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ATR−FTIR Detection of Metal-Sensitive Structural Changes in the Light-Harvesting 1 Reaction Center Complex from the Thermophilic Purple Sulfur Bacterium *Thermochromatium tepidum*

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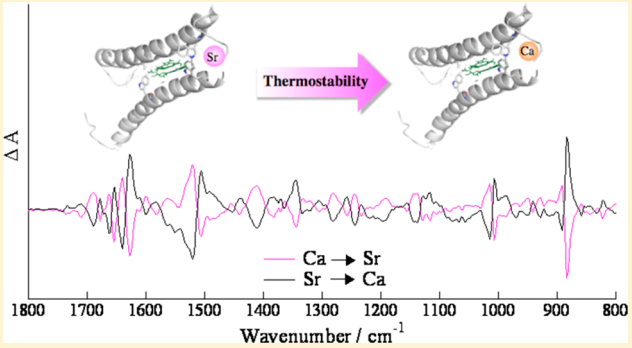
Supporting Information

ABSTRACT: *Thermochromatium tepidum* grows at the highest temperature among purple bacteria, and the light-harvesting 1 reaction center (LH1-RC) complex enhances the thermal stability by utilizing Ca^{2+}, although the molecular mechanism has yet to be resolved. Here, we applied perfusion-induced attenuated total reflection (ATR) Fourier transform infrared (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 in the polypeptide backbones and amino acid residues upon the replacement of native Ca^{2+} with Sr^{2+}. The difference bands also appeared in the following Ca^{2+}/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 *Allochromatium 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 the *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. Possible assignments for the present ATR−FTIR signals and their relation with the molecular mechanism of enhancing the thermal stability of *Tch. tepidum* LH1-RC proteins are discussed on the basis of the recent structural information on the Ca^{2+}-binding site.



Purple photosynthetic bacteria capture light energy with antenna apparatuses known as light-harvesting (LH1 and LH2) complexes and transfer the energy into a reaction center (RC) to initiate a charge separation and the subsequent photosynthetic redox events.[1] An X-ray crystallographic study[2] and scanning probe microscopic analyses[3−7] on mesophilic purple bacteria have demonstrated that the RC is surrounded by a cylindrical LH1 to form a core complex (LH1-RC). 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 as well as the roles of the LH1 complex are not fully understood.

Thermochromatium tepidum is a thermophilic purple sulfur bacterium.[8,9] 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 by an unusual amount (~35 nm) compared to those of the mesophilic counterparts. On the basis of the primary sequence of the LH1 $\alpha\beta$-subunit[10] and structural information of the RC,[11] 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.[11−13] Recently, we demonstrated that Ca^{2+} ions play a key role in the unique properties of the *Tch. tepidum* LH1-RC complexes.[14,15] 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 a 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.[14,15] On the basis of the topological analysis, a putative Ca^{2+}-binding site was expected to exist at the C-

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terminal region of the LH1 α-polypeptides.[16] 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 of 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.[17] In 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,[18,19] transhydrogenase,[20] halorhodopsin,[21] cytochromes *c*[22] and *c* oxidase,[23,24] and V-ATPase[25] as well as upon the redox changes and/or state transitions between intermediates of cytochrome *c* oxidase[26−29] and photosystems I and II.[30,31] As for purple bacteria, the redox-linked structural and/or conformational changes were reported for the cytochrome bc_1 complex of *Rhodobacter capsulatus*[32] and the RC complexes of *Rba. sphaeroides*, *Rhodopseudomonas viridis*,[30] and *Tch. tepidum*.[33] Apart from the perfusion-induced ATR−FTIR investigations, a recent typical ATR−FTIR study[34] 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 metal-bound and metal-depleted forms, and the fine structural modifications of the LH1-RC proteins induced by metal exchanges have not been detected. Here, we report for the first time metal-sensitive fine structural changes of protein backbones and amino acid side chains in the highly purified LH1 and LH1-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 on the basis of the recent structural information on the Ca^{2+}-binding site.

