



Evaluation of the relationship between color-tuning of photosynthetic excitons and thermodynamic stability of light-harvesting chromoproteins

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Abstract

Color-tuning is a critical survival mechanism for photosynthetic organisms. Calcium ions are believed to enhance both spectral tuning and thermostability in obligatory calcium-containing sulfur purple bacteria. This study examined the thermo- and piezo stability of the LH1-RC complexes from two calcium-containing sulfur purple bacteria notable for their extreme red-shifted spectra. The results generally show limited reversibility of both temperature and pressure effects related to the malleability of calcium-binding sites. While the pressure-induced decomposition product closely resembles the calcium-depleted form of the chromoproteins, the thermally induced products reveal monomeric B777 and dimeric B820 forms of bacteriochlorophyll *a*, similar to those seen in non-sulfur purple bacteria treated with detergent. The study further found nearly unison melting of the protein tertiary and secondary structures. Overall, our findings do not support a direct link between color adjustment and thermodynamic stability in light-harvesting chromoproteins.

Keywords: Ca-containing bacteria; circular dichroism; hydrostatic high pressure; LH1-RC complex; purple bacteria.

Introduction

Color-tuning is a basic mechanism for the common thriving of diverse photosynthetic organisms. *Thiorhodovibrio* strain 970 (*Trv.* 970), a photosynthetic sulfur purple bacterium, exhibits the most red-shifted absorption – 973 nm *in vivo* (Permentier *et al.* 2001) – among all the bacteriochlorophyll *a* (BChl) containing bacteria. Responsible for this absorbance are Q_y transitions of multiple BChl molecules cyclically arranged in the light-harvesting 1 (LH1) membrane chromoprotein complex.

The detergent-purified LH1 in association with a reaction center complex named core complex (LH1-RC) absorbs at 960 nm, 35 nm longer as compared to the next most red-shifted LH1-RC complex from *Thermochromatium (Tch.) tepidum*. These two phototrophs are also special because they require Ca²⁺ ions for photosynthetic growth and assembly of core complexes. The ions connect the α -inner and β -outer ring transmembrane α -helices stabilizing the structure of the entire core complex. The LH1-RC structures are available for both *Tch. tepidum* and *Trv.* 970 species, refined to 1.9 Å and 2.82 Å resolution, respectively

Highlights

- Color-tuning of LH excitons does not correlate with chromoprotein stability
- Denaturation by pressure and temperature are qualitatively distinct processes
- *Thiorhodovibrio* strain 970 and *Thermochromatium tepidum* show similar T_m of tertiary and secondary structures

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Abbreviations: BChl – bacteriochlorophyll *a*; Car – carotenoid; CD – circular dichroism; LH1 – light-harvesting complex 1; LH2 – light-harvesting complex 2; P_d – denaturation pressure; RC – reaction center; T_d – denaturation temperature; T_m – melting temperature.

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(Yu *et al.* 2018, Tani *et al.* 2020). Except for the presence of Ca^{2+} , they confirm similar molecular architecture of core complexes that is found in non-sulfur bacterial phototrophs such as *Rhodospirillum (Rps.) rubrum*. In this species, 16 pairs of transmembrane α -polypeptides of LH1 encircle the RC unit and form a closed, slightly elliptical double cylinder, which comprises 32 BChl and 16 carotenoid pigment cofactors.

Upon the Ca^{2+} depletion, the Q_y band of LH1 significantly blue-shifts, while the original spectrum is restored by Ca^{2+} reconstitution (Kimura *et al.* 2008). The complexes deprived of Ca^{2+} also demonstrate reduced thermal stability (Kimura *et al.* 2009, Imanishi *et al.* 2019). On these grounds, attempts have been made to correlate the unusual red shift of the absorption spectra observed in Ca-containing bacteria with their enhanced thermal stability (Kimura *et al.* 2009, Yu *et al.* 2016). This correlation, however, can hardly be universal, because *Tch. tepidum* is classified as a thermophilic organism (*i.e.*, a type of organism that thrives at high temperatures, typically between 45 to 60°C), while *Trv. 970* is a mesophilic organism that grows best at intermediate temperatures between 20 and 40°C.

Besides, one has to consider the different physical origins of the associated phenomena. Whilst the optical properties of chromoproteins are related to the difference between two (ground and excited) electronic states, their thermodynamic stability is a basic ground-state characteristic that refers to the energy difference between the folded and denatured state of the protein in solution (Luke *et al.* 2007, Sancho 2013). Major changes in the secondary, tertiary, and quaternary structures without cleavage of backbone peptide bonds are regarded as denaturation. In contrast, subtle changes in structure, which do not drastically alter the molecular architecture of the protein, are usually viewed as conformational adaptability. Although most folded proteins are only marginally stable, the stability is sufficient to prevent spontaneous unfolding at normal temperatures and pressures.

In photosynthetic chromoproteins, molecular excitons are typically formed when a pigment chromophore absorbs a photon of light. Tuning the bacterial light-harvesting spectra is achieved by a combined effect of the interactions between the transition densities and between each BChl and its surrounding chromoprotein residues (Polyakov *et al.* 2018, Reppert 2023). These conditionally separate tuning contributions are named the exciton shift and site energy shift, respectively. According to Timpmann *et al.* (2021), the key mechanism of the extra spectral red-shift of Q_y excitons in both *Trv. 970* and *Tch. tepidum* is the Ca-facilitated enhancement of exciton couplings between the BChl pigment chromophores. At the same time, a considerable part of the literature reviewed by Kimura *et al.* (2023) seems to favor the site energy shift interpretation.

These inconsistencies met in the previous research prompt the need for a more dedicated investigation into the correlation between the color-tuning of photosynthetic excitons and the thermodynamic stability of light-harvesting chromoproteins. In this study, we conducted

a thorough examination of the thermo- and piezo stability of LH1-RC core complexes from *Trv. 970* and *Tch. tepidum*, covering a temperature range of 20–85°C and pressure range of 0.1–1,200 MPa (100 MPa = 1 kbar). The terms ‘thermostability’ and ‘piezo stability’ used here have traditional (steady-state) meaning. When a protein solution is gradually heated/compressed above a critical temperature/pressure, the proteins undergo sharp transitions from the native state to the denatured state. The temperature/pressure at the transition midpoint, where the concentration ratio of proteins in native and denatured states is one, is known either as the denaturation temperature T_d or denaturation pressure P_d . The used optical spectroscopy techniques, which included absorption, fluorescence, fluorescence excitation, and circular dichroism (CD), were applied across a broad spectral range from far-ultraviolet to near-infrared. The data obtained from Ca-containing sulfur purple bacteria were compared to those of more widely studied non-sulfur purple bacteria such as *Rhodobacter (Rba.) sphaeroides* and *Rps. rubrum*.

