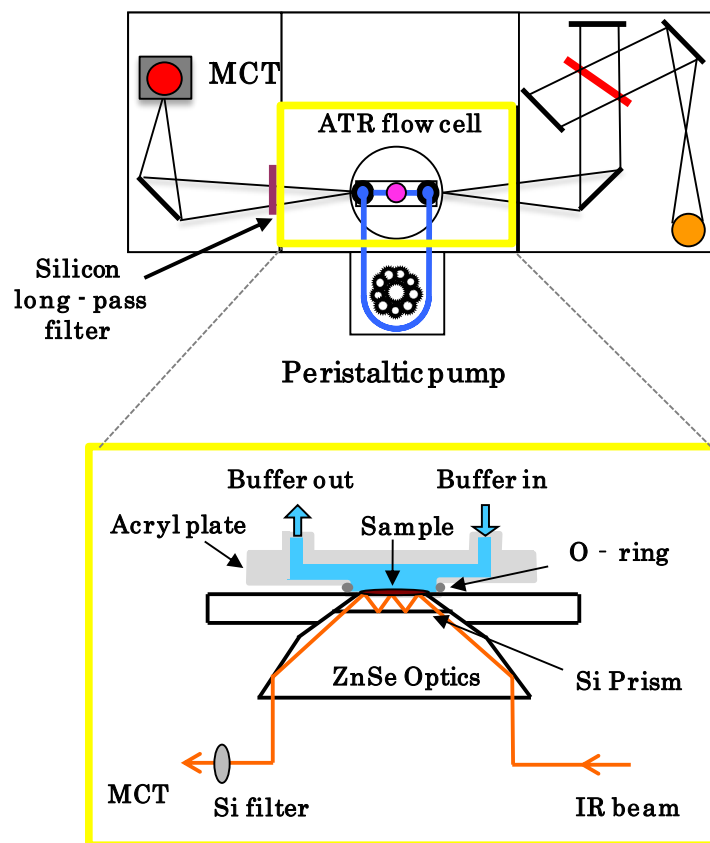


Figure 2-1. A schematic representation of the perfusion-induced ATR-FTIR measurement system. The ZnSe ATR optics with a 3 bounce silicon microprism was incorporated into the FTIR instrument and connected to a flow-attachment and peristaltic pump. A silicon long-pass filter was placed in front of the detector to improve the S/N ratio.

2.3 Results and discussion

2.3.1 Metal-sensitive structural changes in the *Tch. tepidum* LH1-RC complex

Figure 2-2 shows ATR-FTIR absorption spectra of the *Tch. tepidum* LH1-RC complex. The spectrum of the LH1-RC suspension basically exhibited only the H–O–H bending mode of water molecules at $\sim 1640\text{ cm}^{-1}$. Upon drying with a stream of N_2 gas, the film showed the characteristic amide I band (the C=O stretch of backbone polypeptides) at 1653 cm^{-1} , and amide II band (the NH bend coupled with the C–N stretch of backbone polypeptides) at 1543 cm^{-1} together with additional mid-to-low intensity bands originating from buffer components. After the rehydration of the dried film with Ca^{2+} -buffer (20 mM Tris-HCl, 25 mM NaCl, 20 mM CaCl_2 , pH 7.5), the amplitude of the protein bands was slightly decreased but stabilized rapidly. As reported previously [72], extensive removal of detergents from the starting material was required to obtain the stable background of a sample spectrum under a buffer flow. We confirmed that the absorption spectrum of the rehydrated LH1-RC film was little degraded during the measurement.

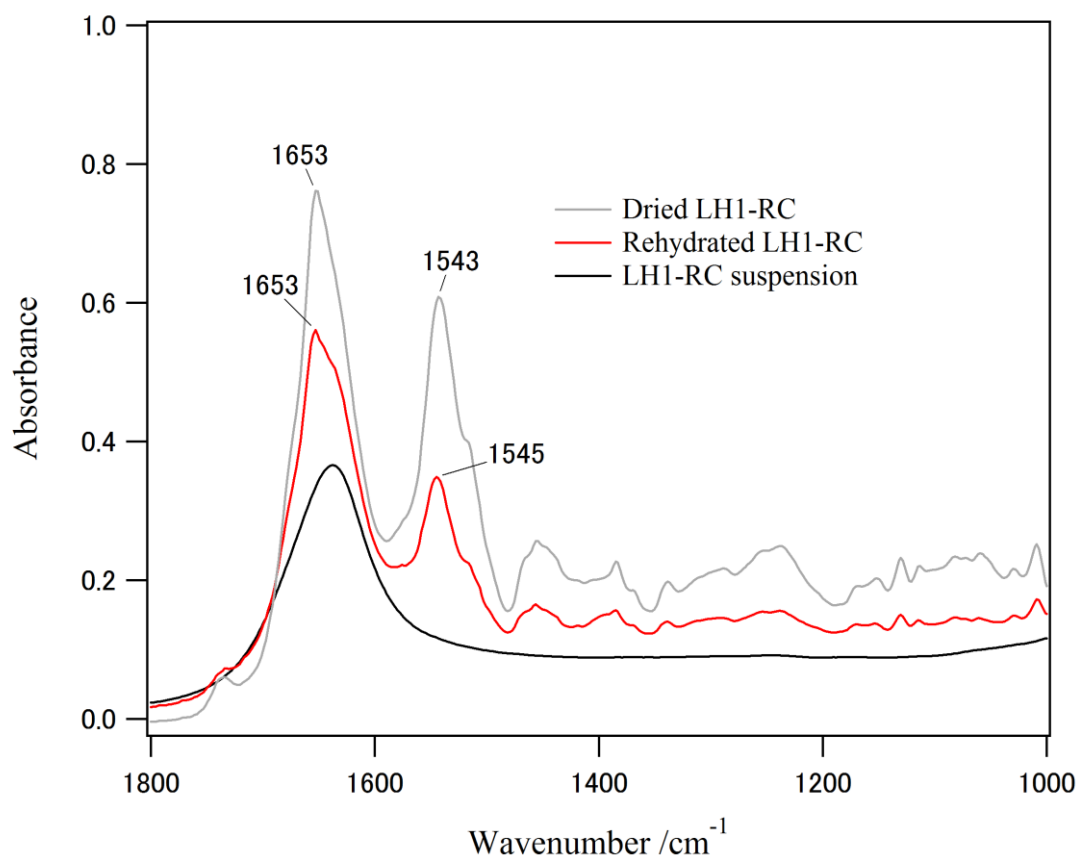


Figure 2-2. Absorption spectra of the LH1-RC complexes from *Tch. tepidum*. An aliquot of the LH1-RC suspension (black) was dried (gray) under a stream of N₂ gas, and rehydrated (red) by the perfusion of the buffer.

Figure 2-3 shows the $\text{Sr}^{2+}/\text{Ca}^{2+}$ ATR-FTIR difference spectrum of the LH1-RC complex from *Tch. tepidum* (a, magenta). Compared to the difference spectrum without the metal exchange (c), characteristic difference bands were clearly observed in the amide I ($\nu\text{C}=\text{O}$, 1700–1600 cm^{-1}) and amide II ($\nu\text{C}-\text{N}$ and $\delta\text{N}-\text{H}$, 1600–1500 cm^{-1}) regions of the polypeptide main chains upon the exchange from Ca^{2+} to Sr^{2+} . In addition, the difference spectrum may include specific vibrational modes of several amino acid side chains that interact directly and/or indirectly with the metal cations [73]. Interestingly, the characteristic bands in the $\text{Sr}^{2+}/\text{Ca}^{2+}$ difference spectrum were observed in the subsequent $\text{Ca}^{2+}/\text{Sr}^{2+}$ difference spectrum (black) with almost identical intensities but inverse signs. The symmetric features in both difference spectra were reproduced in the second and third cycle of the metal exchanges (data not shown), supporting the structural modifications of the LH1-RC complex induced by the metal exchange being fully reversible. In contrast, similar metal-sensitive ATR-FTIR signals were not detected in the LH1-RC complex from the mesophilic counterpart, *Alc. vinosum* (b), although several unassignable bands appeared faintly upon the metal exchange. These results demonstrate that most the ATR-FTIR difference bands are derived from unique structural modifications in the *Tch. tepidum* LH1-RC proteins upon the metal replacement, and not due to nonspecific changes in the proteins, detergents, or buffer molecules. In previous studies, metal-dependent property changes of the *Tch. tepidum* LH1-RC complexes were assessed spectroscopically by monitoring the electronic absorption [12] and emission [74], (magnetic) circular dichroism [12, 75], and (pre)resonance Raman scattering [42, 74] of BChl-*a* molecules bound to the LH1 $\alpha\beta$ -subunits. Although an isothermal titration calorimetry analysis of the *Tch. tepidum* LH1-RC complex indicated exothermic conformational changes due to the binding of Ca^{2+} to the proteins [40], no significant

modification in the secondary structure has been detected in far-UV CD spectra [12]. Therefore, this is the first evidence for the detection of metal-sensitive fine structural changes in the *Tch. tepidum* LH1-RC proteins. Taking into account the metal-dependent property changes in the thermal stability of this complex [40], the present results strongly indicate that metal-sensitive ATR-FTIR bands reflect structural changes that are intimately related with the enhanced thermal stability of the LH1-RC complex from this thermophile.

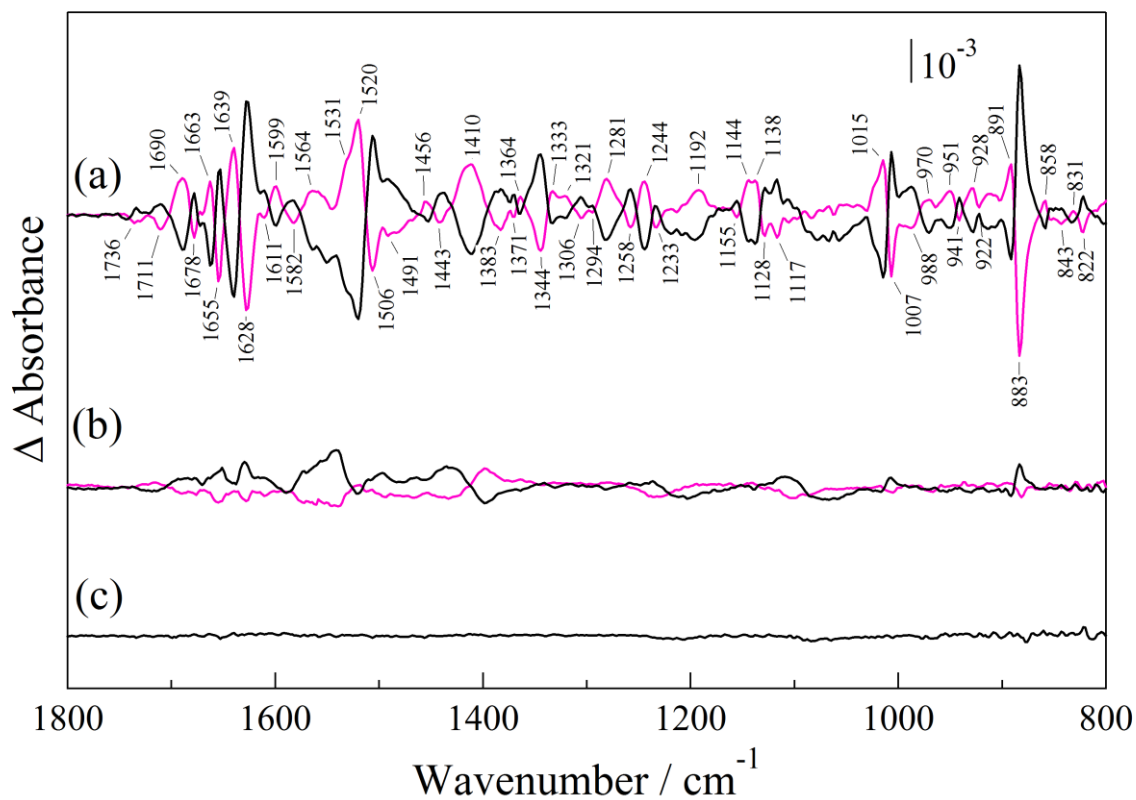


Figure 2-3. ATR-FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* (a) and *Alc. vinosum* (b) induced by switching from Ca^{2+} -buffer to Sr^{2+} -buffer ($\text{Sr}^{2+}/\text{Ca}^{2+}$ difference spectrum, magenta) followed by switching from Sr^{2+} -buffer to Ca^{2+} -buffer ($\text{Ca}^{2+}/\text{Sr}^{2+}$ difference spectrum, black). The difference spectrum of the *Tch. tepidum* LH1-RC complex without buffer switching (c) is presented to show the noise level.

2.3.2 Characterization of the LH1 complex lacking the RC

In contrast to the RC and LH1-RC complexes, the LH1 complex lacking the RC has not been isolated from *Tch. tepidum* yet. In the present study, we purified and characterized the LH1 complex to identify whether the observed vibrational modes originate from the LH1 or RC complex. Figure 2-4 shows the absorption spectra of LH1 (red) and LH1-RC (black) complexes isolated from *Tch. tepidum*. The absorption bands typical of BChl-*a* molecules were comparably observed at 378, 592, and 914 nm in both LH1 and LH1-RC spectra. However, small but significant differences were clearly observed in the band intensities of BPheo-*a*, the accessory BChl-*a*, and the special pair from the RC [76] at ~760, ~800, and ~880 nm, respectively, and the intensities of the bands lower than 450 nm were significantly decreased (Figure 2-4). Consequently, the ratio of Abs₉₁₅/Abs₂₈₀ showing the criteria for the purity of the complexes was larger than 3. The results demonstrate that the purified LH1 complex maintains its native structure even when it is not associated with the RC.

Next, the LH1 complex was characterized using perfusion-induced ATR-FTIR spectroscopy. Figure 2-5 shows the Sr²⁺/Ca²⁺ (a) and Ca²⁺/Sr²⁺ (b) difference spectra of the LH1 (cyan) and LH1-RC (black) complexes from *Tch. tepidum*. Most of the bands were largely coincident between the LH1 and LH1-RC spectra in terms of peak position and intensity although faint differences due to nonspecific changes in the background intensities seemed to be involved. This result demonstrates that the metal-sensitive FTIR signals of the *Tch. tepidum* LH1-RC complex are mostly attributable to structural modifications of the LH1 proteins and the contribution of the RC is negligible under the present experimental conditions.

It is of note that the intensities of difference bands induced by metal exchanges seem to be much larger in the *Tch. tepidum* LH1-RC complex compared with those in

the other protein [77]. This may be attributed to the larger number of Ca^{2+} -binding site in the LH1-RC protein since the LH1 is proposed to be 16-mer of the $\alpha\beta$ -subunit, each of which has a putative Ca^{2+} -binding site at the C-terminal region [12].

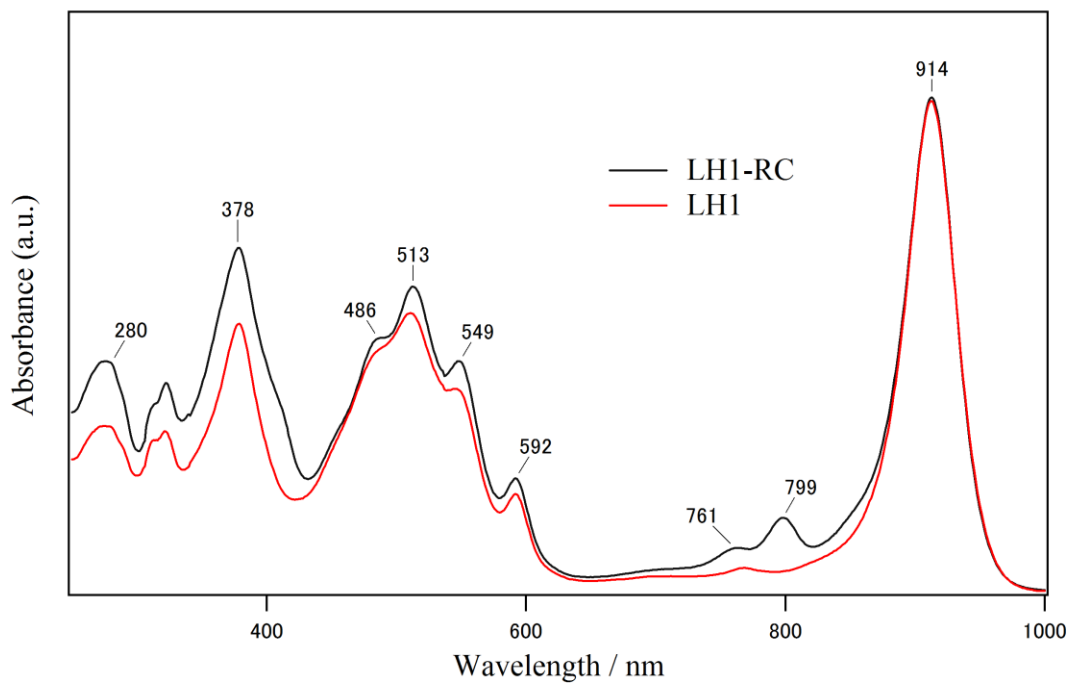


Figure 2-4. Absorption spectra of the LH1 (red) and LH1-RC (black) complexes from *Tch. tepidum*. Both spectra were normalized with respect to the Q_y maximum at 914 nm.