MATERIALS AND METHODS

Preparation of LH1-RC and LH1 Complexes. LH1-RC complexes from *Tch. tepidum* were purified as described previously[35] 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 applying a linear gradient of $CaCl_2$ from 0 to 25 mM, we 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 A_{915}/A_{280} over 3.0 for LH1 complexes and A_{915}/A_{280} 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 vinosum* LH1-RC complexes was conducted as described previously,[36] and fractions with a ratio of A_{884}/A_{280} over 2.0 were collected.

The LH1 or LH1-RC fractions were concentrated with Amicon Ultra 100K filters (Millipore), diluted 10-fold with buffer A (20 mM Tris-HCl, 20 mM $CaCl_2$, pH7.5), and ultracentrifuged at 195 000*g* for 10 min. The resulting pellet was suspended in buffer A containing 0.008% DDPC to result in ∼100 μM of the LH1-RC complex and used in the ATR−FTIR measurements.

ATR−FTIR Measurements. The ATR−FTIR measurement system was constructed as described in the literature by Rich and Breton[37] with modifications. FTIR spectra were recorded on a Prestige-21 spectrophotometer (Shimadzu) equipped with a mercury−cadmium−telluride 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 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 Ca^{2+} buffer (20 mM Tris-HCl, 25 mM NaCl, 20 mM $CaCl_2$, pH 7.5) at a flow rate of 1 mL/min for 30 min. The buffer was switched to Sr^{2+} buffer (20 mM Tris-HCl, 25 mM NaCl, 20 mM $SrCl_2$, 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 Sr^{2+}/Ca^{2+} ATR−FTIR difference spectrum. In a similar manner, Ca^{2+}/Sr^{2+} 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.

RESULTS AND DISCUSSION

Metal-Sensitive Structural Changes in the *Tch. tepidum* LH1-RC Complex. Figure 1 shows the Sr^{2+}/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 (νC═O, 1700−1600 cm^{-1}) and amide II (νC−N and δN−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[17] that interact directly and/or indirectly with the metal cations. Interestingly, the characteristic bands in the Sr^{2+}/Ca^{2+} difference spectrum were observed in the subsequent Ca^{2+}/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

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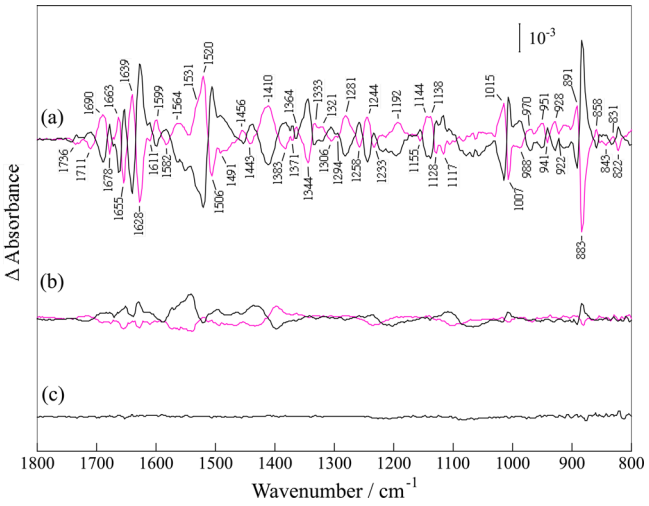

Figure 1. ATR−FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* (a) and *Alc. vinosum* (b) induced by switching from Ca^{2+} buffer to Sr^{2+} buffer (Sr^{2+}/Ca^{2+} difference spectrum, magenta) followed by switching from Sr^{2+} buffer to Ca^{2+} buffer (Ca^{2+}/Sr^{2+} difference spectrum, black). The difference spectrum of the *Tch. tepidum* LH1-RC complex without buffer switching (c) is presented to show the noise level.

