Calcium Ions Are Involved in the Unusual Red Shift of the Light-harvesting 1 Q_y Transition of the Core Complex in Thermophilic Purple Sulfur Bacterium Thermochromatium tepidum

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Thermophilic purple sulfur bacterium, Thermochromatium tepidum, can grow at temperatures up to 58 °C and exhibits an unusual Q_y absorption at 915 nm for the core light-harvesting complex (LH1), an ~35-nm red shift from those of its mesophilic counterparts. We demonstrate in this study, using a highly purified LH1-reaction center complex, that the LH1 Q_y transition is strongly dependent on metal cations and Ca^{2+} is involved in the unusual red shift. Removal of the Ca^{2+} resulted in formation of a species with the LH1 Q_y absorption at 880 nm, and addition of the Ca^{2+} to the 880-nm species recovered the native 915-nm form. Interchange between the two forms is fully reversible. Based on spectroscopic and isothermal titration calorimetry analyses, the Ca^{2+} binding to the LH1 complex was estimated to occur in a stoichiometric ratio of Ca^{2+}/αβ-subunit = 1:1 and the binding constant was in 10^5 M^{-1} order of magnitude, which is comparable with those for EF-hand Ca^{2+}-binding proteins. Despite the high affinity, conformational changes in the LH1 complex upon Ca^{2+} binding were small and occurred slowly, with a typical time constant of ~6 min. Replacement of the Ca^{2+} with other metal cations caused blue shifts of the Q_y bands depending on the property of the cations, indicating that the binding site is highly selective. Based on the amino acid sequences of the LH1 complex, possible Ca^{2+}-binding sites are proposed that consist of several acidic amino acid residues near the membrane interfaces of the C-terminal region of the α-polypeptide and the N-terminal region of the β-polypeptide.

Thermochromatium (Tch.) tepidum is a thermophilic purple sulfur photosynthetic bacterium that can grow at temperatures up to 58 °C, the highest known for purple bacteria (1, 2). The crystal structure of the reaction center (RC) for this bacterium has been determined (3), revealing structural features similar to those of the RCs from other purple bacteria (4, 5). The antenna system in Tch. tepidum contains two types of light-harvesting complex, LH1 and LH2. The LH1 complex has been shown to be tightly associated with RC to form the core complex (6, 7), LH1-RC, and the LH2 complex is supposed to be located in the periphery of the core complex (8, 9). Although a number of high resolution structures are available for the LH2 and LH3 (a spectroscopic variant of LH2) complexes (10–12), information on the molecular structure of the LH1 complex has been lacking. This information is essential for studying its biological functions in terms of excitation energy transfer and reduced ubiquinone transport. The interaction mode between LH1 and RC complexes is also of particular interest because the RC has a structure of 2-fold symmetry (3, 4, 13), whereas low resolution structures of LH1 show a pseudo 14- or 16-fold symmetry depending on the species (14–20). The symmetry issue needs to be taken into account when addressing the possible contacting sites between the LH1 and RC and the factors determining the specific orientation of RC inside the LH1 ring. The crystallographic structure of the LH1-RC complex from Rhodopseudomonas (Rps.) palustris at 4.8 Å resolution shows a slightly elliptical LH1 ring composed of 15 pairs of the αβ-polypeptides with a gap that is associated with an unknown protein W and is proposed to be adjacent to the ubiquinone-binding site in RC (17). However, to gain insight into a more detailed picture of the configuration of LH1-RC, structural information at higher resolutions is required. Recently, the LH1-RC complex from Tch. tepidum has been crystallized (21).

