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Temperature dependent UV-Vis spectral changes in hydrogen- and deuterium-bonded photosynthetic reaction centers of *Rhodobacter sphaeroides*

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Abstract

The UV-Vis absorption spectra of detergent-isolated hydrogen- and deuterium-bonded reaction centers (RCs) from *Rhodobacter sphaeroides* PUC 705Ba were examined as a function of temperature between 20 and 55 °C. The enthalpy and entropy of denaturation for the specimens was determined, revealing that their process of thermal denaturation is significantly different. Deuterium-bonded RCs are most stable at 37 °C, rather than at room temperature, and undergo a "cold denaturation" as the temperature is lowered to room temperature. At room temperature the addition of 1,3,5-heptanetriol brought the deuterium-bonded RC back to its more stable configuration. Hence the hydrogen bonding interactions in the RC do influence its conformation and this is reflected in the microenvironment of its associated pigments.

Additional key words: enthalpy; entropy; ferricyanide; 1,3,5-hexanetriol; photosynthetic bacteria.

Introduction

The reaction center (RC) of purple photosynthetic bacteria is a polymeric membrane complex comprised of several relatively large protein subunits, and a group of chromophores that absorb photon energy and generate the intracellular electrochemical potentials that drive biochemical reactions in the cell. The RC's function is delicately tuned by numerous non-covalent, hydrogen bonding interactions within the complex that help maintain its structure and the relative orientations of the associated chromophores (Thompson *et al.* 1989, Chang *et al.* 1991, Deisenhofer *et al.* 1995). Interactions between the chromophores and nearby amino acids allow for efficient electron transfer from the special pair of bacteriochlorophylls (BChl) to subsequent electron acceptors bacteriopheophytin φ, quinone Q_A, Fe^{2+}, and quinone Q_B (Lancaster *et al.* 2000). Hydrogen bonds between the chromophores and nearby protein directly regulate the redox potential of the special pair of bacteriochlorophylls (BChl), and the rate of this electron transfer (Lancaster *et al.* 2000).

Isotope substitution is a convenient method for evaluating the impact of hydrogen bonding interactions on protein structure and function (Schulz and Schirmer 1979, Yokogaki *et al.* 1995, Scheiner and Cuma 1996). The energetic and bond-distance differences between protonated and deuterated hydrogen bonds are usually sufficient to result in significant structural differences in many proteins. Indeed, studies of electron transfer in hydrogen- and partially deuterium-bonded RCs have shown that the temperature dependence of the rate of electron transfer from room temperature to 5 K is definitely affected by deuteration (Schenck *et al.* 1981, Pashchenko 2000, Yakovlev and Shuvalov 2003).

In order to better understand the overall impact of hydrogen bonding interactions on the thermal stability of the RCs, we examined the temperature dependent changes in the UV-Vis absorption spectrum of hydrogen- and deuterium-bonded, detergent-isolated, RC complexes from *Rb. sphaeroides* PUC 705Ba. The absorbance spectra of the RC's light-absorbing photoactive pigments are very sensitive to changes in the polarity of their local microenvironments (Angerhofer 1991), and can reveal the onset of subtle structural changes that accompany temperature change or isotopic substitution independent of whether electron transfer is affected.

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Materials and methods

Preparation of detergent isolated RCs: To prepare specimens for UV-Vis and EPR measurements, approximately 60 g of *Rb. sphaeroides* PUC705 Ba frozen cell paste originally grown in protonated or deuterated (Crespi *et al.* 1971) medium were thawed overnight at 4 °C, and resuspended in 150 cm^3 of 10 mM Tris (*Sigma*) buffer at pH 7.8 with 0.01 mM EDTA (*Sigma*). Cells were sonicated using *VibraCell* (*Sonics & Materials*) ultrasonic homogenizer at 4 °C for 18 min. Debris was removed by centrifugation at 4 000×*g* at 4 °C (*Sorval RC5*) for 15 min. Membranes were pelleted at 184 000×*g* at 4 °C for another 15 min, using a *Beckman LE-80* Preparative Ultracentrifuge, and resuspended in the same buffer containing 2 % n-octyl-D-glucopyranoside (BOG) (*Anatrace*). The sample was applied to a *DEAE-Sephaceal* column (*Pharmacia LC50*) equilibrated in the same buffer, but with 0.8 % BOG, at 10 ºC and eluted in the dark with a NaCl ionic strength gradient. Purified RCs were eluted at approximately 2.5 % NaCl and were concentrated to an absorbance of 80 cm^{-1} at 803 nm using a *Centricon-30* centrifugal concentrator (*Millipore*). Deuterium-bonded RCs were resuspended in deuterated buffer solution. For absorption spectrophotometry, the concentrated stock solutions were diluted to an absorbance of approximately 1.0 cm^{-1} at 803 nm using 10 mM Tris buffer solution with 0.01 mM EDTA and 0.8 % BOG at pH 7.8. For EPR measurements, the stock sample was used undiluted.

