

Electronic Properties and Thermal Stability of Soluble Redox Proteins from a Thermophilic Purple Sulfur Photosynthetic Bacterium, *Thermochromatium tepidum*

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The electronic properties of soluble redox proteins from a thermophilic purple sulfur photosynthetic bacterium *Thermochromatium* (*T.*) *tepidum* have been studied in terms of electronic absorption, magnetic CD, midpoint redox potential, and electron-transfer behavior. *T. tepidum* possesses one high potential iron–sulfur protein (HiPIP), one cytochrome *c*', and two low potential cytochrome *c*-552's. The two low potential cytochrome *c*-552's can be separated further into a higher and a lower potential species. The HiPIP has a midpoint redox potential of +340 mV at pH 7. From electron-transfer ability shown by flash experiments, the HiPIP was confirmed to be the possible electron-transfer protein to the reaction center under physiological conditions. The HiPIP is thermally stable up to 60 °C, which is much higher than that of the mesophilic photosynthetic bacterium, *Allochromatium* (*A.*) *vinosum*. Cytochrome *c*' has a high-spin protoheme with a redox midpoint potential of +100 mV at pH 7, similar to those of many other photosynthetic bacteria. The molecular weight was determined to be 14147 Da by MALDI-TOF/MS experiments. The higher potential cytochrome *c*-552's have hemes *c*'s with midpoint redox potentials of −15 and +85 mV, and the lower one of −15 mV. The molecular weights for the higher potential cytochrome *c*-552 and the lower potential *c*-552 were found to be 20702 and 47757 Da, respectively. The higher potential cytochrome *c*-552 has an attached flavoprotein with a molecular mass of 42000 Da. The lower potential cytochrome *c*-552 has been found for the first time in *T. tepidum*.

Light energy is transduced into proton-motive force across intracytoplasmic membranes, which is the universal energy source conserved in living cells. Key steps of the energy transduction in photosynthetic bacteria are cyclic electron transfers through photosynthetic reaction centers (RC's) and cytochrome bc_1 complexes mediated by quinone molecules in the membrane and other soluble electron-transfer proteins out of the membrane (i.e., in the intraperiplasmic space), respectively. Quinone molecules reduced in the RC are dislocated to quinone pools and the reduced quinones (quinoles) transfer electrons to cytochrome bc_1 complexes, which in turn reduce the oxidized soluble electron-transfer proteins. The photo-oxidized special pair in the RC obtains electrons from the reduced soluble electron-transfer proteins directly or through tightly bound cytochrome *c*'s. Many purple bacteria (for example, *Rhodobacter sphaeroides* and *Blastochloris viridis*) use cytochrome c_2 as an electron-transfer protein for photosynthetic growth.[1–4] On the other hand, a number of purple bacteria (for example, *Allochromatium* (*A.*) *vinosum* and *Rubrivivax* (*R.*) *gelatinosus*) possess high potential iron–sulfur proteins (HiPIP) as electron-transfer proteins.[5–7] In some photosynthetic bacteria, photo-induced electron transfers between HiPIPs and tightly bound cytochrome *c* subunits in the RC's have been directly observed.[5–11]

Thermochromatium (*T.*) *tepidum* is a thermophilic purple sulfur photosynthetic bacterium that can survive at temperatures up to 58 °C, which is the highest out of all purple photosynthetic bacteria.[12,13] The RC of *T. tepidum* has been isolated and contains L, M, H, and cytochrome *c* subunits.[14] The electron-transfer reaction from the bound cytochrome *c* subunit to the photo-oxidized special pair, that is, the reduction of the photo-oxidized special pair by hemes in cytochrome *c*, has been studied.[15] The three-dimensional structures of the RC and the HiPIP from *T. tepidum* have been solved by X-ray crystallography.[16–20] The surface structures of them indicate that the HiPIP could interact with the most distal heme of the cytochrome subunit by hydrophobic interactions, which suggest that the HiPIP is a possible candidate for the electron-transfer protein to the RC in *T. tepidum*.[18] In this paper, we report the results of isolation and characterization of all of the soluble redox proteins from *T. tepidum* in terms of the physicochemical properties, especially electronic states, redox potentials,

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electron-transfer behavior, and thermal stability, to discuss their feasibilities as electron carrier proteins to the reaction center at temperatures as high as 58 °C.