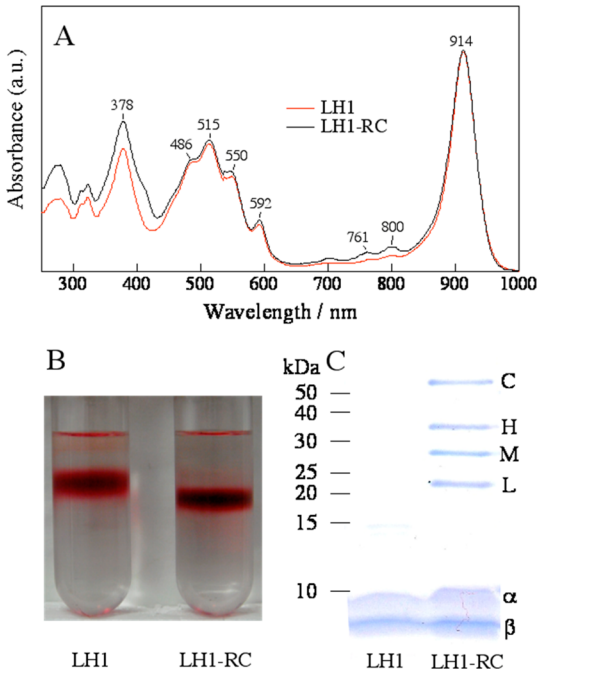

Figure 2. (A) Absorption spectra of the LH1 (red) and LH1-RC (black) complexes from *Tch. tepidum*. Both spectra were normalized with respect to the Q_y maximum at 914 nm. (B) Sedimentation patterns of the LH1 (left) and LH1-RC (right) complexes from *Tch. tepidum* after a continuous 10−40% (w/v) sucrose density gradient ultracentrifugation in 20 mM Tris-HCl buffer (pH 7.5) containing 0.08% DDPC. (C) SDS-PAGE profiles of the LH1 (left) and LH1-RC (right) complexes from *Tch. tepidum* on a 12−22% gradient gel stained with CBB. Four bands for C, H, M, and L subunits from the RC and two bands for *α*- and *β*-polypeptides from the LH1 complex are shown.

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 are 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 (Figure S1)[14] and emission,[38] (magnetic) circular dichroism,[14,39] and (pre)-resonance Raman scattering[36,38] of BChl-*a* molecules bound to the LH1 *αβ*-subunits. Although an isothermal titration calorimetry analysis of the *Tch. tepidum* LH1-RC complex indicated exothermic conformational changes because of the binding of Ca^{2+} to the proteins,[15] no significant modification in the secondary structure has been detected in far-UV CD spectra.[14] It is noteworthy that structural changes of the Ca^{2+}-depleted LH1-RC complexes upon the binding of Ca^{2+} or Mn^{2+} were examined by a typical ATR−FTIR analysis and an upshift of a symmetric carboxylate stretching band from 1403 to 1418 cm^{-1} was observed upon the Ca^{2+} binding.[34] However, the electronic and IR absorption spectra of the LH1-RC complexes used in the previous study[34] were significantly different from those reported previously,[14,39−41] indicating that the samples were degraded in some part because of freeze/thaw processes, dehydration by nitrogen gas, and/or incubation at ambient temperatures in the absence of Ca^{2+}. In the present study, the fresh and highly purified LH1-RC and LH1 samples (Figure 2) were prepared in the presence of Ca^{2+}, and the metal exchanges were conducted on the ATR prism by the perfusion of the buffer containing indicated metal cations, which allowed the detection of metal-sensitive and reversible fine structural changes of protein backbones and amino acid side chains ($\sim 10^{-4}-10^{-3}$ order of changes in absorbance) that are unique for the *Tch. tepidum* LH1-RC proteins. Taking into account the metal-dependent property changes in the thermal stability of this complex,[15] 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.

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*. 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 2A 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. Magnetic circular dichroism spectra of these complexes (Figure S2) also exhibited almost identical spectral feature, supporting strongly that the above electronic transitions are originating from BChl-*a* dimers bound to the LH1 complexes. In addition, there was no difference between LH1 and LH1-RC complexes for carotenoid molecules, predominantly ascribed to spirilloxanthin,[39] at 486, 515, and 550 nm. This result was further supported by resonance Raman data in which intensive $\nu C{=}C$ (1502 cm^{-1}) and $\nu C{-}C$ (1147 cm^{-1}) bands and several minor bands of all-trans spirilloxanthin appeared coincidently in both LH1 and LH1-RC complexes (Figure S3). 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[42] at ∼760, ∼800, and ∼880 nm, respectively, and the intensities of the bands lower than 450 nm were