We have shown that the purified LH1-RC complex of Tch. tepidum is extremely stable (21, 22). Besides the thermal stability, another striking feature is the unusual red shift of the Q_y transition of the LH1 complex. Although the core complex contains only a-type bacteriochlorophyll (BChl), the same as those found in most other purple bacteria, spectroscopically the LH1 complex of Tch. tepidum exhibits a Q_y absorption at 915 nm, ~35 nm red-shifted from those of its mesophilic counterparts. The reason for this red shift has been unclear (2). Two other LH1 complexes from purple bacteria strain 970 and Roseosp-

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3 The abbreviations used are: RC, reaction center; BChl, bacteriochlorophyll; LH, light-harvesting; ITC, isothermal titration calorimetry; OG, n-octyl-β-D-glucopyranoside.
The native LH1-RC complex was passed through a size exclusion column (Sephadex G25M PD10; GE Healthcare) to remove excess salts, followed by incubation at 0 °C for 3 h in darkness in the presence of 1.25 mM EDTA. The sample solution was extensively washed with a buffer containing 20 mM Tris-HCl, pH 7.5, and 0.8% (w/v) OG to remove excess of EDTA. The final concentration of EDTA in the solution was estimated to be lower than 1 mM. The resulting core complex showed an LH1 Q<sub>y</sub> absorption maximum ~880 nm. To examine the effects of metal cations, the B880 sample was incubated at 0 °C overnight after adding various metal cations. Sucrose density gradient centrifugation analysis was conducted by ultracentrifugation at 150,000 × g and 4 °C for 12 h under a 10–40% (w/v) continuous gradient of sucrose concentration in a buffer containing 20 mM Tris-HCl, pH 7.5, and 0.7% (w/v) OG in the presence or absence of 1 mM EDTA.

**Spectroscopic Measurements**—Absorption spectra were recorded on a Beckman DU-640 spectrophotometer at room temperature. Kinetic analysis for the recovering process from B880 to B915 upon the addition of Ca<sup>2+</sup> was performed by monitoring the increase of the absorbance at 937 nm. CD spectra were recorded on a Jasco J-720ws spectropolarimeter in the ranges of 200 to 300 and 400 to 1000 nm. The measurements were conducted under the conditions of 20 nm/min of scan speed, 1.0 nm bandwidth, and 2 s of response time. For the CD measurement between 200 and 300 nm, data were averaged over 10 scans in order to improve the signal-to-noise ratio, while for the measurements between 400 and 1000 nm the spectra were obtained from one scan. The molar extinction coefficient of BChl a dimer in a native αβ-subunit was estimated to be 270 M<sup>−1</sup> cm<sup>−1</sup> by extracting BChl a molecules from purified LH1-RC complexes with acetone/methanol (7/2, v/v) and using ε<sub>770</sub> = 76 M<sup>−1</sup> cm<sup>−1</sup> for the monomeric BChl a (30).

**ITC**—ITC measurements were carried out on a MicroCal VP-ITC microcalorimeter at 25 °C. The LH1-RC samples were dissolved in 20 mM Tris-HCl, pH 7.5, and 0.7% OG at 250 μM. Following thermal equilibration at 25 °C, a total of 14 injections of 50 mM CaCl<sub>2</sub> dissolved in the same buffer were made in 1-μl aliquots into the cell from the syringe using a 300-rpm stirring speed. An injection delay of 10 min was utilized to allow for the baseline to return after each injection. The titration data were deconvoluted based on a binding model containing either one or two sets of noninteracting binding sites by a nonlinear least squares minimization method (31) performed using the MicroCal Origin ITC software.

**RESULTS**

First we confirmed the effect of various metal cations on the absorption spectrum using highly purified LH1-RC complex from the *Tch. tepidum* (supplemental Fig. S1). The native core complex (B915) exhibited LH1 Q<sub>y</sub> absorption at 914 nm as reported previously (21). The Q<sub>y</sub> band was retained at the original position only in the presence of Ca<sup>2+</sup> and blue-shifted by the addition of other cations. The results indicate that the native LH1 form of the *Tch. tepidum* is sensitive to the coexisting metal cations.

