

UV EXCIMER LASER CHEMICALLY INDUCED DYNAMIC NUCLEAR  
POLARIZATION OF AMINO ACIDS AT 360 MHz

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**SUMMARY:** A system has been developed which permits ultraviolet irradiation of samples in the probe of a 360 MHz nmr spectrometer using an excimer laser ( $\lambda = 249$  or  $308$  nm). This system is ideally suited for studying Chemically Induced Dynamic Nuclear Polarization (CIDNP) during photochemical reactions involving amino acids, proteins, and nucleic acids. The photoreagents quinoxaline and p-methoxyacetophenone prove to be the most promising for reactions with His, Trp, and Tyr. Spin polarized spectra are presented and interpreted according to the reaction mechanisms involved, the spin density distributions, and the relative g-factors of the radical intermediates. This system allows the use of a number of new photoreagents for studying protein structure and dynamics by laser CIDNP in solution.

INTRODUCTION

A number of groups have pursued the applicability of CIDNP to probe photochemical reactions of biologically interesting molecules (1-5). CIDNP is the observation of greatly enhanced nmr signal intensities (absorption or emission) in the diamagnetic products of reactions in which radical pairs are intermediates (6,7). Although high magnetic fields are clearly beneficial to improve spectral resolution in these complex molecules, it is very difficult to deliver the required high intensity light from an arc lamp to the high-resolution nmr probe, which is completely buried in the superconducting magnet. Laser excitation is an obvious solution to this problem, as has been demonstrated by Kaptein and in our laboratory using an Ar-ion laser (488 and 514 nm) to excite flavin dyes (4,5). These molecules reversibly attack accessible His, Tyr, and Trp residues, greatly enhancing their nmr signal intensities. We

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Abbreviations Used: CIDNP, Chemically Induced Dynamic Nuclear Polarization; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; esr, electron spin resonance; nmr, nuclear magnetic resonance; PMA, p-methoxyacetophenone; Qx, quinoxaline; UV, ultraviolet.

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1436

have shown that the reaction mechanism for Trp involves electron transfer (5), while that for Tyr involves hydrogen atom abstraction (4,5).

There are several limitations associated with the use of flavins. (i) Flavin photochemistry is complex: the dye is photobleached; the photoexcited flavin reacts with itself, both inter- and intramolecularly (8-10), and with oxygen (11); the flavin singlet excited state is relatively long lived and is quenched by several amino acids (12); the flavin radicals disproportionate and undergo degenerate exchange. Each of these processes reduces the observed polarization and obscures the interpretation of the flavin spin polarization. (ii) Flavins form ground state complexes with other aromatic molecules, particularly aromatic amino acids, which can complicate the interpretation of CIDNP in proteins (13). (iii) Flavin initiated CIDNP results in positive polarization of aromatic His and Trp protons and of the Tyr 2 and 6 protons (4). In protein nmr spectra these resonances frequently overlap, making them difficult to assign to particular amino acid residues.

Spin polarization resulting from direct photoionization of the amino acid and subsequent recombination or escape of the solvated electron would obviate the problem of a photoreagent altogether. One can also imagine many novel experiments which exploit extensive chemical modification of the molecule which initiates CIDNP, for example, to change the magnetic properties of its radical or to place it in a specific environment. Although there is an extensive literature on flavin chemistry, it is far easier to develop new reagents from simpler molecules whose photochemistry is well characterized and which might initiate polarization of additional amino acids as well. Most molecules of this type require UV light for excitation, and, for this reason, we have developed a 360 MHz nmr probe which can be used with a high powered excimer laser.

