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Why do *p*-nitro-substituted aryl azides provide unintended dark reactions with proteins?

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ABSTRACT

Aryl azide-mediated photo cross-linking has been widely used to obtain structural features in biological systems, even though the reactive species generated upon photolysis in aqueous solution have not been well characterized. We have established a mechanistic framework for the formation of adducts between photoactivated 5-azido-2-nitrobenzoyl reagents and protein functional groups. Photolysis of the aryl azide tethered to biotin via an amide linkage yields a cross-link with streptavidin. The ability of the pre-irradiated reagent to form a similar cross-link indicates that it is the long-lived reactive intermediate that contributes to the cross-link formation. The reactive intermediate forms an adduct with tryptophan. The sequence of the labeled peptide is found to be GlyTrp*ThrValAlaTrp*LysAsn, corresponding to residues 74–81 of the streptavidin sequence, where Trp* designates the modified Trp-75 and Trp-79. A peak at *m*/*z* 1455.1 corresponding to the calculated $[M_{peptide}$ + aryl nitrene + 2O$]^+$ molecular ion value has been observed for the labeled peptide. Product structure identification experiments support the assignment that the long-lived reactive intermediate is a *p*-nitro-*N*-arylhydroxylamine, which undergoes a number of transformations in aqueous solution leading to nitroso derivatives. A plausible mechanism of the interaction between tryptophan and nitroso compound is discussed.

1. Introduction

Aryl azide-based photo cross-linking has been widely employed to obtain information about the higher order structure of protein–protein and protein–nucleic acid complexes [1–6]. Despite widespread use, basic features of aryl azide chemistry in the context of an aqueous solutions and biological environment have not been widely investigated [7–9]. In an effort to shed light on some of the problems, we have undertaken a study of the light-induced reaction of the 5-azido-2-nitrobenzoyl reagents. Earlier, we used some of these particular aryl azides in photoaffinity probing [10,11]. Several closely related photocompounds were previously used for the photoaffinity labeling studies in other laboratories (e.g. [5,12,13]). However, the mechanism of the azides action was not clear.

p-Nitro-substituted aryl azides and cross-linking species produced by their photolysis have been studied extensively [9,14–18], although generally in a non aqueous environment [9,14,15]. Four reactive intermediates, the singlet nitrene, triplet nitrene, ketimine, and nitroso compound, resulting from the photolysis of aryl azide, have been identified. In any single system, one or more of these intermediates may be present [14]. Solvents [9,14,16], temperature [14], aryl azide concentration [14], and light source intensity [14] can contribute to the relative yields and reactivity of the potential reactive intermediates. Photolysis of 4-nitrophenyl azide in benzene and acetonitrile (when O_2 is absent) primarily gives 4,4′-dinitroazobenzene; 4-nitroaniline (4%) is formed along with azobenzene in methanol; and when O_2 is present, *p*-dinitrobenzene, 4-nitrosonitrobezene, and 4,4′-dinitroazoxybenzene are also formed. The aryl azide irradiation in acetonitrile under low-power condition (O_2 is present) gives the mixture of azo (20%), nitroso (3%), nitro (12%) and azoxy (28%) compounds. Photolysis of the azide in acetonitrile under high-power conditions (O_2 is present) gives the mixture of azo (97%) and nitroso (3%) compounds. The similar products were observed when 4-nitro-substituted aromatic azides were irradiated in aqueous solution [16–18]. Also, the irradiation of an aqueous deoxygenated solution of this aryl azide gave 4,4′-dinitroazoxy-

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benzene derivative [16–18]. The formation of azoxycompound in the absence of O_2 is an intriguing process. Till now these aryl azide photolysis products were considered to be a result of triplet nitrene chemistry [9,18–20]. Photoactivation of any aryl azide initially produces the corresponding singlet nitrene. The singlet nitrene can undergo intersystem crossing to the triplet. Triplet niternes can react: (i) with O_2 to eventually form the corresponding nitroso, azoxy, and nitro compounds, (ii) with another triplet nitrene or with unreacted precursor azide to form an azo dimer, (iii) via electron abstraction from reducing agents, or (iv) by hydrogen-atom abstraction to form an amino compound.

