"Glass Transition" near 200 K in the Bacterial Photosynthetic Reaction Center Protein Detected by Studying the Distances in the Transient $P^+Q_A^-$ Radical Pair

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The transient radical pair $P^+Q_A^-$ in the photosynthetic reaction center from *Rhodobacter sphaeroides* R26 was studied over a wide temperature range using out-of-phase electron spin−echo envelope modulation (ESEEM) spectroscopy. This method is sensitive to the magnetic dipole−dipole interaction between the two electron spins of the pair and allows precise determination of the distance in the pair $P^+Q_A^-$. The out-of-phase data were complemented by normal in-phase ESEEM spectra from the two stable radicals of P^+ and Q_A^-. The results seem to indicate that the radical pair undergoes a noticeable molecular motion around 200 K that may be characterized by a change in the distance in the pair by ∼0.3 nm. As the two cofactors, P^+ and Q_A^-, are held in a well-defined relative position by the reaction center protein, this means that the protein becomes flexible at 200 K. This effect may be ascribed to a dynamic glass transition around 200 K. The relation with the temperature dependence of the back reaction of $P^+Q_A^-$ is discussed.

Introduction

Proteins may undergo a dramatic change in their dynamical and/or structural properties at approximately 200 K. Above this temperature the amplitude of molecular motion increases remarkably. Such transition in proteins was determined by X-ray crystallography,[1,2] neutron scattering[3−5] and Mössbauer scattering.[6,7] Also, molecular dynamics simulations have shown the abrupt change of atomic motion near 200 K.[8−10] In many respects this transition resembles the behavior of the glass transition of simple molecular glass formers. Such a transition is known to affect the physiological function of proteins.[1,2,11]

Except for proteins, glass transitions have also been reported for biological membranes.[12−14] Also, in living biological organisms the presence of intracellular glasses has been well established.[15−17] For these organisms, such as seed and pollen, the formation of glasses has been correlated with the ability to survive a dry state over a long period.

All the experimental approaches used so far deal with the motion at the atomic scale. Whether such transitions affect the global properties of proteins as well is not known, mainly because experimental techniques to study distances at the nanometer scale are not available. Some molecular dynamic simulations indicate that global changes of the protein structure near the glass transition may occur.[8,10] One may speculate that these global changes are important because they may affect the physiological function of a protein.

In the present paper we provide evidence that the reaction center (RC) protein of photosynthetic bacteria experiences a change of the structure and/or dynamic at the nanometer scale near 200 K. We employ a recently developed electron spin−echo (ESE) approach to study transient radical pairs appearing after absorption of light by the RC.[18−20] In bacterial RCs, the spin correlated radical pair $P^+Q_A^-$ is generated by light, where P denotes the primary electron donor, a bacteriochlorophyll dimer, and Q_A is the primary electron acceptor quinone (for review see, e.g., ref 21). These cofactors are held in a well-defined relative position by the protein. The distance between P and Q_A is about 29 Å.[21] At low temperature the radical pair recombines in about 30 ms and the sample is restored to its initial condition. There is some evidence about an unusual behavior of distances in the pair $P^+Q_A^-$ near 200 K.[18] In the present work we have performed a more comprehensive investigation that includes the study of not only the radical pair but also the individual stable radicals P^+ and Q_A^-, which can be chemically generated in the RCs. For the first time the measurements were done in the temperature range from 30 to 220 K for all three paramagnetic states in the RC protein.

For a two-pulse microwave echo-forming sequence, as the inter-pulse separation τ is varied, the echo intensity is strongly modulated. This phenomenon is called ESE envelope modulation or ESEEM. For the spin-correlated radical pair the echo appears in the out-of-phase channel. The out-of phase ESEEM is mainly determined by the interaction between partners in the radical pair. Sine Fourier transformation of the out of phase ESEEM provides the resonance doublet determined by the magnetic dipole−dipole interaction between the two spins of the pair.[18−20] Two lines of the doublet appear (assuming point-dipole approximation) at the frequencies $\pm(1)/(2\pi)(\gamma^2\hbar)/(r^3)(3\cos^2\theta - 1)$, where γ is the gyromagnetic ratio, r is the distance between the two spins of the pair, θ is the angle between the vector connecting the two partners of the pair and the magnetic field of the EPR spectrometer. Spin exchange interaction between the two electrons is neglected, as it is known to be at least 2 orders of magnitude smaller than dipole−dipole interaction for this radical pair.[20]