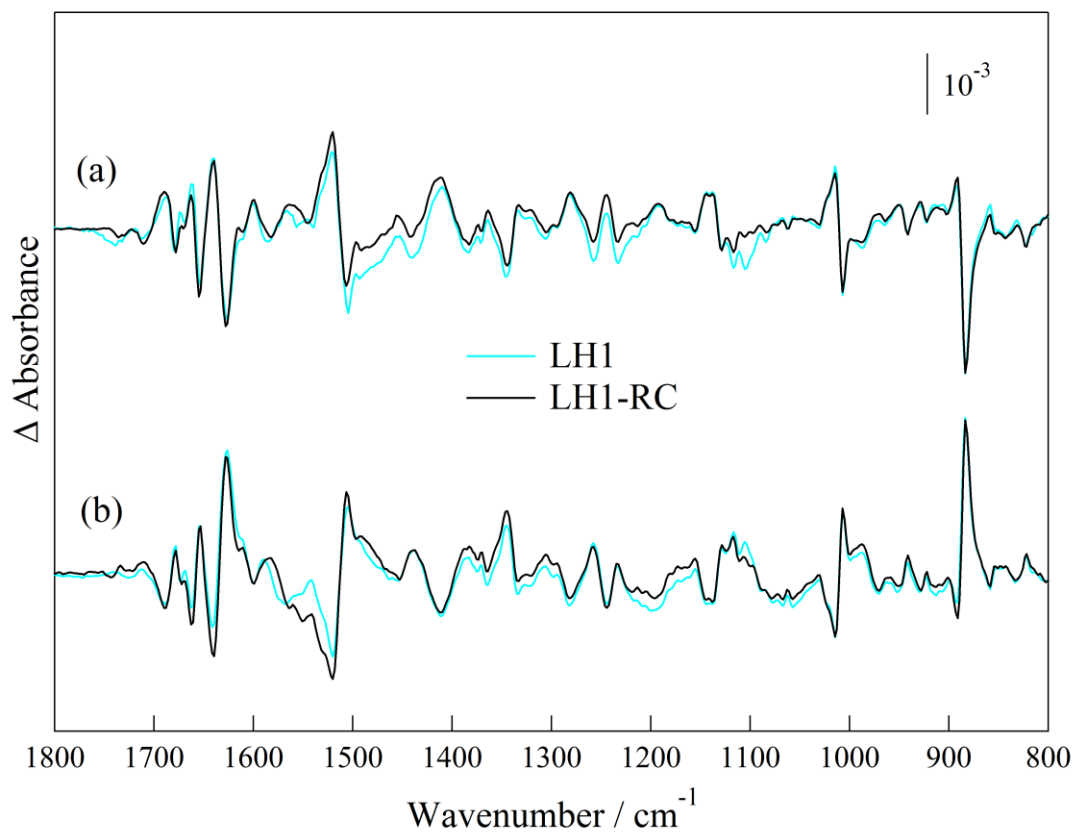


Figure 2-5. $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a) and $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b) difference spectra of the LH1 (cyan) and LH1-RC (black) complexes from *Tch. tepidum*. All spectra were normalized with respect to the $891/883\text{ cm}^{-1}$ differential band.

2.3.3 Properties of the Putative Ca^{2+} -binding Site and its Relevance to the Enhanced Thermal Stability of the *Tch. tepidum* LH1-RC Complexes

The previous study demonstrated that the thermal stability of the *Tch. tepidum* LH1-RC complex largely depends on the metal cation species [40]. Thus, the effects of Ba^{2+} - or Cd^{2+} -substitution for Ca^{2+} were also examined. Figure 2-6A shows the perfusion-induced ATR-FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* obtained by switching from Ca^{2+} -buffer to Sr^{2+} -buffer (a, magenta), Ba^{2+} -buffer (b, green), or Cd^{2+} -buffer (c, purple) along with their respective reversed spectra (black lines). All metal substitutions produced reversible difference spectra, which indicates that metal-sensitive structural changes are fully interconvertible even if Ba^{2+} and Cd^{2+} were employed instead of Sr^{2+} . The $\text{Ba}^{2+}/\text{Ca}^{2+}$ ($\text{Ca}^{2+}/\text{Ba}^{2+}$) difference spectra were almost identical to $\text{Sr}^{2+}/\text{Ca}^{2+}$ ($\text{Ca}^{2+}/\text{Sr}^{2+}$) difference spectra in terms of peak positions and band intensities as clearly seen in the double difference spectra (Figure 2-6B), which were obtained by subtracting $\text{Sr}^{2+}/\text{Ca}^{2+}$ from $\text{Ba}^{2+}/\text{Ca}^{2+}$ difference spectra (a) or by subtracting $\text{Ca}^{2+}/\text{Sr}^{2+}$ from $\text{Ca}^{2+}/\text{Ba}^{2+}$ difference spectra (b). In contrast, $\text{Cd}^{2+}/\text{Ca}^{2+}$ ($\text{Ca}^{2+}/\text{Cd}^{2+}$) difference spectra (Figure 2-6A) showed markedly different spectral features, particularly in the $1750\text{--}1350\text{ cm}^{-1}$ region. Figure 2-6B shows the double difference spectra between $\text{Cd}^{2+}/\text{Ca}^{2+}$ and $\text{Sr}^{2+}/\text{Ca}^{2+}$ differences (c) or $\text{Ca}^{2+}/\text{Cd}^{2+}$ and $\text{Ca}^{2+}/\text{Sr}^{2+}$ differences (d). The double difference bands are prominent at 1653 and 1541 cm^{-1} , and to a lesser extent, at 1703 , 1609 , 1582 , 1520 , 1429 , and 1396 cm^{-1} . These results strongly indicate that Cd^{2+} -substitution induced unique structural modifications in the vicinity of the Ca^{2+} -binding site of the LH1 proteins compared with the Sr^{2+} - or Ba^{2+} -substitutions. Interestingly, the unusual spectral changes by the Cd^{2+} -substitution were also confirmed in aqueous solutions of metal acetate complexes $\text{M}(\text{CH}_3\text{COO})_2$ ($\text{M}=\text{Ca}$, Sr , Ba , or Cd), which are the simple model

compounds for carboxylates ligating metal cations (Figure 2-7A). All the spectra in panel A exhibited the asymmetric and symmetric COO^- stretching bands at $\sim 1551\text{ cm}^{-1}$ and $\sim 1416\text{ cm}^{-1}$, respectively. However, their peak positions and band intensities were altered depending on the metal cations (panel B). The $\text{Cd}^{2+}/\text{Sr}^{2+}$ ($\text{Sr}^{2+}/\text{Cd}^{2+}$) difference spectra exhibited clear differences at 1566, 1441, and 1412 cm^{-1} in contrast to the small changes in the $\text{Ba}^{2+}/\text{Sr}^{2+}$ ($\text{Sr}^{2+}/\text{Ba}^{2+}$) difference spectra. These results are largely compatible with the present ATR-FTIR double difference spectra (Figure 2-6B), supporting the idea that carboxylate stretching bands of the metal-carboxylate complexes are sensitive to the binding property of the metal cation rather than the ionic radius. Furthermore, the spectral shapes in the symmetric COO^- stretching regions in Figure 2-6B ($1429/1396\text{ cm}^{-1}$) are similar with those in Figure 2-7B ($1441/1412\text{ cm}^{-1}$) although the peak positions were deviated by $12\text{-}16\text{ cm}^{-1}$. Therefore, a possible assignment for the $1429/1396\text{ cm}^{-1}$ bands in Figure 2-6B is the symmetric carboxylate stretching bands, and the 1653 and 1541 cm^{-1} bands are amide I and II of the polypeptide main chain. If this is the case, Cd^{2+} may form a bidentate ligation rather than a unidentate ligation judging from the upshift of the symmetric carboxylate stretching bands from 1396 to 1429 cm^{-1} . This change modifies the specific interactions between α - and β -polypeptides at the C-terminus, leading to the destabilization of the LH1-RC complexes upon the binding of Cd^{2+} . Alternatively, an exchange of the ligation partner might occur in the vicinity of the Ca^{2+} -binding site upon the Cd^{2+} substitution since Cd^{2+} prefers softer bases as ligand partners due to their relatively weak Lewis-acids property.

The previous studies have shown that the thermal stability of the Cd^{2+} -substituted LH1-RC complex was strongly decreased to 23.8% compared with those of the LH1-RC complex reconstituted with Ca^{2+} (89.3%) or replaced with Sr^{2+}

(72.0%) and Ba²⁺ (73.2%) [40] despite the fact that Cd²⁺-, Sr²⁺-, and Ba²⁺-bound forms exhibited Q_y peaks at similar positions (887–889 nm) [12]. Therefore, the unique structural changes induced by Cd²⁺-substitution may exert little influence on any interactions that modulate the Q_y absorption properties but are intimately related with the marked deterioration of the thermal stability in the Cd²⁺-substituted LH1-RC complexes.

It is notable that metal-sensitive ATR-FTIR signals were not apparent in the *Alc. vinosum* LH1-RC complex (Figure 2-3) under the present condition even though this complex also possesses a cluster of acidic residues in the C-terminal region in analogy with *Tch. tepidum* (Figure 2-8). A possible factor to interpret the difference of the metal requirement between both species is a deletion at the +7 position of the LH1 α -polypeptide from *Tch. tepidum*. This deletion has been suggested to be indispensable to form the coordination sphere of Ca²⁺. Interestingly, the quite similar sequences including the deletion were confirmed in strain 970 [78] and *Trv. winogradskyi* [79]. However, these species grow at ambient temperatures, and thus, the presence of the deletion alone would not directly verify the enhanced thermal stability of the *Tch. tepidum* LH1-RC complex. Therefore, it is speculative that the presence of the deletion at the +7 position of the LH1 α -polypeptide is a necessary requirement to form a suitable Ca²⁺-affinity site, and that the Ca²⁺-binding induces a key conformational change of the C-terminal amino acid residues of LH α - and/or β -polypeptides.

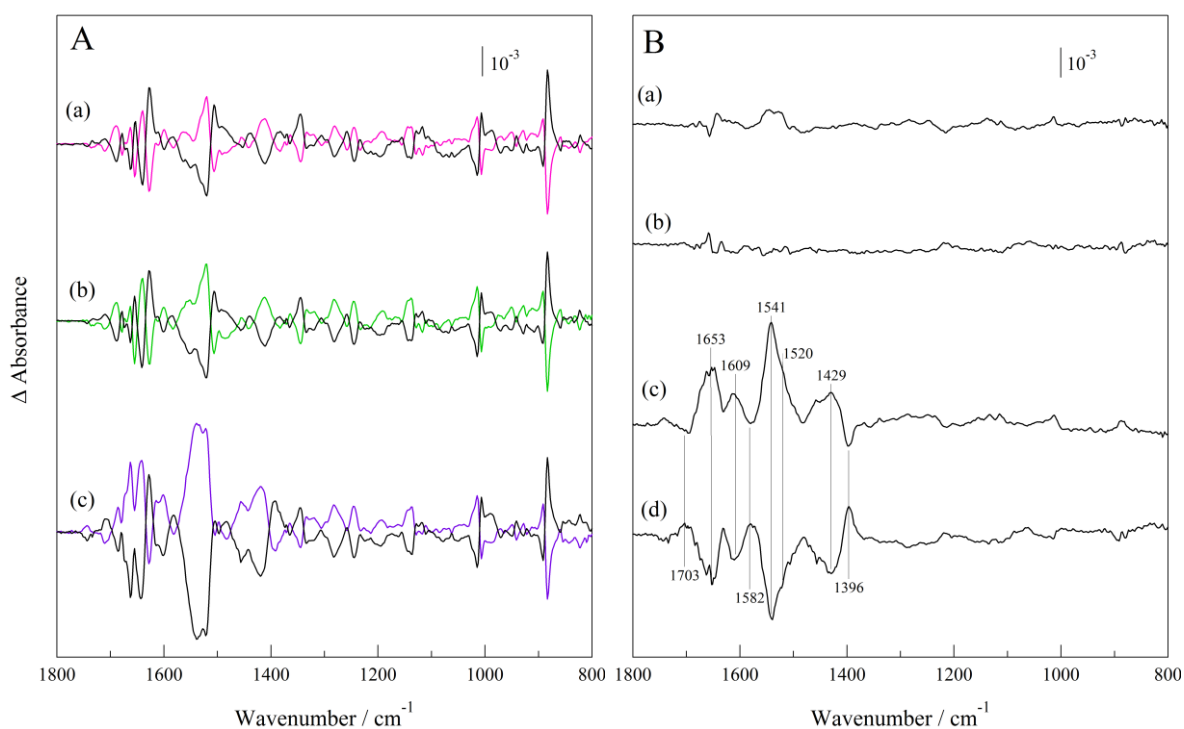


Figure 2-6. (A) ATR-FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* for the Sr²⁺/Ca²⁺ (a, magenta), Ba²⁺/Ca²⁺ (b, green), and Cd²⁺/Ca²⁺ (c, purple) exchanges and their respective ensuing Ca²⁺/Sr²⁺ (a, black), Ca²⁺/Ba²⁺ (b, black), and Ca²⁺/Cd²⁺ (c, black) exchanges. (B) Double difference spectra obtained by subtracting the Ca²⁺/Sr²⁺ spectrum from the Ca²⁺/Ba²⁺ spectrum (a), the Sr²⁺/Ca²⁺ spectrum from the Ba²⁺/Ca²⁺ spectrum (b), the Ca²⁺/Sr²⁺ spectrum from the Ca²⁺/Cd²⁺ spectrum (c), and the Sr²⁺/Ca²⁺ spectrum from the Cd²⁺/Ca²⁺ spectrum (d). All spectra were normalized with respect to the 891/883 cm⁻¹ differential band.

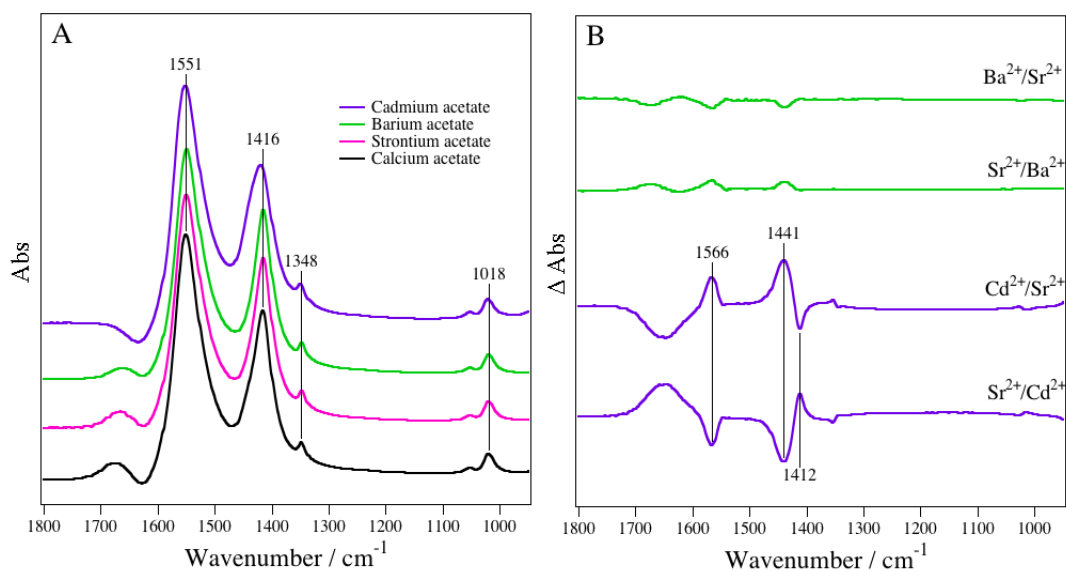


Figure 2-7. (A) ATR-FTIR absorption spectra of calcium acetate (black), strontium acetate (magenta), barium acetate (green), and cadmium acetate (purple) in aqueous solutions. (B) ATR-FTIR difference spectra between Ba^{2+} - and Sr^{2+} -spectra (green) or between Cd^{2+} - and Sr^{2+} -spectra (purple) after the normalization with respect to the peak intensity at 1018 cm^{-1} .