For the first time we proposed *p*-nitrosubstituted *N*-arylhydroxylamine as a precursor of the photolysis products mentioned above [17]. In our previous investigation [16,17], we reported that a reactive intermediate such as *N*-arylhydroxylamine could be formed due to the photolysis of *p*-nitrosubstituted aromatic azide in aqueous solution. A primary aromatic hydroxylamine is susceptible to oxidation, disproportionation and condensation [21]. From the point of view of affinity labeling the most interesting hydroxylamine paths are disproportionation and oxidation by the molecular oxygen. In both cases the nitroso compound, which is reactive towards protein functional groups, may be formed. To provide a framework for interpreting unintended dark reactions of aryl azides with proteins, we have investigated the chemistry of the labeling products produced in aqueous solution upon reactions of the pre-irradiated 5-azido-2-nitrobenzoyl reagents with streptavidin. In the course of this work photointeraction of 5-azido-2-nitrobenzoyl azide with tryptophan residue within the streptavidin and a model compound was investigated. Products of this reaction were revealed and the mechanism was discussed.

2. Materials and methods

2.1. Chemicals

Streptavidin was purchased from Vector (Novosibirsk, Russia); d-biotin, proteinase K and TFA were from Sigma (St. Louis, MO, USA); Gnd·HCl was purchased from Fluka (Buchs, Switzerland); Tris(hydroxymethyl)-aminomethan was obtained from Merck (Darmstadt, Germany); ethyl acetate and EDTA were from Reakhim (Russia); acetonitrile was purchased from Kriochrom (St. Peterburg, Russia); D_2O, methanol-d_4 were obtained from Aldrich (Germany). Other reagents were of analytical grade. Solvents were purified according to standard procedures. d-Biotin-2-nitro-4-sulfophenyl ester was obtained using the method previously described for the protected amino acids [22]. *N*-(5-azido-2-nitrobenzoyl)-1,3-diaminopropane hydrochloride was synthesized and kindly provided by Tamara M. Ivanova (Novosibirsk Institute of Bioorganic Chemistry, Russia). Pentafluorophenolic ester of Boc-Trp was prepared and kindly provided by Vladimir N. Sil'nikov (Institute of Chemical Biology and Fundamental Medicine, Russia).

2.2. General methods

2.2.1. Spectra

Electronic absorption spectra were recorded on a Specord M-40 (Karl Zeiss, Jena, Germany). Infrared (IR) spectra were recorded on a Bruker IFS 66 (Bruker, Rheinstetten, Germany) spectrometer in KBr. ^1H and ^{13}C NMR spectra were recorded on an AV-300 spectrometer (Bruker, Rheinstetten, Germany) at 400.13 and 100.61 MHz frequency, respectively. Recording of the spectra was carried out at 25 °C in 5 mm ampoule. All ^1H chemical shifts were calculated relative to the residual signal of the solvent (D_2O, δ = 4.80 ppm and CHD_2OD, δ = 3.34 ppm). The ^{13}C chemical shifts are reported relative to the solvent (CD_3OD, δ = 49.3 ppm).