Experimental

Purification of Soluble Redox Proteins. *T. tepidum* was grown photosynthetically as previously described.[12,13] Soluble redox proteins were isolated by the method of Bartsch with some modifications as described below.[21,22] Harvested cells were suspended in a Tris-HCl buffer (pH 8.0) and sonicated at 70 W for 20 min at 0 °C. Cell debris was removed by centrifugation at 20000 × g for 15 min. The supernatant with soluble proteins was separated from membrane fractions by ultracentrifugation at 100000 × g for 90 min. The supernatant was charged on a column of 2-(diethylamino)ethyl(:DEAE)-cellulose (DEAE-TOYOPEARL 650S, TOSOH) equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The redox proteins were eluted by a linear gradient of NaCl concentrations between 0 and 250 mM. Further purification of separated redox proteins was achieved by gel filtration chromatography (Sephadex G100, Pharmacia) equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl.

Molecular Weight Measurement. Molecular weights of the purified redox proteins were analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) on 12.5% acrylamide gels and determined by the matrix-assisted laser desorption time of flight mass spectroscopy (MALDI-TOF/MS),[14,23] with a REFLEX III mass spectrometer (Bruker Daltonics). The matrix used was α-cyano-4-hydroxycinnamic acid, and its saturated solution was mixed with a sample of the same volume.[23]

Spectroscopic Measurements. Absorption spectra were measured with a Beckman UD-640 spectrometer at room temperature. Magnetic circular dichroism spectra were measured with a Jasco J-720W spectrometer equipped with an electromagnet at 1.5 T.[24,25] Oxidized and reduced forms of proteins were prepared by the addition of potassium hexacyanoferrate(III), and sodium ascorbate or sodium dithionite, respectively.

Redox Titration of Prosthetic Groups of Proteins. Redox titrations of soluble proteins were carried out in a 1 mm cuvette covered with a septum by the method of Dutton.[26–28] The redox potential was measured by a potentiostat (Hokutodenko HAB-151) with Ag/AgCl as the reference electrode and platinum as working and counter electrodes.[29] The mediators used were 50 μM *p*-benzoquinone, diaminodurol, and 1,4-naphthoquinone and 1 mM potassium hexacyanoferrate(III) for high potential conditions, and 50 μM *p*-benzoquinone, diaminodurol, 1,4-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone for low potential conditions. Under low potential conditions, oxygen molecules in the solution were removed by the reaction of glucose, glucose oxidase, and catalase. The solution was titrated with potassium hexacyanoferrate(III) for oxidation and with sodium ascorbate or sodium dithionite for reduction.

Electron-Transfer Measurements between Soluble Proteins and the RC's. The electron-transfer reactions between soluble redox proteins and the RC's have been observed by a home-made apparatus. The samples were excited at 890 nm with a titanium-sapphire laser pumped by a Nd-YAG laser with a 1 ns duration at ~5 mJ (Lotis). A Xenon flash lamp with a 35 μs half width was used as the observation pulse source. The time control of excited and probe beams was carried out with a digital delay generator (Hamamatsu Photonics DG535). The probe beam was detected by a high sensitive multichannel detector with an image intensifier and a MOS linear image sensor (Hamamatsu Photonics C4560). The electron-transfer reaction was monitored by the α-band absorbance of the hemes in the RC at 556 nm, which is a measure of the redox states of the hemes. Kinetic measurements were performed at room temperature. The time constants of the electron-transfer reactions were estimated by fitting with exponential functions.

Thermal Stability Measurements. The thermal stability of HiPIPs from *T. tepidum* and *A. vinosum* were measured with electronic absorption spectra. Thus, the sample solutions were maintained at respective temperatures and time scan absorption spectral measurements were conducted for them. The results were plotted in the form of a first-order reaction, thus $\ln D_t/D_0$ vs t, where D_t and D_0 represent the absorbance at $t = t$ and $t = 0$. The slope of the plots gave the reaction rate at respective temperatures. Thermodynamic parameters for the denaturation reactions were derived from them using Arrhenius plots with standard methods.[30]