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significantly decreased (Figure 2A). Consequently, the ratio of A_{915}/A_{280} showing the criteria for the purity of the complexes was larger than 3. Figure 2B shows sucrose density gradient ultracentrifugation profiles of the LH1 (left) and LH1-RC (right) complexes. The band of the LH1 complex appears at a slightly higher position than that of the LH1-RC complex,[39] indicating the reduced density of the LH1 complex because of the lack of the RC. Furthermore, the SDS-PAGE profile of the LH1 complex exhibited bands typical for LH1 α- and β-subunits,[39] but almost completely lost were the bands for C, H, M, and L subunits from the RC (Figure 2C). These 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 3 shows the Sr^{2+}/Ca^{2+} (a) and Ca^{2+}/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 cm^{-1} differential band.

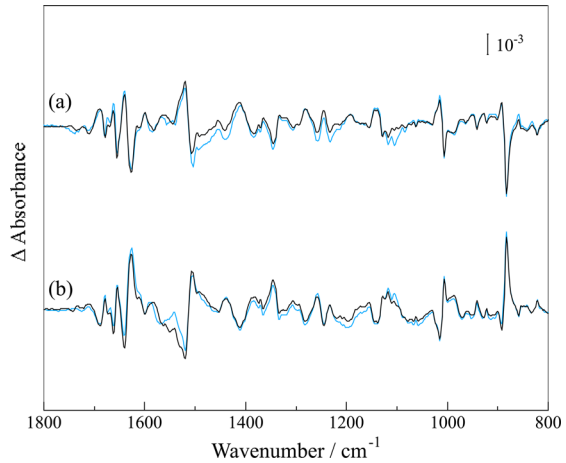

Figure 3. Sr^{2+}/Ca^{2+} (a) and Ca^{2+}/Sr^{2+} (b) difference spectra of the LH1 (cyan) and LH1-RC (black) complexes from *Tch. tepidum*. All spectra were normalized with respect to the 891/883 cm^{-1} differential band.

Ca^{2+} (a) and Ca^{2+}/Sr^{2+} (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 because of 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 that the contribution of the RC is negligible under the present experimental conditions.

It is noteworthy 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.[24] This may be attributed to the larger number of Ca^{2+}-binding sites in the LH1-RC protein because LH1 is proposed to be a 16-mer of the $\alpha\beta$-subunit, each of which has a putative Ca^{2+}-binding site at its C-terminal region.[14]

Tentative Assignments of the Perfusion-Induced ATR−FTIR Difference Bands. On the basis of the primary sequence[10] and a topological analysis,[16] it was proposed that the Ca^{2+}-binding site is composed of several acidic residues located at the C-terminal region of the LH1 $\alpha\beta$-polypeptides.[14,16] Several spectroscopic analyses indicated that the metal-depletion/substitution induced few changes in the secondary and tertiary structures of the LH1-RC complex.[14,38,39] 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 that a new crystallographic structure of the *Tch. tepidum* LH1-RC complex has been very recently presented.[43] As predicted in the previous studies,[14,16,38,39] the LH1 complex is a 16-mer of the $\alpha\beta$-subunit, each of which has a Ca^{2+}-binding site composed of α-Trp_{+10}, α-Asp_{+13}, α-Asn_{+14}, and β-Leu_{+10} located at the C-terminal region of the LH1 $\alpha\beta$-subunit (Figure S4). The carboxylate side chain of α-Asp_{+13} and the C-terminal carboxylate of β-Leu_{+10} coordinate with Ca^{2+} in unidentate and bidentate manners, respectively. Generally, metal-carboxylate complexes exhibit an asymmetric (ν_{as}) COO^- stretching mode at 1675−1515 cm^{-1} and a symmetric (ν_s) COO^- stretching mode at 1495−1260 cm^{-1},[44] and the metal-carboxylate ligation structures are empirically relevant to the value of $\Delta\nu$, which is $\nu_{as} - \nu_s$; $\Delta\nu$ = ∼164 cm^{-1} for the ionic form, $\Delta\nu \gg$ ∼164 cm^{-1} for the unidentate form, $\Delta\nu$ = ∼164 cm^{-1} (but greater than $\Delta\nu$ for bidentate) for the bridging form, and $\Delta\nu <$ ∼164 cm^{-1} for the bidentate form.[45,46] Unfortunately, this is not applicable to the present results because 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 without isotope-edited analyses. In contrast, the symmetric COO^- stretching bands (1495−1260 cm^{-1}) can be analyzed more clearly because 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 and 1390−1370 cm^{-1}) are less sensitive to faint changes in the molecular environment. Thus, the bands at 1443, 1383, 1371, 1306, and 1294 cm^{-1} or 1456, 1410, 1364, 1333, and 1321 cm^{-1} in the Sr^{2+}/Ca^{2+} (Ca^{2+}/Sr^{2+}) difference spectra (Figure 1) are possible candidates for the symmetric COO^- stretching bands of α-Asp_{+13} and β-Leu_{+10} ligating Ca^{2+} or Sr^{2+}. 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.[46] If this is the case, then the ν_s bands of α-Asp_{+13} and β-Leu_{+10} correspond to the lower and higher frequencies, respectively, of the putative bands. However, we 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 $-CONH_2$ from Asn and Gln (1420−1400 cm^{-1}), and the O−H deformation of Tyr, Thr, and Ser (1440−1260 cm^{-1}).[44]