To investigate whether the B915 species is bound to metal ions, metal chelators were employed. Fig. 1 shows an absorb-
tion spectrum obtained with the addition of EDTA to the B915 species. The LH1 Qy band was largely blue-shifted to 876 nm with a spectral shape similar to those observed for the LH1 complexes from other purple bacteria. This clearly indicated that metal ions are involved in the formation of the B915 and removal of the cation by EDTA results in a conformational change of the BChl a molecules in the LH1 complex, leading to a large blue shift of the LH1 Qy band. Similar results were observed using different metal chelators, nitrilotriacetic acid (tetrasodium salt), suggesting that the blue shift was not caused by the specific interaction between EDTA and a large blue shift of the LH1 Qy band (876 nm) of B880. Addition of Ca2+ gave an absorbance change of B880 from 913.5 nm. By increasing the CA2+ concentration, an absorbance change (ΔA) was observed as a function of the Ca2+ concentration as shown in Equation 1.

\[
\Delta A(t) = \Delta A \times [1 - \exp(-k \times t)]
\]  

(Eq. 1)

The binding property of Ca2+ was examined over a wide range of Ca2+ concentrations. Fig. 2 shows the absorption spectra of the EDTA-treated core complex in the presence of Ca2+ at various concentrations. By increasing the Ca2+ concentration, the B915 species was formed accompanied by a decrease of the B880 species. There was an isosbestic point at 895 nm, indicating that the B915 recovered from the B880 upon the addition of Ca2+. The Ca2+-dependent LH1 Qy shifts were plotted in the inset as a function of the Ca2+ concentration, revealing a sigmoid curve that saturated ~50 μM Ca2+. Kinetics of the Ca2+ binding process at room temperature (25°C) was analyzed using the pseudo first-order model. Formation of the B915 species was monitored at 937 nm where we can observe the maximum change in the intensity. Fig. 3 shows the time profile of the absorption change (ΔA) after addition of 5 mM Ca2+ to the B880 complex of Tch. tepidum. Concentration of the LH1 subunit was 16 μM, and residual EDTA in the solution was lower than 1 nM.
range of 2.5 to 100 mM of the Ca\(^{2+}\) concentration (Fig. 3, inset), suggesting that the pseudo first-order kinetic analysis is valid and the conformational changes from B880 to B915 upon the Ca\(^{2+}\) binding occur slowly under the experimental conditions.

The binding affinity of Ca\(^{2+}\) to B880 was evaluated by a non-linear least-square fitting of the spectroscopic data. Fig. 4 shows the plots of \(\Delta A\) at several wavelengths between 930 and 960 nm as a function of the Ca\(^{2+}\) concentration. These wavelengths were selected so as to minimize contribution from the RC that has absorption over 750–950 nm. Assuming that one LH1 subunit of the B880 species binds to one Ca\(^{2+}\) to form one subunit of the B915 species, the absorption changes \(\Delta A\) observed can be expressed by Equation 2 (see supplemental data for details),

\[
\Delta A = I \times (\epsilon_{915} - \epsilon_{880}) \times [(1 + C_0K + Kx) - [(1 + C_0K + Kx)^2 - 4C_0Kx]^{0.5}] / 2K \quad \text{(Eq. 2)}
\]

where \(I\) is the light path length, \(\epsilon_{915}\) and \(\epsilon_{880}\) are molar extinction coefficients of the LH1 subunit in the B915 and B880 forms, respectively, \(C_0\) is the initial concentration of subunit of the B880 species, \(K\) is the Ca\(^{2+}\) binding constant, and \(x\) is the concentration of Ca\(^{2+}\). By using the nonlinear least-square fitting of \(\Delta A\), the Ca\(^{2+}\) binding constant \(K\) was estimated to be 6.5 (±0.3) \(\times 10^4\) M\(^{-1}\). Fig. 4 shows a good agreement between the calculated result and the spectroscopic data. The value of the binding constant is comparable with those reported for typical EF-hand Ca\(^{2+}\)-binding proteins (32).