UV-Vis absorption and Electron Paramagnetic Resonance (EPR) measurements: Absorption spectrophotometric analyses as a function of temperature were performed over the range of 200–1 000 nm using a *Shimadzu 1601* UV-VIS spectrophotometer. Temperature of the sample compartment was adjusted by using a microprocessor-controlled heater (*Thermolyne*, Barnstead) to control the temperature of a circulating water bath. Temperatures were measured in the cuvette using a copper-constantan thermocouple (*Omega*).

The room temperature X-band EPR signal of the special pair radical cations D^+ of both types of RCs (Norris *et al.* 1971, 1972a,b) were obtained as the first derivative of the absorption mode signal using a *Varian E9* EPR spectrometer in a standard *TE102* rectangular cavity with *Varian* platinum heater assembly (*Wilmad*). The temperature in the EPR cavity was controlled using a *LakeShore* temperature controller (±0.1 °C sensitivity). For temperature dependence measurements of the EPR signal, the rate of temperature increase in the cavity was set at approximately 0.5 ºC per minute. All EPR spectra were corrected for the 1/T temperature dependence of EPR signal detection. For the light-induced EPR experiments the special pair radical cation D^+ was produced by steady state irradiation of RCs inside the cavity using a 300 W xenon lamp. A 780 nm cutoff filter and 5 cm path length water filter were used to reduce exposure to UV wavelengths and heating of the sample. No EPR signals were observable in the dark, unless 0.01 M potassium ferricyanide was added to chemically oxidize the special pair.

Results

The UV-Vis absorption spectra of detergent isolated hydrogen- and deuterium-bonded RCs at room temperature (21 °C) are shown in Fig. 1*A*. At this temperature, the most evident spectrophotometric differences between these two specimens were found at the region of protein absorption, between 250–300 nm, and in the near infrared. The spectral peaks in the infrared regions can be assigned to specific light-absorbing pigments (Hayashi *et al.* 1982). For example, 865 nm is assigned to the Q_y transition of the special pair of BChl, 800 nm for the Q_y transition of the accessory BChl, 760 nm for both A and B φs located on either side of the RC, while 280 nm corresponds to the protein backbone.

The features of the absorption spectra changed in a concerted fashion with temperature for protonated RCs, but not for deuterated ones (Fig. 2). For hydrogen-bonded RCs, the absorbances of the special pair of BChl, accessory BChl, and φ were steady up to about 45 ºC, after which those for the special pair of BChl and accessory BChl declined while that for φ increased. At the same time, the absorption difference between 282 and 288 nm, correlated with protein conformation, increased slightly in value up to 40 ºC and increased even more steeply at higher temperatures. In contrast, for deuterium-bonded RCs, the absorbance of the special pair of BChl first increased with the temperature up to 37 ºC, before it decreased. The absorbances corresponding to accessory BChl and φ declined steadily up to about 40 ºC, and only in the case of φ did the trend reverse at higher temperatures. The absorbance difference between 282 and 288 nm (measured in order to reduce the effect of light scattering in the UV portion of the spectrum) was unchanged up to 40 ºC, and increased dramatically above this temperature. For deuterium-bonded RCs, the absorbance at 830 nm is an isoabsorptive point in the spectrum at temperatures above 40 ºC. The absorbances of the special pair of BChl, relative to accessory BChl, coincided best at about 37 ºC, as well as following the addition of 1,3,5-heptanetriol to deuterium-bonded RCs (Fig. 1*B*).

No EPR signal was observed from either type of RC without irradiation, indicating the specimens had not been chemically oxidized as a result of manipulation. When irradiated, signals similar to what have been reported previously were recorded (Norris *et al.* 1972a,b).