Several intensive differential bands (1690, 1678, 1663, 1655, 1639, 1628, and 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 and 1641−1623 cm^{-1} for β-sheet, 1686−1662 cm^{-1} for turns, and 1670−1660 cm^{-1} for random coil.[17,44] However, the contribution of β-sheet structures are excluded because the LH1 $\alpha\beta$-subunits are predominantly composed of transmembrane α-helices as well as turns and random coil structures at the C- and N-terminal sides.[43] Therefore, the bands at 1695−1674 and 1641−1623 cm^{-1} can be assigned to

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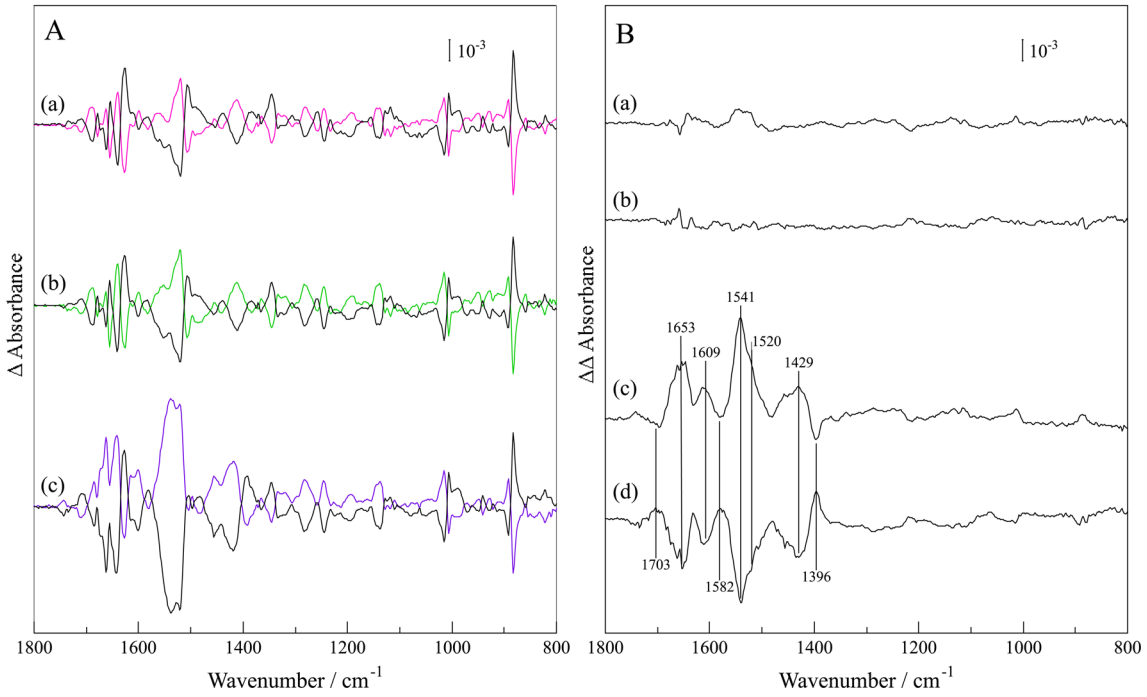