Results

Purification and Characterization of the Soluble Redox Proteins. Four colored soluble proteins were eluted at 10, 65, 140, and 200 mM NaCl concentrations with DEAE-anion exchange chromatography. In the preparation of soluble redox proteins from *A. vinosum* by DEAE-anion exchange chromatography, it was reported that the HiPIP, cytochrome *c*-551 (alternatively called cytochrome c_8), cytochrome *cc*', and cytochrome *c*-552 were eluted at 20–40, 60–80, 80–100, and 140–180 mM NaCl concentrations, respectively.[21,22] The proteins from *T. tepidum* were characterized by absorption spectra. The absorption spectra of the four colored soluble proteins in their reduced and oxidized forms (Fig. 1) show that the proteins eluted with 10, 65, 140, and 200 mM NaCl possess an Fe–S center, protoheme, heme *c*, and heme *c* as their prosthetic groups, respectively.[21,22,31–36] It should be noted that no cytochrome *c*-551 exist in *T. tepidum*. Instead, it has one more cytochorome *c*-552, when compared with *A. vinosum*. The redox spectral changes of the protein with an Fe–S center agree well with those of HiPIP in the previous report.[21,31,32] The redox spectral changes of the protein containing protoheme are very similar to those of cytochrome *c*' from purple bacteria.[32–36] The peak positions of the α band of both proteins with cytochromes *c* are observed at 552 nm. While the cytochrome *c*-552 fractions eluted at 140 mM NaCl can be partially reduced by the addition of sodium ascorbate, the cytochrome *c*-552 fractions eluted at 200 mM NaCl concentration cannot. The results show that the two cytochrome *c*-552's have different midpoint redox potentials. They are referred to as the higher and lower potential cytochrome *c*-552's among the low potential ones throughout this paper.

Figure 2 shows the magnetic circular dichroism (MCD) spectra of the soluble cytochromes in the visible region. The couplet type MCD signals around 550 and 400 nm are assigned to the α and γ bands of the heme chromophore. The spectral profiles, especially the shape, magnitude, and the intensity ratio of the MCD from the α to γ band, reflect the electronic state of hemes (e.g., whether it is in the iron(III) or iron(II) state, and in the high-spin or low-spin state).[37] The intensity ratio for cytochrome *c*' are about 3 and 12 at oxidized and reduced states, respectively, which indicates that cytochrome *c*' has a typical high-spin heme.[37] The ratios for the higher

