Triplet Characterization and Dynamics of a Novel Pyrene Derivative Covalently Linked to Azurin

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The photoexcited triplet state of the thiouredopyrenetrisulfonate (TUPS) covalently linked to (1) α-acetyl lysine (^{3*}TUPS-L) and (2) lysine residue 122 of azurin (^{3*}TUPS-A) were studied by time-resolved EPR spectroscopy. The triplet spectra of ^{3*}TUPS-L and ^{3*}TUPS-A dissolved in water−glycerol (1:1) mixture, at freezing temperatures, show different temperature dependence. While in the case of ^{3*}TUPS-L, no apparent dynamic effects were noticed, ^{3*}TUPS-A exhibits a strong effect at 230 K. The dynamic effects were treated by line shape analysis with the discrete jumps model, suggesting that the environment in which ^{3*}TUPS-A is embedded is not frozen, allowing for fast molecular motion with a correlation time of $\sim 1.8 \times 10^{-10}$ s. These results are explained by the unique properties of the protein-bound water layers, which are kept in the liquid state even at relatively low temperatures, where the bulk water is frozen.

I. Introduction

Introducing artificial photosensitive redox active groups into proteins enables the study of photoinduced processes occurring in these macromolecules, such as electron and energy transfer.[1−4] Recently, thiouredopyrenetrisulfonate (TUPS) was shown to be a suitable chromophore for efficiently initiating electron transfer (ET) in several biological systems, including cytochrome *c*[5,6] and azurin.[5−7] In these studies the ET process was shown to proceed from the photoexcited triplet state of TUPS, which was identified and characterized using optical measurements.[5] It is evident that further characterization of the triplet state calls for EPR spectroscopy, which is capable of providing additional essential data associated with the triplet state.

In the present study we report on the light induced triplet state of 1-isothiocyanatopyrene-3,6,8-trisulfonate (IPTS) in three different configurations: (1) Free IPTS; (2) IPTS covalently linked to the amino groups of α-acetyl lysine, (TUPS-L); and (3) IPTS covalently linked to lysine residue 122 of azurin, (TUPS-A). All systems were dissolved in water−glycerol mixture and studied by X-band (9.5 GHz, 330 mT) time-resolved electron paramagnetic resonance (TREPR) spectroscopy. The high sensitivity of TREPR with respect to spectral and time resolution makes this spectroscopy suitable for detailed investigation of the paramagnetic species which are involved in light-induced reactions at time intervals of 150−200 ns, i.e., far below the triplet lifetime of the chromophores. While the free chromophore (IPTS) exhibits no EPR triplet spectra, the thiouredo adducts, TUPS-L and TUPS-A, clearly show triplet spectra over a wide temperature range of 154−230 K. Furthermore, although both TUPS-L and TUPS-A show TREPR spectra, only the latter system exhibits conspicuous triplet dynamics. These effects are reflected by the temperature-dependent spectra, which were treated via line shape analysis. We show that the spectral changes with respect to temperature can be analyzed in terms of the dynamics of the chromophore bound to the protein macromolecule, which is surrounded by water layers with typical properties different from those of the bulk water.

II. Experimental Section

TUPS-L was prepared by dissolving 37 mg of α-acetyl lysine (Sigma) in 2 mL of distilled water. The pH of the solution was adjusted to 9.5 with 5 M KOH. IPTS (5.5 mg, Lambda Fluorescence, Austria) was dissolved in 0.1 mL distilled water and added to the reaction solution while constantly stirring. The reaction was completed within 10 min. The pH of the resulting TUPS-L solution was brought to 7.0 by the addition of 5 M HCl. The TUPS-L solution was frozen and stored at 253 K until use. The TUPS-A derivative was prepared as described elsewhere.[7] Among the different derivatives obtained, the lysine 122 derivative was chosen for the TREPR experiments. The structure of TUPS-A is shown in Figure 1.

The EPR samples were prepared in 4 mm o.d. quartz tubes, degassed by several freeze−pump−thaw cycles on a vacuum line and sealed under vacuum. Samples (∼1 mM) were dissolved in 1:1 (per volume) water−glycerol mixture (melting point is ∼250 K).[8] Photoexcitation wavelength (355 nm) chosen according to the absorption spectra of TUPS, corresponds to one of its absorption peaks.[5] It is a selective excitation of TUPS, because the peptide and azurin do not absorb in this spectral region. The samples were photoexcited (5−7 mJ/pulse, 20 Hz repetition rate, 10 ns pulse duration) by the third harmonic of a Nd:Yag laser (Continuum 661−20). TREPR measurements were carried out on a Bruker ESP380 EPR spectrometer with field modulation disconnected. The EPR signals were taken from the microwave preamplifier and transferred to a LeCroy 9400 digital oscilloscope after being passed through a low-noise filter and amplified by a fast amplifier (10 dB). The transient traces