LH1 α-polypeptide	-30	-20	-10	0	+10	+20	+30
<i>Alc. vinosum pufB2A2</i>		MHKIQI	FDPRRTL	VALFGFL	FVLGLLI	HFILLSS	PAFNWLSGS
<i>Alc. vinosum pufA1B1</i>		MMPQLY	KIWLAFD	PRMALIGL	GAFLEFAL	LALFIHYML	LRSPFDWLLGPDYAPV
<i>Alc. vinosum pufB3A3</i>		MSPDLW	KIWLVD	PRRILIAV	FAPLTVL	GLAIHMI	LLSTAEFNWLEDGVP
<i>Tch. tepidum</i>		MFTMNAN	LYKIWL	LDPRRVL	SVIVAFQI	VLGLLIH	MIVLST-DLNWLD
<i>Trv. winogradskyi strain 06511</i>		MNESLQ	NLHKVW	LINPAQV	LVALGVFQI	VLGLGIH	MILLST-DLNWLD
<i>Trv. winogradskyi DSM6702</i>		MNDSMQ	NLHKIQI	IINPAQT	LVALGVFQI	VLGLGIH	MILLST-DLNWLD
<i>strain 970 pufB1A1</i>		MNAKSF	DGMHKL	WMIMPF	VTLWAI	FIFQIFL	GLLIHMVVLSS-DLN
<i>strain 970 pufB2A2</i>		MNSDKF	AGMYKL	WTFIDP	RRTLIFIV	AFQIML	GILIHMIVLGS-DLN

LH1 β-polypeptide	-30	-20	-10	0	+10
<i>Alc. vinosum pufB2A2</i>	MANENR	SMSGLTE	DEAREF	HGIFVSS	FVVFTGIV
<i>Alc. vinosum pufA1B1</i>	MADQKS	MTEEEA	KEFHGIF	TQSM	TFGGIVII
<i>Alc. vinosum pufB3A3</i>	MANSMT	GLTEQA	EQEFHGIF	VQSM	TAFPGIVV
<i>Tch. tepidum</i>	MAEQK	SLTGLT	DDEAKE	FHAIFM	QSMYAWF
<i>Trv. winogradskyi strain 06511</i>	MAEKSM	TGLTDA	EAKEFHGIF	MASMS	AYFGLVV
<i>Trv. winogradskyi DSM6702</i>	MAEKSM	TGLTDA	EAKEFHGIF	MSSMT	AYFGLVV
<i>strain 970 pufB1A1</i>	MAEKST	TGLTEA	ESKEFHGIF	MASMT	LWFGLVV
<i>strain 970 pufB2A2</i>	MAEKPT	TGLTEA	ESAKEFHG	LFMASMT	LWFGLVV

Figure 2-8. Amino acid sequences of the LH1 α - and β -polypeptides from purple sulfur bacteria [43, 79]. The amino acid ligands for Ca^{2+} identified in the new crystallographic structure [80] are indicated with red. The numbers represent the relative position of each residue to the conserved His bound to BChl-*a*.

Figure 2-9 shows ATR-FTIR absorption spectra of unlabeled and ^{15}N -labeled LH1-RC complexes from *Tch. tepidum*. The FTIR spectrum of unlabeled LH1-RC complex (black) exhibited prominent amide I and amide II bands at 1653 and 1545 cm^{-1} , respectively. Upon uniform ^{15}N -labeling of the LH1-RC complexes (magenta), the amide I band was slightly downshifted by $\sim 2 \text{ cm}^{-1}$, due to a small coupling with the NH bending vibration, while the amide II band showed significant downshift by 14 cm^{-1} since the latter band is predominantly ascribed to the NH bending and CN stretching vibrations. These isotopic shifts were similar to those reported for PSII from isotope-labeled cyanobacteria [81, 82], strongly indicating that nitrogen atoms in the LH1-RC proteins are biosynthetically replaced with ^{15}N atoms. These results demonstrate that nitrogen atoms in the LH1-RC proteins are biosynthetically replaced with ^{15}N atoms.

Figure 2-10 shows ATR-FTIR difference spectra of ^{15}N -labeled LH1-RC complexes from *Tch. tepidum* upon the metal-exchanges. Although most of spectral features were similar with the corresponding difference spectra of unlabeled LH1-RC complexes (black), significant differences were evident as clearly seen in the $^{14}\text{N}/^{15}\text{N}$ double difference spectrum obtained by subtracting the unlabeled spectrum from the ^{15}N -labeled spectrum after the normalization with respect to the differential band at 891/883 cm^{-1} (Figure 2-11). Most prominent $^{15}\text{N}/^{14}\text{N}$ isotopic bands appeared in the 1600–1500 cm^{-1} region along with several mid-to-low intensity bands. The isotopic signals detected for the exchange from Ca^{2+} to Sr^{2+} , Ba^{2+} or Cd^{2+} were observed in the corresponding reversed spectrum from Sr^{2+} , Ba^{2+} or Cd^{2+} to Ca^{2+} , indicating that the $^{15}\text{N}/^{14}\text{N}$ isotopic bands are originating from the reversible structural changes of polypeptide backbones and amino acid side chains containing nitrogen atoms in the vicinity of the metal-binding site at the C-terminal side of the *Tch. tepidum* LH1

proteins. In addition, the $^{15}\text{N}/^{14}\text{N}$ isotopic bands for the Sr^{2+} -substitution were largely similar with those for the Ba^{2+} -substitution but were distinctively different with those for the Cd^{2+} -substitution in the $1700 - 1500 \text{ cm}^{-1}$ region. This indicates that structural changes of the amide modes containing and/or coupled to ^{15}N were uniquely modified upon the Cd^{2+} -substitution.

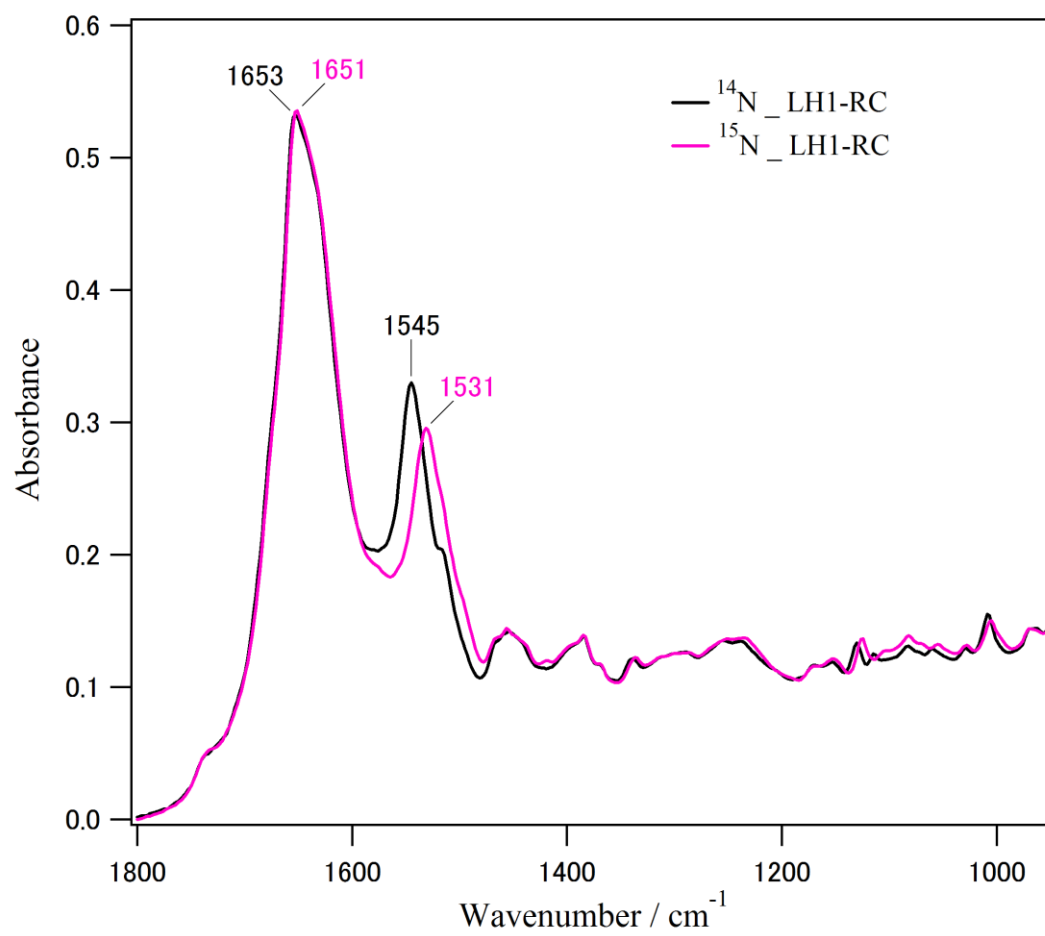


Figure 2-9. Absorption spectra of unlabeled (black) and ¹⁵N-labeled (magenta) LH1-RC complexes from *Tch. tepidum*.

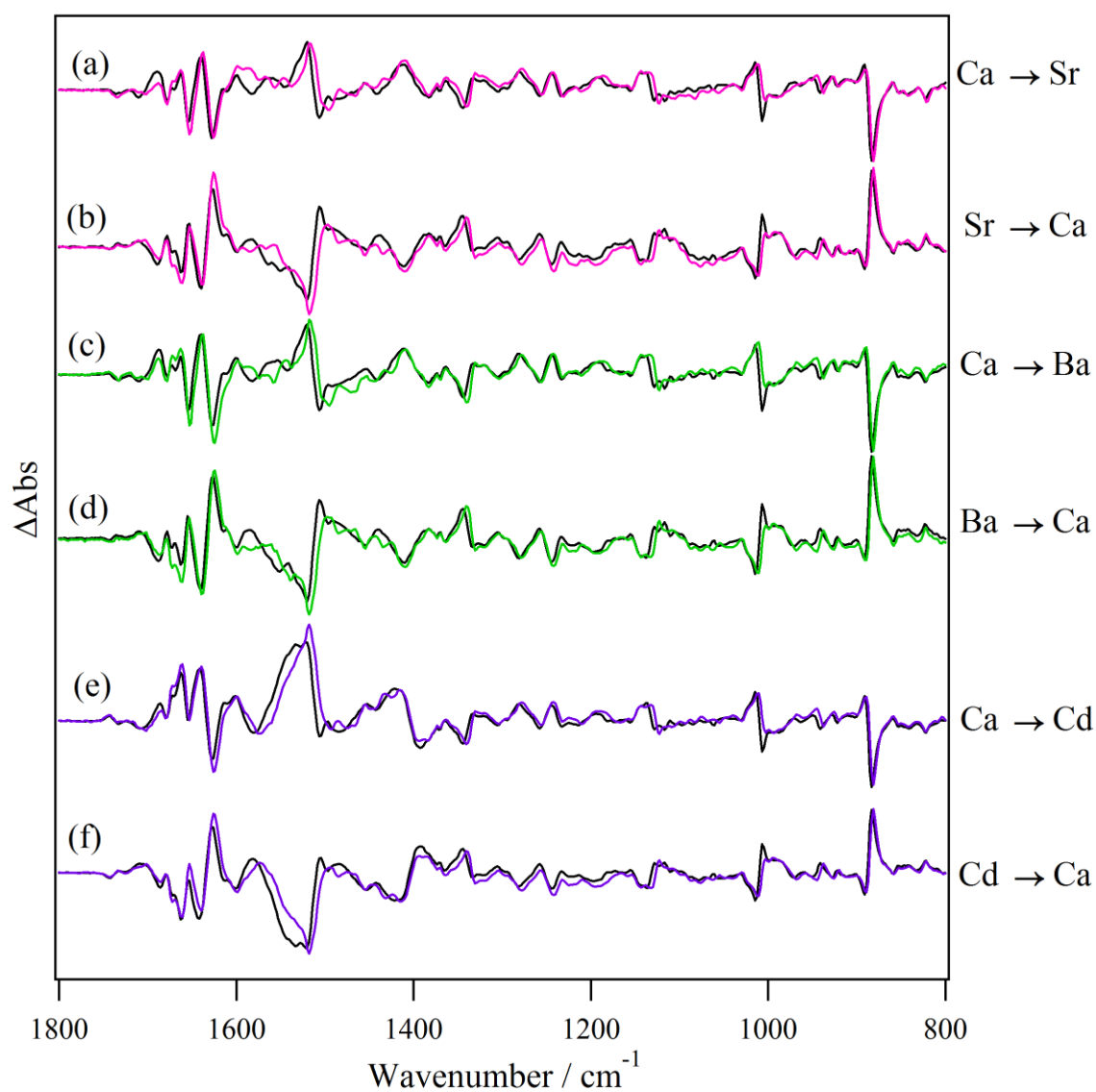


Figure 2-10. ATR-FTIR difference spectra of ^{15}N -labeled LH1-RC complexes from *Tch. tepidum* upon $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a, magenta), $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b, magenta), $\text{Ba}^{2+}/\text{Ca}^{2+}$ (c, green), $\text{Ca}^{2+}/\text{Ba}^{2+}$ (d, green), $\text{Cd}^{2+}/\text{Ca}^{2+}$ (e, purple), and $\text{Ca}^{2+}/\text{Cd}^{2+}$ (f, purple) exchanges. The corresponding difference spectra of unlabeled LH1-RC complexes were presented for comparison (a-f, black).

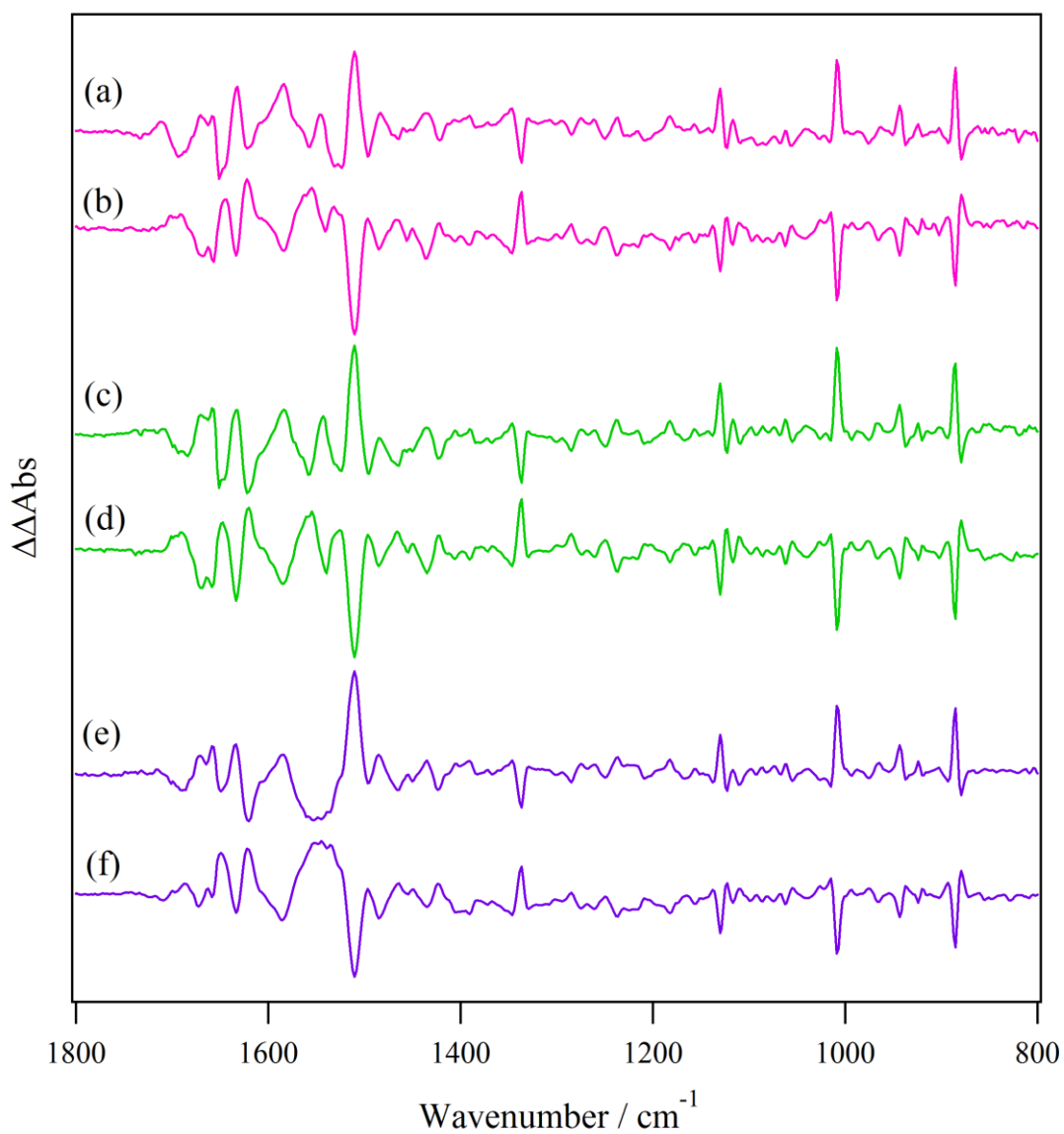


Figure 2-11. $^{15}\text{N}/^{14}\text{N}$ double difference spectra obtained by subtracting the unlabeled spectrum from the ^{15}N -labeled spectrum upon $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a, magenta), $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b, magenta), $\text{Ba}^{2+}/\text{Ca}^{2+}$ (c, green), $\text{Ca}^{2+}/\text{Ba}^{2+}$ (d, green), $\text{Cd}^{2+}/\text{Ca}^{2+}$ (e, purple), and $\text{Ca}^{2+}/\text{Cd}^{2+}$ (f, purple) exchanges after the normalization with respect to the differential band at $891/883\text{ cm}^{-1}$.

Figure 2-12 shows ATR-FTIR absorption spectra of unlabeled and ^{13}C -labeled LH1-RC complexes from *Tch. tepidum*. The FTIR spectrum of unlabeled LH1-RC complex (black) exhibited prominent amide I and amide II bands at 1653 and 1545 cm^{-1} , respectively. Upon uniform ^{13}C -labeling of the LH1-RC complexes (red), the amide I band showed a significant downshift by 37 cm^{-1} , while the amide II band was moderately downshifted by 12 cm^{-1} . These isotopic shifts were similar to those reported for PSII from isotope-labeled cyanobacteria [81, 82], strongly indicating that carbon atoms in the LH1-RC proteins are biosynthetically replaced with ^{13}C atoms.