Materials and methods

Sample preparation and handling: The purified LH1-RC core complexes from *Trv. 970* and *Tch. tepidum* were prepared as described earlier (Suzuki *et al.* 2007, Imanishi *et al.* 2019, Tani *et al.* 2020). The concentrated samples were stored at -78°C in a deep freezer. Prior to the use, the samples were diluted with 20 mM Tris-HCl, pH 8.0 buffer containing 0.05% of n-dodecyl β -D-maltopyranoside (DDM) detergent to prevent aggregation. In order to keep stable saturated Ca^{2+} concentration, 50–100 mM CaCl_2 was added to the samples. The sample temperature between 20–85°C was normally raised at a rate of 1–2°C per min; the temperature was controlled with the precision of $\pm 0.2^\circ\text{C}$. Measurement times per temperature point were about 2 min. For each experimental run, different samples were used.

Spectroscopic methods: Absorption spectra were measured with Cary 60 UV-Vis spectrophotometer (Agilent, USA) equipped with a temperature-controlled cell holder (Quantum Northwest, USA). Fluorescence spectra were recorded using a 0.3-m focal length spectrograph Shamrock SR-303i and a thermo-electrically cooled CCD camera DV420A-OE (both Andor Technology, UK). The fluorescence spectra were corrected for the spectral sensitivity of the set-up. Circular dichroism spectra were measured using a Chirascan Plus spectrophotometer (Applied Photophysics, UK) and thermostabilized quartz cuvettes (Hellma Analytics, Germany) with 1.0- and 10.0-mm path lengths. The fluorescence excitation spectra were recorded by an Andor Kymera 193i spectrometer (Andor Technology, UK) equipped with a DV420A-OE CCD camera using the Chirascan Plus spectrometer as a tunable excitation light source with a fixed bandwidth set to 4 nm. The spectra were corrected to the excitation power, measured by a Newport 1830 power meter using a 918D-UV-OD3 sensor (both Newport Corporation, USA).

High-pressure techniques: A 0.35-mm thick stainless-steel gasket with a 0.3-mm diameter orifice was used to contain the sample between the anvils of a diamond anvil cell (*D-02, Diacell Products, UK*) (Kangur *et al.* 2008). Pressure applied at an average rate of 10–20 MPa per min was determined optically using a ruby micro bead pressure sensor (*RSA Le Rubis SA, France*) directly mounted into the sample volume. The precision of the pressure measurements was ± 20 MPa. Pressure measurements were limited to 1 GPa due to the solidification of the sample and loss of hydrostaticity of the compression. Several independent measurements were carried out to ensure the reproducibility of the data. Reversibility of the effects was checked by recovery of original spectra upon the release of pressure.

Data analyses: The data were analyzed and fitted using the graphing and data analysis software *Origin 9.0 SR1* (*OriginLab, USA*).

Results and discussion

Temperature-dependent absorption and fluorescence spectra of core complexes: Fig. 1 presents an overview of absorption and fluorescence spectra of original and thermally treated LH1-RC core complexes from *Trv.* 970 and *Tch. tepidum* which were recorded at a standard low temperature of 20°C. The dominant absorption bands in untreated samples peaked at 960 nm in *Trv.* 970 and 915 nm

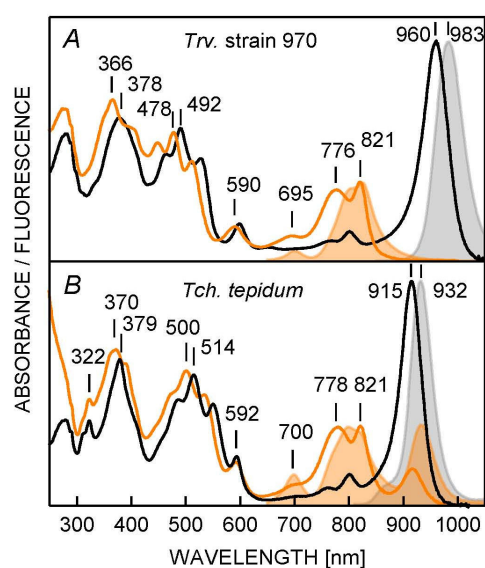


Fig. 1. Overview absorption (*black solid lines*) and fluorescence (*grey shaded shapes*) spectra of isolated LH1-RC core complexes from *Thiorhodovibrio* strain 970 (*A*) and *Thermochromatium tepidum* (*B*) recorded at 20°C. The numbers label the spectral positions of the bands in nanometers. Absorption and fluorescence spectra drawn with *orange lines/shapes* correspond to thermally treated samples recorded at 20°C, *see text* for further explanations. Intensities of the fluorescence spectra excited at 380 nm are normalized according to the highest relevant Q_y absorption peak.

in *Tch. tepidum* are associated with the LH1 component of the core complexes. These lead bands are frequently named Q_y in order to highlight their close relationship with the corresponding singlet electronic transition in individual BChl molecules. In the LH1 complex, the Q_y band represents a manifold of collective excitations (excitons) in the assembly of 32 closely coupled BChls (Polyakov *et al.* 2018). The group of weak bands visible around 730 and 820 nm overlapping with the high-energy tail of the LH1 excitons represents the Q_y transitions of pigment cofactors contained in the RC complex; the band at ~ 760 nm belongs to two bacteriopheophytin *a* (BPheo) molecules, while the band at ~ 800 nm is due to overlapping spectra of two accessory BChls of RC.

Further absorption bands towards shorter wavelengths are also of mixed origin. Nonetheless, they have traditionally been assigned as the main contributor to the band's intensity. So, the Q_x bands visible in both species at around 590 nm and the Soret band at 380 nm are governed by LH1 BChls, as are the carotenoid bands seen in untreated samples at 465, 492, and 527 nm in *Trv.* 970 and at 488, 514, and 550 nm in *Tch. tepidum*. The protein aromatic amino acid residues and secondary-structure peptide bond chromophores apparently dominate in the far-ultraviolet (195–220 nm) part of the chromoprotein absorption spectra. Yet the evaluation of the main donor into the ultraviolet absorption band around 280 nm requires special investigation because most of the pigment chromophores of LH1-RC contribute to this band along with the protein aromatic amino acid residues. The fluorescence spectra of Fig. 1 were obtained using excitation at 380 nm. This light is mainly absorbed by the Soret band of BChls and partly by carotenoid pigments, which pass some of this energy to low-energy exciton states of the LH1 antenna complex. The fluorescence of untreated samples peaks at 983 nm in *Trv.* 970 and at 932 nm in *Tch. tepidum*. The data obtained on untreated samples validate the previous data recently reviewed by Kimura *et al.* (2023).