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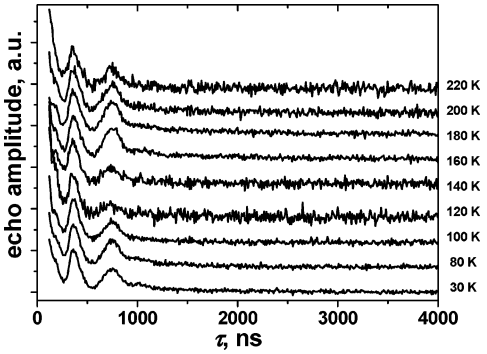

Figure 1. Two-pulse electron spin−echo signal as a function of time separation τ between microwave pulses at different temperatures for P^+ stable radical in RCs from *Rb. sphaeroides* R26. The first 100 ns of the echo modulation are obscured by the spectrometer dead time. All curves are normalized to the first maximum.

The two characteristic frequencies of the frequency resonance pattern, $f_\perp$ and $f_{||}$, correspond to the singularity at the perpendicular orientation ($\theta = \pi/2$) and to the edge of the frequency spectra attained at the parallel orientation ($\theta = 0$), respectively:

$$f_\perp = \pm \frac{1}{2\pi} \frac{\gamma^2 \hbar}{r^3} \qquad (1)$$

$$f_{||} = \mp \frac{1}{\pi} \frac{\gamma^2 \hbar}{r^3}$$

The plus and minus correspond to two components of the doublet. With eq 1 the distance r can be determined with accuracy better than 0.4 Å.[18−20,22]

For isolated stable radicals, ESEEM appears because of electron−nuclear hyperfine interaction (a normal in-phase echo for radicals in thermal equilibrium). Cosine Fourier transformation then provides a resonance pattern reflecting nuclear transitions.[23]

Experimental Section

RCs of *Rhodobacter (Rb.) sphaeroides* R26 were isolated according to ref 24. For the ESEEM measurements, the native Fe^{2+} ion ($S = 2$), which forms a magnetically coupled complex with the Q_A^- radical, was replaced with the diamagnetic Zn^{2+}.[25] A typical EPR sample contained 66% (v/v) of glycerol to provide a transparent glass when freezing. Electron spin−echo measurements were carried out on an Elexsys E-680X/E-580E FT EPR spectrometer equipped with a dielectric cavity (Bruker ER 4118 X-MD-5) inside an Oxford Instruments CF 935 liquid helium flow cryostat. Dead time caused by resonator ringing was about 100 ns. As a light source for sample irradiation inside the EPR cavity a Continuum Surelite I laser was used. The excitation wavelength was 532 nm. The repetition rate of the laser flashes of ca. 4 ns duration was 10 Hz. Microwave pulses were delayed after the laser flash by 1 μs. Q_A^- stable radical was generated in the RCs by reduction with sodium dithionite in the dark followed by rapid freezing.[26] To obtain the P^+ radical, the primary donor in the RCs was oxidized by adding $KFe(CN)_4$.

Results

Figures 1 and 2 show ESEEM time traces for samples with isolated P^+ and Q_A^- free radicals, respectively. The ESEEM from P^+ arises from interaction of the unpaired electron with nitrogen atoms of the dimer.[27,28] For Q_A^-, the ESEEM arises from interaction of the unpaired electron and the nearby nitrogen of the protein.[29] These are the first measurements of two pulse ESEEM for P^+ and Q_A^- in RCs in such wide temperature range (30−220 K).

