JOURNAL OF PHYSICAL ORGANIC CHEMISTRY
J. Phys. Org. Chem. 2006; **19:** 291–294
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/poc.1031

Fast reactions of arylnitrenium ions with amino acids and proteins: a laser flash photolysis study

Selina I. Thomas and Daniel E. Falvey*

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland, USA

Received 5 December 2005; revised 20 January 2006; accepted 21 January 2006

ABSTRACT: Laser flash photolysis was used to examine the reaction of *N*-methyl-*N*-(4-biphenylyl)nitrenium ion with various amino acids and proteins in aqueous media. This nitrenium ion was found to react rapidly ($>10^8\,M^{-1}\,s^{-1}$) with tryptophan, tyrosine, methionine and cysteine, more slowly (10^7–$10^8\,M^{-1}\,s^{-1}$) with lysine, histidine, and arginine. Rapid reaction was also seen with several representative proteins including bovine serum albumin, lysozyme, and chymotrypsin. These results suggest that reaction with proteins is likely to be a significant pathway in the reactions of nitrenium ions generated in vivo. Copyright © 2006 John Wiley & Sons, Ltd.
Supplementary electronic material for this paper is available in Wiley Interscience at http://www.interscience.wiley.com/jpages/0894–3230/suppmat/

KEYWORDS: nitrenium ion; *N*-methyl-*N*-(4-biphenylyl)nitrenium ion; amino acids, proteins; hydrazine dimer; methionine; photochemistry; arylnitrenium ion

INTRODUCTION

Arylnitrenium ions are short-lived, electrophilic intermediates characterized by a positively charged, dicoordinate nitrogen atom which is bound to one or two aromatic groups. A series of elegant experiments carried out several decades ago showed that the carcinogenic effects of aromatic amines could be attributed to enzymatic oxidation of the amines followed by esterification of the resulting hydroxylamine.[1–3] The key DNA damaging step was inferred to be heterolysis of this ester, forming an arylnitrenium ion, which in turn reacts with guanine bases in DNA forming covalent adducts.[4–7] Subsequent kinetic studies using both competitive trapping and laser flash photolysis (LFP) confirmed this general mechanism.[6,8–10] In particular those arylnitrenium ions derived from highly carcinogenic amines were found to react very rapidly with guanine, but to be far less reactive toward water.

There is far less information regarding the stability of nitrenium ions toward other biological molecules. Novak and others have shown that glutathione can rapidly trap nitrenium ions.[11] However, we are unaware of any direct kinetic data regarding the stability of arylnitrenium ions toward proteins or their component amino acids. Earlier studies from this laboratory on *N*-methyl-*N*-4-biphenylylnitrenium ion (**2**) showed that this intermediate was trapped rapidly by amines, and electron-rich arenes.[12,13] This caused us to consider the possibility that nitrenium ions generated in vivo might also be trapped by nucleophilic and/or readily oxidized amino acids in proteins. The LFP experiments described below support this prediction. This nitrenium ion is quenched by tryptophan, tyrosine, methionine, and cysteine, at or near the diffusion limit and somewhat more slowly by serine, histidine, lysine, and arginine. LFP experiments carried out with: bovine serum albumin (BSA), bovine pancreatic nuclease, lysozyme (lyz), insulin (Ins), and chymotrypsin (Chym) show that native proteins are also reactive toward this nitrenium ion.

**Correspondence to:* D. E. Falvey, Department of Chemistry and Biochemistry, Building 091, University of Maryland, College Park, MD 20742, USA.
E-mail: falvey@umd.edu

RESULTS AND DISCUSSION

The method for the photochemical generation and detection of **2** has been described elsewhere.[14] Briefly this reactive intermediate is generated by photolysis of 1-(*N*-methyl-*N*-4-biphenylyl)-2,4,6-trimethylpyridinium tetrafluoroborate (**1**) using the third harmonic (355 nm, 20 mJ, 4 ns) of a Nd:YAG laser. The resulting singlet nitrenium ion can be detected through its absorption at 460 nm. In aqueous solution, the latter lives for 590 ns. Its typical decay reactions have been previously shown to include: (a) addition of nucleophiles to the aromatic ring carbons; (b) addition of electron rich arenes to the nitrogen and the *ortho* carbon on the proximal phenyl ring: and a net reduction of the nitrenium ion through what is inferred to be a relatively long-lived complex with the arene.

