p-AZIDOPHENYLGLYOXAL: A HETEROBIFUNCTIONAL PHOTOSENSITIVE REAGENT

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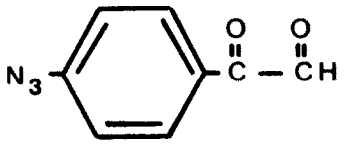
Received 11 November 1980

1. Introduction

Heterobifunctional reagents provide a versatile tool for probing the spacial arrangements of macromolecular structures ([1] and references therein); one such reagent is *p*-azidophenylglyoxal [I]. Examples of similar 1,2-dicarbonyl compounds such as glyoxal and kethoxal react specifically with guanine nucleotides [2–6]. By coupling a dicarbonyl moiety to an aromatic nucleus specificity of reaction toward the arginyl guanidino group in proteins or peptides is assured [7]. We have extended these studies and have shown the aryl glyoxal molecule to react specifically with the guanine nucleotides under mild conditions to form adducts not readily reversed.



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Specificity of reaction toward a single type of amino acid or base residue facilitates localization of the reaction site in the appropriate polymer. Since the specificity is determined by the 1,2-dicarbonyl group, the second functional moiety should ideally display wide chemical reactivity and controlled activation if it is to react with any residues in its vicinity during crosslinking experiments. Most phenyl azides are one such group, which are stable to long wavelength light but generate highly reactive nitrenes when irradiated with ultraviolet light.

A preliminary report of this work appeared in [8].

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2. Materials and methods

p-Azidophenylglyoxal (I) was synthesized from *p*-aminoacetophenone by way of the intermediate *p*-azidoacetophenone as follows: to *p*-aminoacetophenone (0.2 mol) in 1 l of 2 M HCl at 4°C, $NaNO_2$ (0.22 mol) in 40 ml H_2O was added dropwise with vigorous stirring over 20 min. The mixture was then left for an additional 10 min at 4°C. The azido-derivative was then formed by the addition of NaN_3 (0.22 mol) in 60 ml H_2O at 4°C with vigorous stirring over 40 min. After an additional 20 min at 4°C the reaction mixture was cooled to −15°C and allowed to stand overnight to further precipitate the *p*-azidoacetophenone. After thawing the precipitate was collected, recrystallized from petroleum ether (b.p. 60–110°C) and dried under vacuum. The isolated compound migrated as a single ultraviolet-absorbing spot on silicic acid thin-layer chromatography (R_F = 0.70) in an ethyl acetate:acetic acid:benzene (20:1:80) solvent and had a m.p. of 45–47°C.

The conversion of *p*-azidoacetophenone to *p*-azidophenylglyoxal was essentially as in [9]. The final product was homogeneous when examined by several chromatographic methods and gave the predicted spectra when examined by ^{13}C NMR; ^{1}H NMR, before and after D_2O exchange; IR; and mass spectroscopy. The UV spectrum had a λ_{max} of 291 nm with an $\epsilon_M = 2.4 \times 10^4$ in 95% ethanol. Upon thin-layer chromatography on silicic acid plates, *p*-azidophenylglyoxal had an R_F of 0.51 in the earlier noted solvent.

The reaction of *p*-azidophenylglyoxal with 5′-[^{14}C]-GMP was carried out in 10 mM Bicine (pH 8.0). After reacting 8 h at room temperature an aliquot was applied to a DEAE-cellulose column equilibrated with 0.10 M ammonium formate–0.35 M HCOOH (pH 3.15). Elution was carried out isocratically with the

same buffer. Material in the late eluting peak was pooled, diluted, and applied to a small DEAE-cellulose column equilibrated with 0.1 M triethylamine–CH_3COOH (pH 5). Bound material was eluted with 2 M triethylamine–CH_3COOH (pH 5).

HPLC of the 5′-GMP adduct was carried out on a column (0.15 × 60 cm) of corasil AX resin (Waters) equilibrated and eluted with an isocratic solvent of 20 mM ammonium phosphate (pH 3.3); 10% ethanol at room temperature. For additional details on the system see [10].

The reactions of *p*-azidophenylglyoxal and phenylglyoxal with unlabeled nucleotides were carried out at room temperature. They were buffered with either 10 mM MES (pH 6.6) or 10 mM Bicine (pH 8.0). Both *p*-azidophenylglyoxal and phenylglyoxal were added as ethanol solutions with final reaction mixtures containing 20% ethanol.

The nucleoside triplet ApUpGoH was reacted with *p*-azidophenylglyoxal for 3 h in 10 mM Bicine (pH 8). Chromatography of ApUpGoH and its adduct was carried out using a SAX-Partisil column (Whatman) (0.45 × 25 cm) at room temperature. For analytical work linear gradients running from 50 mM potassium phosphate: 10% methanol (pH 4) to 250 mM potassium phosphate: 10% methanol: 0.5 M HCl were used. The ApUpG-adduct was recovered following chromatography on the SAX-Partisil column and a linear gradient running from 0.5 M triethylamine–CH_3COOH: 10% methanol (pH 5) to 1.5 M triethylamine–CH_3COOH: 10% methanol (pH 5). Solvent and salts were eliminated by evaporation.