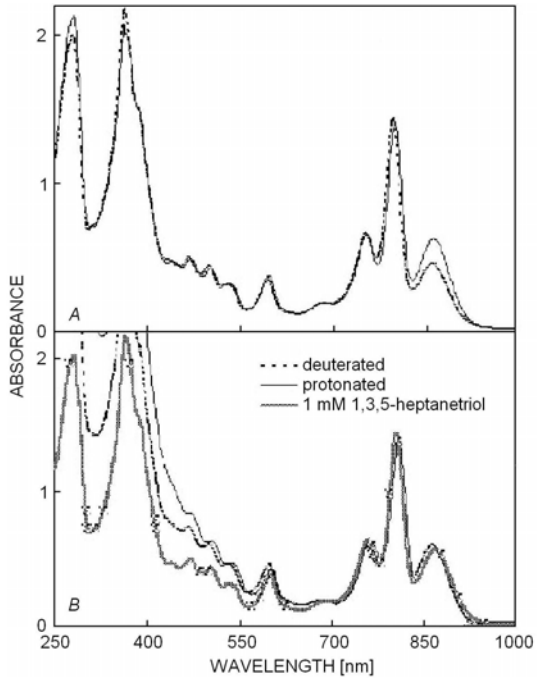

Fig. 1. (*A*) Room temperature (21 °C) absorption spectra of hydrogen- (*solid line*) and deuterium-bonded (*dotted line*) reaction centers (RCs) of *Rb. sphaeroides* PUC705Ba from 250–1 000 nm. (*B*) Absorption spectra of hydrogen- (*solid line*) and deuterium-bonded (*dotted line*) RCs at 37 °C, and deuterium-bonded RCs to which 1 mM 1,3,5, heptanetriol was added (*hatched line*).

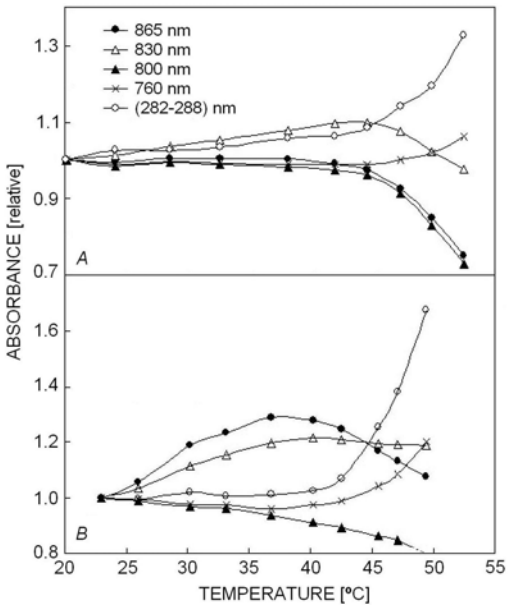

Fig. 2. Temperature dependence of pigment and protein absorbances in (*A*) hydrogen-bonded reaction centres (RCs) and (*B*) deuterium-bonded RCs. The spectral peak at 865 nm is assigned to the Q_y transition of the special pair of bacteriochlorophyll (BChl), 800 nm for the Q_y transition of the accessory BChl, 760 nm for both A and B φs located on either side of the RC, and the absorbance difference between 282 and 288 nm for the protein backbone. The absorbance at 830 nm is an isoabsorptive point in the spectrum of deuterium-bonded RCs at temperatures above 40 °C.

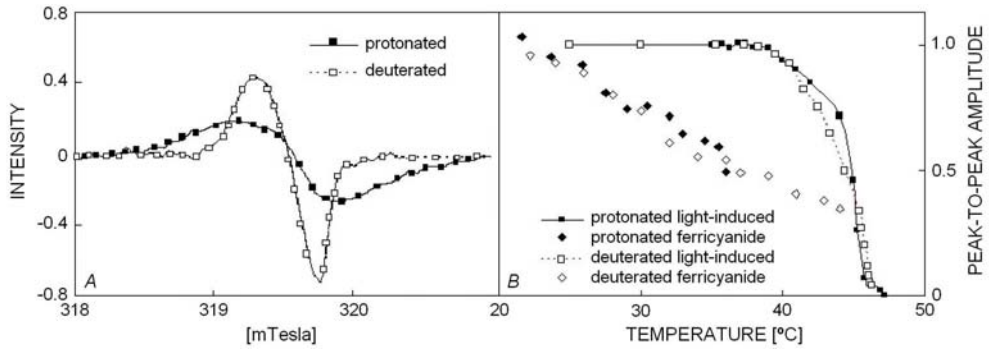

Fig. 3. (*A*): Typical X-band EPR signal from irradiated protonated (*solid squares*) and deuterated reaction centres (RCs) (*open squares*) obtained as the first derivative of the absorption mode signal. Similar traces could be obtained in the dark when RCs were chemically oxidized using 0.01 M potassium ferricyanide. (*B*): The temperature dependence of the peak-to-peak height of the X-band EPR signals for each of these samples: protonated with irradiation (*solid squares*), deuterated with irradiation (*open squares*), protonated oxidized with potassium ferricyanide (*solid diamonds*), and deuterated oxidized with potassium ferricyanide (*open diamonds*).