Figure 2-13 shows ATR-FTIR difference spectra of ^{13}C -labeled LH1-RC complexes from *Tch. tepidum* upon the metal-exchanges. Most of the bands were significantly downshifted upon the ^{13}C -labeling as compared with the corresponding difference spectra of unlabeled LH1-RC complexes (black). The differences were more clearly seen in the $^{13}\text{C}/^{12}\text{C}$ double difference spectrum obtained by subtracting the unlabeled spectrum from the ^{13}C -labeled spectrum after the normalization with respect to the differential band at 891/883 cm^{-1} (Figure 2-14). Intensive $^{13}\text{C}/^{12}\text{C}$ isotopic bands appeared in the 1700 – 1500 cm^{-1} and 900 – 850 cm^{-1} regions along with several mid-to-low intensity bands in the 1500 – 900 cm^{-1} region. The isotopic signals detected for the exchange from Ca^{2+} to Sr^{2+} , Ba^{2+} or Cd^{2+} were observed in the corresponding reversed spectrum from Sr^{2+} , Ba^{2+} or Cd^{2+} to Ca^{2+} , indicating that the $^{13}\text{C}/^{12}\text{C}$ isotopic bands are originating from the reversible structural changes of polypeptide backbones and amino acid side chains containing carbon atoms in the vicinity of the metal-binding site at the C-terminal side of the *Tch. tepidum* LH1 proteins. In contrast to the ^{15}N -isotope effects, the $^{13}\text{C}/^{12}\text{C}$ isotopic bands were modified depending on the cation species; effects by the Sr^{2+} -substitution were slightly different from those by the Ba^{2+} -substitution in the 1700 – 1500 cm^{-1} region,

and largely by the Cd^{2+} -substitution in the 1700 –1350 cm^{-1} region. These results suggest that the binding modes of several residues and/or polypeptide main and side chains comprising the metal binding site are differently affected depending on the metal cations.

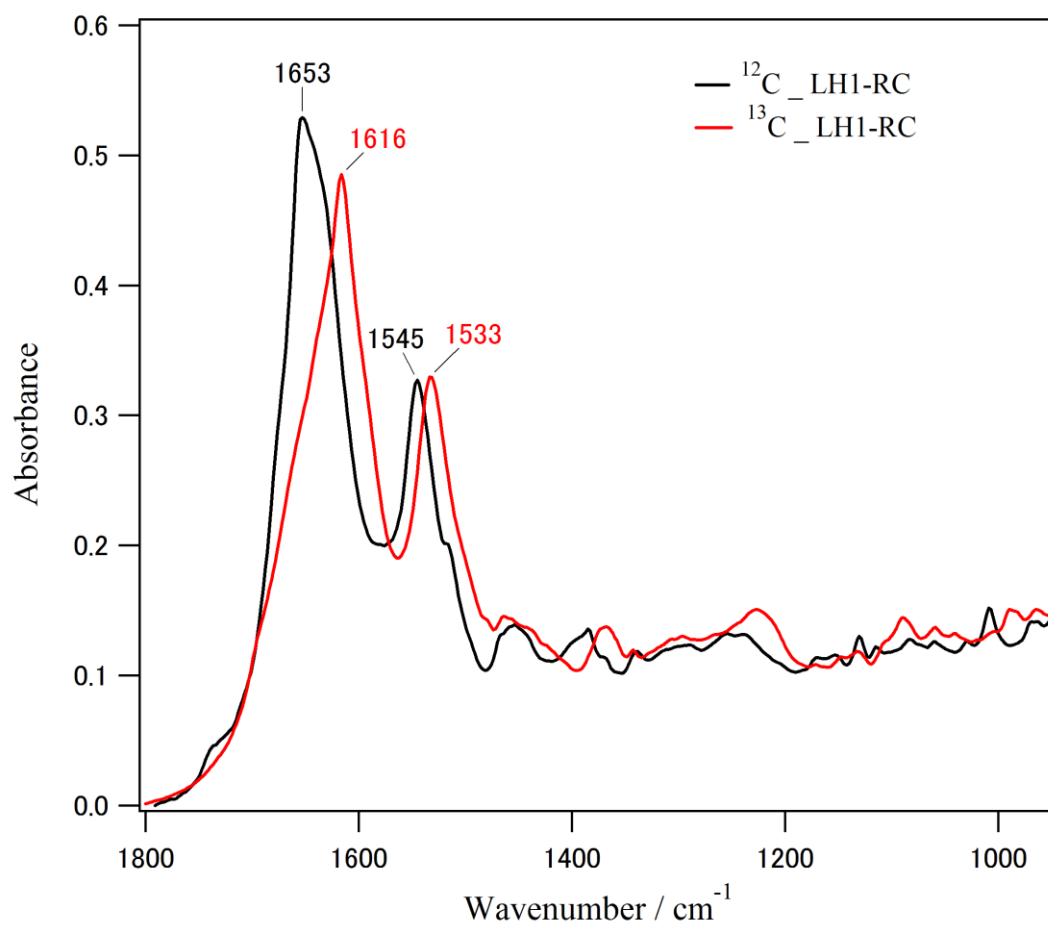


Figure 2-12. Absorption spectra of unlabeled (black) and ¹³C-labeled (red) LH1-RC complexes from *Tch. tepidum*.

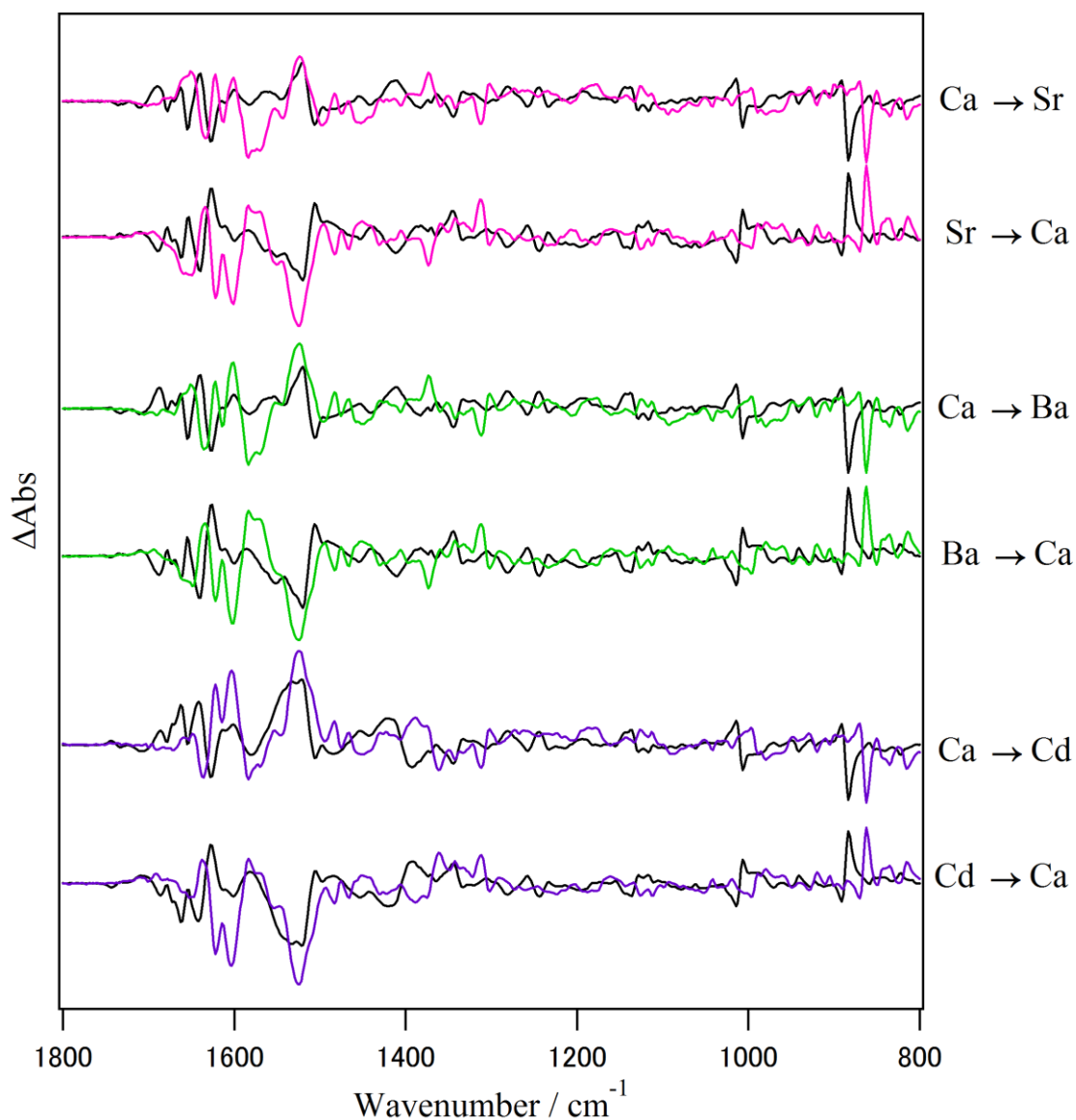


Figure 2-13. ATR-FTIR difference spectra of ¹³C-labeled LH1-RC complexes from *Tch. tepidum* upon Sr²⁺/Ca²⁺ (a, magenta), Ca²⁺/Sr²⁺ (b, magenta), Ba²⁺/Ca²⁺ (c, green), Ca²⁺/Ba²⁺ (d, green), Cd²⁺/Ca²⁺ (e, purple), and Ca²⁺/Cd²⁺ (f, purple) exchanges. The corresponding difference spectra of unlabeled LH1-RC complexes were presented for comparison (a-f, black).

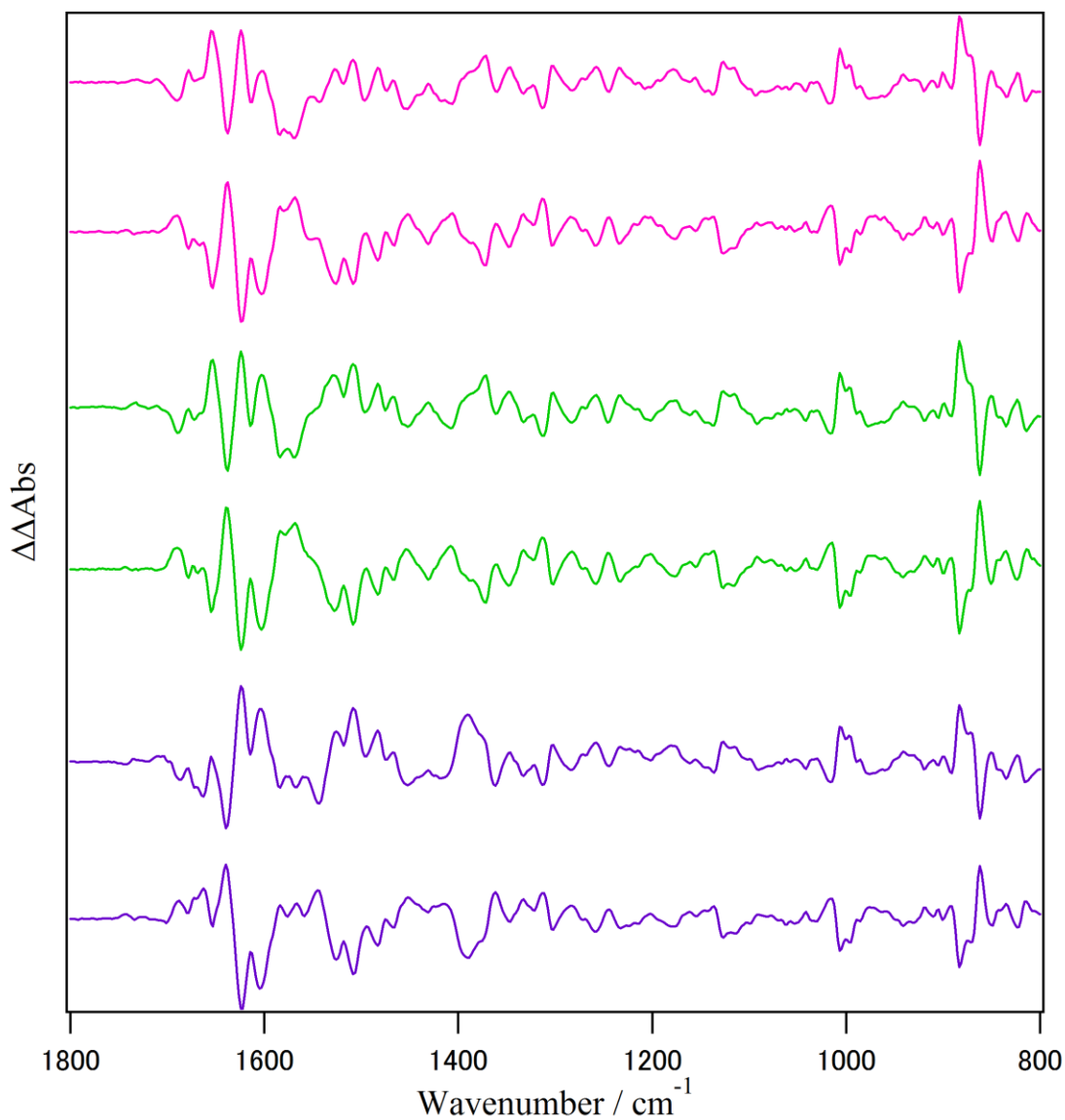


Figure 2-14. $^{13}\text{C}/^{12}\text{C}$ double difference spectra obtained by subtracting the unlabeled spectrum from the ^{13}C -labeled spectrum upon $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a, magenta), $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b, magenta), $\text{Ba}^{2+}/\text{Ca}^{2+}$ (c, green), $\text{Ca}^{2+}/\text{Ba}^{2+}$ (d, green), $\text{Cd}^{2+}/\text{Ca}^{2+}$ (e, purple), and $\text{Ca}^{2+}/\text{Cd}^{2+}$ (f, purple) exchanges after the normalization with respect to the differential band at $891/883\text{ cm}^{-1}$.

Figure 2-15 shows absorption spectra of unlabeled (black) and ^2H -labeled (blue) LH1-RC complexes from *Tch. tepidum*. The intensities of typical amide I and II bands at $\sim 1653\text{ cm}^{-1}$ and $\sim 1545\text{ cm}^{-1}$, respectively, were reduced and new bands appeared at $\sim 1454\text{ cm}^{-1}$ for amide II' mode (νCN and δND) of deuterated polypeptide backbones and at $\sim 1206\text{ cm}^{-1}$ for the DOD bending mode. These spectral changes are largely compatible with that reported previously for the PSII complex from *Thermosynechococcus elongatus* [83], indicating that most of hydrogen atoms in the LH1-RC complexes are replaced with deuterium.

Figure 2-16 shows ATR-FTIR difference spectra of ^2H -labeled LH1-RC complexes from *Tch. tepidum* upon the metal-exchanges. The bands in the $1700\text{--}1500\text{ cm}^{-1}$ and $1300\text{--}1200\text{ cm}^{-1}$ regions were significantly affected by the ^2H -labeling as compared with the corresponding difference spectra of unlabeled LH1-RC complexes (black). However, several bands in the $1450\text{--}1300\text{ cm}^{-1}$ and $1150\text{--}850\text{ cm}^{-1}$ regions were little changed upon the deuteration. The differences were more clearly seen in the $^2\text{H}/^1\text{H}$ double difference spectrum obtained by subtracting the unlabeled spectrum from the ^2H -labeled spectrum after the normalization with respect to the differential band at $891/883\text{ cm}^{-1}$ (Figure 2-17). The prominent $^2\text{H}/^1\text{H}$ double difference bands were apparent in the $1700\text{--}1500\text{ cm}^{-1}$ region which potentially includes amide I and II modes, $\nu\text{C=O}$ of Asn or Gln, $\nu_{\text{as}}\text{CN}_3\text{H}_5^+$ of Arg, $\delta_{\text{as}}\text{NH}_3^+$ and $\delta_{\text{s}}\text{NH}_3^+$ of Lys, δNH_2 of Asn and Gln, several vibrational modes of Trp, Tyr, and His residues [73]. On the basis of the isotopic shifts by ^{15}N -, ^{13}C -, and ^2H -labelings, the difference bands detected upon Sr^{2+} -substitution are tentative assigned and summarized in Table 2-1.