Figure 4. (A) ATR−FTIR difference spectra of the LH1-RC complexes from *Tch. tepidum* for the Sr^{2+}/Ca^{2+} (a, magenta), Ba^{2+}/Ca^{2+} (b, green), and Cd^{2+}/Ca^{2+} (c, purple) exchanges and their respective ensuing Ca^{2+}/Sr^{2+} (a, black), Ca^{2+}/Ba^{2+} (b, black), and Ca^{2+}/Cd^{2+} (c, black) exchanges. (B) Double difference spectra obtained by subtracting the Sr^{2+}/Ca^{2+} spectrum from the Ba^{2+}/Ca^{2+} spectrum (a), the Ca^{2+}/Sr^{2+} spectrum from the Ca^{2+}/Ba^{2+} spectrum (b), the Sr^{2+}/Ca^{2+} spectrum from the Cd^{2+}/Ca^{2+} spectrum (c), and the Ca^{2+}/Sr^{2+} spectrum from the Ca^{2+}/Cd^{2+} spectrum (d). All spectra were normalized with respect to the 891/883 cm^{-1} differential band.

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}).[17,47,48] In particular, the contribution of α-Asn_{+14} to the metal-sensitive FTIR bands at 1677−1678 and 1612−1622 cm^{-1} is likely to be involved because its carbonyl side chain serves as a direct ligand for Ca^{2+}.[43]

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.[43] 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[49] and are proposed to be 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.[50,51] 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.[36] On the basis of these results, it is possible that conformational changes of α-Trp_{+10} residues and/or BChl molecules (1760−1680 cm^{-1})[52] 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, and 1506 cm^{-1} and mid-to-low intensity bands at 1599, 1582, and 1564 cm^{-1} are the amide II modes of the polypeptide main chain (1577−1507 cm^{-1}),[44,53,54] the asymmetric stretching modes of COO^- from α-Asp_{+13} side chain and C-terminal carboxylate of β-Leu_{+10} (1675−1515 cm^{-1}, depending on the ligation structure),[45,46] and/or the CC stretching of Tyr (1602−1594 and 1518−1516 cm^{-1}).[17] 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 possibly involved in this region.[17]

The present ATR−FTIR study successfully detected significant metal-sensitive bands of the *Tch. tepidum* LH1-RC protein, but it can not achieve the correct assignments for respective bands because many vibrational modes from the structures associated with the metal exchange are overlapping. Therefore, further ATR−FTIR analyses in combination with ^{15}N-, ^{13}C-, and ^{2}H-isotope labeling are in progress to verify the detected metal-sensitive vibrational modes of the *Tch. tepidum* LH1 protein.

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.[15] Thus, the effects of Ba^{2+} or Cd^{2+} substitution for Ca^{2+} were also examined. Figure 4A 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 Ba^{2+}/Ca^{2+} (Ca^{2+}/Ba^{2+}) difference spectra were almost identical to Sr^{2+}/Ca^{2+} (Ca^{2+}/Sr^{2+}) difference spectra in terms of peak positions and band intensities, as can be clearly seen in the double difference spectra (Figure 4B) that