#### MATERIALS AND METHODS

The probe configuration and pulse sequence used in conjunction with the 360 MHz nmr spectrometer at the Stanford Magnetic Resonance Laboratory have been previously described (5). The light source was a Lumonics model TE-861 excimer laser: KrF, 249 nm, pulse repetition rate 50 Hz, average power 5 W at the laser. The laser was under computer control providing 1 sec irradiation prior to each

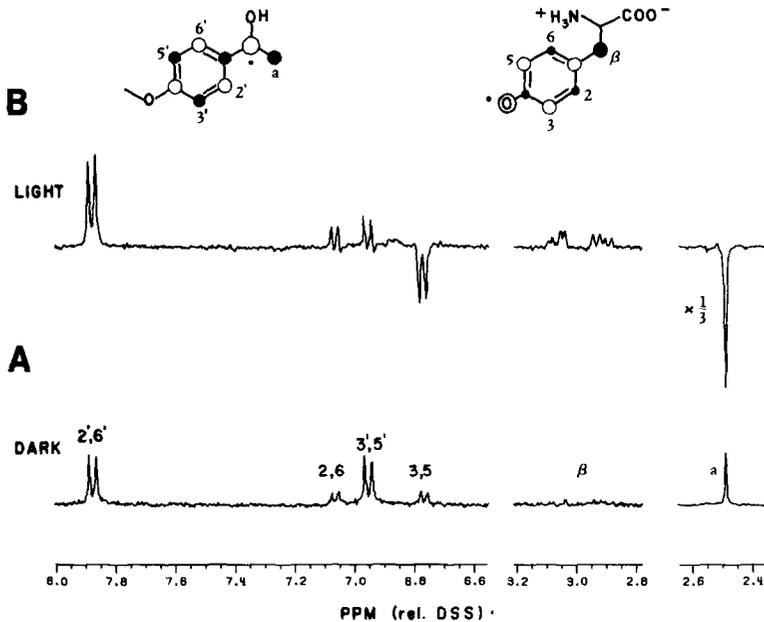


Figure 1: 360 MHz  $^1\text{H}$  nmr spectra of 9mM PMA and 2.5mM L-Tyr in  $[\text{}^2\text{H}]\text{H}_2\text{O}$ . (A) Normal dark spectrum; (B) spectrum immediately following 1 sec irradiation (249 nm). The weak emission on the upfield side of peaks 2,6 and 3,5 are the 3',5' protons of the two diastereomers of the pinacol. ○ and ● indicate the relative magnitudes of positive and negative spin density, respectively, in the illustrated radical intermediates.

rf pulse and data acquisition scan (single scan unless otherwise noted). Light was delivered to the sample in a quartz nmr tube via a quartz light pipe with an optically contacted  $90^\circ$  prism (5). For low field experiments, a probe was modified to allow irradiation of the sample in a Varian XL100 nmr spectrometer. The light source was a focused 300 W high-pressure xenon arc lamp (aluminum reflector) used in conjunction with a computer controlled shutter and directed through a water filter and quartz light pipe into the probe. Samples were not degassed, and all compounds were obtained commercially.

### RESULTS AND DISCUSSION

Direct photolysis of Trp, His, and Phe in unbuffered  $[\text{}^2\text{H}]\text{H}_2\text{O}$  with the excimer laser produces no spin polarization in the amino acid, and no new products are detected. Thus, it appears that simple photoionization to form the hydrated electron and the amino acid radical cation followed by recombination does not lead to CIDNP under these conditions. Direct photolysis of Tyr produces very weak polarization of Tyr itself, possibly by OH bond cleavage and recombination.

