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Resonance Raman Spectroscopy of the Neutral Radical Trp_{306} in DNA Photolyase

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Amino acid radicals play important roles in enzyme catalysis.[1] Tryptophan (Trp) and tyrosine are of special interest because of their role in electron-transfer (ET) processes, e.g., in DNA photolyase and ribonucleotide reductase (RNR).[1−3] Trp radicals have also been detected in cytochrome *c* peroxidase,[4] catalase-peroxidase,[5] and azurin.[6] These radicals have been studied by EPR and absorption spectroscopy, but these techniques sometimes lack time resolution or structural information content for investigations in real time. Vibrational spectroscopy has been identified as a powerful tool to study Trp radicals that can differentiate between cationic and neutral radicals and between radicals on different Trp residues.[7,8] Time-resolved and continuous-wave resonance Raman (RR) spectroscopy of Trp have been used to study allosteric changes and protein unfolding,[9,10] by using the sensitivity of several Trp vibrations to conformation, hydrogen-bonding, and protein environment.[11,12] The same strategy can be used with time-resolved RR (TR^3) spectroscopy to distinguish between radicals on conformationally different Trp residues and to follow radical migration in proteins in real time. However, experimental data to provide a basis for such studies and to test the predicted vibrational modes are scarce.[13−15] Here, we report the first RR characterization of a Trp neutral radical in a protein.

Escherichia coli photolyase repairs cyclobutane pyrimidine dimers of DNA in a light-driven, ET mechanism and contains two cofactors: flavin adenine dinucleotide (FAD), the active site, and 5,10-methenyltetrahydrofolate polyglutamate (MTHF), a light-harvesting pigment.[2] Isolated photolyase contains a catalytically inactive neutral radical semiquinone ($FADH^•$), which can be photoreduced to catalytically active $FADH^-$ with Trp_{306} as terminal electron donor.[2,16−22] There is no evidence that this process plays a role in DNA repair in vivo,[2,20] and the exact ET pathway for photoreduction is controversial.[2,22,23] $Trp_{306}^•$ is formed within 1 μs after excitation of $FADH^•$ and, in the absence of external electron donors, has a pH-dependent lifetime that ranges from 10 ms at pH 7.4 to <1 ms at pH 5.4.[17,18,21,22,24]

The RR spectra of DNA photolyase at pH 7.4 are shown in Figure 1.[25] Selective excitation of $FADH^•$ with 527 nm pulses initiates charge separation.[17] New vibrations are observed at a 1.5 kHz laser repetition rate due to excitation of the non-recombined fraction of $FADH^-−Trp_{306}^•$ in a previously excited sample volume after one full rotation of the spinning sample cell (Supporting Information). We hypothesize that the new vibrations are due to $Trp_{306}^•$, which is excited in its absorption band ($\epsilon_{525} \approx 2000\ M^{-1}\ cm^{-1}$) and resonantly enhanced by the 527 nm pulses.[22,26] At 0.5 kHz repetition rate, the new vibrations can be suppressed, and the spectra are identical to those previously reported for photolyase in its ground state and contain contributions from $FADH^•$ and MTHF.[27,28] The new vibrations are clearly enhanced after removal of buffer contributions and by subtracting the 0.5 kHz ($FADH^•$) spectrum from the 1.5 kHz spectrum after normalization on the

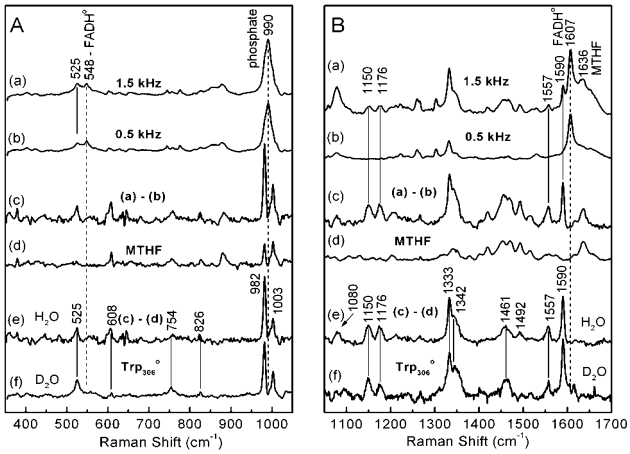

Figure 1. Low- (A) and high-frequency (B) resonance Raman spectra of *E. coli* photolyase obtained with 10 mW pulsed excitation at 527 nm at 1.5 (a) and 0.5 kHz (b) repetition rates. (c) 1.5 kHz − 0.5 kHz difference spectra after buffer correction. (d) Reduced photolyase: Raman spectra of MTHF. (e) $Trp_{306}^•$: spectrum c corrected for MTHF contributions. (f) $Trp_{306}^•$ in D_2O. Dashed lines: removal of $FADH^•$ and buffer vibrations.

1607 and 548 cm^{-1} modes of $FADH^•$. This difference spectrum (spectrum c) contains vibrations that are associated with the charge-separated pair $FADH^−−Trp_{306}^•$. $FADH^-$ has no absorbance above 500 nm,[29] and nonresonance Raman scattering of $FADH^-$ is very weak.[30] Although MTHF is not excited at 527 nm, its vibrations are enhanced because of reduced absorption of $FADH^•$ at 527 nm following its conversion to $FADH^-$. The Raman spectrum of MTHF (spectrum d) was obtained by reducing photolyase. Subtraction of this spectrum from spectrum c ($Trp_{306}^•$ and MTHF) results in the spectrum of an intermediate that we tentatively assign to $Trp_{306}^•$ (spectrum e). The spectrum in D_2O buffer (spectrum f) was obtained similarly.