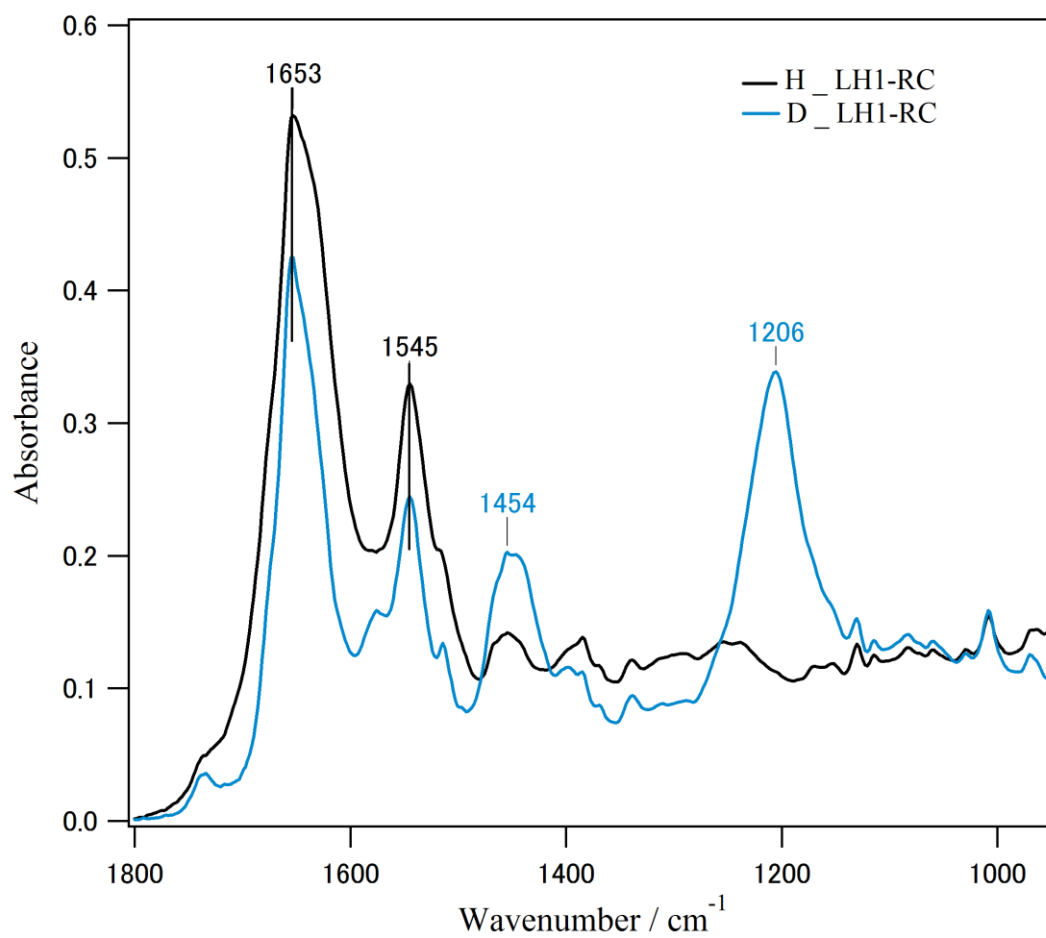


Figure 2-15. Absorption spectra of unlabeled (black) and ^2H -labeled (blue) LH1-RC complexes from *Tch. tepidum*.

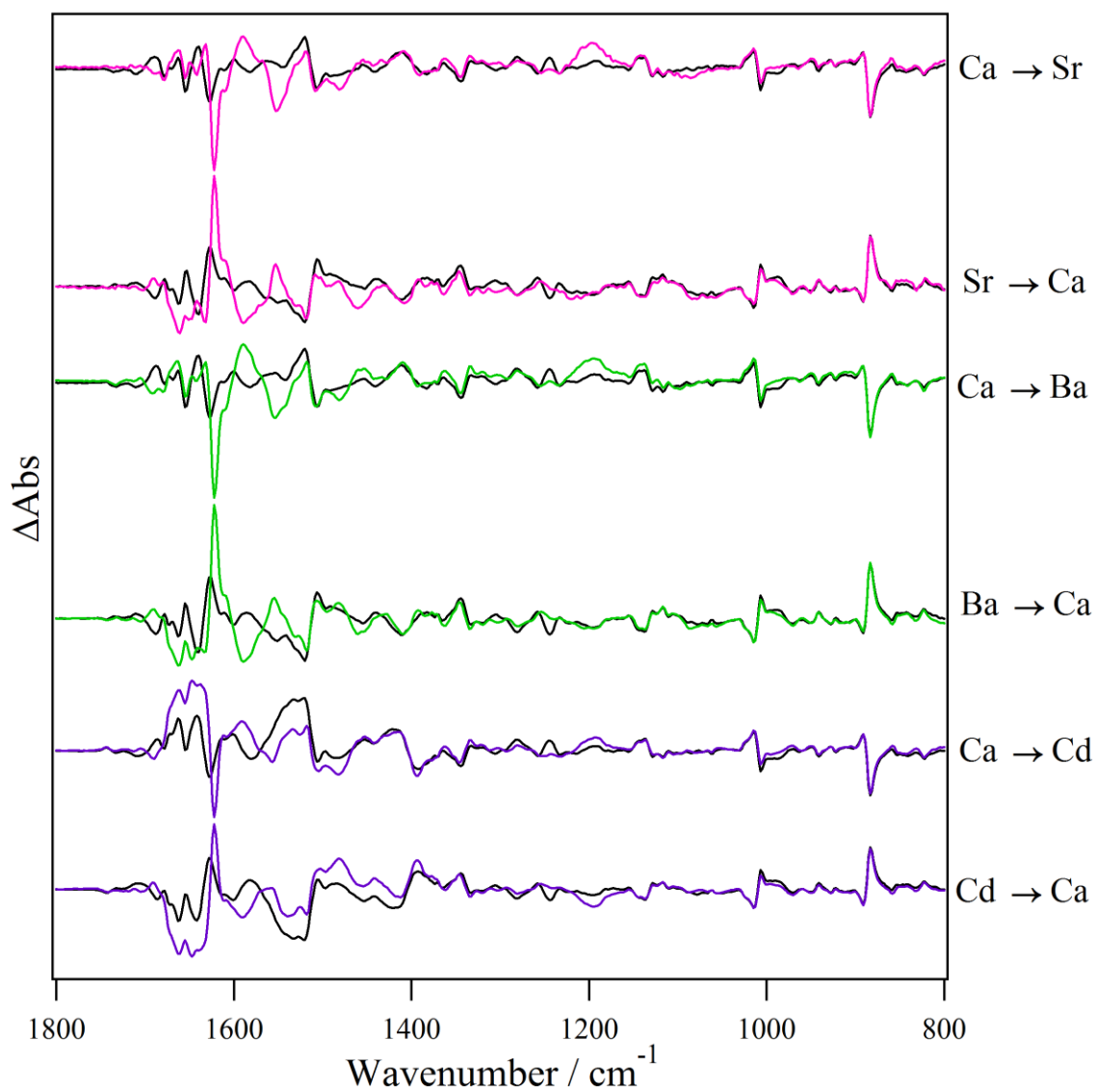


Figure 2-16. ATR-FTIR difference spectra of ^2H -labeled LH1-RC complexes from *Tch. tepidum* upon $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a, magenta), $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b, magenta), $\text{Ba}^{2+}/\text{Ca}^{2+}$ (c, green), $\text{Ca}^{2+}/\text{Ba}^{2+}$ (d, green), $\text{Cd}^{2+}/\text{Ca}^{2+}$ (e, purple), and $\text{Ca}^{2+}/\text{Cd}^{2+}$ (f, purple) exchanges. The corresponding difference spectra of unlabeled LH1-RC complexes were presented for comparison (a-f, black).

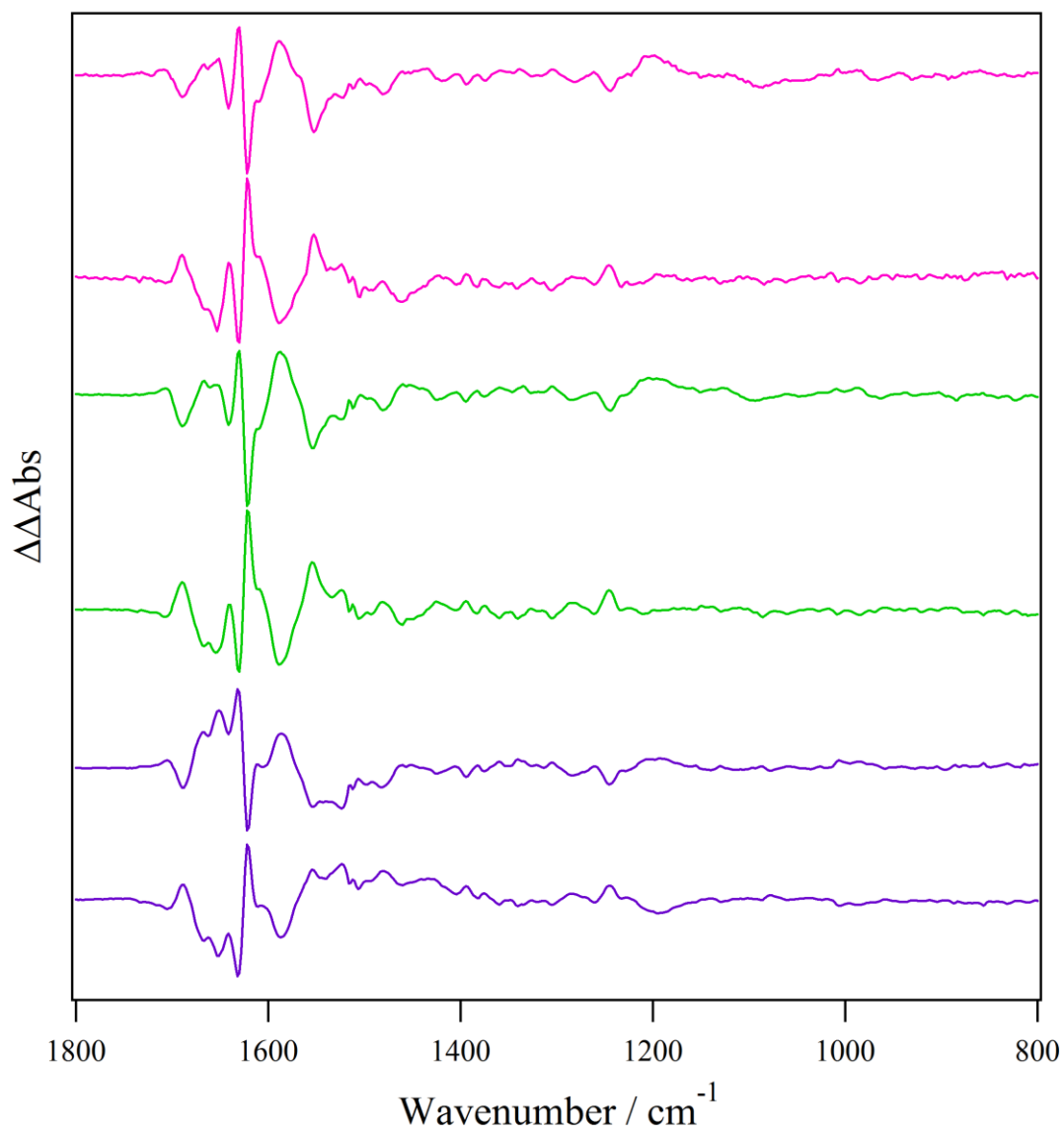


Figure 2-17. $^2\text{H}/^1\text{H}$ double difference spectra obtained by subtracting the unlabeled spectrum from the ^{13}C -labeled spectrum upon $\text{Sr}^{2+}/\text{Ca}^{2+}$ (a, magenta), $\text{Ca}^{2+}/\text{Sr}^{2+}$ (b, magenta), $\text{Ba}^{2+}/\text{Ca}^{2+}$ (c, green), $\text{Ca}^{2+}/\text{Ba}^{2+}$ (d, green), $\text{Cd}^{2+}/\text{Ca}^{2+}$ (e, purple), and $\text{Ca}^{2+}/\text{Cd}^{2+}$ (f, purple) exchanges after the normalization with respect to the differential band at $891/883\text{ cm}^{-1}$.

In the present study, I selected metal cations (Sr^{2+} , Ba^{2+} , and Cd^{2+}) as useful metal probes in the *Tch. tepidum* LH1-RC complex because of the following reasons: (1) Sr^{2+} is the sole metal cation which is biosynthetically replaceable with Ca^{2+} [41], (2) spectroscopic and thermodynamic effects by Ba^{2+} -substitution are very similar with those by Sr^{2+} -substitution [12, 40], and (3) Cd^{2+} has the divalent cation with almost identical ionic radius with Ca^{2+} [50]. However, the most appropriate metal cation for monitoring the Ca^{2+} -binding site is $^{44}\text{Ca}^{2+}$, the stable isotope of Ca^{2+} , which induces a minimum perturbation in the binding site and is possible to detect selectively the structural changes within the first coordination sphere. Therefore, I examined the effects of $^{44}\text{Ca}^{2+}$ -substitution on the ATR-FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* (spectra a in Figure 2-18). Several bands due to the changes of the background appeared in the $^{44}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ ($^{40}\text{Ca}^{2+}/^{44}\text{Ca}^{2+}$) difference spectra. However, no significant $^{44}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ bands were detected in comparison with the difference bands detected upon the Sr^{2+} -substitution (spectra b). A possible explanation is that each interaction between Ca^{2+} and the ligand is not strong and the increased mass of Ca^{2+} little affect on the vibrational modes of polypeptide main chain and amino acid side chains appearing in the present mid-frequency region. If this is the case, some Ca^{2+} -ligand modes appearing in the low-frequency region ($< 800 \text{ cm}^{-1}$) may exhibit significant isotope shifts upon the $^{44}\text{Ca}^{2+}$ -substitution.

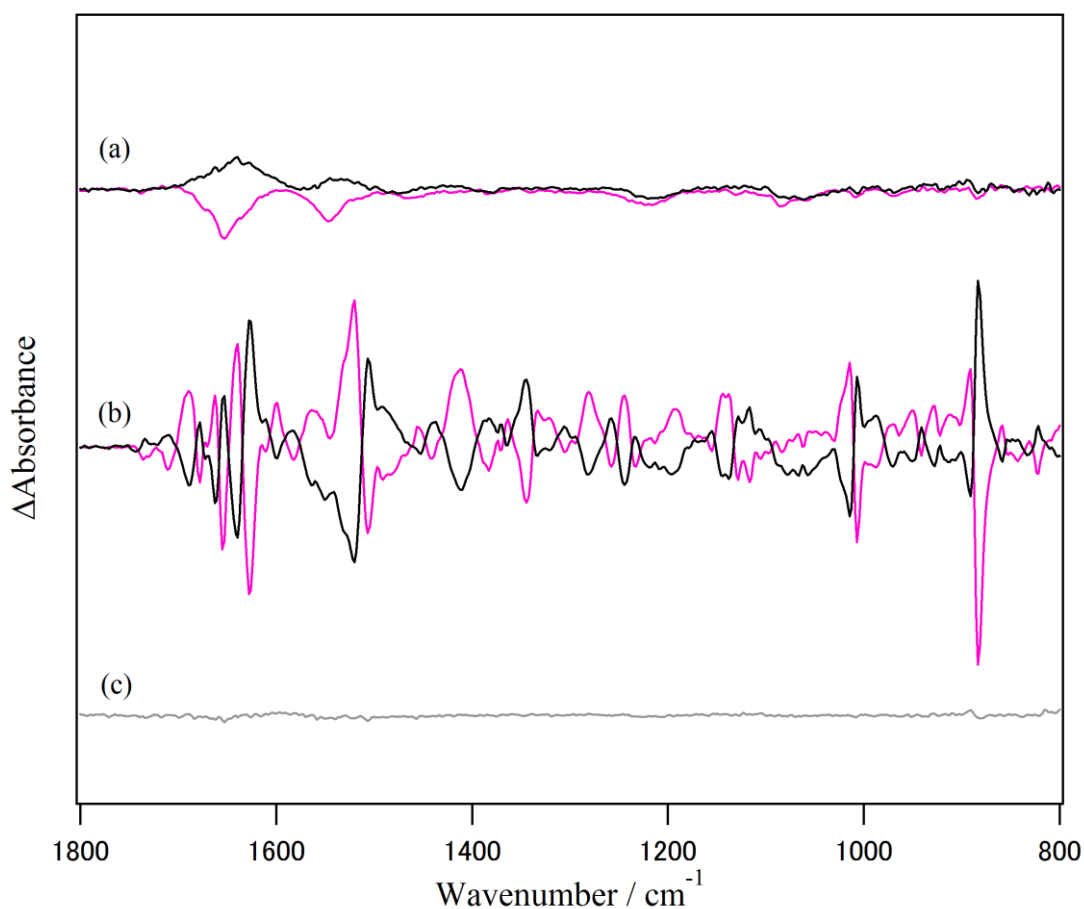


Figure 2-18. ATR-FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* induced by switching from Ca^{2+} -buffer to $^{44}\text{Ca}^{2+}$ -buffer ($^{44}\text{Ca}^{2+}/^{40}\text{Ca}^{2+}$ difference spectrum, a, magenta) and the following reversed spectrum ($^{40}\text{Ca}^{2+}/^{44}\text{Ca}^{2+}$ difference spectrum, a, black), and difference spectrum(b). The difference spectra of the LH1-RC complex induced by the Sr^{2+} -substitution (b) and the noise level (c) are presented for comparison.

Table 2-1. ATR-FTIR difference bands of the *Tch. tepidum* LH1-RC complexes upon Sr²⁺-substitution and tentative assignments of each band based on the isotopic shifts upon uniform ¹⁵N-, ¹³C- and ²H-labelings.