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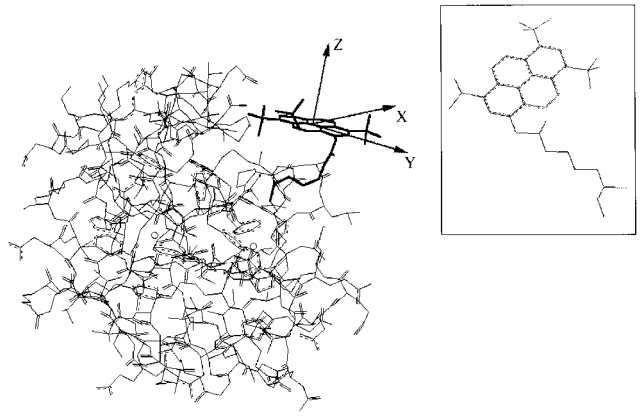

Figure 1. Computer drawn stick diagram depicting the minimum energy orientation of TUPS bound to lysine 122 of *Pseudomonas aeruginosa* azurin, as predicted by molecular modeling. TUPS and lysine residue 122 are outlined in bold. The inset to the figure shows an enlarged diagram of TUPS covalently linked to the ϵ-amine of lysine.

of the EPR signals were acquired and accumulated on a PC compatible Gateway 2000 through a GPIB interface. The spectra at relevant time windows after the laser pulse were reconstructed from the full set of data over a chosen magnetic field range. The response time of the EPR signal detection is 150−200 ns. The temperature was controlled by a Bruker variable-temperature unit, model ER411 VT.

Line shape analysis of the TREPR spectra of randomly oriented triplets, which do not depend on temperature, was carried out via the density matrix formalism, given elsewhere.[9,10] In this case such an analysis does not require dynamic parameters to account for the spectra. On the other hand, when the line shape depends on temperature, it is conceivable that dynamics may also be associated and should be taken into account.[11,12]

Two mechanisms could describe the dynamic processes: (1) rotational diffusion of the chromophores, which assumes random Brownian diffusion;[13] and (2) discrete solidlike jumps model, which assumes exchange between two principal magnetic axes of the dipolar tensor of the chromophore molecule about the third principal axis.[11,12,14,15] The latter process may account for triplet hopping between two sites, A and B, characterized by separate frames of reference, i.e., X, Y, Z and X′, Y′ ,Z′. Thus, orientational changes can be expressed in terms of the apparent triplet hopping that occurs between A and B. The relationship between the two principal axis systems of these two sites is expressed by the Euler rotation matrix.[11] In this case, line shape analysis provides the triplet magnetic parameters, the relative orientations of two configurations, and the triplet hopping rate k (or motion correlation time, $\tau = 1/k$). Although formally both mechanisms provide similar results for finite rotation angles, the discrete jumps model is more suitable for relatively low concentrations of chromophores dissolved in viscous or frozen media like water−glycerol at sufficiently low temperatures.[16,17] The EPR signal decay rates were extracted by fitting the experimental magnetization time profiles, $M_y(t)$, with the biexponential expression given elsewhere.[18]

Molecular dynamics calculations were performed to identify the optimal orientation of the TUPS residue on the protein molecule as described elsewhere.[19,20]

III. Results and Discussion

TUPS Bound to α-Acetyl Lysine (TUPS-L). Photoexcitation of the frozen solution of TUPS-L at 186 K gives rise to the spin-polarized triplet spectra, ^{3*}TUPS-L, with the polarization pattern *a,a,a,e,e,e,* from low to high field, where *a* stands for absorption and *e* for emission (Figure 2a). Line shape analysis of this triplet spectrum provides with the following zero-field splitting (ZFS) parameters: $|D| = (82.5 \pm 4)$ mT, $|E| = (17.5 \pm 1)$ mT, and relative population rates of the triplet sublevels via intersystem crossing, i.e., $T_i \leftarrow S_1$ (i = x, y, z) A_x:A_y:A_z = 1.0:0.0:1.15. Increasing the temperature to 230 K results in a slightly different triplet spectrum (Figure 2b). The analysis of this spectrum at 230 K gives the same ZFS parameters as at 186 K and relative population rates A_x:A_y:A_z = 0.5:0.5:1.15. In this temperature interval the slight spectral modification of ^{3*}TUPS-L does not indicate a noticeable dynamic effect. The change in population rates may be attributed to some molecular fluctuations of the chromophore at 230 K, as was observed in other systems as well.[17] The failure of observing the triplet EPR spectra at temperatures above 230 K is probably because of matrix softening.

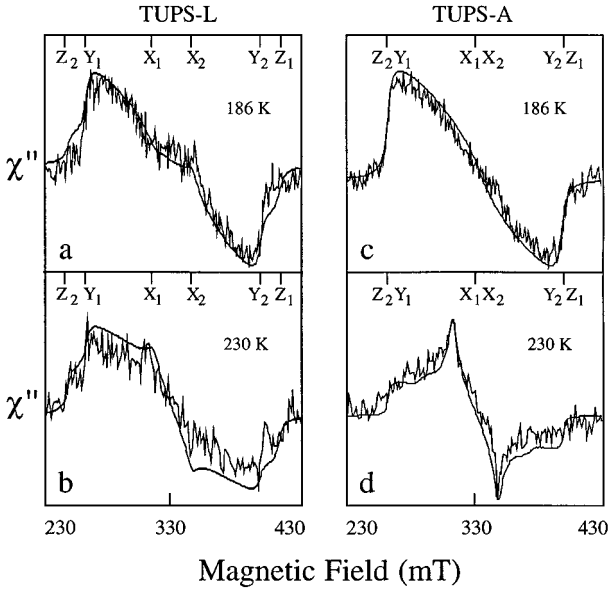

Figure 2. (a, c): TREPR (continuous wave, direct detection mode) triplet spectra of photoexcited TUPS-L and TUPS-A in water−glycerol (1:1) mixture at 186 K. (b, d): The same as (a, c) but at 230 K. Spectra taken at microwave power of 57 mW. The smooth lines superimposed on the experimental spectra are computer simulations.