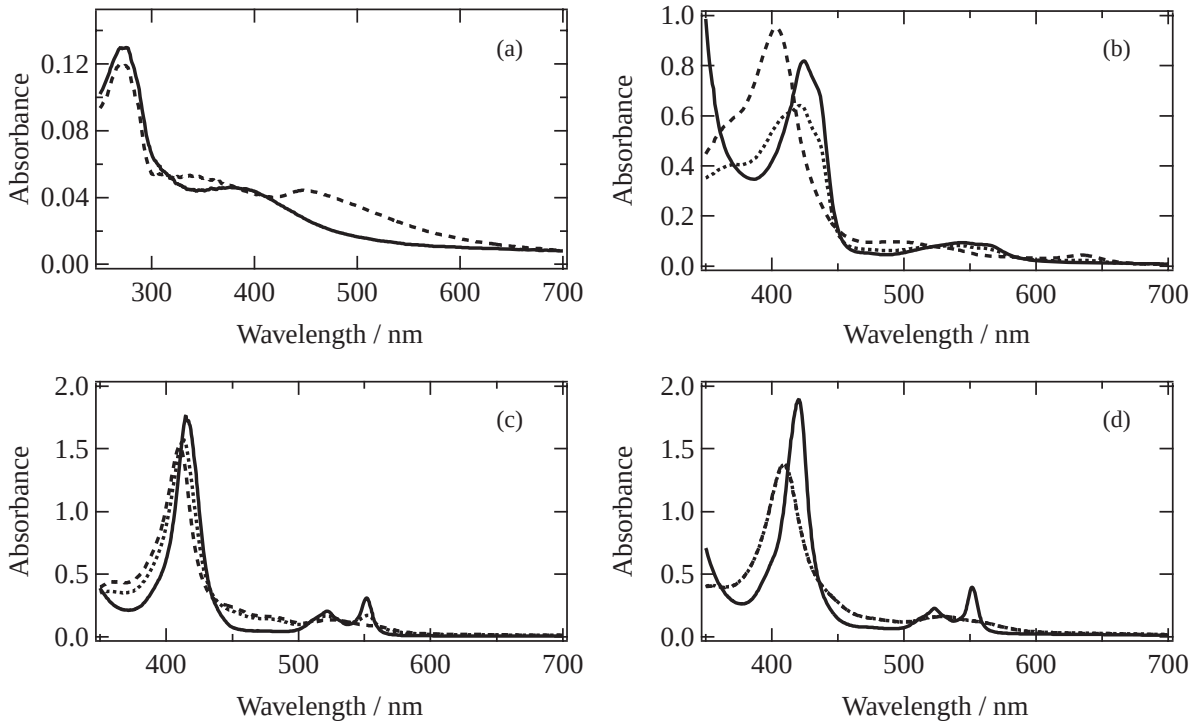

Fig. 1. Absorption spectra of HiPIP (a), cytochrome *c*' (b), higher potential cytochrome *c*-552 (c), and lower potential cytochrome *c*-552 (d) from *T. tepidum* at oxidized (broken line) and reduced (dotted line and solid line) state. Soluble proteins were solved in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl in the absence of redox reagents (oxidized state) and in the presence of sodium ascorbate (dotted line) or sodium dithionite (solid line) (reduced state).

and lower potential cytochrome *c*-552 are 8.5 and 14, and 0.25 and 0.06 at oxidized and reduced states, respectively. This result indicates that the higher potential cytochromes *c*-552 contain typical low-spin hemes. However, the lower potential cytochrome *c*-552 could not be explained by a typical iron(II) low-spin state profile in the γ band for a monomeric heme. As discussed later, molecular weight and the titration profile imply the presence of more than one heme. The non-typical MCD as a monomeric heme in the γ band may be interpreted as the combination of more than one low-spin heme.

Figure 3 shows the redox titration curves of four purified soluble redox proteins in the form of the Nernst plot. The midpoint redox potentials and the *n* values at pH 7.0 are determined to be +340 mV (vs normal hydrogen electrode: NHE) and 1.1 for the HiPIP, +100 mV (vs NHE) and 0.8 for the cytochrome *c*', −15 and +85 mV (vs NHE) and 0.9 at the ratio of 3:1 for the higher potential cytochrome *c*-552, and −15 mV (vs NHE) and 1.55 for the lower potential cytochrome *c*-552, respectively.

The molecular masses of the soluble redox proteins estimated by SDS-PAGE are 12, 15, 20, and 45 kDa, respectively, for the HiPIP, cytochrome *c*', the higher potential cytochrome *c*-552, and the lower potential cytochrome *c*-552 (Fig. 4). Precise mass values of them were determined by MALDI-TOF/MS to be 8786, 14147, 20702, and 47757 Da, respectively. The molecular weight of the HiPIP agrees well with that estimated from the primary structure.[38]

Figure 5 shows the plot of the absorbance change vs time for the reduced cytochrome *c* in the RC at 556 nm to detect the electron-transfer reaction in the presence of the RC and the HiPIP. We can see that the bleached absorbance of the RC heme was recovered when the HiPIP was present. The exponential simulation gave a time constant of 52 ms for the absorbance change.

Figure 6 shows the absorption changes of the HiPIP plotted in first-order reaction kinetics at respective temperatures. Thus, the time dependent relative absorption changes at respective temperatures were plotted in log scale vs time. It was disclosed that the HiPIP from *T. tepidum* was stable up to 60 °C (data not shown), which is as high as the temperature for growth. This is compared with the thermal stability of HiPIP from *A. vinosum*, which becomes denatured at 50 °C (Fig. 6).