The spectra obtained after gradual heating of the samples up to 80 (in case of *Trv.* 970) or 85°C (in case of *Tch. tepidum*) and subsequent cooling down to the recording temperature of 20°C are in Fig. 1 drawn by an orange-colored line. In contrast to thermophilic *Tch. tepidum*, where a number of complexes remains intact even after achieving 85°C, the Q_y band in mesophilic *Trv.* 970 almost disappears already at 60°C, *see Fig. 2*. This observation is consistent with the significantly higher thermal denaturation temperature determined by differential scanning calorimetry in the complexes of *Tch. tepidum* (75.0°C) compared to that of *Trv.* 970 (61.5°C) (Kimura *et al.* 2009, Yu *et al.* 2016).

The cooling-down phase is in both samples accompanied by the development of a distinct 821-nm band alongside a broader band peaking at 776 nm (*Trv.* 970) or at 778 nm (*Tch. tepidum*). While the latter structure evidently belongs to monomeric solubilized BChls units called B777, the 821-nm band resembles the spectrum of a detergent-solubilized B820 subunit from non-sulfur purple bacterium *Rps. rubrum* (Chang *et al.*

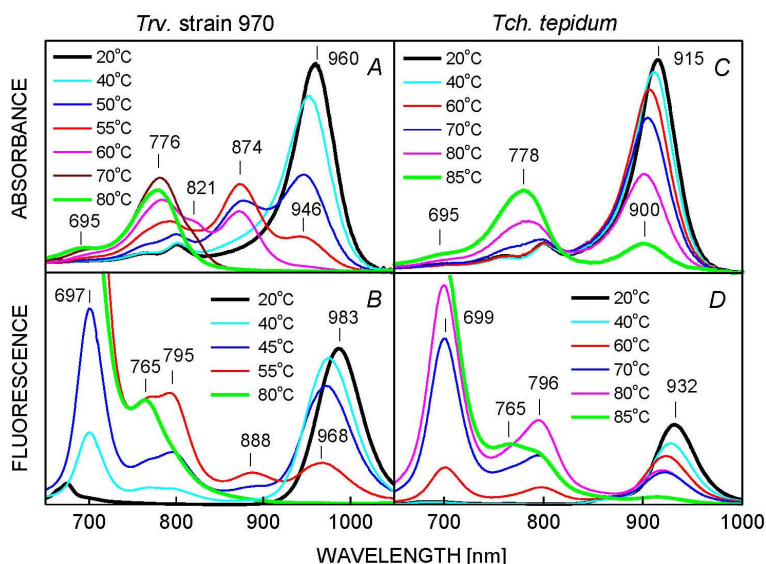


Fig. 2. Temperature-dependent pigment absorption (A,C) and fluorescence (B,D; excitation at 407 nm) spectra of LH1-RC complexes from *Thiorhodovibrio* strain 970 (left panels) and *Thermochromatium tepidum* (right panels). Bold lines correspond to initial and final spectra recorded at 20°C and 80/85°C, respectively.

1990, Pandit *et al.* 2003, Fiedor and Scheer 2005, Michalik *et al.* 2019). The B777 form that consists of BChl bound to either α or β polypeptide chain is typically obtained by further dissociation of B820 using an increased amount of detergent (Parkes-Loach *et al.* 1988). According to resonance Raman spectroscopy, the dissociated B820 form is in many ways different from dimeric apoprotein subunits in native core complexes (Visschers *et al.* 1993). A shift to shorter wavelengths and modification of the Car and Soret bands as a result of thermal treatment is also worth noting.

More detailed dependences of the absorption and emission spectra on temperature can be observed in Fig. 2. In both samples, the main Q_y absorption band continuously blue-shifts with raising the temperature, while its intensity gradually decreases. In *Trv.* 970, the fading Q_y band is replaced by a series of new bands peaking around 874, 821, 776, and 695 nm. Among them, the former two are just transiently visible, while the latter two that persist through the highest applied temperatures appear to belong to the final products of thermal disintegration. According to the literature (Imanishi *et al.* 2019), the transiently generated 874-nm band can be ascribed to the Ca^{2+} -depleted form of the LH1-RC complex (see Figs. 3 and 6 below), while the 776-nm band is most likely related to B777. Distinctly from *Trv.* 970, there is no evidence for the appearance of the transient Ca^{2+} -depleted form of the *Tch. tepidum* complex or the B820 subunit. This means that most of the LH1-RC complexes dissociate directly into monomeric B777 units, which in this case absorb around 778 nm. In *Tch. tepidum* the B820 subunits are thus only formed in the B777 association process following cooling (Fig. 1B).

The fluorescence spectra of Fig. 2B,D excited at 407 nm are well consistent with the respective absorption spectra in Fig. 2A,C at corresponding temperatures. In *Trv.* 970, the rise of temperature from 20°C up to 55°C shifts the Q_y fluorescence band from 983 to 968 nm, while new bands around 888, 795, 765, and 697 nm emerge. The 888-nm fluorescence band is absent from

the spectrum of the *Tch. tepidum* complex and it obviously corresponds to the 874 nm transient absorption of Ca^{2+} -depleted complexes. The 795-nm fluorescence band is visible also in *Tch. tepidum* and can be associated with the absorbance of the B777 BChls at 776 nm. The emission peaking at 697/699 nm most probably originates from oxidized BChls [which by analogy with Smith and Calvin (1966) and Leiger *et al.* (2019) can be identified as the 3-acetyl chlorophyll *a* (3-acChl)], while the 765-nm feature is its vibrational sideband. In the absorption spectra of Fig. 1, the presence of a new spectral feature at around 405 nm related to 3-acChl is also evident.

Evaluation of thermally-induced intermediates by fluorescence excitation spectra: As can be seen from Fig. 2, in the cooled-down samples after reaching the highest temperature of 80/85°C, the emission of oxidized BChls practically dominates the fluorescence spectra of core complexes. This is an incomplete image, though, because of the used 407-nm light source that preferentially excites oxidized BChls. A more balanced representation can be attained by measuring fluorescence excitation spectra. The spectra in Fig. 2 also confirm significant oxidation of the samples only at elevated temperatures, above about 40°C in *Trv.* 970 and about 60°C in *Tch. tepidum*. Keeping maximum heating temperatures close to these values, *i.e.*, reducing the number of oxidized complexes, should improve the exposure of weaker fluorescence components. This is exactly what is observed in Fig. 3, where the bottom red-line fluorescence spectra recorded along the 50/20°C and 85/20°C heating/cooling cycle comprise either 5 (*Trv.* 970) or 4 (*Tch. tepidum*) clearly separable bands. The fluorescence excitation spectra measured by selective recording at individual bands thus have a potential for unique identification of the emitting components.