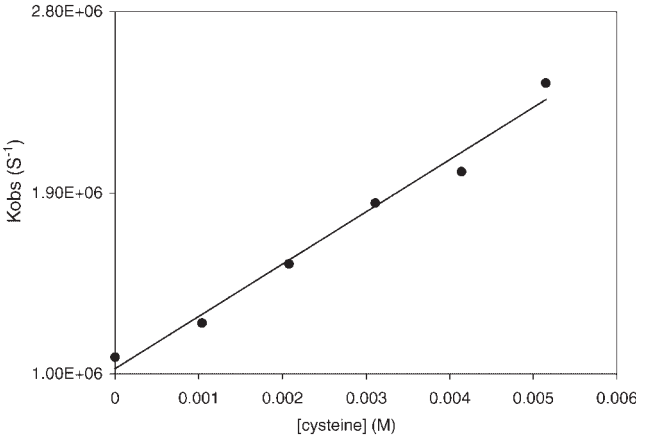


Figure 1. Dependence of the pseudo-first order decay rate of nitrenium ion **2** on the concentration of cysteine, determined by laser flash photolysis (355 nm, 6 ns, 20 mJ) of **1**

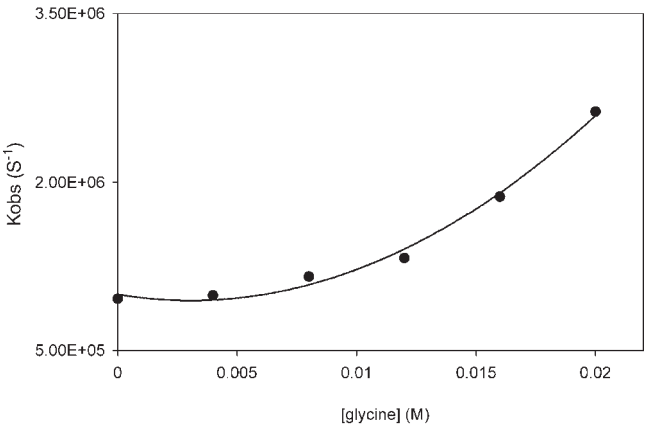


Figure 2. Dependence of the pseudo-first order decay rate constant (k_{obs}) of nitrenium ion **2** on the concentration of glycine, determined by laser flash photolysis (355 nm, 6 ns, 20 mJ) of **1**

The reactivity of the nitrenium ion toward the various amino acids was determined by LFP. Specifically the decay of the nitrenium ion's absorbance at 460 nm was measured in the presence of varying concentrations of each amino acid (typically 0–10 mM). In most cases the decays could be fit to a first order decay function. The bimolecular rate constants were derived from the dependence of these pseudo-first order rate constants on the concentration of the amino acid. Typical data are shown in Fig. 1 and all of the second order rate constants are compiled in Table 1. For the amino acids with aliphatic side chains, carboxylic side chains, and amide side chains, along with phenylalanine, threonine, and proline, no change in the lifetime of the nitrenium ion could be detected. The short lifetime of the nitrenium ion, coupled with the limited solubility of most amino acids in the 10% aqueous acetonitrile medium makes it impossible to characterize trapping rate constants less than $10^5\,M^{-1}\,s^{-1}$, thus the latter is assumed to be the upper limit for these amino acids.