Discussion

HiPIP. As is apparent from the absorption spectra in Fig. 1, the protein eluted at 10 mM is an iron–sulfur protein. Together with the value of the midpoint redox potential this protein was determined to be a HiPIP. In fact, the HiPIP was crystallized, and its three-dimensional structure has been determined up to 0.8 Å resolution.[17,18,20] Comparison of the three-dimensional structure of HiPIPs from *T. tepidum* and *A. vinosum* reveals that they are very similar to each other, but there exist some distinct differences between them. These include the loop region between Asn52 and Asp57, which results in one less net overall charge.[20]

It has been determined that the redox potential for the bound cytochrome *c* of *T. tepidum* is 380 mV for the higher potential ones. Though the precise values for the redox potential of cytochrome c_1 of cytochrome bc_1 in *T. tepidum* has not been determined, it is anticipated to be around 280 mV in *T. tepidum*

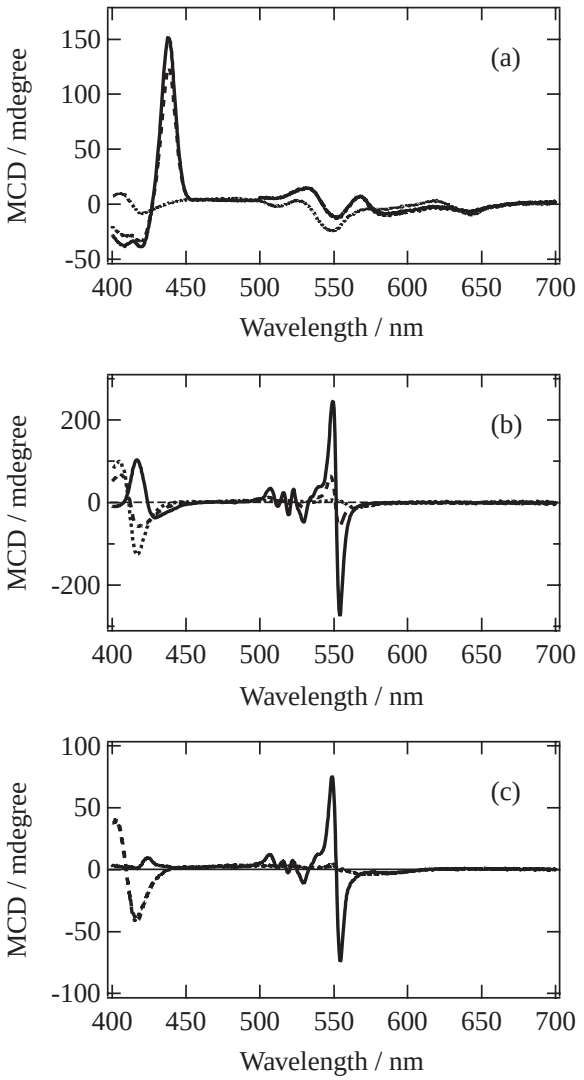

Fig. 2. Magnetic circular dichroism spectra of cytochrome *c*' (a), higher potential cytochrome *c*-552 (b), and lower potential cytochrome *c*-552 (c) from *T. tepidum* in the absence of redox reagent (broken line) and in the presence of sodium ascorbate (dotted line) and sodium dithionite (solid line) in 20 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl.

as in many other purple bacteria.[1,2,39] Therefore, the HiPIP is possibly reduced by cytochrome c_1 and can reduce the heme *c* in a bound cytochrome in the RC of *T. tepidum*. This is in contrast to the fact that it may be difficult for cytochrome *c*' and cytochrome *c*-552's to be reduced by cytochrome c_1 of cytochrome bc_1 complex, judging from the redox potentials mentioned above.

Cytochrome *c*'. It has been found that purple bacteria contain cytochrome *c*' in their periplasmic space composed of two identical subunits.[2,21,33–36] Cytochrome *c*'s have a molecular weight of around 14 kDa in the monomeric state and the midpoint redox potential values of the hemes exist between −10 and +100 mV (vs NHE),[2,4,21,33–36] in which the value of cytochrome *c*' from *T. tepidum* exists. The functional role of cytochrome *c*' remains rather elusive. Studies on *R. gelatinosus* showed that cytochrome *c*' could reduce the photo-oxidized special pair in the mutants RC with tetraheme cytochrome depleted. This fact suggests that cytochrome *c*' acts as an electron donor to the special pair when the cytochrome subunit of the RC has been lost from the RC.[40]