The binding properties of Ca\(^{2+}\) to the LH1 complex were further examined by thermodynamic analysis. Fig. 5A shows the ITC profile obtained with titration of Ca\(^{2+}\) to the B880 species. Large exothermic change was observed for each injection, providing independent evidence for the strong Ca\(^{2+}\) binding. A remarkable feature in the thermogram is that each exothermic signal clearly revealed a two-phase change: a rapid return immediately after the injection and a slow recovery, as shown typically in the inset of Fig. 5A. The two exothermic components can be interpreted in terms of a fast Ca\(^{2+}\) binding to the B880 and a slow process of conformational rearrangement within the core complex, respectively. Based on a two-component analysis, the time constants were determined to be 20 and 369 s for the fast and slow processes, respectively. The latter is quite consistent with the rate constant obtained from spectroscopic measurement (Fig. 3). Fig. 5B shows a plot of the integrated heat/mol of injectant (Ca\(^{2+}\)) as a function of the molar ratio of Ca\(^{2+}\)/(B880 subunit). The plot exhibited a sigmoid-like curve with a stoichiometrically equivalent point ~1.0, suggesting that the Ca\(^{2+}\) binding phenomenon can be described by a simple one-site model. From the model analysis, several thermodynamic parameters were obtained: the number of binding site/subunit \(n = 0.97\), the binding constant \(K = 9.6 \times 10^4\) M\(^{-1}\), and enthalpy change \(\Delta H = -4.0\) kcal mol\(^{-1}\). Using these values, the changes of Gibbs free energy \(\Delta G\) and entropy \(\Delta S\) were evaluated to be \(-6.8\) kcal mol\(^{-1}\) and 9.36 cal mol\(^{-1}\) K\(^{-1}\), respectively, from Equation 3,

\[
\Delta G = \Delta H - T\Delta S = -nRT \ln K \quad \text{(Eq. 3)}
\]

where \(T\) is the absolute temperature, \(n\) is the mole number, and \(R\) is the gas constant, 8.315 J mol\(^{-1}\) K\(^{-1}\). These thermodynamic parameters indicate that the Ca\(^{2+}\)-binding site has a high affinity with a 1:1 stoichiometric ratio and the Ca\(^{2+}\) binding is driven both enthalpically and entropically.
Structural differences in conformation and assembly of the core complexes between the B915 and B880 species were investigated by CD spectroscopy and sucrose density gradient centrifugation. Fig. 6 shows the CD spectra. As reported previously, the CD spectrum of B915 was characterized by small and non-conservative signals in the Qy region (21), a typical feature for the core complexes from purple bacteria (33, 34). Upon removal of Ca2+, the CD signals for the Qy and Qx bands were blue-shifted in accordance with the changes of absorption spectra, whereas other signals from the RC and carotenoids remained unchanged. These results support that Ca2+-dependent spectral changes originate from the change in configuration of the BChl a molecules in the LH1 complex. We also examined the effect of Ca2+ on the secondary structure of the apoproteins of the LH1-RC complex by measuring the CD spectra in the far UV region (Fig. 6, inset). Spectra for both the B915 and B880 revealed almost the same shape and can be characterized by highly α-helical structures. This suggests that there was no change in the secondary structures of the polypeptides.Sucrose density gradient ultracentrifugation exhibited a single band for both B915 and B880 at identical positions (supplemental Fig. S3), indicating that the B880 is not a decomposed form of B915 and the whole assembly of the core complex remains unchanged.

All results described above were obtained using the purified LH1-RC complex. In Fig. 7, we show that similar phenomena can be observed for Tch. tepidum chromatophores. The chromatophore membrane contains a large amount of LH2 complex, as revealed by the intensive absorption maxima at 800 and 850 nm, and a small amount of LH1 complex with the Qy transition at 915 nm. In the presence of CaCl2, there was no change in the absorption spectrum of the chromatophore. However, upon the addition of EDTA or MgCl2, the LH1 915-nm band was shifted toward 880 nm and merged with the LH2 absorptions. The result implied that the Ca2+-binding site of the LH1 complex exists in a hydrophilic part or at the membrane interface where metal cations and EDTA are readily accessible.

**DISCUSSION**

The present study demonstrated that the LH1 Qy transition at 915 nm of the core complex from *Tch. tepidum* is induced by the binding of Ca2+ to the LH1 complex. Removal of the Ca2+ resulted in a blue shift of the LH1 Qy transition to 876 nm, the wavelength similar to those of other mesophilic purple bacterial. The B915 native form can be recovered from the B880 by the addition of Ca2+. The result indicates that Ca2+ is an indispensable cofactor for retaining the native structure of the *Tch. tepidum* LH1 complex. The Ca2+ binding property of the *Tch. tepidum* LH1 complex is considered to be closely related to the living environment of this organism. Mammoth Hot Springs in Yellowstone National Park, from where the bacterium was collected (1), is known to contain rich mineral calcium carbonate deposited over millions of years to form thick layers of sedimentary limestone. This bacterium cannot grow in a Ca2+-depleted culture (data not shown).