The nitrenium ion reacts rapidly (10^8–$10^9\,M^{-1}\,s^{-1}$) with the electron-rich aromatic amino acids, tyrosine and tryptophan, but slowly, if at all, with phenylalanine. The same nitrenium ion has been shown to react very rapidly with electron rich arenes, such as 1,3,5-trimethoxybenzene, and *N*,*N*-dimethylaniline, but showed no measurable reactivity toward unactivated or weakly activated arenes (e.g., toluene).[12] The nitrenium ion reacts measurably, but more slowly with amino acids having amine (lysine, arginine, and histidine) and hydroxyl (serine) side chains. The rate constants we observe are somewhat lower than seen for comparable amines in aprotic solvents. This is readily explained by H-bonding of water to the traps, which ought to diminish the reactivity of these groups. It is interesting to note that serine is weakly reactive (as seen for simple alcohols), but threonine shows no measurable quenching. However, the previous studies have shown that the reactivity of the alcohols is significantly retarded by increasing steric bulk. While we suspect that threonine is somewhat reactive, it is apparently too slow to detect by our method.

Table 1. Bimolecular trapping rate constants of 2 by amino acids

Amino Acids	k_q ($M^{-1}\,s^{-1}$)
L-Glycine	(a)
L-Serine	$(7.93 \pm 0.7) \times 10^6$
L-Cysteine	$(2.79 \pm 0.2) \times 10^8$
L-Methionine	$(1.29 \pm 0.7) \times 10^9$
L-Tyrosine	$(2.95 \pm 0.7) \times 10^8$
L-Tryptophan	$(2.91 \pm 0.1) \times 10^9$
L-Histidine	$(5.39 \pm 0.5) \times 10^7$
L-Lysine	$(8.04 \pm 1.6) \times 10^7$
L-Arginine	$(3.04 \pm 0.5) \times 10^7$

The remaining amino acids tested showed no measurable quenching.
(a) A non-linear pseudo-first order plot was obtained (Fig. 2).

It was also observed that glycine quenches the nitrenium ion. This is surprising, given the lack of a reactive side chain on this amino acid. However, a careful examination of the pseudo-first order dependence of this process (Fig. 2) indicates that it is not a simple bimolecular reaction, but apparently involves reaction of two or more glycine molecules. This process was not investigated in any more detail.

The reactivity of the sulfide-bearing amino acids, methionine and cysteine is interesting. The current LFP experiments show that these amino acids react with **2** with rate constants comparable to that for tyrosine and tryptophan. Examples of arylnitrenium ion trapping by sulfides seems to be largely confined to the reactions of glutathione with those nitrenium ions implicated in DNA damaging mechanisms.[6,15,16] For example, Novak and Lin report addition of the sulfur atom of glutathione to the ring carbons of *N*-acetyl-*N*-biphenylylnitrenium ion.[11] These products were also accompanied by formation of the parent amide, 4-biphenylylacetamide. The latter product was attributed to initial N–S bond formation followed by SN2 displacement of the glutathione residue

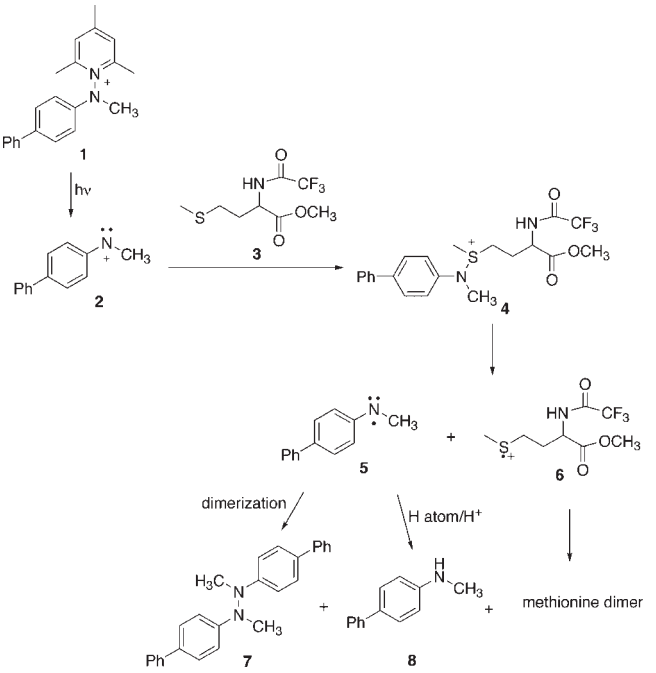
by another glutathione molecule, forming the disulfide and the amide. It seems reasonable to assume that cysteine is reacting via similar pathways. With methionine, however, the reaction pathway is less clear as this lacks the acidic S–H bond.

