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Aryl Azide Photochemistry in Defined Protein Environments

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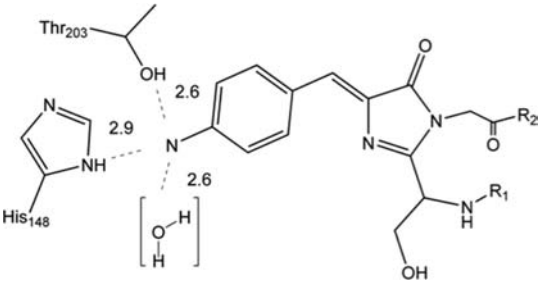
ABSTRACT



A genetically encoded precursor to an aryl nitrene, *para*-azidophenylalanine, was introduced site specifically into proteins to deduce if distinct environments were capable of caging a reactive organic intermediate. Following photolysis of mutant T4 lysozyme or green fluorescent proteins, EPR spectra showed, respectively, the presence of a triplet nitrene and an anilino radical.

Chemists have long been intrigued by the reactivity of phenyl nitrene and closely related derivatives.[1] Early product studies demonstrated the widely varying reactivity of nitrenes.[2,3] Subsequent attempts to unravel the chemistry of photochemically and thermally activated aryl azides were to an extent impeded by the fact that different experimental techniques sometimes gave incomplete and even misleading clues about the underlying mechanism.[4,5] Now, with detailed spectroscopic and mechanistic analysis the reaction pathways of many aryl azides are known under a variety of conditions.[4,6−10] The obvious benefits extend to the many bioconjugate[11] and cross-linking applications of nitrenes.[12]

The work presented here also found nitrenes, in the form of the genetically encoded[13] noncanonical amino acid (ncAA) *p*-azidophenylalanine (AzPhe),[14] useful in evaluating a novel approach for generating and caging a reactive intermediate in proteins. Previously, a variety of structures have been used to cage nitrenes, including: calixarenes,[15] hemicarcerands,[16,17] cavitands,[18] and cryptands.[19] Our approach differs from these reports in that our chemical

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synthesis relied not on multistep organic synthesis, but rather was performed using standard protein expression protocols; also, because our method is easily amended with standard recombinant techniques, it allows exploration of chemical space that would be difficult to access by synthetic organic methods.

To begin, three microenvironments were identified from crystallographic data to test the nitrene caging hypothesis: (1) the surface of a protein, (2) the core of a protein in close proximity to hydrogen-bond donors, and (3) within a protein cavity (Figure 1). Since photolysis of phenyl azides at 77 K can favor the formation of the triplet nitrene,[20] continuous wave (CW) EPR was used to probe for such intermediates.[19,20] It is also important to note that any nitrene generated under caging conditions is incapable of the bimolecular chemistry (i.e., dimerization with azo formation) that has historically plagued identification of many phenyl nitrene intermediates.

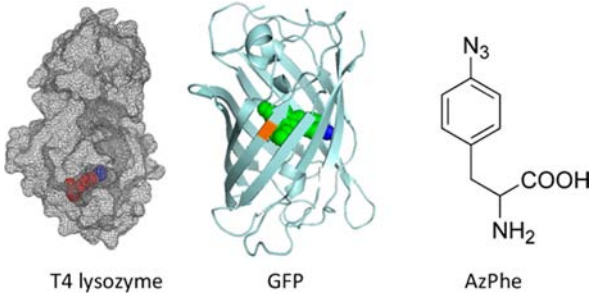

Figure 1. Target proteins showing the positioning of AzPhe. The AzPhe residue is colored red in T4 lysozyme and is shown in green as part of the chromophore in GFP. The putative nitrene N is colored blue in both structures. The location of the surface AzPhe mutant of GFP is shown in orange.

T4 lysozyme is noted for its well-defined cavities,[21] and indeed, the enzyme was shown to tolerate *p*-iodophenylalanine (iodoPhe) at residue 153 within nonpolar cavity 3.[22] Using the same strategy, we generated a Phe153AzPhe T4 lysozyme mutant.[23] The site-specific incorporation of AzPhe (Figure S1) was typical of a high fidelity ncAA system where high fidelity entails two competing scenarios:[24,25] (a) AzPhe is incorporated at the recoded position or (b) AzPhe is not incorporated, and protein synthesis ceases at residue 152 due to the presence of the recoded UAG stop codon at 153. The latter scenario results in truncated material that can be easily purified away full-length (as is any residual free AzPhe) using standard affinity purification, guaranteeing that the mutant protein possesses AzPhe at the desired position.[26]

Irradiation of AzPhe153 T4 lysozyme at 77 K showed a typical ($S = 1$) triplet nitrene EPR spectrum (Figure 2a).[19,20] Although EPR cannot detect other species known to form, exposure was limited to 2 min to minimize their formation.[27] The internuclear distances between the putative aryl nitrene N and each H-donor may be estimated from crystallographic data (Figures 3 and S3). Calculations[28] have suggested a significant barrier for triplet phenyl nitrene to abstract a hydrogen, which may explain why the triplet was observed.