3. Results and discussion

p-Azidophenylglyoxal (I) in 95% ethanol has an A_{291} max, with an $\epsilon_M = 2.75 \times 10^4$. Photolysis of the compound with short wavelength ultraviolet light (253.4 nm) for increasing time periods leads to rapid spectral changes, as expected upon photolysis of an aryl azide (fig.1). In aqueous solution buffered with 10 mM Hepes (pH 7), the λ_{max} of *p*-azidophenylglyoxal peaks at 297 nm before photolysis.

Ion-exchange chromatography of the reaction mixture containing 5′-[^{14}C]GMP and *p*-azidophenylglyoxal gave 3 new ultraviolet-absorbing peaks which were not seen when GMP was omitted from the mixture. The third component, eluting as a broad peak between 8–10 column volumes, was identified as the

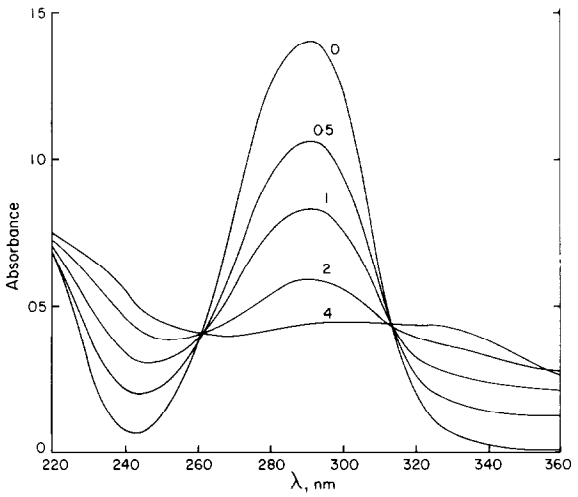

Fig.1. Ultraviolet spectra of *p*-azidophenylglyoxal before and after photolysis with ultraviolet light (λ = 253.4 nm) for 0, 0.5, 1, 2, and 4 min. λ_{max} = 291 nm with λ_{min} = 245 nm for zero-time sample (in 95% ethanol).

p-azidophenylglyoxal–5′-[^{14}C]GMP adduct from its decrease in specific activity over the 5′-[^{14}C]GMP, its spectrum, and its sensitivity toward ultraviolet irradiation. Rechromatography of an aliquot of the adduct by HPLC revealed one major component representing ~80% of the total ultraviolet absorbing material, and a minor, later eluting shoulder. HPLC of the *p*-azidophenylglyoxal–GMP reaction mixture directly, also produced this double peak which is not the result of photolyzed material.

p-Azidophenylglyoxal presumably reacts with the guanine base moiety in a manner similar to that of phenylglyoxal, and therefore should have similar chromatographic properties. When reaction mixtures containing 5′-GMP and either *p*-azidophenylglyoxal or phenylglyoxal were examined chromatographically, the newly generated GMP-adducts for both phenylglyoxal and *p*-azidophenylglyoxal did elute in similar positions. However, unlike *p*-azidophenylglyoxal, the phenylglyoxal reaction mixture gave a product which eluted as a single peak. When the pH of the reaction mixture for both *p*-azidophenylglyoxal and phenylglyoxal was lowered from pH 8–6.6, the reactions occurred much slower, as determined by direct HPLC of the mixtures. The reactivity of phenylglyoxal with guanosine residues was also found to be slow or non-detectable at acidic pH-values in [2,11].

Most guanine-adducts resulting from reaction with

1,2-dicarbonyl-containing compounds have been shown to be unstable under alkaline conditions [2,3, 12]. To determine the stability of *p*-azidophenylglyoxal–5′-GMP, it's reversal was examined in parallel with that of kethoxal–guanosine and kethoxal–GMP adducts [3]. There was no apparent breakdown of the *p*-azidophenylglyoxal–5′-GMP adduct after 12 h incubation at 37°C in 10 mM $(NH_4)_2CO_3$ (pH 10) as determined by HPLC (5% reversal should have been detected). In contrast, the kethoxal derivatives showed >50% breakdown after only 1 h as monitored by thin-layer chromatography and ΔA_{270}. Composition, concentration, and pH of the buffers had significant effects on the rates of hydrolysis of the kethoxal adducts. For example, only minimal reversal was noted with kethoxal–guanosine after 75 min in 10 mM $(NH_4)_2CO_3$ at pH 6, 7 or 8; whereas increasing to pH 10 or higher resulted in essentially complete reversal. Substitution of CAPS buffer for $(NH_4)_2CO_3$ slowed this breakdown at all pH-values examined, indicating that free primary amines promote the reversal.