In order to understand the reaction pathway followed with methionine, we carried out a larger scale reaction by generating the nitrenium ion in the presence of a protected analog of methionine (**3**). The protecting groups were required to circumvent solubility limits of this amino acid. LFP experiments wherein the nitrenium ion was generated in the presence of the protected methionine in CH_3CN showed that the trapping reaction occurs with a rate constant ($1.6 \times 10^9\,M^{-1}\,s^{-1}$) comparable to that observed in aqueous solution.

Three products were isolated from preparative runs: the parent amine (**8**), a dimeric hydrazine (**7**), and an oxidative dimeric coupling product from the amino acid. The parent amine has been previously characterized. The hydrazine dimer (**7**) is a new compound, not previously detected in the trapping reactions of the nitrenium ion and was characterized by NMR and MS.

The proposed mechanism for the reaction of the nitrenium ion with the protected methionine is shown in Scheme 1. It is assumed that the initial reaction is N–S coupling, forming a relatively unstable amino-sulfonium ion (**4**). Homolysis of the N–S bond in this adduct would produce the aminyl radical (**5**) and the cation radical of methionine (**6**). The parent amine forms when **5** abstracts H atoms from the a-position of unreacted methionine molecules. The same parent amine has also been observed in the reactions of the nitrenium ion with arenes, it is similarly considered to result from a slow dissociation of a pi-complex followed by a subsequent H atom transfer to the resulting aminyl radical.

Because **7** was not observed in the arene experiments, it seems likely that it is generated from a different pathway. In particular, we suggest that the intermediate sulfonium ion reacts with a neutral amine molecule producing the hydrazine (following deprotonation) and regenerates the methionine.



Scheme 1

Aside from the aforementioned products, ^{1}H-NMR of the reaction mixture showed a complex mixture of several minor products. It is assumed that these include various products of methionine oxidation and perhaps minor adducts of the nitrenium ion. Because of the low yields, these were not characterized.

To determine if the reactivity toward these amino acids was manifest in native proteins, LFP experiments were carried out with several readily available proteins known to contain various amounts of the reactive amino acids. Table 2 shows rate constants observed for the reactions of **2** with BSA, bovine pancreatic ribonuclease (BPR), lyz, Ins, and Chym. The observed rate constants ranged from 5 to $70 \times 10^8\,M^{-1}\,s^{-1}$. These are comparable to the rates for the trapping of 2-fluorenylnitrenium ion by single-stranded DNA and significantly faster than the reactions of the same nitrenium ion with double-stranded DNA.[8] Also shown in

Table 2. Bimolecular trapping rate constants of 2 measured for each proteins and the molecular weights, number of reactive amino acids and the percentage of reactive amino acids for each proteins (based on k_q of amino acids $\geq 10^8\,M^{-1}\,s^{-1}$)

Proteins	k_q ($M^{-1}\,s^{-1}$)	Molecular weight of protein (Da)	Number of Met	Number of Cys	Number of Tyr	Number of Trp	Percentage of reactive amino acids
BSA	$(8.22 \pm 0.9) \times 10^8$	66 465.8[b]	4	35	20	2	10.5[b]
BPR	$(4.59 \pm 0.4) \times 10^8$	13 686[c]	4	8	6	0	14.5[d]
Lysozyme	$(5.79 \pm 0.6) \times 10^8$	14 388[e]	2	8	3	6	14.7[e]
Insulin[a]	$(8.96 \pm 0.8) \times 10^8$	5800[f]	0	6	4	0	19.6[f]
Chymotrypsin[a]	$(7.71 \pm 0.8) \times 10^9$	25 000[g]	2	10	3	8	9.9[g]