The vibrations of the intermediate occur at 1590, 1557, 1492, 1461, 1342, 1333, 1176, 1150, and 1080 cm^{-1}. The low-frequency vibrations are weaker at 1003, 982, 826, 754, 605, and 525 cm^{-1}. A 1590 cm^{-1} vibration has also been reported in pump−probe RR studies of Trp and tryptamine neutral radicals in solution obtained with 532 nm probe pulses, while their overall spectra are very similar to the spectrum of our intermediate.[14] However, no additional vibrational frequencies were reported, and further comparison between the solution and protein data is not possible. Density functional theory (DFT) calculations of the indole neutral radical predict vibrational frequencies that are very similar to the ones determined in our study.[7] Finally, none of the vibrations of the intermediate are sensitive to H/D exchange, which is expected because the indole proton is absent in the neutral radical form. Given the light-induced formation of the $Trp_{306}^•$ intermediate in photolyase[17,20,21] and the agreement between our data and those reported in the literature,[7,14] we assign the intermediate to $Trp_{306}^•$ of photolyase, the first RR spectrum of a protein Trp radical.

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Table 1. Tentative Assignment of $Trp_{306}^{\bullet}$ Vibrational Modes (cm^{-1})

mode	W1	W2	W3	W4	W5	W7	W10	W16	W17	W18
$Trp_{306}^{\bullet}$ [a]	1590	1557	982	1461	1492	1342/ 1333	1080	1003	826	754
Trp[b]	1622	1575	1555	1496	1462	1361/ 1342	1238	1016	880	762

[a] This work. [b] References 11 and 35.

Our results are only in some agreement with a Trp intermediate previously reported in UV saturation RR experiments on Trp in solution.[13] The differences may be due the different excitation wavelengths and the fact that Raman saturation spectroscopy can give rise to multiple intermediates,[31] e.g., singlet excited state, triplet state, a charge-transfer intermediate, $Trp^{\bullet}$, and $Trp^{\bullet+}$.[26,32,33] In our case, the Trp radical is formed by intraprotein ET, not by direct photoionization via two-photon excitation as determined from the energy dependence of the intensity of the vibrations (Supporting Information), and we rule out that additional Trp intermediates contaminate our spectra. Low-temperature FTIR experiments on UV-irradiated Trp showed vibrations at 1612, 1465, and 1071 cm^{-1} that were assigned to $Trp^{\bullet}$.[15] We see no evidence for such vibrations in our RR spectra, but IR-active modes are usually weak or not active in Raman spectroscopy. Others have argued that the FTIR data are due to $Trp^{\bullet+}$, not $Trp^{\bullet}$.[7]

In an effort to assign the observed $Trp_{306}^{\bullet}$ vibrations, we compare our data to the theoretically determined frequencies for indole and 3-methylindole.[7,8] Some of the predicted frequencies are close to our experimentally determined frequencies, but this does not necessary mean that they describe the same vibrational mode. No experimental or theoretical isotope shifts are available, and we have used the theoretically predicted frequency difference between the parent molecule and its neutral radical for assignment of specific vibrational modes (Supporting Information). The W# normal-mode notation has been described previously.[34] Several modes are of special interest, because they are sensitive to Trp conformation (W3), hydrogen-bonding (W4, W6, W17), and protein environment (W7),[11,12] and can potentially be used to distinguish between conformationally different Trp radicals. Our tentative assignments are given in Table 1.

The W2, W4, W5, W16, W17, and W18 modes have very good agreement with both theoretical studies on the basis of experimentally determined and theoretically predicted frequency changes. For the W1 mode, one model predicts a frequency downshift[7] and the other a frequency upshift.[8] We find our data more in agreement with the former study. Significant differences are also predicted for W6 and W13, but our current data cannot discriminate between the two models. The W3 mode is of great interest, because it is sensitive to the torsion angle between the indole ring and the $C_{\beta}-C_{\alpha}$ bond and can be used to discriminate between conformationally different Trp residues.[11,12] Both theory studies predict a significant frequency downshift for W3 of $Trp^{\bullet}$, -571 and -442 cm^{-1},[7,8] and the $Trp_{306}^{\bullet}$ vibrations at 982 and 1150 cm^{-1} are good candidates. Because of the strong intensity of the Trp W3 mode, we slightly favor the 982 cm^{-1} mode of $Trp_{306}^{\bullet}$ because of its stronger intensity.[11]

In summary, we have obtained the first RR spectrum of a Trp radical in a protein, and our results demonstrate that visible excitation can be used to obtain these data, even though photolyase contains 15 Trp residues,[2] and $Trp_{306}^{\bullet}$ has a low extinction coefficient at 525 nm.[22] Biosynthetic isotopic labeling experiments are being prepared for accurate mode assignment. The current and future data will be of great importance for theoretical predictions of the properties of tryptophan radicals. Furthermore, TR[3] spectroscopy with visible excitation has also great potential to study migration of Trp radicals in proteins, especially in RNR, photolyase, and cryptochrome.[1−3,22,36]

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Supporting Information Available: Comparison of Trp and $Trp^{\bullet}$ vibrational modes, experimental conditions for observation of $Trp_{306}^{\bullet}$, and energy dependence of signal. This material is available free of charge via the Internet at http://pubs.acs.org.

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