Through the spectroscopic and ITC analyses, binding of the Ca2+ to the LH1 αβ-subunit was found to occur in a one-to-one ratio with a binding constant of ~10^6 order of magnitude. The high binding affinity is comparable with that for the well known EF-hand Ca2+-binding proteins (32, 35). Generally, Ca2+ is classified as a hard acid and prefers to interact with a hard base. In the proteins with an EF-hand Ca2+-binding site, the ligands are mainly composed of oxygen atoms from acidic amino acid residues (Asp and Glu) as well as those from main chain carbonyl groups and water molecules (35). Inspection of the primary sequences of the *Tch. tepidum* LH1 polypeptides revealed that three Asp in the α-polypeptide are located in the C-terminal region and are close to the BChl a-coordinating His residue (Fig. 8) (6, 7). Three similar residues (two Glu and one Asp) are found in the C terminus of the LH1 α-polypeptide from the mesophilic purple bacterium *Allochromatium (Ach.) vinosum*. However, a deletion exists in this region of the *Tch. tepidum* LH1 α-polypeptide that is thought to be essential for the formation of a Ca2+-binding site and the specific interaction between the polypeptide and pigment molecule. Positions of the deletion and the three Asp residues are estimated to be on.
Ca\textsuperscript{2+} Binding to the LH1 complex of Tch. tepidum

| LH1-α | Tch. tepidum | MPTMNALYK1WILDPPRULVSIAFQQLVGLLH2TVLVL-STD2LMLDDNP3FYQALGKK |
|-------|--------------|---------------------------------------------------------------|
|       | Ach. vinousum | MSPDLWKL1VILVPRIL1AFAVFLTVGLAIH31LSTA5FMNLEDQVPA            |

| LH1-β | Tch. tepidum | AFXKLGLTDLDDAKFHA1PMQGGMGSMVLVIAH4LMMQRPWLL |
|-------|--------------|------------------------------------------------|
|       | Ach. vinousum | NNSYTQLTQAE7HPQGMAFQGLVTVIAH5LMLQRPWLL |

FIGURE 8. Comparison of the amino acid sequences of LH1 α- and β-polypeptides from Tch. tepidum and Ach. vinousum (6, 7). The sequences are aligned relative to the BChl α-coordinating histidine residues (shadow fonts). Acidic amino acid residues in the C termini of α-polypeptides and N termini of β-polypeptides are indicated by bold fonts, and a deletion in the Tch. tepidum α-polypeptide is indicated by an arrow. Underlined regions represent hydrophobic membrane-spanning domains predicted by SOSUI.

the membrane interface or in the hydrophilic part of the α-polypeptide. Similarly, several neighboring acidic residues are found in the N-terminal domain of the β-polypeptide, which might also serve as a potential bonding site. Possibilities for other residues around the BChl α that are capable of hydrogen bonding (27, 29) and/or Ca\textsuperscript{2+} binding need to be further investigated.

Despite the high affinity between the Tch. tepidum LH1 complex and Ca\textsuperscript{2+}, the structural changes induced by the Ca\textsuperscript{2+} binding occurred slowly as revealed from the spectroscopic and the ITC measurements. Therefore, the conformational change from B880 to B915 is the rate-limiting step under the experimental conditions. Previous study showed that the Tch. tepidum LH1-RC complex was purified in a monomeric form, with the LH1 ring composed of 16 αβ-subunits (21). In the present work, the Ca\textsuperscript{2+} binding to the LH1 complex was estimated to occur in a stoichiometric ratio of Ca\textsuperscript{2+}/αβ-subunit = 1:1. This means that the B915 form may not be completed from the B880 form until all the 16 binding sites are occupied by the Ca\textsuperscript{2+} ions, because the Q\textsubscript{y} transition is a consequence of cooperative interaction between the exciton states formed by the 32 BChl α molecules. In the case where there are less Ca\textsuperscript{2+} ions than LH1-αβ pairs, the original state of the exciton interaction in the LH1 ring will be altered and become heterogeneous. As a result, a broadening in the Q\textsubscript{y} transition could be expected. Such broadening was actually observed at low Ca\textsuperscript{2+} concentrations up to 3.2 mM (Fig. 2 and supplemental Fig. 54). At higher Ca\textsuperscript{2+} concentrations than 3.2 mM, the width at half maximum decreased rapidly with increasing Ca\textsuperscript{2+} concentration. Taking into account the total molecular weight of ~330,000 for the core complex, the slow structural change may be interpreted in terms of the large size of the membrane proteins associated with a considerable amount of the surrounded detergent molecules. Other factors include the relatively long time required for the fine-tuning of the 16 Ca\textsuperscript{2+}-bound αβ-subunits within the LH1 complex to form a stabilized conformation that can also contribute to the thermal stability of the Tch. tepidum.