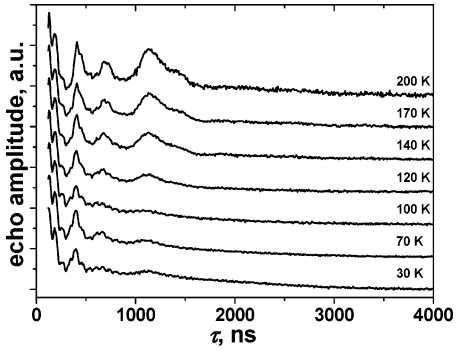

Figure 2. Same as in Figure 1, for Q_A^- stable radical.

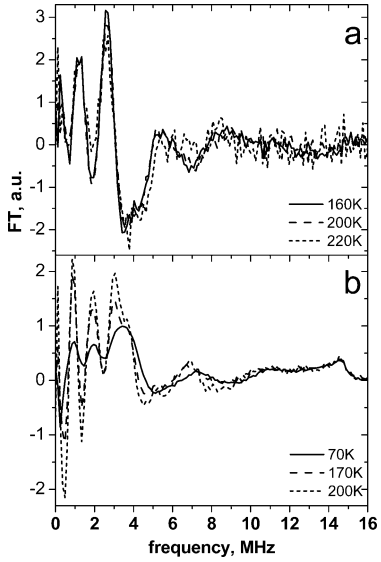

Figure 3. Cosine Fourier transformation of the time traces shown in Figures 1 and 2 at several temperatures for P^+ (a) and for Q_A^- (b) radicals (without dead time correction).

We only observed a slight temperature dependence for the modulation pattern of P^+ (Figure 1). Some shortening of the decay around 120 K is observed. This temperature is typical for echo relaxation induced by intramolecular rotation of methyl groups.[30] As the primary donor P contains several methyl groups, this explanation is reasonable. Cosine Fourier transformation of ESEEM time traces obtained after subtraction of the fitted exponential decay (electron−nuclear ESEEM contains nonmodulated contribution which normally decays exponentially) above 160 K is shown in Figure 3a.

The temperature dependence of the ESEEM pattern for the Q_A^- radical is more pronounced, showing that the oscillations become remarkably sharper above 100 K (Figure 2). Figure 3b shows the cosine Fourier transform of these ESEEM time traces (cf. with data in ref 29) and again shows that the resonance frequencies remain nearly unchanged between 30 and 200 K, but that the lines become sharper. Two different effects may induce the sharpening of lines with increasing temperature: (1) molecular motion that can average hyperfine interaction with the nuclei and (2) narrowing of the distribution of static conformations of the supramolecular complex of Q_A (or Q_A^-) and the protein backbone. Evidence for both mechanisms has been found. The temperature dependence of the distance distribution of the radical pair $P^+Q_A^-$ has been investigated.[20]

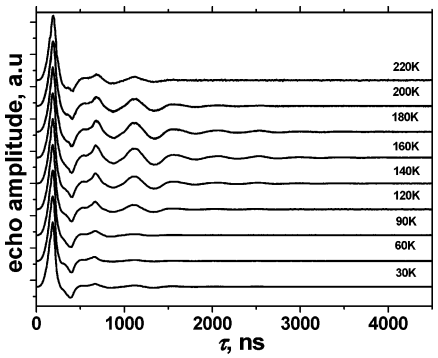

Figure 4. Same as in Figures 1 and 2, for the out-of-phase echo of the $P^+Q_A^-$ spin-correlated radical pair. In the dead time (~100 ns) signal was restored by parabolic interpolation.[20]

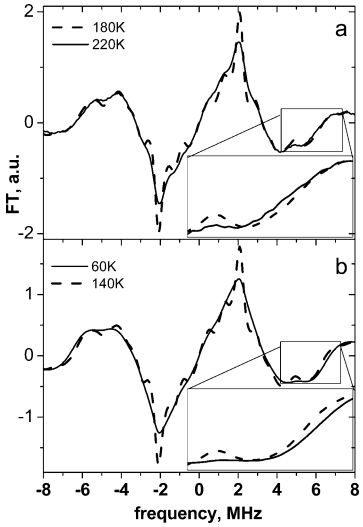

Figure 5. Sine Fourier transformation of the time traces shown in Figure 4 for two different temperature regions 180−220 K (a) and 60−140 K (b). The right-side wings are enlarged for better visualization.