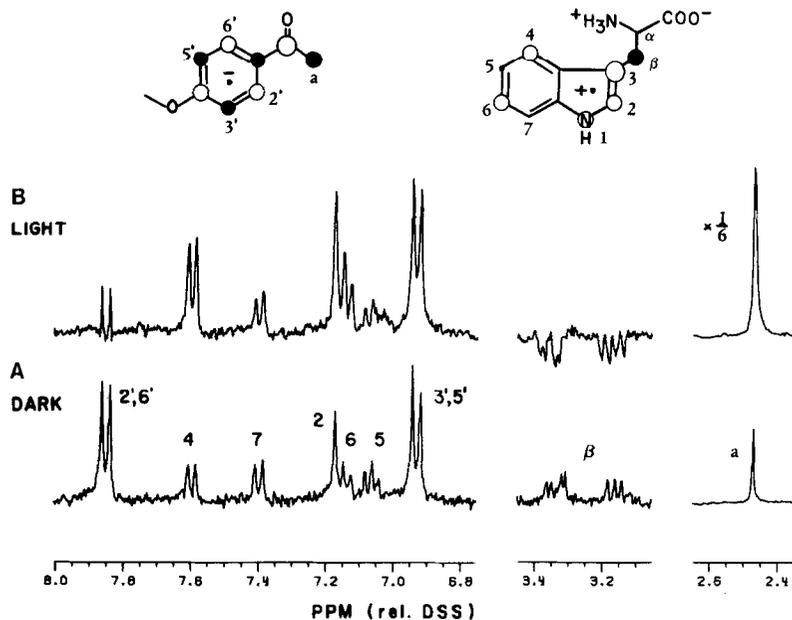


Figure 2: 360 MHz  $^1\text{H}$  nmr spectra of 13mM PMA and 10mM L-Trp in  $[\text{}^2\text{H}]\text{H}_2\text{O}$ . (A) Normal dark spectrum; (B) spectrum immediately following 1 sec irradiation (249 nm).  $\odot$  and  $\bullet$  indicate the relative magnitudes of positive and negative spin density, respectively, in the illustrated radical intermediates.

360 MHz CIDNP spectra resulting from UV irradiation of aqueous solutions of p-methoxyacetophenone (PMA) with Tyr and Trp are shown in Figures 1 and 2, respectively. It is well established that acetophenones are photoreduced by alcohols via hydrogen atom abstraction (14,15). This mechanism is entirely consistent with the results for Tyr, assuming a reversible reaction from the triplet state of the ketone, where the polarization is dominated by the geminate recombination pathway. The g-factor of  $\text{PMAH}^\bullet$  is probably only slightly greater than that of the corresponding acetophenone radical,  $g = 2.0031$  (16), and the g-factor for the Tyr radical is 2.0041 (17). Thus, the known spin density distribution shown for the Tyr radical in Figure 1 leads to the correct signs of the polarization according to the rules used to predict CIDNP spectra (6,7). In this reaction system the polarization of the photoreagent can be readily observed and interpreted, in contrast to the situation with flavins. Based on the mechanism above and the spin density distribution for  $\text{PMAH}^\bullet$  illustrated in

Figure 1, one predicts the observed spin polarization pattern. This spin density distribution is entirely consistent with  $^{13}\text{C}$  CIDNP data from other systems (18), esr data (19), and molecular orbital calculations (19).

In contrast, the reaction between photoexcited PMA and Trp appears to involve reversible electron transfer. This conclusion is supported by the observation that l-methylTrp shows the same polarization pattern. In this case the g-factor of  $\text{Trp}^{\dot{+}}$  is less than that of  $\text{PMA}^{\dot{-}}$ , thus, the aromatic proton polarization of Trp, which is associated with positive spin density at an adjacent carbon atom, is opposite in sign to that found for Tyr. Consistent with this, the PMA polarization is exactly reversed. Photoexcited p-methyl-, p-chloro-, p-hydroxyacetophenone, and acetophenone itself also produce the identical polarization patterns for Trp and Tyr and analogous polarization patterns for the acetophenones; we find that use of PMA gives the strongest CIDNP signals. His shows very weak polarization with PMA in  $[\text{}^2\text{H}]\text{H}_2\text{O}$  under conditions where the His aromatic protons would be strongly enhanced absorption with flavins. This selectivity should be sufficient to permit the preferential detection of Trp when both His and Trp are present in a protein.

Spin polarization is also detected in the pinacol formed from coupling of escaping acetophenone radicals (this is only detected with laser excitation, apparently because of the larger radical concentration). Since both DL and meso products are formed, two sets of peaks are detected. These consist of two singlets for the methyl protons (1.3-1.5 ppm) and both sets of aromatic multiplets. Consistent with the proposed mechanism, the methyl protons are in emission during photolysis with Trp, but in enhanced absorption with Tyr. .