An AzPhe34 GFP mutant was included as a control, wherein the triplet nitrene formed would likely be solvent-exposed on the protein's surface. Here, no EPR signal was detected upon irradiation of this mutant.[29] A second GFP control, an AzPhe66 mutant, also did not indicate any evidence of triplet nitrene after photolysis but, surprisingly, generated a spectrum that was unusually wide (*ca.* 330 G) for an $S = 1/2$ organic spin system, possessing an anisotropic profile in the frozen matrix (Figure 2b). The spectrum is assigned to an anilino radical.

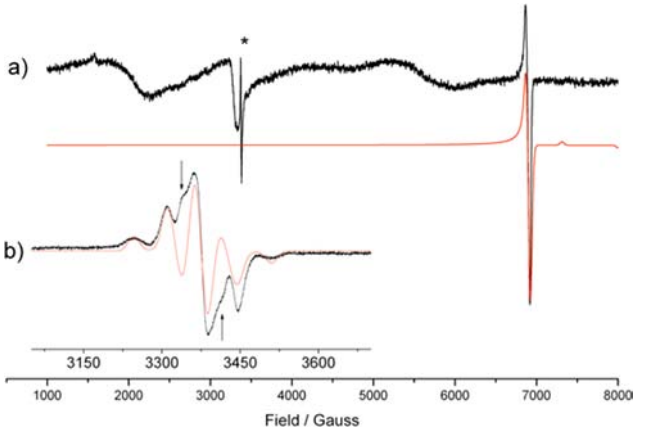

Figure 2. X-band CW EPR spectra (10K) obtained after UV photolysis at 77 K of (a) AzPhe153 T4L and (b) chromophore bound AzPhe66 GFP. [Arrows indicate contribution from unspecified radical; * = cavity artifact in the free spin region; red traces = simulated spectra.]

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(29) Clearly, this only rules out species that are EPR active (i.e., triplet or anilino radical, etc.). Mass spectrometry shows the loss of 28 Da, indicating the azide was indeed activated.

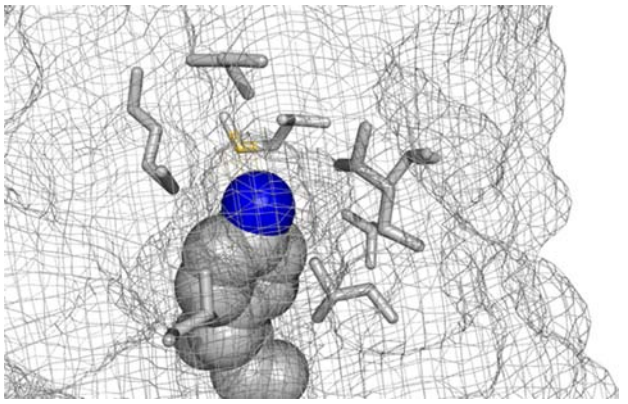

Figure 3. A model of the T4 lysozyme cavity showing the putative orientation of AzPhe153 with its six nearest residues. The nearest side chains are all nonpolar residues with approximate separations ranging from 3.3 to 5.5 Å. The nitrene N (blue) was modeled by replacing the iodoPhe residue for AzPhe from a known PDB entry (ID:1T6H).

The spectrum shown in Figure 2b is dominated by an intense profile with an apparent five-line pattern that upon first inspection does not appear to arise from a radical pair, owing to both the absence of any $\Delta ms = \pm 2$ transition and the presence of the intense central feature in the experimental spectrum. A smaller background contribution assigned to a second unknown radical (marked with the arrows in Figure 2b) is also observed. Platz and co-workers reported a related EPR spectrum which they assigned to a radical pair arising from an aryl nitrene abstracting a hydrogen within a frozen ethanol matrix.[30] However, no simulation or half field transition was given to support their assignment. Direct comparison with Platz's work is also complicated by the differences in experimental design. Their experiments were performed in homogeneous ethanol solutions with (electron withdrawing) 2,6-difluorophenyl nitrene vs our variable matrix and a previously unknown nitrene strucuture based around incorporating an (electron donating) imidazolidinone ring system in conjugation with the nitrene (Figure 4).