Generally, metal binding to proteins is known to be driven entropically because the dehydration enthalpy of divalent cations is highly endothermic. However, overall enthalpy could become exothermic if the metal binding is thermodynamically coupled to the conformational change that eventually could result in a thermal stabilization of the protein (36). In calmodulins from bovine brain (37–39) and wheat germ (40), the Ca\textsuperscript{2+} binding reactions were reported to be driven solely by a large favorable entropy change despite unfavorable enthalpy change. In contrast, for the troponin C (41–43) and parvalbumins (32, 44, 45) the Ca\textsuperscript{2+} binding reactions were mostly favorable in both entropy and enthalpy except for several specific sites where endothermic- and entropy-driven reactions occur in a similar way to the calmodulins (42–44). For the Tch. tepidum LH1-RC complex, the Ca\textsuperscript{2+} binding is driven both entropically and enthalpically, similar to the cases of troponin C and parvalbumins. Therefore, the large exothermic changes of the Tch. tepidum core complex strongly support that the Ca\textsuperscript{2+} binding to the LH1 complex caused a conformational change that largely stabilized the structure of the LH1-RC complex.

The Ca\textsuperscript{2+} binding in calmodulins was reported to induce a conformational transition of random coils to α-helical structures based on the results of CD measurement (46–48). For the Tch. tepidum core complex, the far UV-CD spectra did not show any change in the Ca\textsuperscript{2+} depletion. This means that conformational change of the LH1 complex may occur mainly in the tertiary structure or the change in the secondary structure, if there is any, may be too small to be detected. Similar results were reported for troponin C from rabbit skeletal muscle (41). In EF-hand proteins (35), the Ca\textsuperscript{2+}-binding sites are located in the loop of a helix-loop-helix motif and large conformational changes are accompanied with the binding of Ca\textsuperscript{2+}. In contrast, the putative Ca\textsuperscript{2+}-binding sites in the LH1 complex are located at the terminal regions of the polypeptides. Furthermore, the number of acidic residues involved in the binding site and their arrangement seem to be different from those of EF-hand Ca\textsuperscript{2+}-binding proteins (32). As a result, the slight structural change in LH1 can only be detected by its Q\textsubscript{y} transition that is extremely sensitive to the conformational change of the BChl α molecules. The changes in LH1 Q\textsubscript{y} transition for different alkaline earth cations appeared to be partly related to the ionic radius, as the cations with larger (Sr\textsuperscript{2+}, Ba\textsuperscript{2+}) and smaller (Mg\textsuperscript{2+}) ionic radii than that of Ca\textsuperscript{2+} showed the cation-dependent Q\textsubscript{y} bands between 885 and 889 nm. However, substitution of Cd\textsuperscript{2+} that has an ionic radius almost the same as that of Ca\textsuperscript{2+} also induced a red shift to 887 nm. The results indicate that the Ca\textsuperscript{2+}-binding site in the LH1 complex is highly selective and can distinguish the cations not only by the ionic radius but also by other properties such as binding mode and coordination number (49) as well as the hard-soft nature. In this regard, the complex mechanism of molecular recognition of the Tch. tepidum LH1 is expected to be explored by detailed structural information, and the effort toward determining a high-resolution structure of the core complex is in progress (21).

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