Unlabeled	¹⁵ N-labeled ($\Delta^{15}\text{N}$)	¹³ C-labeled ($\Delta^{13}\text{C}$)	D-labeled (ΔD)	Tentative assignment
1736	1734(-2)	1695(-41)	1690(-46)	BChl vC=O
1690	1688(-2)	1651(-39)	1663(-27)	Asn vC=O, Amide I turn (Trp), random coil
1663	1663(0)	1622(-41)	1663(0)/1649(-14)	Amide I α -helix, turn, random coil
1655	1653(-2)	1613(-42)	1655(0)/1641(-14)	Amide I α -helix, turn, random coil
1640	1638(-2)	1601(-39)	1632(-8)	Amide I α -helix
1628	1626(-2)	1584(-44)	1622(-6)	$\nu_{\text{as}} \text{COO}^-$
1599	1599(0)	1553(-46)	1589(-10)	Tyr vC=C
1582	1557(-25)	1545(-37)	1481(-101)	Amide II
1564	1545(-9)	1524(-40)	1454(-110)	Amide II
1520	1518(-2)	1483(-37)	1520(0)	$\nu_{\text{as}} \text{COO}^-$, Tyr vC=C
1506	1495(-11)	1476(-30)	1508(+2)	Amide II, Trp vCN
1412	1410(-2)	1373(-39)	1410(-2)	$\nu_{\text{s}} \text{COO}^-$
1383	1383(0)	1360(-23)	1391(+8)	$\nu_{\text{s}} \text{COO}^-$
1373	1373(0)	1350(-23)	1373(0)	$\nu_{\text{s}} \text{COO}^-$
1364	1364(0)	1323(-41)	1364(0)	$\nu_{\text{s}} \text{COO}^-$
1344	1341(-3)	1317(-27)	1344(0)	$\nu_{\text{s}} \text{COO}^-$
1333	1331(-2)	1302(-23)	1335(+2)	$\nu_{\text{s}} \text{COO}^-$
1281	1279(-2)	1260(-12)		$\nu_{\text{s}} \text{COO}^-$ $\nu_{\text{s}} \text{COO}^-$, Trp, Try
1258	1256(-2)	1246(-10)		Tyr δCOH
1244	1242(-2)	1234(-10)		Tyr δCOH
1233	1231(-2)	1219(-14)		Tyr δCOH
1144	1144(0)	1136(-8)	1144(0)	vC-O
1138	1134(-4)	1123(-15)	1138(0)	vCN
1128	1123(-5)	1094(-34)	1128(0)	His vCN
1117	1105(-12)	1082(-35)	1117(0)	His vCN
1015	1013(-2)	997(-18)	1015(0)	$\nu_{\text{as}} \text{CNC}$
1007	1003(-4)	990(-17)	1007(0)	$\nu_{\text{as}} \text{CNC}$
891	891(0)	870(-21)	891(0)	$\nu_{\text{s}} \text{CNC}$
883	881(-2)	862(-21)	883(0)	$\nu_{\text{s}} \text{CNC}$
858	860(+2)	851(-9)	858(0)	$\nu_{\text{s}} \text{CNC}$

2.3.4 Tentative Assignments of the Perfusion-induced ATR-FTIR Difference Bands

Based on the primary sequence [43] and a topological analysis [70], it was proposed that the Ca^{2+} -binding site is comprised of several acidic residues located at the C-terminal region of the LH1 $\alpha\beta$ -polypeptides [12, 70]. Several spectroscopic analyses indicated that the metal-depletion/substitution induced few changes in the secondary and tertiary structures of the LH1-RC complex [12, 74, 75]. However, the present ATR-FTIR study detected clear metal-sensitive difference bands in the 1800–800 cm^{-1} region which includes characteristic vibrational modes of polypeptide main chains and amino acid side chains in the LH1-RC complex. These results indicate that small but significant conformational modifications upon the metal exchange occur in the structures that are directly and/or indirectly interacting with Ca^{2+} in the LH1 C-terminal region.

It is noteworthy here that very recently a new crystallographic structure of the *Tch. tepidum* LH1-RC complex has been presented [80]. As predicted in the previous studies [12, 70, 74, 75], the LH1 complex is 16-mer of the $\alpha\beta$ -subunit, each of which has a Ca^{2+} -binding site comprised of $\alpha\text{-Trp}_{+10}$, $\alpha\text{-Asp}_{+13}$, $\alpha\text{-Asn}_{+14}$, and $\beta\text{-Leu}_{+10}$ locating at the C-terminal region of the LH1 $\alpha\beta$ -subunit (Figure 2-8). The carboxylate side chain of $\alpha\text{-Asp}_{+13}$ and the C-terminal carboxylate of $\beta\text{-Leu}_{+10}$ coordinate with Ca^{2+} in unidentate and bidentate manners, respectively. Generally, metal-carboxylate complexes exhibit asymmetric (ν_{as}) COO^- stretching mode at 1675–1515 cm^{-1} and symmetric (ν_{s}) COO^- stretching mode at 1495–1260 cm^{-1} [84], and the metal-carboxylate ligation structures are empirically relevant to the value of $\Delta\nu$ ($=\nu_{\text{as}}-\nu_{\text{s}}$); $\Delta\nu = \sim 164 \text{ cm}^{-1}$ for ionic form, $\Delta\nu \gg \sim 164 \text{ cm}^{-1}$ for unidentate form, $\Delta\nu = \sim 164 \text{ cm}^{-1}$ (but greater than $\Delta\nu$ for bidentate) for bridging form, and $\Delta\nu < \sim 164 \text{ cm}^{-1}$

for bidentate form [85, 86]. Unfortunately, this is not applicable to the present results since the intensive bands in the amide I and II regions are largely overlapping with the asymmetric COO^- bands, and therefore, the close inspection of the ν_{as} bands is hard to achieve dispense with isotope-edited analyses. In contrast, the symmetric COO^- stretching bands (1495–1260 cm^{-1}) can be analyzed more clearly since the intensive amide I and II bands are absent in this region, and CH_2 scissoring (1480–1440 cm^{-1}) and CH_3 deformation modes (1465–1440 cm^{-1} and 1390–1370 cm^{-1}) are less sensitive to faint changes in the molecular environment. Thus, the bands at 1443/1383/1371/1306/1294 cm^{-1} and 1456/1410/1364/1333/1321 cm^{-1} in the $\text{Sr}^{2+}/\text{Ca}^{2+}$ ($\text{Ca}^{2+}/\text{Sr}^{2+}$) difference spectra (Figure 2-3) are possible candidates for the symmetric COO^- stretching bands of $\alpha\text{-Asp}_{+13}$ and $\beta\text{-Leu}_{+10}$ ligating Ca^{2+} and Sr^{2+} , respectively. In addition, the symmetric COO^- stretching bands of carboxylates in a unidentate ligation tend to appear at lower frequencies than that in a bidentate ligation [86]. If this is the case, the ν_{s} bands of $\alpha\text{-Asp}_{+13}$ and $\beta\text{-Leu}_{+10}$ correspond to the lower and higher frequencies, respectively, of the putative bands. However, I can not exclude the possibility that the 1495–1260 cm^{-1} region includes contribution from other vibrational modes; the symmetric COO^- stretching of another Asp residue indirectly coupled with the Ca^{2+} -binding site (no Glu residue is located at the C-terminal side), the C-N stretching of $-\text{CONH}_2$ from Asn and Gln (1420–1400 cm^{-1}), and the O-H deformation of Tyr, Thr, and Ser (1440–1260 cm^{-1}) [84].

Several intensive differential bands (1690/1678/1663/1655/1639/1628/1599 cm^{-1}) in the 1700–1600 cm^{-1} may be assigned to the amide I mode of the main chains (1695–1623 cm^{-1}). It is known that the frequencies of the amide I bands reflect the secondary structures; 1657–1648 cm^{-1} for α -helix, 1695–1674 cm^{-1} and 1641–1623 cm^{-1} for β -sheet, 1686–1662 cm^{-1} for turns, and 1670–1660 cm^{-1} for random coil [73,

84]. However, the contribution of β -sheet structures are excluded since the LH1 $\alpha\beta$ -subunits are predominantly comprised of transmembrane α -helices as well as turns and random coil structures at the C- and N-terminal sides [80]. Therefore, the bands at 1695–1674 cm^{-1} and 1641–1623 cm^{-1} can be assigned to other bands including the C=O stretching of Asn (1678–1677 cm^{-1} , or up to 1704 cm^{-1} in proteins) and Gln (1687–1668 cm^{-1}), the asymmetric and symmetric CN_3H_5^+ stretching of Arg (1695–1652 and 1663–1614 cm^{-1} , respectively), the NH_3^+ deformation of Lys (1629–1626 cm^{-1}), and the NH_2 bending of Asn (1622–1612 cm^{-1}) [73, 87, 88]. In particular, contribution of α -Asn₊₁₄ to the metal-sensitive FTIR bands at 1677–1678 cm^{-1} and 1612–1622 cm^{-1} is likely to be involved since its carbonyl side chain serves as a direct ligand for Ca^{2+} [80].

In addition, the carbonyl main chain of α -Trp₊₁₀ exists in the first coordination sphere of the Ca^{2+} -binding site and is involved in the turn structure at the C-terminal region of the LH1 α -polypeptide [80]. Therefore, the 1686–1662 cm^{-1} bands are putatively ascribed to the main chain of α -Trp₊₁₀. The α -Trp₊₁₀ and β -Trp₊₉ residues are highly conserved among purple bacteria at the C-terminal region of LH1 polypeptides [79], and proposed as hydrogen-bonding partners of the C3-acetyl C=O groups of BChl-*a* molecules for tuning the LH Q_y transition energy through the hydrogen-bonds [89, 90]. A previous Raman study strongly indicated that the hydrogen-bonding interactions between BChl-*a* and LH1 α -Trp₊₁₀/ β -Trp₊₉ are modulated by the metal cations in the *Tch. tepidum* LH1-RC complex [42]. Based on these results, it is possible that conformational changes of α -Trp₊₁₀ residues and/or BChl molecules (1760–1680 cm^{-1}) [91] are also involved in the metal-sensitive ATR-FTIR signals.

In the 1600–1500 cm^{-1} region, possible candidates for the intensive bands at

1531(shoulder)/1520/1506 cm^{-1} and mid-to-low intensity bands at 1599/1582/1564 cm^{-1} are the amide II modes of the polypeptide main chain (1577–1507 cm^{-1}) [81, 82, 84], the asymmetric stretching modes of COO^- from $\alpha\text{-Asp}_{+13}$ side chain and C-terminal carboxylate of $\beta\text{-Leu}_{+10}$ (1675–1515 cm^{-1} , depending on the ligation structure) [85, 86], and/or the CC stretching of Tyr (1602–1594 cm^{-1} and 1518–1516 cm^{-1}) [73]. In addition, other bands for the symmetric deformation of NH_3^+ from Lys (1527–1526 cm^{-1}), and the CN stretching and CH/NH bending of Trp (1509 cm^{-1}) are possible to be involved in this region [73].

2.4 Conclusion

Thermochromatium (Tch.) tepidum is a purple sulfur photosynthetic bacterium which can grow at 58°C, the highest temperature among purple bacteria, and exhibits an unusually red-shifted Q_y transition of the light-harvesting 1 reaction center (LH1-RC) complex at 915 nm (B915). These unique properties are closely related with an inorganic cofactor, Ca^{2+} , of which the putative binding site is proposed to be residing at the C-terminal region of the LH1 α -subunits. However, the molecular mechanism how this organism acquired the enhanced thermal stability and unusual spectroscopic property by utilizing Ca^{2+} is still an open question.

In the present study, I detected for the first time metal-sensitive fine structural changes of protein backbones and amino acid side chains by means of perfusion-induced ATR-FTIR spectroscopy, and provide structural indications responsible for the enhanced thermal stability of the *Tch. tepidum* LH1-RC complex upon the binding of Ca^{2+} . The essential points of this study are as follows:

1. I constructed perfusion-induced ATR-FTIR measuring system and detected for the first time the structural changes of polypeptide backbones and amino acid side chains in the *Tch. tepidum* LH1-RC proteins upon the replacement of native Ca^{2+} with other metal cations (Sr^{2+} , Ba^{2+} , Cd^{2+}).
2. The metal-sensitive ATR-FTIR bands were tentatively assigned based on the isotope shifts upon uniform ^{15}N , ^{13}C , and 2H -labelings.

3. The structural changes induced by the exchange between two metal cations were fully reversible and specific for the *Tch. tepidum* LH1-RC complex based on a comparative analysis using the mesophilic counterpart, *Allochromatium vinosum*.
4. The LH1 complex lacking the RC was first isolated and characterized in *Tch. tepidum*. The ATR-FTIR signals for the LH1 complex were almost identical with those of the LH1-RC complex. These and previous results strongly indicated that the metal-sensitive ATR-FTIR bands originate from polypeptide backbones and amino acid residues near the putative Ca^{2+} -binding site located at the C-terminal side of the *Tch. tepidum* LH1 $\alpha\beta$ -polypeptides.
5. The structural modifications induced by the Ba^{2+} -substitution were basically identical with those by the Sr^{2+} -substitution. In contrast, the Cd^{2+} -substitution exhibited unique structural modifications, particularly in the amide I and II, and carboxylate modes, which may be responsible for the severely deteriorated thermal stability of the Cd^{2+} -substituted complex and are intimately related with the molecular mechanism for the enhanced thermal stability of *Tch. tepidum*.

Chapter 3 Structural and functional roles of calcium ions and extrinsic proteins in the oxygen-evolving complex of photosystem II

3.1 Introduction

Oxygenic phototrophs convert photon energy into chemical energy through a series of light-induced electron transfer reactions initiated with charge separation of chlorophyll special pairs located in the central part of photosystem I and II (PSI and PSII). The reducing power is transferred from PSII to PSI through cytochrome *b₆f*, and finally utilized for reduction of NADP⁺ to assimilate CO₂. The oxidized equivalents accumulated on the PSII donor side are neutralized by substrate water molecules to release protons for driving ATP synthase and O₂ molecules as a by-product. This water oxidation takes place in the oxygen-evolving complex (OEC) of PSII [34, 92]. The OEC assembly is largely similar between cyanobacteria and higher plants, except for a critical difference in the composition of extrinsic proteins [26]. In cyanobacteria, PsbO, PsbV, and PsbU residing on the luminal side of PSII play significant roles in the regulation and stabilization of the water oxidation machinery. Higher plants possess major nuclear gene-encoded extrinsic proteins named PsbO, PsbP, and PsbQ. PsbO is a common extrinsic protein highly conserved among the oxygenic phototrophs. PsbP and PsbQ are indigenous to plant PSII and have been proposed as the functional equivalents of PsbV and PsbU in bacterial PSII, having replaced them during the course of evolution from ancestral cyanobacteria to higher plants. These proteins play significant roles in the regulation and stabilization of the photosynthetic water oxidation [26, 27, 33] although the details of their function(s) are still a matter of debate.

In this chapter, I describe the structural-functional roles of extrinsic proteins in the plant PSII. The effects of extrinsic proteins on the photosynthetic function of the

Mn₄CaO₅ cluster and the structural stability of the OEC core complex were investigated by spectroscopic and biochemical analyses. Based on the results presented here and reported previously, the structural and functional roles of extrinsic proteins in the regulation and stabilization of photosynthetic functions are discussed.

3.2 Materials and methods

3.2.1 Sample preparation

In the present study, I used two types of PSII membranes lacking functional Ca^{2+} with or without extrinsic proteins (Ex) including PsbP and PsbQ as shown in Figure 3-1. BBY-type PS II membranes (untreated PSII, A) were prepared from spinach according to the method described previously [93]. The O_2 -evolving activity was $\sim 550 \mu\text{moles of O}_2/\text{mgChl/h}$. For depletion of Ca^{2+} , PsbP, and PsbQ, the membranes were suspended in medium A (2 M NaCl, 10 mM MES/NaOH, and pH 6.5) at 0.5 mg of Chl per ml and gently stirred on ice under weak light ($10 \mu\text{mol/s/m}^2$) for 30 min. Next, the following procedures were carried out in complete darkness or dim green light unless otherwise noted: EDTA was added to the suspension to achieve a final concentration of 1 mM, followed by 10-min incubation in the dark. The suspension was centrifuged and extensively washed with Chelex-treated medium B (400 mM sucrose, 20 mM NaCl, 20 mM MES/NaOH, and pH 6.5) to yield PSII membranes depleted of Ca^{2+} , PsbP, and PsbQ (Ex Ca^{2+} -depleted PSII, B). For depletion of PspP and PsbQ proteins, PS II membranes were suspended in medium A at 0.5 mg of Chl per ml, and gently stirred on ice in darkness for 30 min. The extracted PsbP and PsbQ proteins were reconstituted into the NaCl/EDTA-treated PSII to obtain Ca^{2+} -depleted PSII (C).

Alternatively, the PSII membranes were washed with medium C (400 mM sucrose, 20 mM NaCl, 0.1 mM MES-NaOH, and pH 6.5) and then treated with low-pH medium D (400 mM sucrose, 20 mM NaCl, 20 mM citrate-NaOH, pH 3.0) at 2 mg of Chl per ml. After 5 min incubation on ice in darkness, the suspension was diluted with medium D (400 mM sucrose, 20 mM NaCl, 500 mM MOPS-NaOH, pH 7.5), and incubated for 10 min to facilitate the rebinding of extrinsic proteins. Then,

the sample was washed with medium E (400 mM sucrose, 20 mM NaCl, 40 mM MES/NaOH, 0.5 mM EDTA, pH6.5) to obtain PSII membranes depleted of only Ca^{2+} (Low-pH-treated PSII, D). Finally, the resulting Low-pH-treated PSII membranes were treated with medium A to produce PSII membranes depleted of both Ca^{2+} and extrinsic proteins (Ex Ca^{2+} -depleted PSII, E).

3.2.2 O_2 -evolving activity

O_2 -evolving activities of the PSII preparations were measured at 25°C with a Clark-type oxygen electrode in medium B (400 mM sucrose, 20 mM NaCl, 20 mM MES/NaOH, and pH 6.5) containing 20 mM CaCl_2 with 0.5 mM of phenyl-p-benzoquinone (PpBQ) as electron acceptor.