The dominant features in Figure 2b were qualitatively simulated based on large −NH (^{1}H, ^{14}N) and phenyl ($^{1}H_{ortho}$) hyperfine couplings (hfc's) of $^{H}a_{iso}$ = 78.5G (220 MHz), $^{N}a_{iso}$ = 31.5G (88 MHz), and $^{Hortho}a_{iso}$ = 10G (28 MHz) respectively (Supporting Information, Table S1). The putative background radical was not added to the simulation (as the hfc's were not sufficiently well resolved), hence the subtle discrepancy in the fit between the experimental and simulated spectra. The calculated hfc's are 3−4 times larger than those reported for the simpler parent $C_6H_5NH\bullet$ anilino radical ($^{H}a_{iso}$ = 16.85G, $^{N}a_{iso}$ = 8.85G, and $^{Ho,p}a_{iso}$ = 6.47G) generated in a solid

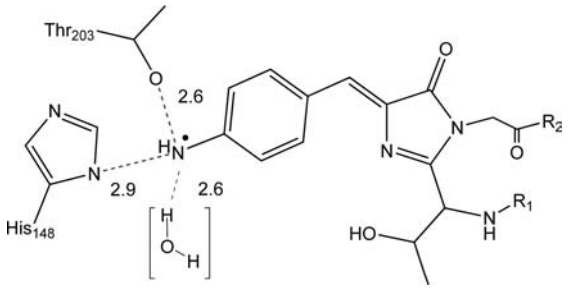

Figure 4. A model of the GFP-bound AzPhe66 anilino radical. Distances (in Å) between the nitrene (anilino) N and the nearest residues were estimated from crystallographic data of hydrogen bonds to Tyr66 in the wild type GFP (PDB 1EMA). [R_1 signifies residues N-terminus-to-64, R_2 residues 68-to-C-terminus].

crystal.[31] DFT calculations on the chromophore bound anilino radical suggest the difference in hfc's is partially due to the electronics of the imidazolidinone ring system (Supporting Information). These calculations revealed not only spin delocalization over the chromophore but also a slight electron-donating influence for the imidazolidonone ring, which in turn increased the spin density on the terminal N atom. This implies that the larger −NH hfc's observed are an expected trend for this previously unknown nitrene.

The literature provides several mechanisms that could account for an anilino radical (Figure S4). Importantly, each of these mechanisms culminates in the formation of an amine as one potential product.[32] An amine should impart significant effects on the fluorescent properties of a protein such as GFP. Indeed, the final fluorescence spectra of photolyzed AzPhe66 GFP are fully consistent with the spectra reported for *p*-aminophenylalanine substituted at the same 66 position by Schultz and co-workers (Figure S2),[33] thus lending support for an overall mechanism: nitrene → anilino radical → aniline (i.e., aminoPhe).

Although the photophysical and photochemical properties of GFP and aryl azides are routinely revised in the literature, support for our general mechanism comes from a number of disparate sources, including electron−proton transfer studies of hydrogen bonded dyes,[34] the excited state dynamics[35] and photoreductive power[36] of GFP, and the direct photoreduction of aryl azides.[37,38] All of these are suggestive of features of the same mechanism.

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More direct support can be found in the nitrene literature with both inter- and intramolecular nitrene reactions,[28,39–42] including one involving a protein.[43] To account for the final amine product, a variety of differing mechanisms have been offered, including electron transfer,[44,45] direct photoreduction of the aryl azide,[38] and tunneling.[39,46,47] Work in our group is currently directed at dissecting the complicated interplay between GFP photophysics and nitrene photochemistry. However, our initial contribution may simply be in confirming what has long been speculated in the nitrene literature: that such "dramatic" EPR line broadening effects, as reported here, are: "...probably a preliminary to a nitrene-hydrogen abstraction reaction."[48]

In summary, *p*-azidophenylalanine has been genetically encoded to probe the ability of a protein to cage a highly reactive intermediate. Evidence suggests a triplet aryl nitrene and an anilino radical have been observed. Generating a reactive intermediate site selectively within a protein opens up intriguing possibilities for studying processes such as tunneling in protein radical chemistry[49] or in directly reducing aryl azides.[37] Since the composition of the caging matrix appears to correlate well with the species observed, these results suggest a general approach for possibly studying other reactive intermediates using an expanded genetic code.

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Supporting Information Available. Experimental details and spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

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The authors declare no competing financial interest.