The excitation spectra obtained in *Trv.* 970 and *Tch. tepidum* samples are in parallel demonstrated in the upper part of Fig. 3. Note also that similar-origin spectra

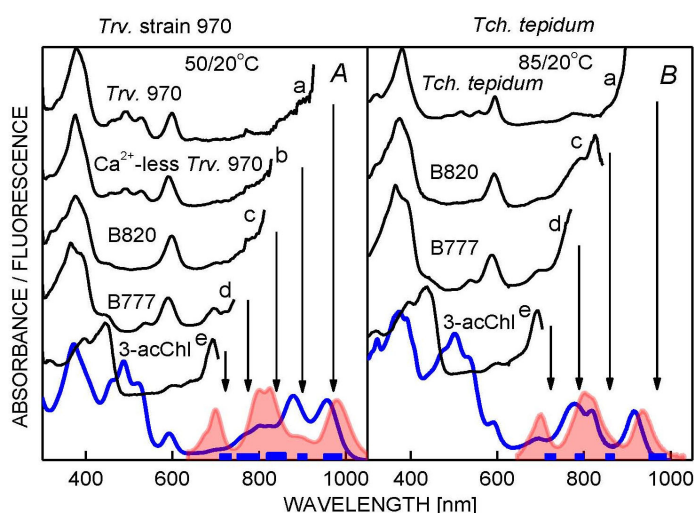


Fig. 3. Fluorescence excitation spectra (vertically shifted black lines) of LH1-RC complexes of *Thiorhodovibrio* strain 970 (A) recorded after heating up to 50°C and cooling down to 20°C and *Thermochromatium tepidum* (B) after heating up to 85°C and cooling down to 20°C. The assigned fluorescence was selectively detected at (a) 950–990 nm, (b) 890–910 nm, (c) 820–860 nm, (d) 750–800 nm, and (e) 710–735 nm in case of *Trv.* 970, and at (a) 950–990 nm, (c) 850–870 nm, (d) 780–800 nm, and (e) 710–735 nm in case of *Tch. tepidum*, as indicated by the bottom blue bars and central arrows. The bottom absorption (blue line) and fluorescence (red-shaded shape, excited at 380 nm) spectra are shown for reference.

are in parts A and B marked by the same letter. Although all the spectra prove typical features of the BChl absorbance such as the presence of Q_y , Q_x , and Soret bands, they also reveal several differences. Firstly, only top (a) spectra that correspond to intact LH1-RC complexes show involvement of carotenoids. The reduced (relative to corresponding absorption spectra) contribution of carotenoids into the excitation spectrum of intact LH1-RC complexes confirms the previously known (Permentier *et al.* 2001) diminished excitation energy transfer efficiency between the carotenoid and BChl excitons. The still weaker carotenoid response in the Ca^{2+} -free LH1-RC complex of *Trv.* 970 (spectrum b) indicates that the accompanying damage of the native protein structure also leads to a partial loss of carotenoids. Carotenoids are completely lost from the B820 complexes (spectrum c) during the thermally-induced dissociation process, similar to the detergent-induced dissociation of core complexes from *Rps. rubrum* and *Rba. sphaeroides* (Visschers *et al.* 1992). The remaining (d and e) spectra that correspond to B777 and 3-acChl, respectively, reveal typical features of BChl and chlorophyll *a*, well approving the above preliminary interpretation of the bands.

Variations of the circular dichroism spectra induced by sample heating: Circular dichroism spectroscopy, which provides information on the chirality of individual chromophores and the interactions between multiple chromophores, was applied for further elucidation of the intermediate steps of thermal decomposition of the LH1-RC complexes. The CD and absorption spectra of the samples heated up to specific temperatures and subsequently cooled down were in parallel recorded at 20°C (Fig. 4).

Fig. 4A,D demonstrates the reference pigment range CD and absorption spectra of LH1-RC recorded at 20°C. The CD spectra comprise a relatively weak (see comparative Fig. 5) and nonconservative Q_y signal of LH1 complexes at 976/930 nm in *Trv.* 970/*Tch. tepidum* as commonly observed in all core complexes of purple bacteria (Georgakopoulou *et al.* 2006, Suzuki *et al.* 2007, Kangur *et al.* 2020). The negative lobe at 815/818 nm and

positive at 798/797 nm have been assigned to accessory BChls in the RC complex (Nozawa *et al.* 1987). The largely positive peak at 598 nm in *Trv.* 970 corresponds to Q_x transitions of all BChl pigment chromophores in LH1-RC. In *Tch. tepidum*, the CD signal related to the Q_x transition is distorted by the overlapping spectrum of carotenoids. In the carotenoid and Soret regions, the CD signal remains roughly conservative, revealing a number of positive and negative peaks. The protein range CD spectra recorded at 20°C and shown in Fig. 6 have also rather similar shapes, typical for polypeptides in α -helical conformation (Matsuo *et al.* 2007).

Fig. 4B–F shows the spectra of the samples that were first heated up to indicated elevated temperatures and then cooled down to the recording temperature of 20°C. As a general principle, not many changes are expected upon rising the temperature up to the optimal growth temperature of the bacteria. Reduced thermostability of *Trv.* 970 in comparison with that of *Tch. tepidum* (Suzuki *et al.* 2007, Kangur *et al.* 2020) is clearly seen in this figure. In *Trv.* 970, already mild heating to 50°C results in partial degradation of the naturally occurring pigment structures (Fig. 4B), while heating to 70°C totally destroys the functionally competent light-harvesting complexes (Fig. 4C). In *Tch. tepidum*, in contrast, some native spectral features remain even after heating to 85°C (Fig. 4F).

The most notable spectral modifications observed in the LH1-RC complexes of *Trv.* 970 induced by the modest heating to 50°C comprise the loss of the original negative 976-nm CD band, the appearance of a new negative CD band at 880 nm, and a significant (314 cm^{-1}) shift of the Q_x band from 598 to 587 nm. According to Fig. 2A,B, these synchronized spectral changes are associated with the partial degradation of the native LH1 complexes to their Ca^{2+} -depleted form. The carotenoid and Soret signals also drop to almost half their initial value, although their position virtually persists. Such behavior is consistent with a loosened structure of the ‘hot’ protein and a partial release of the pigments into the solvent phase. Note that the optical activity of the solubilized pigments is either much weaker (in the case of BChls, as shown in Fig. 5) or