3.2.3 Pulse amplitude modulation (PAM) fluorescence measurement

The maximal photochemical quantum yield of PSII (F_v/F_m) was measured using a pulse-modulated fluorometer (Junior-PAM, Heinz Walz, Germany). The minimum fluorescence (F_0) was determined after a weak far red modulated light. Then the maximum fluorescence (F_m) was reached by exposing the sample suspension to a saturating light pulse. The maximum quantum yield of PS II (F_v/F_m) was calculated by the following equation.

$$F_v/F_m = (F_m - F_0)/F_m$$

3.2.4 ATR-FTIR spectroscopy

FTIR spectra were measured on an FTIR8600PC (SHIMADZU) spectrophotometer equipped with a ZnSe ATR optics. A solution of the extrinsic proteins (50~100 μl) with or without 5 to 10 mM of Ca^{2+} was dehydrated under a stream of dry air to form a dry film, and a single-beam spectrum (64 scans) for each film was recorded at 4 cm^{-1} resolution and room temperature.

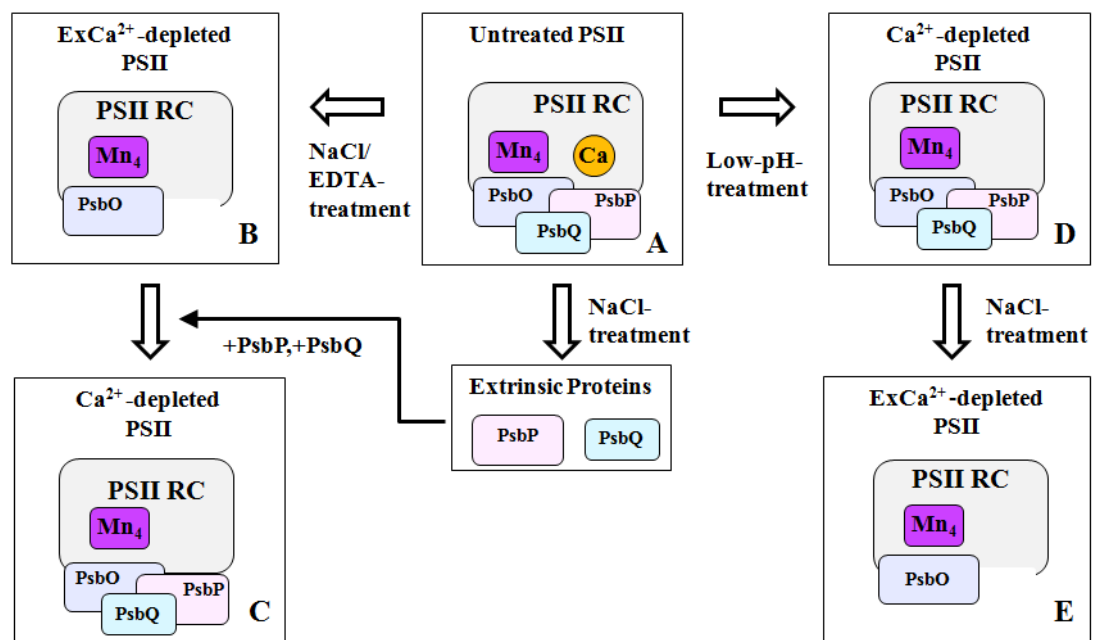


Figure 3-1. Schematic representation for the sample preparations of two types of PSII membranes lacking functional Ca²⁺ with or without PsbP and PsbQ.

3.3 Results and discussion

3.3.1 Interaction of extrinsic proteins with the Mn_4CaO_5 cluster in the OEC

The PSII membranes prepared by the different methods were assessed by O_2 -evolving activity and Fv/Fm values of chlorophyll fluorescence and the resulting data are summarized in Table 3-1. The O_2 -evolving rate of the untreated PSII was decreased to 17% when PsbP, PsbQ and Ca^{2+} were depleted by the NaCl/EDTA treatments (B). The decreased activity was restored to 83% by reconstituting Ca^{2+} , as reported previously [93, 94]. However, the addition of PsbP and PsbQ to the ExCa^{2+} -depleted PSII in the absence of Ca^{2+} lowered the O_2 -evolving rate to ~0% (C). Furthermore, the O_2 -evolving activity was almost completely lost upon Ca^{2+} depletion by the Low-pH treatment (D) but restored to 79% by adding Ca^{2+} . Notably, the lost activity was partially restored by the further depletion of PsbP and PsbQ to 25% (E). These results indicate that PsbP and PsbQ proteins completely suppress O_2 evolution in the absence of functional Ca^{2+} . Similar effects are also evident in the chlorophyll fluorescence measurements: the Fv/Fm values were much lower in the Ca^{2+} -depleted PSII (45%) than in the ExCa^{2+} -depleted PSII (64%), and both values were recovered to ~80% after the supplementation with Ca^{2+} . In the Ca^{2+} -depleted PSII, the partial recovery to 68% was induced by the following depletion of the extrinsic proteins. Since Fv/Fm values are related to O_2 -evolving activity, this strongly suggests that the functions of the OEC are disturbed by the extrinsic proteins in the absence of Ca^{2+} .

Table 3-1. Effects of Ca²⁺ and extrinsic proteins (PsbP and PsbQ) on the properties of the OEC.

PSII preparation	Additives	O ₂ -evolving activity	Fv/Fm	FTIR S ₂ /S ₁ carboxylate bands	Thermoluminescence Q-band (°C)	S ₂ EPR multiline signal
Untreated PSII (A)	No addition	100%	100%	Normal ^b	Normal ^{e,f}	Normal ^f
	No addition	17%	64%	Normal ^b	Normal ^{b,d,e}	Normal ^d
ExCa ²⁺ -depleted	+Ca ²⁺	83%	79%	Normal ^b	Normal ^{b,d,e}	Normal ^d
	+PsbP, +PsbQ (C)	~0%	— ^a	— ^a	Abnormal ^e	Modified
PSII (B)	+PsbP	~0%	— ^a	— ^a	Abnormal ^{e,f}	— ^a
	No addition	~0%	45%	Abnormal ^c	Abnormal ^{e,f}	Modified ^f
Ca ²⁺ -depleted PSII (D)	+Ca ²⁺	79%	81%	Normal ^c	Normal ^{e,f}	Normal ^f
	-PsbP, -PsbQ (E)	25%	68%	— ^a	Normal ^e	Normal

^aNot reported, ^b[93], ^c[94], ^d[92], ^e[95], ^f[46].

Next, the effects of extrinsic proteins and Ca^{2+} on the thermal stability of the OEC were examined. Figure 3-2 shows the relative absorbance at 680 nm of the untreated control PSII (circle), ExCa^{2+} -depleted (triangle), and Ca^{2+} -depleted (square) PSII membranes during incubation at 50°C. The relative band intensity of the control PSII remained at ~85% after 64 min incubation, but was slightly decreased to ~75% in the ExCa^{2+} -depleted PSII and was markedly decreased to 50% in the Ca^{2+} -depleted PSII. This is consistent with the effects seen in the O_2 -evolving activity and Fv/Fm values. These results strongly support the idea that PsbP and PsbQ lower the structural stability and disturb the normal functioning of the OEC in the absence of Ca^{2+} .

The present data are largely in agreement with previous findings, as shown in Table 3-1. FTIR spectroscopy provides valuable information on the structure and interactions within the OEC. The ligation geometry around the Mn_4CaO_5 cluster is mostly similar between untreated and ExCa^{2+} -depleted PSII, at least in the S_1 - and S_2 -states [94]. However, Ca^{2+} -depleted PSII exhibited marked deterioration in the carboxylate bands, which are thought to be from putative amino acid residues coordinating to the Mn_4CaO_5 cluster [95]. Furthermore, the redox potential of the Mn_4CaO_5 cluster has been reported to be abnormal when the extrinsic proteins bound to the PSII core in the absence of Ca^{2+} , as indicated by elevated peak temperatures of the thermoluminescence band for the S_2Q_A^- recombination [46, 96]. Additional support for this view was obtained from electron paramagnetic resonance (EPR) studies which demonstrated abnormal magnetic structures of PSII lacking Ca^{2+} but retaining the extrinsic proteins as revealed by modified S_2 -state multiline signals. These results are largely compatible with the present findings that the appropriate binding of extrinsic proteins in the presence of functional Ca^{2+} is required for the normal functioning of the OEC.

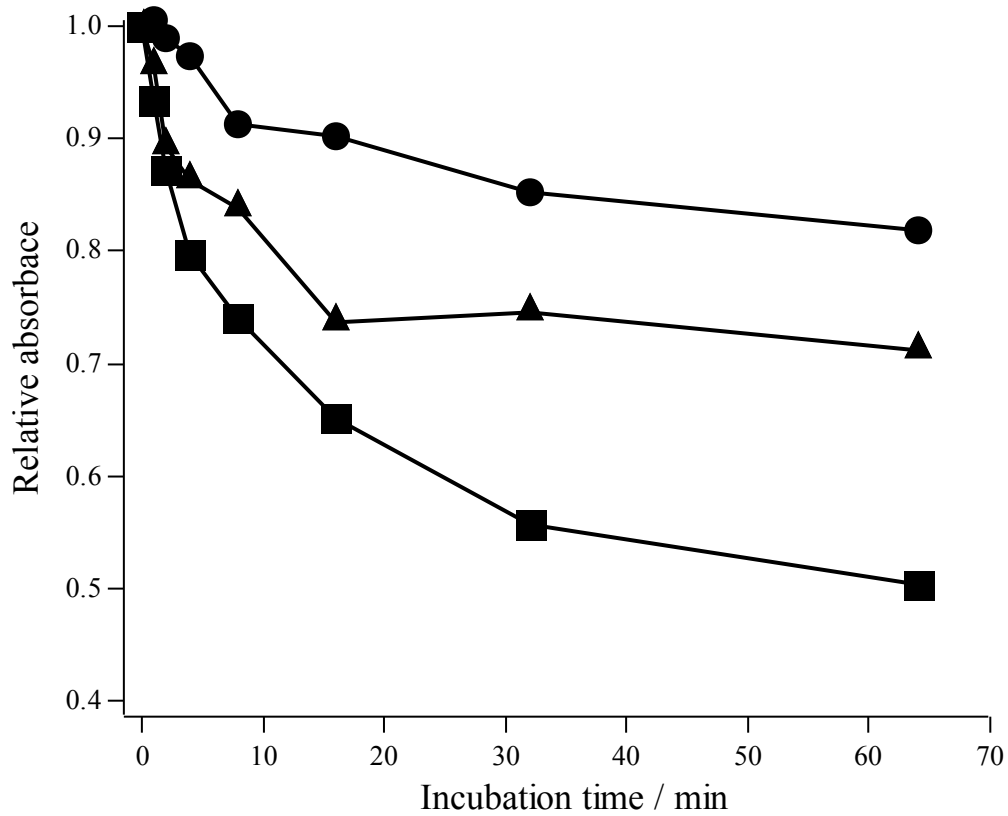


Figure 3-2. Plots of relative absorbance at 680 nm for the untreated (circle), ExCa²⁺-depleted (triangle), and Ca²⁺-depleted (square) PS II membranes as a function of incubation time at 50°C.

To understand function of these extrinsic proteins in the OEC, structural changes of the PsbP and PsbQ proteins induced by Ca^{2+} were observed by ATR-FTIR spectroscopy. Figure 3-3 shows ATR-FTIR spectra of isolated PsbP and PsbQ (spectrum a) and those supplemented with Ca^{2+} (spectrum b). The control spectrum a exhibited characteristic bands for amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) vibrational modes from backbone polypeptides of the OEC. These bands were significantly modified when Ca^{2+} was added to the extrinsic proteins as can be clearly seen in the difference spectrum (lower part of Figure 3-3). The IR bands at 1693, 1659 and 1539 cm^{-1} are decreased and new bands are visible at 1641 and 1566 cm^{-1} , strongly indicating that PsbP and/or PsbQ are metal-binding proteins that alter their secondary structures upon the binding of Ca^{2+} . Similar structural changes were evident in the spectrum of the purified PsbP protein (Figure 3-4). Although high-resolution crystallographic studies have revealed the structure of the PsbP protein in *Nicotiana tabacum* [97], this protein lacks the N-terminal region which are thought to contain the Ca^{2+} -binding site, and therefore, the relationship between PsbP and Ca^{2+} remains unclear [98]. However, Bondarava et al. hypothesized that PsbP acts to reserve Mn^{2+} or Ca^{2+} ions [36]. These results strongly support the idea that the PsbP protein is a metal-binding protein that directly and/or indirectly interacts with the catalytic center of the OEC in the absence of sufficient Ca^{2+} .

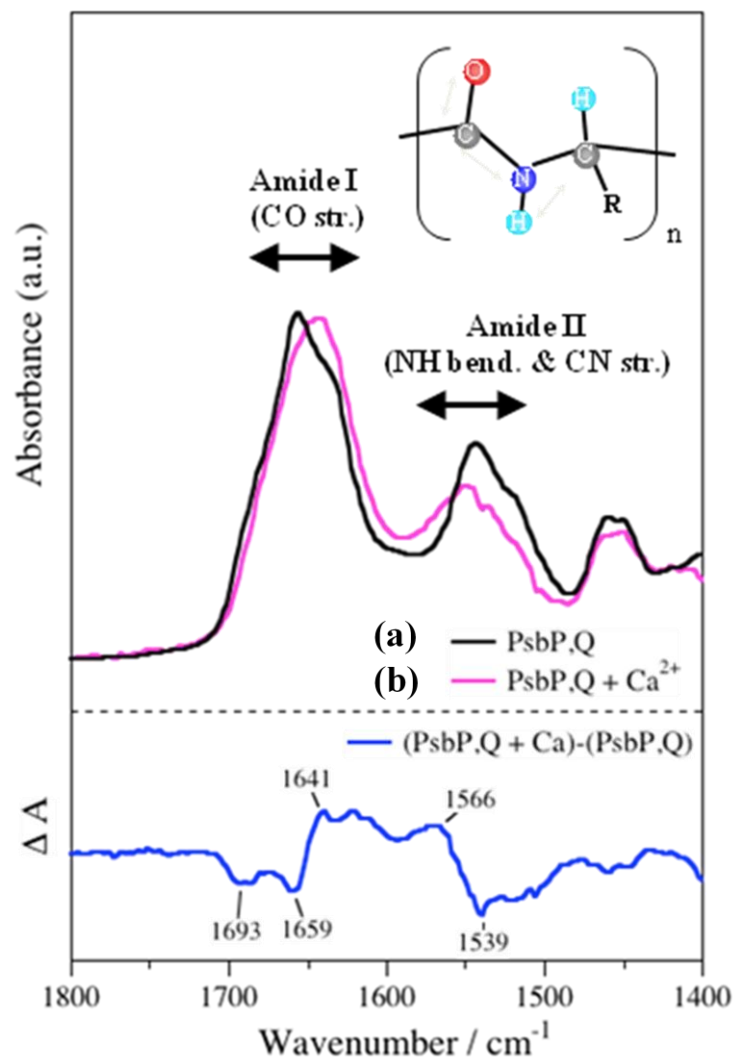


Figure 3-3. ATR-FTIR spectra of isolated PsbP and PsbQ proteins in the absence (a, black) and presence of Ca^{2+} (b, magenta). The difference spectrum obtained by subtracting spectrum a from spectrum b is shown in the lower panel.

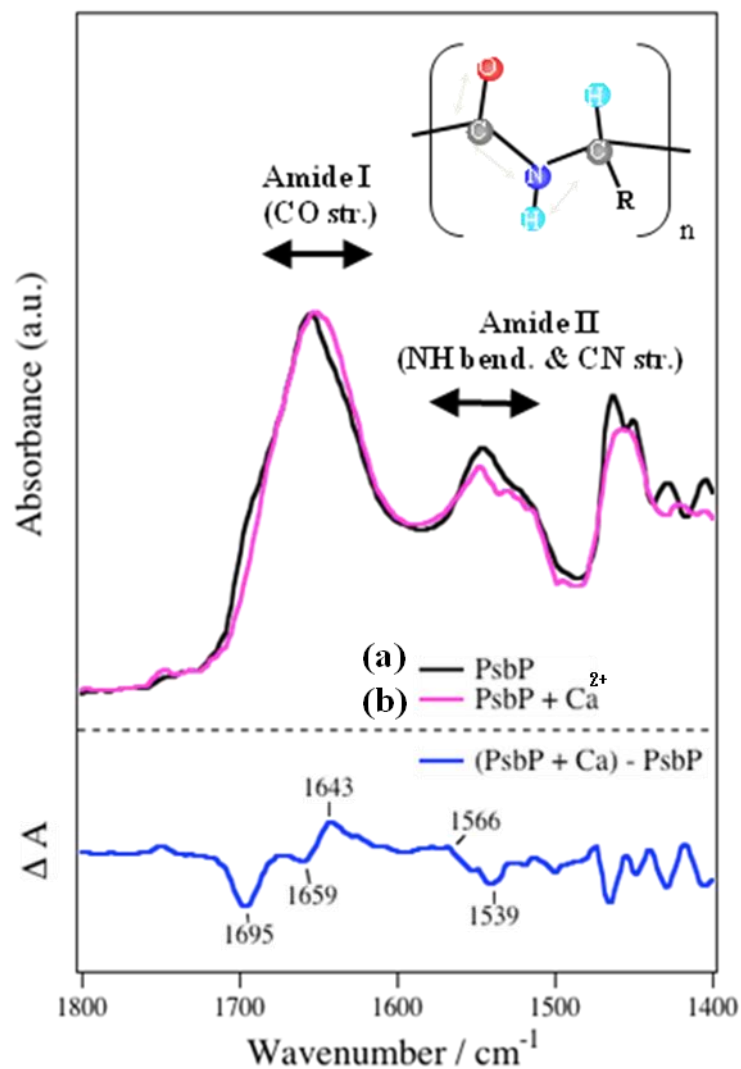


Figure 3-4. ATR-FTIR spectra of isolated PsbP proteins in the absence (a, black) and presence of Ca^{2+} (b, magenta). The difference spectrum obtained by subtracting spectrum a from spectrum b is shown in the lower panel.