Apart from the magnetic parameters, the spin−lattice (T_1) and spin−spin (T_2) relaxation times, $T_1 = (4.3 \pm 0.4) \times 10^{-6}$ s and $T_2 = (1.8 \pm 0.2) \times 10^{-7}$ s, respectively, were calculated from the corresponding kinetic profiles (not shown). These values were found within the experimental error to be temperature independent.

TUPS Bound to Azurin (TUPS-A). Figure 2c shows the TREPR spectrum of ^{3*}TUPS-A at 186 K. Comparison of this spectrum with that of ^{3*}TUPS-L shows that the triplet line shape, the polarization pattern, and relaxation times ($T_1 = (4.5 \pm 0.5) \times 10^{-6}$ s, $T_2 = (2 \pm 0.2) \times 10^{-7}$ s) are very close. However, the ZFS parameters ($|D| = (68.5 \pm 3)$ mT, $|E| = (22.8 \pm 1)$ mT) and relative population rates (A_x:A_y:A_z = 0:0:1) are significantly different from those of ^{3*}TUPS-L at 186 K. The smaller $|D|$ value indicates increased delocalization of the triplet electrons in the protein-bound TUPS molecule. Such delocalization might result from the different surroundings of the chromophore and, in turn, its different interactions with the neighboring groups of the azurin polypeptide chain. These interactions could also be responsible for the change in the population rates. The triplet line shape was found to be

unchanged between 186 and 230 K and could be simulated without any necessity of dynamic effects.

Unlike ^{3*}TUPS-L, the EPR spectrum of ^{3*}TUPS-A exhibits the sharp noticeable changes at 230 ± 2 K (Figure 2 c,d) as compared to 186 K. Attempts to simulate the high-temperature spectrum with the parameters extracted from those at 186 K failed. This implies that at 230 K chromophore motion starts to occur and affect the spectrum. This motion takes place in the water layers bound to the protein macromolecule. The hydration shells exist in the liquid phase despite the fact that the bulk temperatures correspond to the frozen state of water. This phenomenon has been suggested by a number of independent studies.[21−24] These hydration layers allow local conformational mobility, "breathing" of the proteins, and, consequently, functional activity of biomolecules even at relatively low temperatures.[25−32] Under such conditions, dynamics are allowed in the TUPS-A system at 230 K.

In terms of the ZFS principal axes and the dynamic part of the spin Hamiltonian,[11,17] three modes of discrete jumps are possible about the X-, Y-, and Z-axes. This leads to interchange between different dipolar axes and results in changes in the overall EPR line shape. The analysis was carried out under the assumption of a planar TUPS and that the Z-axis is perpendicular to the TUPS plane, whereas the X- and Y-axes lie in the TUPS plane, in such a way that the Y-axis lies along the bond connecting TUPS with azurin. The results of the simulations are given in Figure 2d. For this temperature, the simulations show discrete jumps of ∼5° about the Y-axis and of ∼60° about the Z-axis, with an exchange rate of $k \sim 5.6 \times 10^9$ s^{-1}. Such a process results in a significant exchange between the X- and Y-axes and a slight exchange between the X- and Z-axes. It is noteworthy that 230 K, the temperature where TUPS-A exhibits fast motion, coincides with the temperature where myoglobin conformational mobility was found to be sufficient for effective functioning.[25]

The predicted orientation of TUPS within the protein complex, calculated using molecular modeling[19,20] allows us to qualitatively estimate the possible extent of the rotation of TUPS along the given axes. It seems that rotations about the Y-axis, which lies along the bond connecting TUPS with lysine 122, are limited because of strike restrictions imposed by the polypeptide backbone, while rotation about the Z-axis, which is perpendicular to the molecular plane, can occur to a wider extent (Figure 1). Thus, there seems to be good agreement between the orientation and possible motions of TUPS as estimated from molecular modeling and as experimentally deduced by EPR.

To summarize, this work is a first EPR investigation employing TUPS chromophore which, when photoexcited, generates the triplet state with high quantum yield. With its redox properties, it may serve as an efficient electron donor in biological and biomimetic systems, as well as a probe for studying the dynamics of water−protein matrices. This report demonstrates the ability of TREPR spectroscopy to characterize the magnetic, kinetic, and dynamic parameters of chromophores and, thus, to complement and extend the data obtained by optical spectroscopy. The dynamic experimental results achieved are in very good agreement with those predicted by the molecular modeling.

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