The Higher Potential Cytochrome *c*-552 of the Low Potential Ones. The higher potential cytochrome *c*-552 has heme proteins with a molecular mass of 20702 Da. The absorption spectra in the reduced and oxidized state for the higher potential cytochrome *c*-552 eluted from a DEAE chromatogram showed the presence of a flavin prosthetic group.[41–43] SDS-PAGE of both 20 and 42 kDa fractions reveal that this cytochrome is combined with a protein with a molecular mass of about 42 kDa.[21,41–45] Therefore, this cytochrome does work as a flavocytochrome. A similar flavocytochrome was found in *A. vinosum* and its function is considered to be to metabolize the sulfide anion.[2,21,46] As the flavocytochrome from *A. vinosum* has two hemes with identical redox potentials, we simulated the titration data of the flavocytochrome from *T. tepidum* with two different redox potentials and the molecular absorption coefficient ratio.[44,45,47,48] The best fit of redox potential of the diheme of flavocytochrome of *T. tepidum* exists at −15 and 85 mV (vs NHE) at the ratio of 3:1. This showed that the environments of the hemes of flavocytochrome from *T. tepidum* are different from each other.

The Lower Potential Cytochrome *c*-552 of the Low Potential Ones. The lower potential cytochrome *c*-552 of the low potential cytochrome *c*-552's from *T. tepidum* has been found for the first time and the redox potential was −15 mV (vs NHE), which would be too low to get electrons from cytochrome bc_1. Therefore, it may not be used in the cyclic electron-transport chain. The molecular mass of 47757 Da implies that this cytochrome may have more than one heme. Actually, the oxidation–reduction titration curve could be fitted well by the Nernst equation with $n = 1.55$ (Fig. 3). The abnormal Soret MCD also suggests the presence of more than one heme as discussed above.

Low potential cytochromes with a redox potential of around 0 mV (vs NHE) were isolated from *R. gelatinosus* and called the low potential cytochrome c_8 (formerly low potential cytochrome *c*-551), whose redox midpoint potential is about +50 mV (vs NHE).[8,40,49]

Possible Electron Carrier Proteins. Figure 5 shows that the HiPIP from *T. tepidum* can actually transfer electrons to the oxidized cytochrome tightly bound in the RC. No other soluble proteins have been found to have a suitable reduction potential to function as the electron-transfer carrier for cytochrome bc_1 to the RC with cytochrome. Therefore, the HiPIP is not only a possible candidate, it is the only candidate for this electron-transfer protein.

Cytochrome c_2 and the HiPIP have been reported to be electron-transfer proteins in purple photosynthetic bacteria. High potential cytochromes c_8 (formerly high potential cytochromes *c*-551) have been shown to be electron-transfer proteins on some purple bacteria, *Rhodocyclus tenuis*, *Rhodoferax fermentans*, *R. gelatinosus*, and *A. vinosum*.[5–11,49–51] The midpoint potential of the high potential cytochromes c_8 is +405 mV (vs NHE) for *R. tenuis*, +285 mV (vs NHE) for *R. fermentans*, +330 mV (vs NHE) for *R. gelatinosus*, and +240 mV (vs NHE) for *A. vinosum*, respectively.[5–11,49–51] The redox poten-