The most important physiological role of PsbO is to stabilize the binding of the Mn_4CaO_5 cluster, which is essential for oxygen-evolving activity [53]. The PsbO protein can be dissociated from the PSII by a variety of chemical treatments including washing with alkaline Tris buffer, a high concentration of CaCl_2 , and chaotropic agents [99, 100]. In particular, Lys residue-modifying chemicals such as *N*-succinimidyl propionate and 2,4,6-trinitrobenzene sulfonic acid caused release of PsbO from PSII and loss of oxygen-evolving activity [101], suggesting that the positive charge of Lys is important for the electrostatic interaction between PsbO and PSII. Alternatively, the release of PsbO can be caused by thermal denaturation. However, PsbO itself is a thermostable protein [102], and therefore, other factors might also be responsible for the release of PsbO as described later in this chapter.

Several spectroscopic studies using isolated PsbO reported different Ca^{2+} -binding properties between higher plants and cyanobacteria. It has been suggested that plant PsbO can bind Ca^{2+} , which induces slight changes in secondary structure from a β -sheet to a loop or disordered structure, and facilitated the association of PsbO with the PSII core [54, 55]. However, an EPR study indicated that the functional Ca^{2+} ion was not involved in the binding to PsbO [56]. In cyanobacteria, PsbO does not bind Ca^{2+} , at least before the protein associates with the PSII core, since no significant conformational change upon the Ca^{2+} -binding was induced in isolated PsbO [57]. In contrast, the low-affinity Ca^{2+} -binding site in PsbO located at the luminal exit of the proton channel has been suggested to be responsible for water oxidation [58, 59]. These results strongly indicate that the structural-functional role of PsbO is not identical between higher plants and cyanobacteria. Interestingly, thermal stability was enhanced when plant PsbO proteins were replaced with thermally stable homologues from thermophilic *Phormidium laminosum* [103]. Therefore, slight variation in the

primary structure and/or the protein folding pattern is possibly responsible for the difference in thermal stability of PsbO between higher plants and thermophilic cyanobacteria.

3.3.2 Protective role of extrinsic proteins in regulation and stabilization of photosynthetic functions

The present study revealed that PsbP significantly affects the structure and function of the Mn_4CaO_5 cluster in the OEC only in the absence of sufficient Ca^{2+} in the OEC. This result is compatible with the previous analyses that involved FTIR, thermoluminescence, and EPR spectroscopies [46, 93-96]. In addition, it has been reported that PsbP has Ca^{2+} -binding sites in the N-terminal region [98] and functions as a reserver of Mn^{2+} or Ca^{2+} ions to supply them as needed by the impaired OEC [36]. Therefore, it is possible that the PsbP completely eliminates functional Ca^{2+} or interacts with the Mn_4CaO_5 cluster directly and/or indirectly to inhibit the O_2 -evolving activity and modify the ligation geometry, redox potentials and magnetic structures of the Mn_4CaO_5 cluster.

It has been suggested that normal functioning of PSII requires 15 highly conserved residues in the N-terminal region of the PsbP protein as well as the PsbQ protein for retention of functional Ca^{2+} [104]. A recent FTIR study indicated that the PsbP protein, but not the PsbQ protein, has an effect on S_2/S_1 conformational changes of the intrinsic polypeptide backbone around the Mn_4CaO_5 cluster through the N-terminal region of the PsbP [105]. In addition, little change was found in characteristic carboxylate stretching modes from putative amino acid ligands for the Mn_4CaO_5 cluster in the presence of Ca^{2+} when PsbP and PsbQ were depleted by NaCl washing, or all the extrinsic proteins were eliminated by CaCl_2 washing [105]. Based on these results, it is possible that the PsbP protein interacts with intrinsic proteins, which may be closely related to the Mn_4CaO_5 cluster, and preserves the OEC functions appropriately in the presence of Ca^{2+} , but modifies the properties of the cluster directly and/or indirectly through intrinsic proteins in the absence of Ca^{2+} .

It is intriguing to note that PsbV in cyanobacteria exhibits functional similarity with PsbP in higher plants, although their primary and 3D crystallographic structures are largely different [97, 106]. The apparent inconsistency in the structural-functional consequence may reflect the fact that PsbP and PsbV in plant and cyanobacterial PSII are not involved in specific interactions between the protein and the Mn_4CaO_5 cluster, but serve to maintain indispensable inorganic cofactors in the proximity of the cluster and to protect it from invasion of reductants. Additionally, PsbQ and PsbU also play a key role for tuning O_2 -evolving activity and enhancing structural stability through the interaction with PsbP and PsbV, respectively [107, 108].

In higher plants, PSII is much more susceptible to high temperatures than PSI [109]. The thermal stability of the PSII core is closely related to the acquisition of cellular thermal tolerance in oxyphototrophs. The thermosensitivity of oxygen evolution in higher plants has been studied through simple experiments using PSII particles or isolated thylakoid membranes. Previous in-vivo and in-vitro studies have estimated the heat-labile properties of the OEC [109-111]. These studies demonstrated that the release of PsbO occurs first, followed by liberation of two of the four Mn^{2+} ions from the Mn_4CaO_5 cluster of the OEC [100, 112, 113], and finally by the loss of oxygen evolution at high temperatures [100, 114].

Another form of damage to the physiological function of the PSII can be caused by reactive oxygen species (ROS) generated under high light conditions. The D1 proteins are degraded by the ROS species and inhibited in their ability to repair the photodamaged PSII by suppressing the synthesis of D1 proteins [115]. The ROS are thought to arise from heat-induced inactivation of a water-oxidizing manganese complex and through lipid peroxidation [116]. On the other hand, saturation of polyunsaturated fatty acids (PUFAs) contributes to the acquisition of heat tolerance of

photosynthesis by altering physicochemical properties [117-119]. The increased saturation of PUFAs raises the temperature at which lipids phase-separate into non-bilayer structures, providing the proper assembly and dynamics of PSII tolerant to higher temperatures [120].

Recently, I reported biochemical evidence that the biological effect of reactive carbonyls such as malondialdehyde (MDA) and acrolein is greatly enhanced under heat-stressed conditions. [121]. PsbO is one of the proteins most frequently modified by MDA, which is an end-product of peroxidized polyunsaturated fatty acids. Detailed biochemical experiments indicated that the modification of PsbO by MDA affects its binding to the PSII complex and causes inactivation of the OEC (Figure 3-5). Purified PsbO and PSII membranes, from which extrinsic proteins had been eliminated, of the oxygen-evolving complex (PSII Δ OEE) of spinach were separately treated with MDA. The binding was diminished when both PsbO and PSII Δ OEE were modified, but when only PsbO or PSII Δ OEE was treated, the binding was not impaired. In an experiment using thylakoid membranes, the release of PsbO from PSII and a corresponding loss of oxygen-evolving activity were observed when thylakoid membranes were treated with MDA at 40°C but not at 25°C. In spinach leaves treated at 40°C under light, the maximum efficiency of PSII photochemistry (Fv/Fm ratio of chlorophyll fluorescence) and oxygen-evolving activity decreased. Simultaneously, the MDA content of the heat-stressed leaves increased, and PsbO and PSII core proteins (including 47 kDa and 43 kDa chlorophyll-binding proteins) were modified by MDA. In contrast, these changes were less profound when these experiments were performed at 40°C in the dark. Thus, MDA modification of PSII proteins likely causes the release of PsbO from PSII, an effect that is particularly marked in heat and oxidative conditions.

First, ROS attack trienoic fatty acids in thylakoid membranes, resulting in the generation of MDA. MDA attaches to critical Lys residues of PsbO and PsbB (CP47) for the interaction between PsbO and PSII in a temperature-dependent manner. When both sides of PsbO and PSII are modified by MDA, PsbO is released from PSII. Finally, the Mn_4CaO_5 cluster is spontaneously released from PSII, causing loss of oxygen-evolving activity.

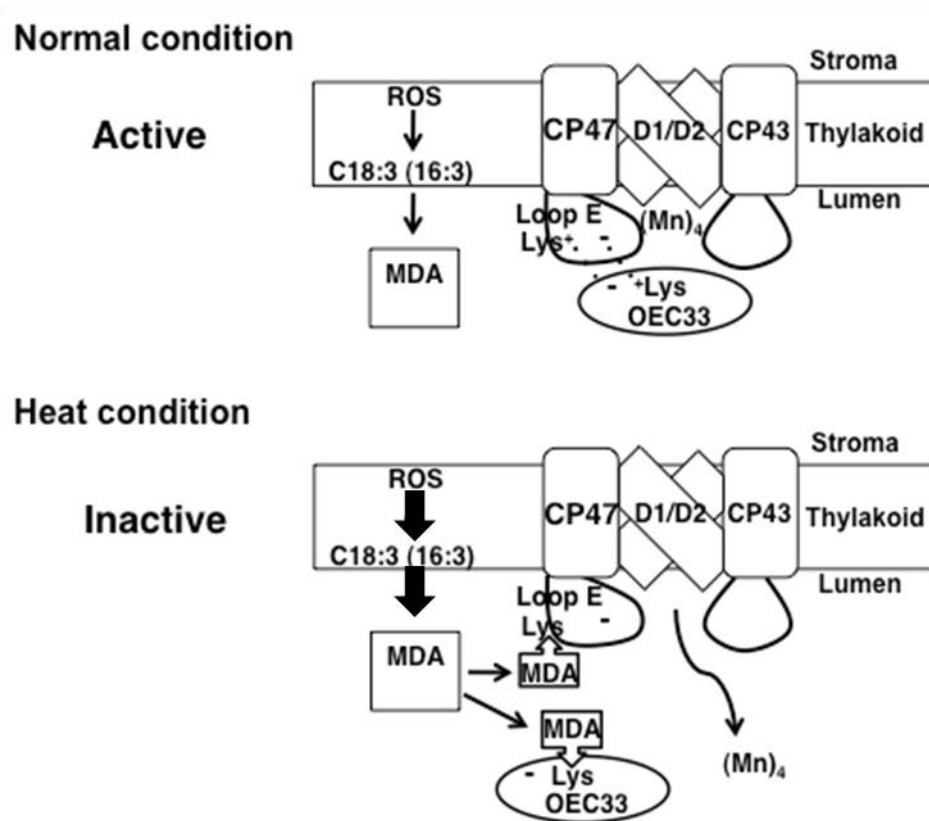


Figure 3-5. A schematic model of MDA-induced loss of oxygen evolution in heat-stressed spinach PSII complexes.

3.4 Conclusion

In this chapter, I focused on the structural and functional roles of extrinsic proteins in the plant PSII. Since PSII is an integrated pigment-protein complex embedded in plant membranes, the structures and interactions of these extrinsic proteins in the membrane interface are of significance for protecting the RC. This involves protecting the Mn_4CaO_5 cluster from exogenous invasion of reductants and/or alteration of physiological conditions. However, based on the results presented here and reported previously, I consider it very likely that the extrinsic protein itself is also responsible for the deterioration of the normal functioning of the OEC under inappropriate conditions. Further studies on the plant PSII, including high-resolution crystallographic studies, will be required for understanding the functions of extrinsic proteins in the structural stability and the water oxidation chemistry in PSII.

Chapter 4 Summary

In this doctoral dissertation, the structural and functional roles of calcium ions were investigated using photosynthetic organisms with type II RC. PSII is evolutionarily related to purple bacteria, as evidenced by the similarities in their heterodimeric RC and quinone-mediated electron transport system. The purple bacterial photosystem lacks Mn_4CaO_5 clusters, the catalytic center for the water oxidation, and certain extrinsic proteins which reside ubiquitously in the periphery of PSII. In purple bacteria, the circular pigment-protein complex known as LH1 is closely associated with the RC to maintain the structures and functions of the bacterial photosystem. Calcium ions are intimately related to the differences between the ancestral and evolved phototrophs with type-II reaction centers. Therefore, the roles of calcium are significant to understand how the photosynthetic organisms utilize calcium ions in response to their living environment and during the process of the evolution from the ancestral to evolved photosynthetic organisms with type-II reaction centers.

In Chapter 2, the structural and functional roles of calcium ions in purple bacteria, the ancestral photosynthetic organism with type-II reaction centers, were investigated. The light-harvesting 1 reaction center (LH1-RC) complex from purple sulfur photosynthetic bacterium *Tch. tepidum* is believed to enhance thermal stability by binding of Ca^{2+} to the C-terminus of the LH1-polypeptides. However, structural roles of Ca^{2+} and details of the Ca^{2+} -binding site are remained to be resolved. Here, I applied perfusion-induced ATR-FTIR spectroscopy to highly purified LH1-RC complexes from *Tch. tepidum*, and detected for the first time metal-sensitive fine structural changes involved in the enhanced thermal stability of this complex. The *Tch. tepidum* LH1-RC complex exhibited Sr^{2+}/Ca^{2+} ATR-FTIR difference bands that

reflect changes of polypeptide backbones and amino acid residues upon the replacement of native Ca^{2+} with Sr^{2+} . The difference bands also appeared in the following $\text{Ca}^{2+}/\text{Sr}^{2+}$ difference spectra with almost identical intensities but inverse signs, demonstrating that the structural changes induced by the metal exchange are fully reversible. In contrast, these ATR-FTIR signals were faintly detected in the mesophilic counterpart, *Alc. vinosum*. A comparative analysis using LH1 complexes lacking the RCs strongly indicated that the metal-sensitive bands originate from polypeptide backbones and amino acid residues near the putative Ca^{2+} -binding site at the C-terminal region of *Tch. tepidum* LH1 complexes. Structural changes induced by Sr^{2+} - and Ba^{2+} -substitutions were essentially identical. However, Cd^{2+} -substitution exhibited unique structural modifications, which may be responsible for the severely deteriorated thermal stability of Cd^{2+} -substituted complexes. Furthermore, the observed FTIR signals were tentatively assigned based on the isotopic shifts by uniform ^{15}N , ^{13}C and ^2H -labelings of LH1-RC complexes. The molecular mechanism enhancing the thermal stability of *Tch. tepidum* LH1-RC proteins is proposed based on the possible assignments of the ATR-FTIR signals and the recent structural information on the Ca^{2+} -binding site.

In Chapter 3, the structural and functional roles of calcium ions in PS II from higher plants, the evolved photosynthetic organism with type-II reaction centers, were discussed in connection with the extrinsic proteins that are lacking in the ancestral purple bacteria. Photosynthetic oxygen evolution occurs in an OEC of PSII although details of the reaction mechanism are not fully understood. It is known that one OEC includes one or two Ca^{2+} , depletion of which results in the loss of oxygen-evolving ability. In addition, an extrinsic protein PsbP is closely related with binding properties of the functional Ca^{2+} . Therefore, it is significant to understand functional and

structural roles of Ca^{2+} and extrinsic proteins for elucidating the reaction mechanism of the photosynthetic oxygen evolution. In the present study, effects of depletion of Ca^{2+} and/or extrinsic proteins from the PSII on the oxygen-evolving ability, pulse amplitude modulation fluorescence measurement, and thermal stability of the OEC were examined using Ca^{2+} -depleted PSII samples prepared by different biochemical procedures. The oxygen-evolving activity of NaCl/EDTA preparation in which Ca^{2+} and extrinsic proteins (PsbP, PsbQ) are depleted was decreased to ~20% compared with untreated preparations. On the other hand, the oxygen evolution was completely suppressed when only Ca^{2+} was removed from the PSII by Low-pH treatment. In addition, Low-pH preparation was less stable than NaCl/EDTA preparation. Based on these findings, I proposed that the extrinsic protein, particularly PsbP, plays a significant role to protect the OEC in the presence of Ca^{2+} , whereas the PsbP may act as a metal-binding protein in the absence of Ca^{2+} to decompose the catalytic Mn_4CaO_5 cluster, resulting in the loss of O_2 -evolving activity and the deterioration of the structural stability.

Acknowledgments

Firstly, I would like to express my sincere appreciation to my adviser, Professor Takashi Ohno (Graduate School of Agricultural Science, Kobe University) for giving me this precious opportunity to take the degree of Doctor of Agriculture in his laboratory. I especially would like to express my deepest appreciation to my supervisor, Assistant Professor Yukihiro Kimura for his elaborated guidance, considerable encouragement and invaluable discussion that make my research of great achievement.

I would like to express my gratitude to thesis committee members, Professor Yukihiro Sugimoto (Graduate School of Agricultural Science, Kobe University) and Associate Professor Kengo Kanamaru (Graduate School of Agricultural Science, Kobe University) for their through reviews and valuable comments on this thesis.

I wish to express earnest thankfulness to Professor Zheng-Yu Wang-Otomo (Faculty of Science, Ibaraki University) and Professor Taka-aki Ono (Graduate School of Engineering Faculty Engineering, Ibaraki University) for their valuable advice and cooperation. I thank to Assistant Professor Yasuo Yamauchi (Graduate School of Agricultural Science, Kobe University) for his technical support during this study.

Finally, I am very grateful to Mr. Yuki Yura for his valuable cooperation in my experiments. I also express my deep gratitude to all the members of Ohno laboratory at Kobe University for their encouragement.

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