[a] NH_4OH was added to aid the solubility of the proteins in buffer.
[b] Brown JR. *Fed. Proc. Fed. Am. Soc. Exp. Biol*. 1975; 34: 591; Abstract 2105.
[c] Raines RT. *Chem. Rev.* 1998; 98: 1045-1065.
[d] Smyth DG, Stein WH, Moore S. *J. Biol. Chem.* 1963; 238: 227-234.
[e] Canfield RE. *J. Biol. Chem.* 1963; 238: 2698-2707.
[f] Ryle AP, Sanger F, Smith LF, Katai R. *Biochem. J.* 1955; 60: 541.
[g] Hartley BS. *Nature*. 1964; 201, 1284-1287.

Table 2 are the molecular weight, number of reactive amino acids (methionine, cysteine, tyrosine, and tryptophan), and the percentage of reactive amino acids for each protein. There is no obvious correlation of these rate constants with any of these properties. It is likely that the reactivity of a given protein depends on more subtle structural factors, such as the number of reactive amino acids that are readily accessible to the nitrenium ion, and perhaps the ability of the proteins tertiary structure to assist in any oxidation or addition reactions by the nitrenium ion.

CONCLUSIONS

The LFP experiments described herein demonstrate that **2** reacts rapidly with amino acids and proteins. In most cases, this results in a complex mixture of products. Thus, it is unclear what the biological consequences of such protein damage would be. However, given the rapid reactions of arylnitrenium ions with several representative proteins, it is clear that any quantitative description of nitrenium ion toxicity will need to take protein reactivity into account.

Supplementary material

Experimental descriptions of the kinetic measurements and isolation and characterization of **7** are available in Wiley Interscience.

REFERENCES

1. Cramer JW, Miller JA, Miller EC. *J. Biol. Chem*. 1960; **235**: 885–888.
2. Robbins RJ, Laman DM, Falvey DE. *J. Am. Chem. Soc*. 1996; **118**: 8127–8135.
3. Huffmann GR, Fuchs RPP. *Chem. Res. Toxicol*. 1997; **10**: 347–359.
4. Miller JA, Miller EC. In *Physico-Chemical Mechanisms of Carcinogenesis, The Jerusalem Symposia on Quantum Chemistry and Biochemistry*, Bergmann ED, Pullman B (eds). The Israel Academy of Sciences and Humanities: Jerusalem, 1969; 237–261.
5. Bahl OP, Gutmann HR. *Biochem. Biophys. Acta*. 1964; **90**: 391–393.
6. McClelland RA, Ahmad A, Dicks AP, Licence VE. *J. Am. Chem. Soc*. 1999; **121**: 3303–3310.
7. McClelland RA, Gadosy TA, Daniel R. *Can. J. Chem*. 1998; **76**: 1327–1337.
8. Novak M, Kennedy SA. *J. Phys. Org. Chem*. 1998; **11**: 71–76.
9. Kennedy SA, Novak M, Kolb BA. *J. Am. Chem. Soc*. 1997; **119**: 7654–7664.
10. McClelland RA, Kahley MJ, Davidse PA. *J. Phys. Org. Chem*. 1996; **9**: 355–360.
11. Novak M, Lin J. *J. Am. Chem. Soc*. 1996; **118**: 1302–1308.
12. Chiapperino D, McIlroy S, Falvey DE. *J. Am. Chem. Soc*. 2002; **124**: 3567–3577.
13. Kung AC, Chiapperino D, Falvey DE. *Photochem. Photobiol. Sci*. 2003; **2**: 1205–1208.
14. Srivastava S, Ruane PH, Toscano JP, Sullivan MB, Cramer CJ, Chiapperino D, Reed EC, Falvey DE. *J. Am. Chem. Soc*. 2000; **122**: 8271–8278.
15. van den Goorbergh JAM, Meerman JHN, de Wit H, Mulder GJ. *Carcinogenesis*. 1985; **6**: 1635–1640.
16. Kazanis S, McClelland RA. *J. Am. Chem. Soc*. 1992; **114**: 3052–3059.