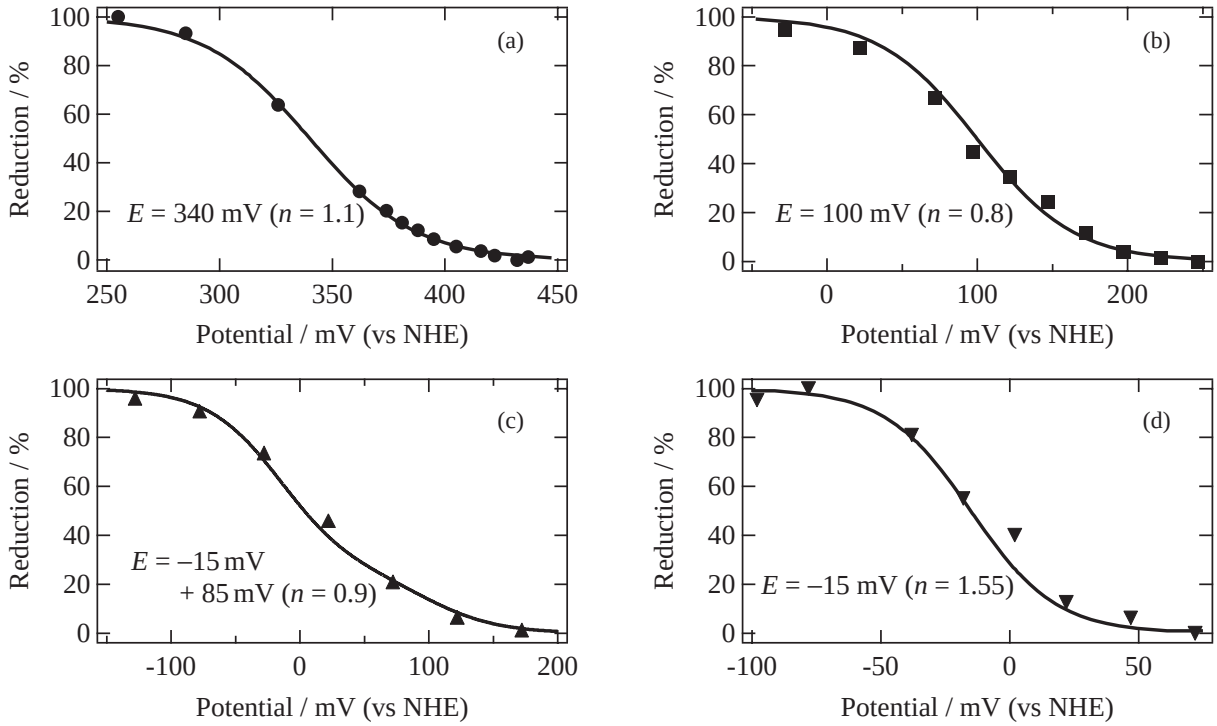

Fig. 3. Redox titration curve of HiPIP (a), cytochrome *c*' (b), higher potential cytochrome *c*-552 (c), and lower potential cytochrome *c*-552 (d) from *T. tepidum*. The solid lines represent the best fit of the data by Nernst equation. Mediators are used 50 μM *p*-benzoquinone, diaminodurol, and 1,4-naphthoquinone and 1 mM potassium hexacyanoferrate(III) at high potential condition and 50 μM *p*-benzoquinone, diaminodurol, 1,4-naphthoquinone, and 2-hydroxy-1,4-naphthoquinone at low potential condition.

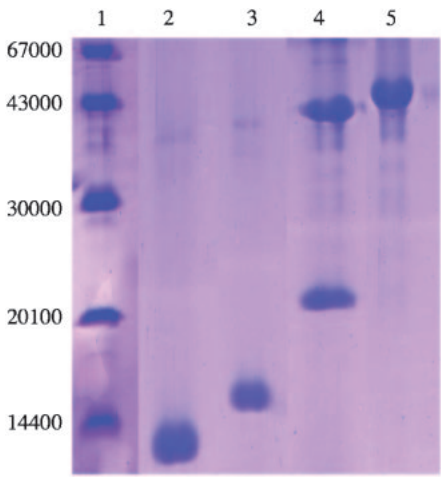

Fig. 4. Photograph of SDS-PAGE at 12.5% acrylamide gel. Lane 1 is molecular weight marker, Lane 2, 3, 4, and 5 is HiPIP, cytochrome *c*', higher potential cytochrome *c*-552, and lower potential cytochrome *c*-552 from *T. tepidum*, respectively.