Specificity of the reaction of *p*-azidophenylglyoxal with the guanine moiety was examined by incubating the 5′-monophosphates of adenosine, cytidine and uridine with the glyoxal derivative at room temperature at pH 8 for ≤7 h. Mixtures containing 5′-GMP took on a faint yellowish tinge, while others, including the blank remained colorless. Subsequent HPLC of the mixtures also gave no evidence of any reaction with nucleotides other than 5′-GMP.

Reaction of *p*-azidophenylglyoxal with the trinucleotide ApUpGoH, results in the appearance of a new peak that elutes between 8–11 min from a SAX-Partisil column (fig.2). The peak eluting between 2–8 min represents unreacted ApUpGoH. To recover the ApUpGoH-adduct free of salts, a large reaction mixture was applied to the same Partisil column and eluted with triethylamine–CH_3COOH buffer (see section 2). The spectra of the ApUpGoH-adduct is shown in fig.3, before and after photolysis. The reaction product shows a λ_{max} similar to that of ApUpGoH (λ_{max} 259 nm) before photolysis and shifts to a slightly shorter wavelength upon photolysis, with a concomitant decrease in the $A_{280-320}$ (fig.3). Unreacted ApUpGoH showed no spectral changes following irradiation for 4 min under identical conditions. These findings indicate that *p*-azidophenylglyoxal is capable of reacting with guanine residues in other than the monomer state. Preliminary results also indicate that in addition to guanine residues, *p*-azidophenylglyoxal

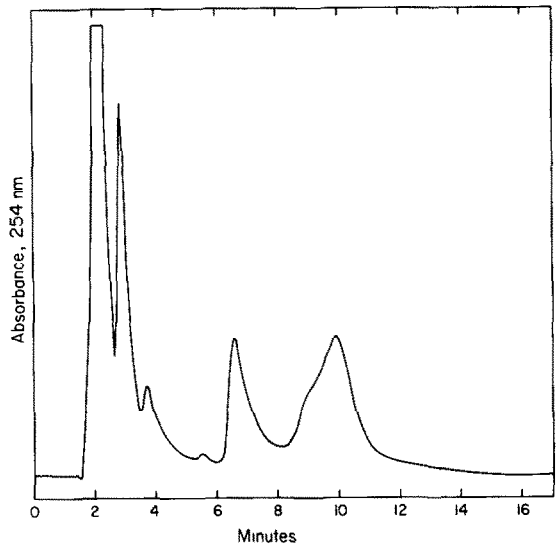

Fig.2. Reaction of *p*-azidophenylglyoxal with ApUpGoH (peak at 6.5 min) results in the formation of a new product eluting after the trinucleotide (peak at 10 min).

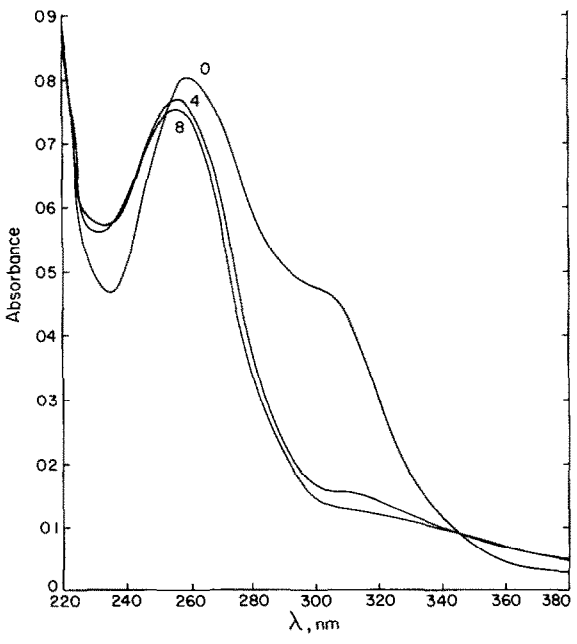

Fig.3. Ultraviolet spectra of the *p*-azidophenylglyoxal–ApUpGoH adduct (collected between 8.5–11 min, fig.2), before and after photolysis with ultraviolet light for 4 and 8 min. Buffer was 10 mM potassium phosphate (pH 7.0).

shows reaction specificity with the guanidinium-group found in arginine.

We describe the synthesis of a new heterobifunctional photosensitive probe that reacts specifically with guanine moieties in either the monomeric or oligomeric form. The analog, *p*-azidophenylglyoxal, reacts at room temperature and at relatively low concentrations to form an ultraviolet-sensitive product that shows considerably more stability than related, kethoxal-guanosine adducts. The highly reactive nitrene generated upon photolysis is non-specific and forms stable covalent linkages with virtually any nearby molecule. This reagent should prove to be extremely useful in probing a variety of macromolecular structures.

Acknowledgement

This work was supported by National Institutes of Health Grant GM 20326. Journal paper no. JA 1076 of the Wyoming Experimental Station.

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