Mass spectra were recorded on a Vision 2000 (Thermo Bio Analysis, Manchester, UK) MALDI time-of-flight (TOF) mass spectrometer. A nitrogen laser (VSL-337ND, Laser Science, Newton, MA, 337 nm with 3-ns pulse width) was used. 2,5-Dihydroxybenzoic acid was used as a matrix. A 0.1–0.5 mM aqueous solution of the modified peptides (0.4 μL), compounds 5-azido-2-nitro-*N*-{3-[5-(2-oxohexahydrothieno[3,4-d]imidazol-6-yl)pentanoylamino]propyl}benzamide (**I**), *N*-{3-[2-amino-3-(1H-indol-3-yl)-propylamino]-propyl}-5-azido-2-nitrobenzamide (**II**) and photogenerated products were added to 0.3 μL of 2.5-dihydroxybenzoic acid solution (20 mg/mL in 20% (v/v) aqueous acetonitrile). The mixture was deposited on the stainless steel multiprobe and allowed to dry before being introduced into the mass spectrometer.

2.2.2. Chromatographic methods

Analytical TLC was carried out on DC-Alufolien Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany) using 9:1 (v/v) chloroform–methanol (system A) and 3:2 (v/v) chloroform–methanol (system B) as eluents.

Gel filtration chromatography, analytical ion-exchange microcolumn chromatography (analysis of the affinity of compound **I** toward streptavidin) and reversed-phase liquid chromatography (HPLC) (purification of the biotin derivative **I**, analysis of *N*-{3-[2-amino-3-(1H-indol-3-yl)-propylamino]-propyl}-5-azido-2-nitrobenzamide (**II**) photoproducts and isolation of the labeled peptides from the streptavidin hydrolysis mixture) were performed on a Milichrom-4 chromatograph (Nauch. Pribor, Orel, Russia) [23]. The relative amounts of photoproducts were estimated by integration of the corresponding chromatographic peaks, which were registered by multiwave detection using a program supplied with the chromatograph (the author is A.P. Zenkov, Institute of Chemical Biology and Fundamental Medicine, Russia).

Gel filtration chromatography was carried out on a Sephadex G-25 column (5.5 × 150 mm, Pharmacia Fine Chemicals, Sweden). Deionized water was used as eluent at a flow rate of 0.1 mL min^{-1}.

Analytical ion-exchange microcolumn chromatography was performed using a Polisil-SA 15 μm column (1.5 × 45 mm, Vector, Novosibirsk Russia) and a gradient of NaCl in 0.01 M Tris–HCl (from 0 to 0.25 M, pH 7.5, 50 μL min^{-1}).

Purification of the photobiotin analogue **I** was performed using a Silosorb C_{18}, 10 μm column (1.5 × 45 mm, Merck, Darmstadt, Germany), and a linear gradient of acetonitrile (from 0% to 70% for 20 min, 100 μL min^{-1}).

The analysis of the reaction mixtures obtained after hydrolysis of the photomodified and nonmodified streptavidin, separation of the labeled peptides, analysis of *N*-{3-[2-amino-3-(1H-indol-3-yl)-propylamino]-propyl}-5-azido-2-nitrobenzamide (**II**) photoproducts and their transformation products were performed using Nucleosil 100-10 C_{18}, 10 μm column (1.5 × 45 mm, Macherey–Nagel, Duren, Germany). Products photogenerated from *N*-{3-[2-amino-3-(1H-indol-3-yl)-propylamino]-propyl}-5-azido-2-nitrobenzamide (**II**) were separated using a linear gradient of acetonitrile in 0.1% TFA (from 0% to 70% for 40 min, 50 μL min^{-1}).

Preparative isolation of all the compounds obtained after irradiation of **II** was performed using Waters 600E chromatograph (USA). All procedures were made on a LiChrsorb-RP18 column (4.6 × 250 mm, Merck, Darmstadt, Germany) using a linear gradient of acetonitrile in 0.1% TFA (from 0% to 70% for 120 min, 300 μL min^{-1}).

2.2.3. UV irradiation of the samples

A high-pressure mercury lamp (DRSh-1000, 1000 W, Russia) was used as a light source. A mercury line (313 nm) was isolated by a combination of glass filters JS-3 and UFS-2 (LZOS, Russia). The intensity of the incident light was measured using a Hatchard-Parker ferrioxalate actinometer [24] and it was