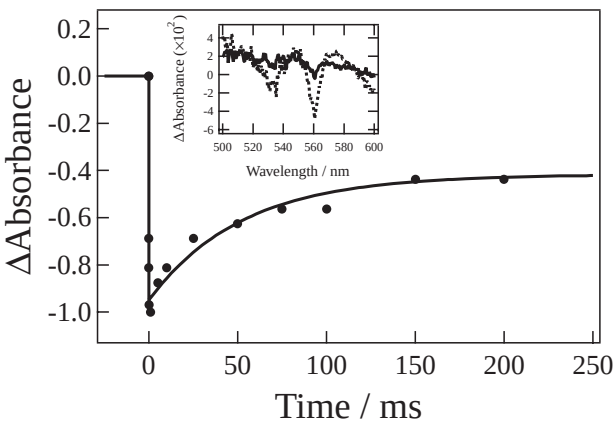

Fig. 5. Kinetics of light-induced absorbance change at 556 nm in the presence of HiPIP. The concentrations of RCs and HiPIP were adjusted at 5 μM, respectively, and suspended in 15 mM phosphate buffer (pH 7.0) containing 0.8% octyl-β-D-glucoside and 100 mM potassium chloride. Solution potentials were adjusted at 280 mV (vs normal hydrogen electrode: NHE) by addition of sodium ascorbate in the presence of 50 μM diaminodurol and 1,4-naphthoquinone as mediators. Insert: light-induced difference absorption spectra without (dash line) and with (solid line) HiPIP after 100 ms.

tial of the higher potential cytochrome *c*-552's from *T. tepidum* (+20 mV) is much lower than that from other purple bacteria. Thus, there might be no possibility for this cytochrome to take the role of electron carrier.

Thermal Stability. We have measured the thermal stability of the HiPIP. The data shown in Fig. 6 show that the HiPIP from *T. tepidum* is stable enough at the maximum cultural temperature of 58 °C for *T. tepidum*. As discussed above, it is inferred that the HiPIP is the only possible candidate for the electron-transfer protein to the tightly bound cytochrome *c* in

Table 1. Thermodynamic Parameters for Thermal Denaturation at 60 °C of HiPIPs from *T. tepidum* and *A. vinosum*

	$\Delta E_a^{\ddagger}$ /kJ mol^{-1}	$\Delta H^{\ddagger}$ /kJ mol^{-1}	$T\Delta S^{\ddagger}$ /kJ mol^{-1}	$\Delta G^{\ddagger}$ /kJ mol^{-1}	$\Delta\Delta G^{\ddagger}$ /kJ mol^{-1}
HiPIP (*T. tepidum*)	365.6	362.8	240.4	122.4	18.6
HiPIP (*A. vinosum*)	156.4	153.6	49.82	103.8	

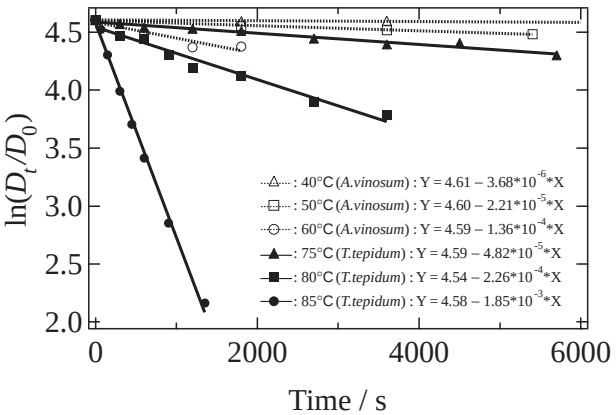

Fig. 6. Time course of the denaturation in the first-order reaction plot of the HiPIPs from *T. tepidum* (solid line) and *A. vinosum* (dash line) at respective temperatures shown in the figure. The denaturations of both HiPIPs were monitored at 390 nm in the transition of the Fe–S center of HiPIP. Both HiPIPs were solved in 20 mM Tris-HCl buffer (pH 8.0) at $OD_{390} = 0.25$. The resulted equations simulated as the first-order reactions were given in the figure.

the RC, the thermal stability data of this protein giving additional support for this argument. As shown in Fig. 6, the spectral changes of HiPIP were well fitted to the first-order reaction plot, which gave the reaction rate of the denaturation reaction. The Arrhenius plot, i.e., the reaction rates vs the inverse temperature, yielded the activation energy for the reaction, from which thermodynamic parameters listed in Table 1 were calculated. The values, especially $\Delta E_a^{\ddagger}$ and $\Delta G^{\ddagger}$, confirmed a much higher thermal stability for *T. tepidum* in comparison with that for *A. vinosum*. The differences in thermal stability between *T. tepidum* and *A. vinosum* have been discussed to be caused by rather subtle changes in the structures.[20]

Conclusion

Four soluble redox proteins have been isolated from *T. tepidum* and characterized. The midpoint redox potentials as well as electron-transfer experiments suggest that HiPIP is the possible electron carrier protein for the RC. The thermal stability of the protein also supports this conclusion.

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