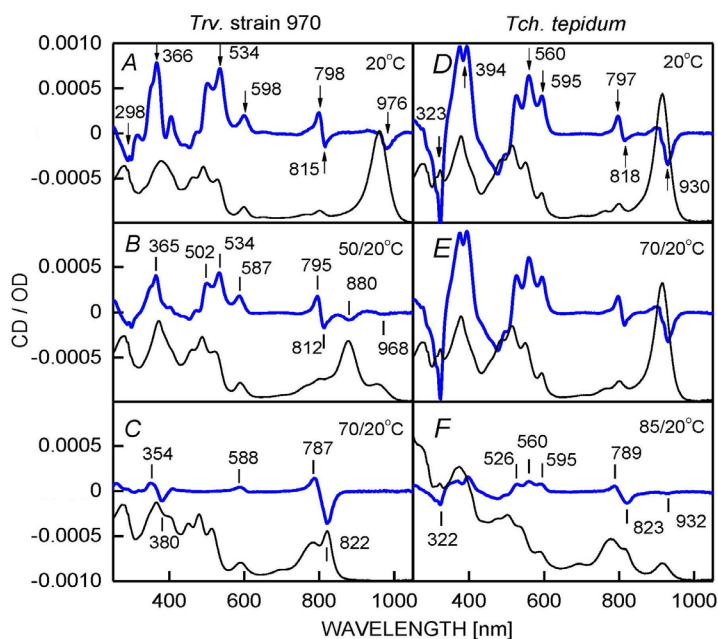


Fig. 4. Pigment-range CD (blue line) and absorption (black line) spectra of LH1-RC complexes from *Thiorhodovibrio* strain 970 (left column) and from *Thermochromatium tepidum* (right column) measured at 20°C (A,D) and after heating the samples up to either 50/70°C (B/E) or 70/85°C (C/F) and then cooling down. The spectra are normalized to the Q_y absorbance maximum at 20°C. The black-line absorption spectra in all panels were multiplied by a factor of 10^{-3} and shifted down to show absorption and CD spectra in comparable frames.

entirely missing [in the case of carotenoids, Cogdell *et al.* (1997)] compared to ordered pigments in light-harvesting complexes.

Further heating to 70°C leads to total wiping out of the CD response around 850–1,000 nm, the Q_y region of LH1 complexes, as well as between 450–550 nm, the position of carotenoid transitions. Also, the signal related to Soret transitions was reduced to the level of monomers, see Fig. 5. Note that the carotenoid absorbance almost persisted. However, the blue-shifted position of the absorption band (see Fig. 1) and missing CD indicate that the remaining absorbance is just due to random pigments in the solvent phase. Although the shape of the CD spectrum around 800 nm appears very similar to the spectrum of the detergent-isolated B820 subunit of LH1 from *Rsp. rubrum* (Chang *et al.* 1990), it most probably has a mixed RC-B820 identity.

The thermal stability of a material is typically determined by the temperature at which it begins to decompose or react. In protein physics, the measure of thermal stability often used is the melting temperature T_m , which is defined as the temperature at which the concentration of the protein in its folded state equals the concentration in the unfolded state. Because most folded proteins have a hierarchic structure, it is essential to investigate the correlation between the melting of major (secondary and tertiary/quaternary) protein conformations. To achieve this, we utilized a spectroscopic approach that involved monitoring the temperature-dependent amplitudes of specific CD lines associated with various pigment and protein chromophores (as shown in Fig. 7). This approach originally applied in Rätsep *et al.* (2018) turned out to be both convenient and informative in analyzing thermal stability of photosynthetic membrane proteins.

Already a first glance at Fig. 7 confirms the higher thermal stability of the complexes from *Tch. tepidum*

compared to those from *Trv.* 970. All the plots representing different pigment and protein CD lines describe similar three stages: an almost constant stage within the physiologically competent range of temperatures, a dropping signal stage between the maximum photosynthetic growth temperature of the bacteria and calorimetric thermal denaturation temperature of the core complexes, and finally, a no-signal stage past the denaturing temperature. From these data, $T_m \approx 60 \pm 2^\circ\text{C}$ for *Trv.* 970 and $T_m \approx 80 \pm 2^\circ\text{C}$ for *Tch. tepidum* was obtained in qualitative agreement with the calorimetric thermal denaturation temperatures T_d also indicated in Fig. 7. Corresponding temperatures of the CD amplitude collapse are about 67 and 83°C.

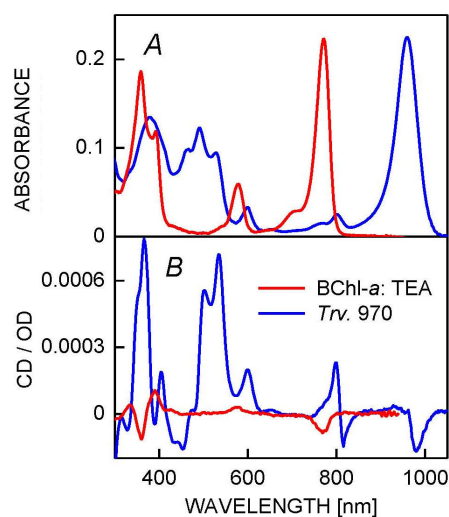


Fig. 5. Comparison of absorption (A) and CD (B, normalized to the Q_y absorbance) spectra of *Thiorhodovibrio* strain 970 core complex (blue line) and monomeric BChl *a* dissolved at molar concentration of 2×10^{-6} in triethylamine (TEA, red line) at 20°C.

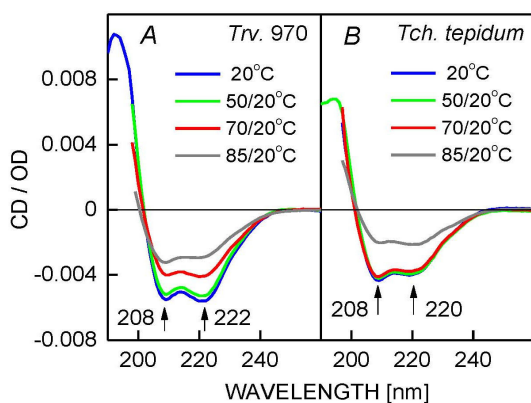


Fig. 6. Protein range CD spectra of *Thiorhodovibrio* strain 970 (A) and *Thermochromatium tepidum* (B) measured at 20°C, and after heating up the samples to various temperatures indicated and cooling them down. The reference CD spectrum is drawn in blue. All the spectra are normalized to the Q_y BChl exciton absorbance.