We find that a very different photoreagent, quinoxaline (Qx), gives especially strong polarization with His and Tyr (Trp is also polarized, but more weakly). The photo-CIDNP spectrum of Tyr and Qx is shown in Figure 3. As in the case with PMA, the polarization patterns can be interpreted as arising from reversible hydrogen abstraction. If the reaction is conducted in  $[\text{}^2\text{H}](\text{CH}_3)_2\text{SO}$

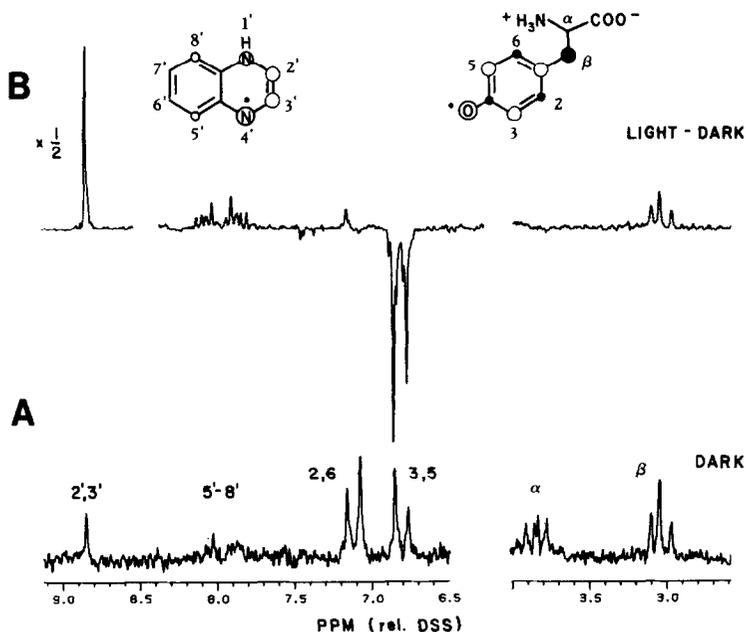


Figure 3: 100 MHz  $^1\text{H}$  nmr spectra of 1mM Qx and 2.5mM L-Tyr in  $\text{D}_2\text{O}$ . (A) Dark spectrum, 48 pulses signal averaged; (B) spectrum immediately following 1 sec irradiation (arc lamp).  $\odot$  and  $\ominus$  indicate the relative magnitudes of positive and negative spin density, respectively, in the illustrated radical intermediates.

rather than  $[\text{^2H}]\text{H}_2\text{O}$ , the phenolic proton of Tyr shows strong positive polarization. This is consistent with the proposed mechanism, as this proton is expected to reside on the Qx nitrogen in the  $\text{QxH}^\bullet$  radical, which is known to have a large positive spin density (20,21).

For the reaction between Trp and photoexcited Qx, we find the same pattern of polarization for Trp as observed in Figure 2, which is consistent with an electron transfer mechanism. The sign of the polarization indicates that the  $g$ -factor of  $\text{Trp}^\dagger$  is less than that of  $\text{Qx}^\ominus$ . If the reaction is carried out in  $[\text{^2H}](\text{CH}_3)_2\text{SO}$ , positive polarization of the Trp 1H proton is observed. The  $\beta$  protons of Trp show a multiplet effect (absorption/emission) superimposed on a negative net effect at 100 MHz, suggesting that the  $g$ -factor difference between these radical intermediates is smaller than that in the corresponding flavin reactions. Reaction with photoexcited Qx produces very strong positive

polarization for His. The g-factor of the imidazole radical is 2.00226 (22), thus, the spin polarization pattern indicates that the aromatic ring proton hyperfine coupling constants are negative in the His radical. The Qx polarization is completely consistent with this assignment.

In summary, we have demonstrated the utility of excimer laser photo-CIDNP with appropriate reagents to study photochemical reactions involving residues in proteins. Since PMA leads to very weak spin polarization of His, while Qx produces strong polarization of this amino acid, it should be possible to distinguish these residues from overlapping positively polarized Trp and Tyr residues in the photo-CIDNP spectra of proteins. Although we have used the KrF excimer emission at 249 nm for the initial experiments reported here, sufficient power is also available from XeCl (308 nm), which would minimize photochemical damage to the protein. This technology greatly extends the range of photochemical reactions which can be studied by the photo-CIDNP method at high field, and a number of applications to proteins will be reported shortly.

#### ACKNOWLEDGEMENTS

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