Temperatures below 100 K resulted in a broader distribution of distances between P and Q_A, approaching ~4 Å at 15 K. Above 100 K the line shape became much narrower. Alternatively, anisotropic librational motion of the Q_A^- radical was found at 115 K −120 K.[31,32] Also, probing the local dynamics of the RC with a nitroxide spin label has shown the onset of librational motion at 100 K.[33] Such librational motion may induce averaging of the hyperfine interaction with nearby nuclei and can result in a more pronounced modulation pattern as seen in Figure 2 at temperatures above 120 K.

Figure 4 presents the out-of-phase ESEEM for the transient spin-correlated radical pair $P^+Q_A^-$. Noticeable changes occur in two temperature regions: the oscillations become sharper when the temperature is increased from 90 to 120 K and become more damped from 180 to 220 K.

Figure 5 shows the sine Fourier transform of the ESEEM time traces in temperature regions from 180 to 220 K (Figure 5a) and from 90 to 120 K (Figure 5b). The spectra show two main peaks at the frequency $f_\perp$; see eq 1. In Figure 5b additional structure around these peaks appears with increasing temperature. This structure may be readily assigned to the combination frequencies of electron−electron and electron−nuclear interactions.[34] Indeed, the offset frequencies of the small spectral features seen around the two main peaks in Figure 5 coincide with the peak positions seen at Figure 3b for the stable radical Q_A^-. Also, the onset of these features with temperature coincides with the onset of the sharp oscillations seen in Figure 2. Therefore the line narrowing between 90 and 120 K (Figure 5b) may be assigned to a narrowing of the corresponding electron−nuclear ESEEM frequency peaks.

The electron−nuclear ESEEM for P^+ radical may also influence the electron−electron ESEEM of the $P^+Q_A^-$ radical pair. However, this influence may be obscured by line broadening in the pair below 100 K and above 200 K and by contribution of ESEEM of Q_A^-.

Discussion

At temperatures above 180 K the behavior of the electron−electron ESEEM for the $P^+Q_A^-$ radical pair is clearly different from the electron−nuclear ESEEMs of the isolated radicals P^+ and Q_A^-. For the radical pair, the temperature increase results in clear line broadening in the frequency domain and in the loss of the additional structure around the two main peaks (Figure 5a), whereas the spectrum for Q_A^- becomes better resolved (Figure 3b) and the resolution for P^+ is almost unchanged (Figure 3a). Therefore, the trivial explanation of line broadening in the pair by shortening of transverse spin relaxation may be ruled out because it should be the same for the isolated radicals. (Note that some shortening of the decay seen for P^+ above 200 K (Figure 1) does not influence much on the line resolution in the frequency domain (Figure 3a)).

So, we may conclude that the damping of the ESEEM pattern seen in Figure 4 above 180 K, and corresponding broadening of two major peaks seen in Figure 5a, is induced solely by a change of electron−electron interaction in the $P^+Q_A^-$ radical pair.

Two possible mechanisms may induce this effect: (1) broadening of the distribution of pair conformations and (2) molecular motion of the pair resulting in a fluctuation of the radius-vector connecting the two cofactors. Both these phenomena reflect some structure and/or dynamical changes occurring in the protein matrix at the nanometer range of distances. In the case of broadening of the static distribution, an estimation of the distance changes could be done on the basis of computer simulation as performed in ref 20. The decrease of the intensity of the main peaks in Figure 5a between 180 and 220 K is around 30%. The same effect was found in computer simulation, for an increase of the distance distribution by 0.2−0.3 nm.

However, broadening of the distribution would result also in broadening of the spectral wings.[20,35] This is clearly seen in Figure 5b, where broadening of two major peaks at lower temperature is accompanied also by broadening of the spectral wings. The opposite effect is seen in Figure 5a: the broadening of two major peaks is accompanied by a small but still detectable shift of the spectral edges toward the center. Therefore, it is likely that the spectral changes seen in Figure 5a for increasing temperature are induced by molecular motion.