Typical X-band EPR traces obtained as the first derivative of the absorption mode signal from protonated and deuterated RCs using irradiation (*squares*) or chemical oxidation (*diamonds*) are shown in Fig. 3*A*. The peak to peak height of the EPR signals collected for these samples as a function of temperature is shown in Fig. 3*B*. In this figure, the EPR spectra were adjusted to compensate for the 1/T dependence in detection of the EPR signal. The first point of each series of data was normalized to one for convenient comparison of the temperature-dependent trends. No unusual change in efficiency in the yield of charge separation (as measured by magnitude of the D^+ radical cation EPR signal) was observed for the case of deuterated RCs between room temperature (about 20 ºC) and 37 ºC, despite the fact that the absorbance at 865 nm seems to change over this region. Above 40 ºC, there was a small difference in the temperature dependence of EPR signal intensity

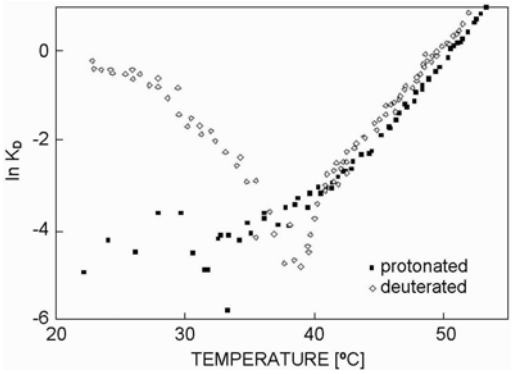

Fig. 4. Ln K_D *versus* temperature [ºC] for hydrogen- (*open diamonds*) and deuterium-bonded (*solid squares*) reaction centers. See text for explanation of K_D.

loss, which shows that the deuterated sample undergoes functional deactivation at a slightly lower temperature, as would be consistent with the weakened deuterated hydrogen bonding interactions. For comparison is also plotted the temperature-dependent change in radical cation EPR intensity obtained in the dark for both protonated and deuterated RCs using the oxidant ferricyanide presented at a concentration of 0.01 M. In this case, the mechanisms responsible for loss of the EPR signal are complicated, since both thermal denaturation, oxidative damage, and denaturation assisted by oxidative damage take place simultaneously. Therefore it is not unexpected these samples have a completely different temperature dependence.

Discussion

The specific conformation of the RC cannot be known from simple room temperature absorbance studies, but the temperature-dependence of the absorbances of the various pigments in the RC should generally coincide with the manner in which the subunit structures shift as the temperature is raised. The absorbance changes at 865 nm, which are related to the dimeric character of the special pair of BChl, can be expressed in terms of K_D, a spectrophotometric equilibrium constant for denaturation defined assuming a two state model for denaturation:

$$K_D = [\text{denatured}]/[\text{native}] = \frac{A(BChl)_N - A(BChl)}{A(BChl) - A(BChl)_D}.$$

In this equation we have followed convention in describing the two state model in terms of native and denatured states, by assigning the lowest energy state as 'native' and higher energy states as 'denatured'. The absorbance $A(BChl)_D$ is assumed to be equal to zero at the 'denatured state' in which the dimeric nature of the special pair is absent, and the absorbance in the 'native state' $A(BChl)_N$ is the temperature at which the special pair dimer absorbance is maximum. In this context, we can assume that when the absorption spectra in the NIR are similar for the two, their overall conformations are also similar. No assumption about the electron transfer functionality of the special pair in these conformations is implied.