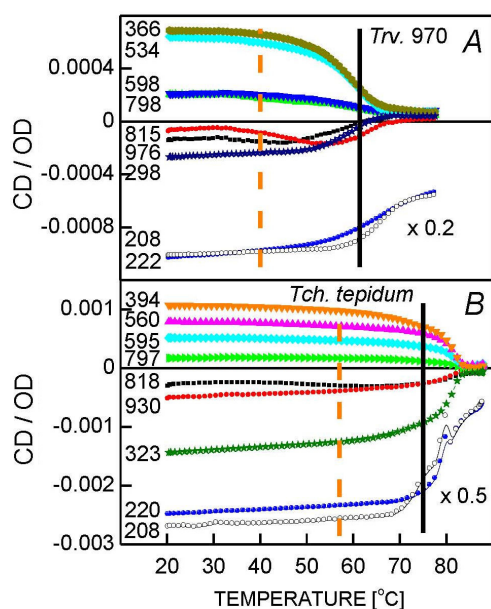


Fig. 7. Temperature dependence of the CD spectra of LH1-RC complexes from *Thiorhodovibrio* strain 970 (A) and *Thermochromatium tepidum* (B, data modified from Kangur *et al.* 2020) was recorded with a resolution of 2 nm at the indicated wavelengths in nanometers. The selected lines are in Figs. 4 and 6 marked by arrows. Note the different y-scale applied in the case of pigment and protein range signals. The sample temperature was raised with a rate of 1°C min⁻¹; measurement time per temperature point was ~1 min. Vertical dashed and solid lines, respectively, designate the maximal growth temperature, which characterizes the thermal stability of the bacterial cells (Di Bari *et al.* 2023), and the thermal denaturation temperature of the LH1-RC complexes determined by differential scanning calorimetry (Kimura *et al.* 2009, Imanishi *et al.* 2019). Extra modulations of the CD amplitudes in panel A related to Q_y transitions of BChls in LH1 (at 976 nm) and RC (at 815 nm) complexes are due to the shifting/overlapping of different spectral lines along increasing the temperature.

Differently from the CD amplitudes related to pigments, the protein amplitudes as presented in Figs. 6 and 7 do not completely vanish even at the highest experimental temperatures. This residual signal may in principle be attributed to surviving secondary structure elements, but alternatively, to peptide bond chromophores in random coil conformations or to individual aromatic conformers such as tryptophan (Štěpánek and Bouř 2014). The *Trv.* 970 and *Tch. tepidum* complexes have a similar number of tryptophans, 136 and 123, respectively (Yu *et al.* 2018, Tani *et al.* 2020). We tend to believe that the enhanced contribution of random coil conformations is the most probable explanation because of two reasons. Firstly, there is a visibly greater residual signal level in the less thermostable *Trv.* 970 complexes. Secondly, in complexes of *Trv.* 970, there is a deviation in the temperature dependence of the 222-nm and 208-nm protein CD signals around 60°C, absent in *Tch. tepidum* complexes, which can be explained by the growing contribution of random coil conformations.

Since the tertiary structure governs the formation of all pigment-binding sites of the chromoprotein, the orchestrated loss of BChl and carotenoid signals observed can be directly linked to the melting of the tertiary structure. It thus turns out that tertiary and secondary structures in the chromoproteins of both *Trv.* 970 and *Tch. tepidum* melt nearly simultaneously, despite their significantly different thermostability.

The core complexes from mesophilic non-sulfur purple bacteria show qualitatively similar thermal behavior, with an additional suggestion that the B820 subunit is only formed in detergent-isolated complexes; membrane-protected complexes generally do not show such an intermediate (data not shown). Available literature data, furthermore, reveal a much-enhanced resilience of peripheral LH2 light-harvesting complexes relative to LH1 or LH1-RC complexes, which rests on subtle structure differences of these complexes (Georgakopoulou *et al.* 2006, Suzuki *et al.* 2007, Rätsep *et al.* 2018, Kangur *et al.* 2020). Yet, the physiological significance of this result awaits to be understood.

Spectral responses to hydrostatic high-pressure compression: Protein stability against hydrostatic pressure refers to the ability of a protein to maintain its structural and functional integrity under elevated pressure conditions. The mechanisms that allow some proteins to be stable against pressure-induced denaturation are not fully understood, but they likely involve a combination of factors, such as changes in protein flexibility, increased hydrophobic interactions, and stabilization of secondary as well as higher-order structural elements.

Some of the authors of this paper recently conducted a thorough hydrostatic high-pressure perturbation spectroscopy study to investigate the role of multiple Ca²⁺-binding sites in the destabilization of the LH1-RC complexes from *Trv.* 970 and *Tch. tepidum* (Timpmann *et al.* 2023). By using the Q_y exciton absorption band as an optical probe, it was possible to distinguish between

the native (Ca-saturated, Ca⁺) and denatured (Ca-depleted, Ca⁻) phases of these complexes. These findings showed that pressure-induced denaturation of the complexes resulted in the failure of the protein Ca-binding pockets and the related breakage of tertiary structure hydrogen bonds between pigment chromophores and the protein environment. This cooperative process involved all or most of the Ca²⁺-binding sites and was irreversible. Furthermore, this study revealed strong hysteresis in the spectral and kinetic characteristics of phase transitions along the compression and decompression pathways, suggesting an asymmetry in the relevant free energy landscapes and activation free energy distributions.

To focus our analysis, we primarily examined the pressure dependencies of LH1-RC complexes from *Trv.* 970 and *Tch. tepidum*. The data in Fig. 8 are conveniently presented side-by-side which facilitates easy comparison.

One striking feature of Fig. 8 is the sudden blue shift and broadening of the absorption spectra that occurs between 400 and 600 MPa upon increasing the pressure (as seen in Fig. 8A–D). Another key observation is the pressure dependencies of the LH1-RC complexes from *Trv.* 970 are quite similar to those of *Tch. tepidum*, with the main difference being the relative shift of their absorption spectra. The spectral changes seen in these experiments are consistent with the depletion of LH1 from Ca, which

characterizes the denaturation of the core complexes (as seen in Fig. 8E,F, and in Timpmann *et al.* 2023).

It is worth noting that denaturation by pressure appears to be different from denaturation by temperature, as pressure-induced denaturation produces a single denatured product (that of a Ca-free LH1-RC complex), while temperature-induced denaturation generates at least two stable structures (B777 and B820), along with the Ca-free LH1-RC intermediate (in *Trv.* 970, but not in *Tch. tepidum*). Furthermore, while denaturation by temperature is truly irreversible, denaturation by pressure is in principle reversible, although kinetically hindered, because of the slow kinetics of Ca rebinding compared to the typical (seconds to minutes) time scale of the present spectral measurements. After a sufficiently long waiting time, however, initial states of the chromoproteins appear to almost perfectly recover (as can be seen in Fig. 8C,D, and in Timpmann *et al.* 2023).