Some model calculations of the dipolar line shape for a moving radical pair were done in ref 36. For a pair of two electron spins tethered so that they may freely diffuse between two boundaries, an inner and outer radial limit, the set of spectra were calculated. Calculations show that for a moving pair the spectral singularities are broadened, and spectral edges are shifted toward the center of the spectrum. Spectral changes seen in Figure 5a qualitatively resemble those calculated in ref 36 for a diffusion coefficient between 10^{-10} and 10^{-8} cm^2/s, and for a ratio of maximum and minimum separations about 1.1 (i.e., these distance separations differ by 0.3 nm).

Finally, it is interesting to discuss also the probable relation of the found peculiarities of the RC protein dynamics and the

known behavior of the back recombination in the $P^+Q_A^-$ radical pair. Several studies of the temperature dependence of the back reaction rate of $P^+Q_A^-$ seem to indicate sudden changes around 200 K.[39−43] Many photoreactions have an "inflection point" between 150 and 190 K,[39] which was explained by electron tunneling with a temperature-dependent barrier width. The temperature dependence of the $P^+Q_A^-$ back reaction in aqueous solution was about 100 ms at 300 K and decreased to about 20 ms at 200 K.[40] In dehydrated films, no temperature dependence was found and the decay time was about 20 ms for the whole temperature range (50−300 K). These effects were ascribed to one or two phase transitions taking place between 200 and 300 K.[40] Simulations of the temperature dependence of $P^+Q_A^-$ back reaction using a model in which the back reaction is coupled to vibrational modes in the protein[41] showed that from 200 K and higher, the data could not be simulated with the model, which was ascribed to thermal expansion in the temperature of 200−300 K (see also ref 42). The $P^+Q_A^-$ lifetime in a set of mutants with different P/P^+ redox potentials was found to suddenly change around 200 K.[43]

Conclusions

The study of dipole−dipole interaction in radical pairs separated by several nanometers can provide new insights into the nature of protein dynamics. As was mentioned in the Introduction, the onset of molecular motion with increasing temperature above 200 K is clearly seen for many proteins by different techniques sensitive to motion at the atomic scale. From our present study we conclude that in bacterial RCs around 200 K a noticeable protein dynamics appears at the nanometer scale of distances. These nanoscale dynamics may be related to a glass transition occurring in the protein. The preliminary estimation of the spatial scale of the motion gives a value around 0.3 nm, at the total length of 3 nm.

The temperature dependence of the electron−nuclear two-pulse ESEEM of Q_A^- also indicates increased motion above 200 K at the atomic scale, as is evident from the line narrowing in the frequency domain with increasing temperature. Note also that recent investigation of molecular dynamics of spin labels in bacterial RC has revealed an increase of the spin label libration angle[33] above 200 K. Two other dynamical and/or structural transitions were reported also for this system at cryogenic temperatures. At temperatures below 100 K, the distance distribution between P and Q_A is broadened by ∼4 Å at 15 K.[20] In the present work we obtained from ESEEM of Q_A^- evidence that the distance distribution between Q_A and the neighboring protein also becomes larger below 100 K. Probably this distribution is averaged above 100 K by librational motion that becomes active for Q_A at this temperature:[31−33] i.e., motion averages anisotropic hyperfine interaction, sharpening therefore the lines in the frequency domain. However, the alternative explanation is also valid, that this effect is induced by a more ordered state above 100 K.[20] Note also that for these RCs ENDOR study on surrounding hydrogen-bonded protons shows an onset above 100 K of the decreasing of hyperfine interaction couplings.[37]

Another transition was found around 15 K that resulted in narrowing of the distribution of distances between P and Q_A as temperature decreases.[35] This was interpreted as the onset of a more ordered state at low temperatures, probably occurring by protein folding via low-height barriers determining the protein energy landscape. Recently, data obtained by K-edge X-ray absorption fine structure (XAFS) spectroscopy have shown evidence for protein matrix expansion near the Fe^{2+} site because of lowering the temperature to 15 K.[38] This explanation probably reflects the onset of a more ordered state postulated in ref 35.

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