Plotted in Fig. 4 is ln K_D *vs*. T over the entire temperature range for both hydrogen- and deuterium-bonded RCs. From the slope of this plot, an estimated value for the enthalpy and entropy of denaturation of the RC can be obtained. For hydrogen-bonded RCs: $\Delta H/R = -4\,153$ J mol^{-1} and $\Delta S/R = 12.8$ J mol^{-1} K^{-1}, while for deuterium-bonded RCs the situation was more complex: between room temperature and 37 ºC, $\Delta H/R = 3\,149$ J mol^{-1} and $\Delta S/R = 10.6$ J mol^{-1} K^{-1}, while for temperatures above 37 °C, $\Delta H/R = -4\,186$ J mol^{-1} and $\Delta S/R = 13.0$ J mol^{-1} K^{-1}. The values obtained for hydrogen-bonded RCs and for deuterium-bonded RCs at temperatures above 37 ºC are similar to what has been reported for a number of denaturing proteins (Brandts 1969). The change in sign for the enthalpy and entropy of denaturation at 37 ºC indicated that the most stable structure of deuterium-bonded RCs is found at this temperature. So-called 'cold' denaturations are not unusual in biology, and occur most frequently in enzymes that possess quaternary structure (Klotz *et al.* 1974, Babu *et al*. 2004). Generally, cold denaturation is exothermic and hot denaturation is endothermic (Bohr 2003), which is what is observed here for the case of the RC. This is the first time cold denaturation has been reported in detergent-isolated photosynthetic RCs at temperatures above room temperature. The fact that following the addition of 1,3,5-heptanetriol, the UV-Vis absorbance spectra of both hydrogen- and deuterium-bonded RCs converged, also supports the conclusions that structural differences exist between these two types of RCs at room temperature.

The lowest energy state for the deuterated RCs need not be the most photosynthetically active, or functional state. In fact, as Fig. 3*B* shows both the room temperature and 37 ºC states appear to be able to support the early stages of light-induced electron transfer with about the same efficiency. This observation suggests that hydrogen bonding interactions in the RC complex are strategically placed in such a way that temperature or deuteration-induced bond weakening minimally affect that part of the structure responsible for initial electron transfer. Further it also shows that the intensity of the 865 nm band may be also connected with conformational changes not important for the initial stages of electron transfer. A small structural shift at the periphery of the complex may leave the relative orientations of adjacent pigments unchanged and lead to no obvious effect on the RC electron transfer function.

Calorimetry measurements are the accepted way for evaluating temperature dependent structural change in

proteins. Unfortunately, the amount of material needed for these measurements poses a problem in the case of deuterated RCs. Further, although these measurements clearly identify loss of tertiary structure, for example alpha helix unwinding, it is uncertain whether subtle changes in the quaternary structure of the three subunit RC complex could be detected.

Changes in hydrogen bond stability could affect the accessibility of the soluble quinone binding site in the RC between deuterated and protonated RCs. An EPR study of the exchange of protonated and deuterated quinone in photosystem 1 (Ostafin and Weber 1997) indicated that while the binding conformation of the two molecules was the same, the exchange process efficiency was correlated to the growth maximum of the organism from which the RC was extracted. Whether deuteration has affected *R. sphaeroides* optimal temperature range for growth (close to 30 ºC for protonated RC's) is not known. Correlating electron transfer efficiency to quinone accumulation over this temperature range should be done to reveal whether regulation of photosynthetic output over this temperature range occurs at the end stages of the light-driven RC reaction loop. This correlation could be further applied to interpret the effect of temperature stress on the photosynthetic output of various photosynthetic purple bacteria, and to explain population shifts in temperature-fluctuating micro-environments. This assumes that the effects being considered here also occur in membrane-bound RCs and in whole cells.

A smoothly varying stability profile, such as seen in hydrogen-bonded RCs, may be an adaptation advantage for photosynthetic organisms, which must survive daily or seasonal temperature fluctuations, and be able to quickly recover once conditions improve. Thus, being able to alter the RC conformation smoothly back to an optimum form could be a survival advantage. In contrast, deuterium-bonded RCs with two distinctly different conformations and a sharp transition in energetics could encounter complications. To regain the smooth dependence of conformational change with temperature seen in hydrogen-bonded RCs, a reoptimization of the distribution of non-covalent interactions in the RC might be required.

In conclusion, we show that deuterium-bonded RC complexes of *R. sphaeroides* PUC 705Ba have different temperature dependent conformations than their hydrogen-bonded counterparts. Deuterium-bonded RCs are most stable at 37 ºC, rather than at room temperature, and undergo a "cold denaturation" as the temperature is lowered to room temperature. The conformation of the deuterium-bonded RC complex can be changed at room temperature by the addition of amphiphiles as 1,3,5-heptanetriol which appears to bring the complex back to its more stable configuration.

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