The robustness against high pressure of the core complexes that miss Ca as an obligatory structure element is much greater than the ones of Ca-containing bacteria (Freiberg *et al.* 1993, 2012). So, the denaturation related to the breakage of tertiary structure hydrogen bonds in core complexes from wild type *Rba. sphaeroides* requires a pressure as high as 1.09 GPa to take place (Freiberg *et al.* 2012). Also, the pressure-induced dissociation of isolated LH1 complexes from the same species into the

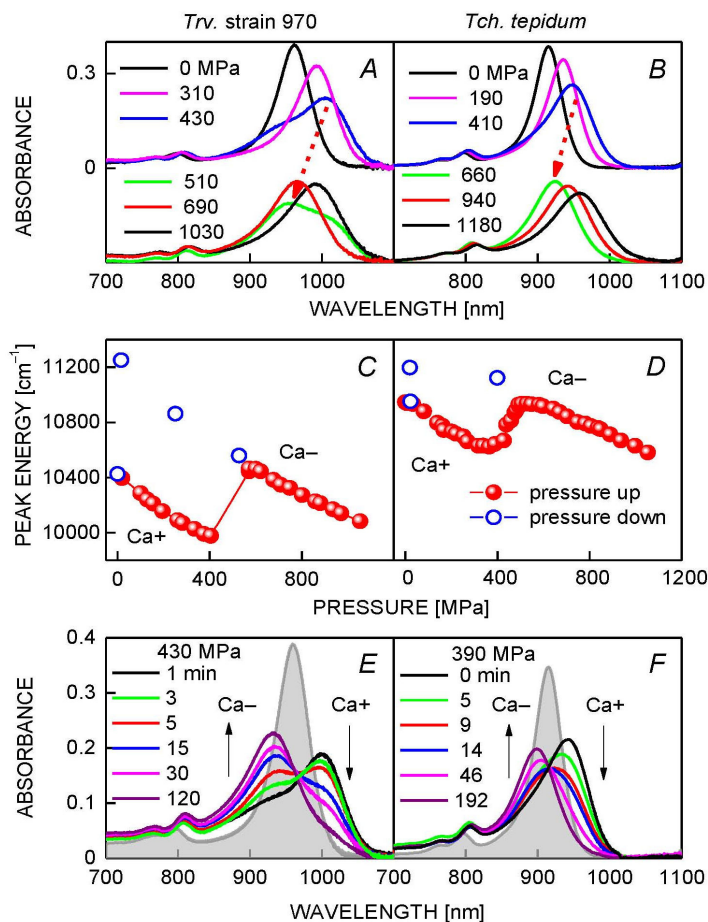


Fig. 8. Pressure dependence of the Q_y absorption spectra (A,B), band maxima (C,D), and spectral kinetics (E,F) of LH1-RC complexes from *Thiorhodovibrio* strain 970 (A,C,E) and *Thermochromatium tepidum* (B,D,F) at an ambient temperature of 22°C. The spectra in panels A and B are recorded along increasing the pressure; furthermore, groups of spectra that are measured above the denaturation pressure are for better visibility shifted by 0.3 optical density units down with respect to the groups of higher-pressure spectra. Panels C and D represent data that are recorded along both increasing (red balls) and decreasing (blue rings) pressure. The lines connecting scattered data points are drawn for leading the eye. Panels E and F present the time dependences of the spectra recorded at fixed pressures within the range of apparent denaturation pressures. They show a decrease in the native Ca-containing complexes designated by Ca⁺ and a correlated increase of the denatured Ca-depleted complexes denoted by Ca⁻. The shaded contour represents the reference absorption spectrum measured at ambient pressure.

B777 and B820 subunits could only be demonstrated at high destabilizing detergent concentrations (Puusepp *et al.* 2015).

Summary and conclusions

In this study, we investigated the resilience of LH1-RC core complexes purified from Ca-containing sulfur purple bacteria to super-physiological heating and hydrostatic compression. Our aim was to assess the suggested correlation between the color-tuning of photosynthetic excitons and the thermodynamic stability of these proteins. We conducted parallel measurements of absorption, fluorescence, fluorescence excitation, and CD spectra over a broad spectral range that enabled us to differentiate between the responses of the protein and pigment subsystems of the chromoproteins.

Our results provide conclusive evidence of limited reversibility of the effects of both temperature and pressure due to the malleability of Ca-binding sites. Yet, we found that denaturation by pressure is distinctly different from denaturation by temperature. While the sole product of the former process is a Ca-depleted form of the LH1-RC complex, the latter process produces at least two stable products (B777 and B820).

Based on spectral criteria, we observed that the complexes from *Trv.* 970 were less thermally robust compared to those from *Tch. tepidum*. Interestingly, the CD markers of protein tertiary and secondary structure showed nearly simultaneous melting in both complexes, unlike the LH2 complexes of non-sulfur purple bacteria, where different protein structures display varying stabilities.

The core complexes from *Trv.* 970, which has the most red-shifted spectrum, showed lower thermal stability compared to those from *Tch. tepidum*, while having similar piezo stability. The results of this study thus do not support the idea that the stability of light-harvesting chromoproteins might be directly related to the color-tuning of photosynthetic excitons.