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were obtained by subtracting the Sr^{2+}/Ca^{2+} from Ba^{2+}/Ca^{2+} difference spectra (a) or by subtracting the Ca^{2+}/Sr^{2+} from Ca^{2+}/Ba^{2+} difference spectra (b). In contrast, the Cd^{2+}/Ca^{2+} (Ca^{2+}/Cd^{2+}) difference spectra (Figure 4A) showed markedly different spectral features, particularly in the 1750−1350 cm^{-1} region. The double difference spectra (Figure 4B) between Cd^{2+}/Ca^{2+} and Sr^{2+}/Ca^{2+} (c) or Ca^{2+}/Cd^{2+} and Ca^{2+}/Sr^{2+} (d) exhibited prominent bands 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 $M(CH_3COO)_2$ (M = Ca, Sr, Ba, or Cd), which are simple model compounds for carboxylates ligating metal cations (Figure S5). All of the spectra in panel A exhibited the asymmetric and symmetric COO^- stretching bands at ∼1551 and ∼1416 cm^{-1}, respectively. However, their peak positions and band intensities were altered depending on the metal cations (panel B). The Cd^{2+}/Sr^{2+} (Sr^{2+}/Cd^{2+}) difference spectra exhibited clear differences at 1566, 1441, and 1412 cm^{-1} in contrast to the small changes in the Ba^{2+}/Sr^{2+} (Sr^{2+}/Ba^{2+}) difference spectra. These results are largely compatible with the present ATR−FTIR double difference spectra (Figure 4B), supporting the idea that the ligation structure of the Sr^{2+}−carboxylate complex is almost identical with that of the Ba^{2+}−carboxylate complex but significantly different from that of the Cd^{2+}−carboxylate complex. Furthermore, the spectral shapes in the symmetric COO^- stretching regions in Figure 4B (1429 and 1396 cm^{-1}) are similar with those in Figure S5B (1441 and 1412 cm^{-1}), although the peak positions deviated by 12−16 cm^{-1}. Therefore, a possible assignment for the 1429 and 1396 cm^{-1} bands in Figure 4B is the symmetric carboxylate stretching bands, and the 1653 and 1541 cm^{-1} bands are ascribed to amide I and II of the polypeptide main chain, although the contribution of asymmetric carboxylate stretching bands cannot be excluded. If this is the case, then 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}. The recent structural information strongly indicated that one of the critical roles of Ca^{2+} is a binder between the loop (α-Trp_{+10}, α-Asp_{+13}, and α-Asn_{+14}) at the C-terminal side of an α-polypeptide and the C-terminal β-Leu_{+10} of the adjacent β-polypeptide to connect two $\alpha\beta$-subunits.[43] Therefore, one of the possible interpretations for the largely deteriorated thermal stability of the Cd^{2+}-substituted LH1-RC complex is that the unique binding mode of Cd^{2+}, which prefers the bidentate ligation, weakened the specific interaction between two adjacent $\alpha\beta$-subunits at the C-terminal region, leading to the destabilization of the LH1 complexes upon the binding of Cd^{2+}. However, we cannot exclude a possibility that an exchange of the ligation partner might occur in the vicinity of the Ca^{2+}-binding site upon Cd^{2+} substitution because Cd ions favor softer bases as ligand partners because of their relatively weak Lewis-acid properties.

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 Ba^{2+} (73.2%)[15] despite the fact that Cd^{2+}-, Sr^{2+}-, and Ba^{2+}-bound forms exhibited Q_y peaks at similar positions (887−889 nm).[14] Therefore, the unique structural changes induced by Cd^{2+} 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 Cd^{2+}-substituted LH1-RC complexes.

It is notable that metal-sensitive ATR−FTIR signals were not apparent in the *Alc. vinosum* LH1-RC complex (Figure 1) under the present conditions even though this complex also possesses a cluster of acidic residues in the C-terminal region in analogy with *Tch. tepidum* (Figure S4). 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 Ca^{2+}. Interestingly, the quite similar sequences including the deletion were confirmed in strain 970 and *Trv. winogradskyi*,[49] which exhibit unusual shifts of the LH1 Q_y bands toward the red (963 nm)[55] and blue (867 nm),[49] respectively. 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 Ca^{2+}-affinity site and that the Ca^{2+} binding induces a key conformational change of the C-terminal amino acid residues of LH1 α- and/or β-polypeptides. Further isotope-edited ATR−FTIR studies will provide valuable insight to reveal the details of the molecular mechanism enhancing the thermal stability of LH1-RC complexes from *Tch. tepidum* by utilizing Ca^{2+}.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Absorption spectra of the metal-bound LH1-RC complexes from *Tch. tepidum*, magnetic circular dichroism spectra of the LH1 and LH1-RC complexes from *Tch. tepidum*, resonance Raman spectra of the LH1 and LH1-RC complexes from *Tch. tepidum*, amino acid sequences of the LH1 α- and β-polypeptides from purple sulfur bacteria, and ATR−FTIR absorption spectra of metal acetate complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

BChl, bacteriochlorophyll; LH, light harvesting; RC, reaction center; DDPC, dodecylphosphocholine; ATR, attenuated total reflection; FTIR, Fourier transform infrared

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