References

- Chang M.C., Callahan P.M., Parkes-Loach P.S. *et al.*: Spectroscopic characterization of the light-harvesting complex of *Rhodospirillum rubrum* and its structural subunit. – *Biochemistry* **29**: 421-429, 1990.
- Cogdell R.J., Isaacs N.W., Freer A.A. *et al.*: The structure and function of the LH2 (B800–850) complex from the purple photosynthetic bacterium *Rhodospseudomonas acidophila* strain 10050. – *Prog. Biophys. Mol. Biol.* **68**: 1-27, 1997.
- Di Bari D., Timr S., Guiral M. *et al.*: Diffusive dynamics of bacterial proteome as a proxy of cell death. – *ACS Centr. Sci.* **9**: 93-102, 2023.
- Fiedor L., Scheer H.: Trapping of an assembly intermediate of photosynthetic LH1 antenna beyond B820 subunit: Significance for the assembly of photosynthetic LH1 antenna. – *J. Biol. Chem.* **280**: 20921-20926, 2005.
- Freiberg A., Ellervee A., Kukkk P. *et al.*: Pressure effects on spectra of photosynthetic light-harvesting pigment-protein complexes. – *Chem. Phys. Lett.* **214**: 10-16, 1993.
- Freiberg A., Kangur L., Olsen J.D., Hunter C.N.: Structural implications of hydrogen-bond energetics in membrane proteins revealed by high-pressure spectroscopy. – *Biophys. J.* **103**: 2352-2360, 2012.
- Georgakopoulou S., van der Zwan G., Olsen J.D. *et al.*: Investigation of the effects of different carotenoids on the absorption and CD signals of light harvesting 1 complexes. – *J. Phys. Chem. B* **110**: 3354-3361, 2006.
- Imanishi M., Takenouchi M., Takaichi S. *et al.*: A dual role for Ca²⁺ in expanding the spectral diversity and stability of light-harvesting 1 reaction center photocomplexes of purple phototrophic bacteria. – *Biochemistry* **58**: 2844-2852, 2019.
- Kangur L., Rätsep M., Timpmann K. *et al.*: The two light-harvesting membrane chromoproteins of *Thermochromatium tepidum* expose distinct robustness against temperature and pressure. – *BBA-Bioenergetics* **1861**: 148205, 2020.
- Kangur L., Timpmann K., Freiberg A.: Stability of integral membrane proteins against high hydrostatic pressure: the LH2 and LH3 antenna pigment-protein complexes from photosynthetic bacteria. – *J. Phys. Chem. B* **112**: 7948-7955, 2008.
- Kimura Y., Hirano Y., Yu L.-J. *et al.*: 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*. – *J. Biol. Chem.* **283**: 13867-13873, 2008.
- Kimura Y., Tani K., Madigan M.T., Wang-Otomo Z.-Y.: Advances in the spectroscopic and structural characterization of core light-harvesting complexes from purple phototrophic bacteria. – *J. Phys. Chem. B* **127**: 6-17, 2023.
- Kimura Y., Yu L.-J., Hirano Y. *et al.*: Calcium ions are required for the enhanced thermal stability of the light-harvesting-reaction center core complex from thermophilic purple sulfur bacterium *Thermochromatium tepidum*. – *J. Biol. Chem.* **284**: 93-99, 2009.
- Leiger K., Linnanto J.M., Rätsep M. *et al.*: Controlling photosynthetic excitons by selective pigment photooxidation. – *J. Phys. Chem. B* **123**: 29-38, 2019.
- Luke K.A., Higgins C.L., Wittung-Stafshede P.: Thermodynamic stability and folding of proteins from hyperthermophilic organisms. – *FEBS J.* **274**: 4023-4033, 2007.
- Matsuo K., Sakurada Y., Yonehara R. *et al.*: Secondary-structure analysis of denatured proteins by vacuum-ultraviolet circular dichroism spectroscopy. – *Biophys. J.* **92**: 4088-4096, 2007.
- Michalik M., Zbyradowski M., Heriyanto, Fiedor L.: Tuning the photophysical features of self-assembling photoactive polypeptides for light-harvesting. – *Materials* **12**: 3554, 2019.
- Nozawa T., Trost J.T., Fukada T. *et al.*: Properties of the reaction center of the thermophilic purple photosynthetic bacterium *Chromatium tepidum*. – *BBA-Bioenergetics* **894**: 468-476, 1987.
- Pandit A., van Stokkum I.H.M., Georgakopoulou S. *et al.*: Investigations of intermediates appearing in the reassociation of the light-harvesting 1 complex of *Rhodospirillum rubrum*. – *Photosynth. Res.* **75**: 235-248, 2003.
- Parkes-Loach P.S., Sprinkle J.R., Loach P.A.: Reconstitution of the B873 light-harvesting complex of *Rhodospirillum rubrum* from the separately isolated α - and β -polypeptides and bacteriochlorophyll *a*. – *Biochemistry* **27**: 2718-2727, 1988.
- Permentier H.P., Neerken S., Overmann J., Ames J.: A bacteriochlorophyll *a* antenna complex from purple bacteria absorbing at 963 nm. – *Biochemistry* **40**: 5573-5578, 2001.
- Polyakov I.V., Khrenova M.G., Moskovsky A.A. *et al.*: Towards first-principles calculation of electronic excitations in the ring of the protein-bound bacteriochlorophylls. – *Chem. Phys.* **505**: 34-39, 2018.
- Puusepp M., Kangur L., Freiberg A.: Dissociation of the light-harvesting membrane protein complex I from *Rhodobacter*

- sphaeroides* under high hydrostatic pressure. – High Press. Res. **35**: 176-180, 2015.
- Rätsep M., Muru R., Freiberg A.: High temperature limit of photosynthetic excitons. – Nat. Commun. **9**: 99, 2018.
- Reppert M.: Bioexcitons by design: How do we get there? – J. Phys. Chem. B **127**: 1872-1879, 2023.
- Sancho J.: The stability of 2-state, 3-state and more-state proteins from simple spectroscopic techniques... Plus the structure of the equilibrium intermediates at the same time. – Arch. Biochem. Biophys. **531**: 4-13, 2013.
- Smith J.R.L., Calvin M.: Studies of the chemical and photochemical oxidation of bacteriochlorophyll. – J. Am. Chem. Soc. **88**: 4500-4506, 1966.
- Štěpánek P., Bouř P.: Multi-scale modeling of electronic spectra of three aromatic amino acids: Importance of conformational averaging and explicit solute-solvent interactions. – Phys. Chem. Chem. Phys. **16**: 20639-20649, 2014.
- Suzuki H., Hirano Y., Kimura Y. *et al.*: Purification, characterization and crystallization of the core complex from thermophilic purple sulfur bacterium *Thermochromatium tepidum*. – BBA-Bioenergetics **1767**: 1057-1063, 2007.
- Tani K., Kanno R., Makino Y. *et al.*: Cryo-EM structure of a Ca²⁺-bound photosynthetic LH1-RC complex containing multiple $\alpha\beta$ -polypeptides. – Nat. Commun. **11**: 4955, 2020.
- Timpmann K., Kangur L., Freiberg A.: Hysteretic pressure dependence of Ca²⁺ binding in LH1 bacterial membrane chromoproteins. – J. Phys. Chem. B **127**: 456-464, 2023.
- Timpmann K., Rätsep M., Kangur L. *et al.*: Exciton origin of color-tuning in Ca²⁺-binding photosynthetic bacteria. – Int. J. Mol. Sci. **22**: 7338, 2021.
- Visschers R.W., Nunn R., Calkoen F. *et al.*: Spectroscopic characterization of B820 subunits from light-harvesting complex I of *Rhodospirillum rubrum* and *Rhodobacter sphaeroides* prepared with the detergent *n*-octyl-*rac*-2,3-dipropylsulfoxide. – BBA-Bioenergetics **1100**: 259-266, 1992.
- Visschers R.W., van Grondelle R., Robert B.: Resonance Raman spectroscopy of the B820 subunit of the core antenna from *Rhodospirillum rubrum* G9. – BBA-Bioenergetics **1183**: 369-373, 1993.
- Yu L.-J., Kawakami T., Kimura Y., Wang-Otomo Z.-Y.: Structural basis for the unusual Q_y red-shift and enhanced thermostability of the LH1 complex from *Thermochromatium tepidum*. – Biochemistry **55**: 6495-6504, 2016.
- Yu L.-J., Suga M., Wang-Otomo Z.-Y., Shen J.-R.: Structure of photosynthetic LH1-RC supercomplex at 1.9 Å resolution. – Nature **556**: 209-213, 2018.