Photochemically Induced Nuclear Polarization Probes for Thiol Groups in Peptides and Proteins

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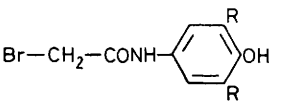
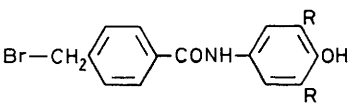
4-Hydroxyphenyl substituted protein labelling reagents have been developed as photo-CIDNP probes for accessible SH groups of cysteine residues in peptides and proteins.

We report on four sensitive probes for the study of cysteinyl peptides and proteins by $^{\cdot}H$ atom phototransfer-CIDNP (Chemically Induced Dynamic Nuclear Polarization) introduced by the alkylation of thiol groups by reagents (**1**)—(**4**).† Although alkylation at the cysteine residues is preferred,[1] less reactive nucleophilic sites such as in histidine could also potentially be modified. Because of the large enhancement of ^1H n.m.r. signals of phenols provided by photo-CIDNP[2,3] (see also below), probes such as (**1**)—(**4**) should facilitate significantly n.m.r. studies of cysteine residues and their environments in peptides and proteins under physiological conditions.

Thus far, H atom phototransfer-CIDNP[2-5] has been successfully applied to the study of three amino-acid residues[2-6] (chiefly tyrosine,[3-5] but also histidine[5] and tryptophan[4,5]) in proteins and peptides. It was shown to hold promise for



(**1**) R = H (Br—AAP)
(**2**) R = Me (Br—AADP)



(**3**) R = H (Br—MBAP)
(**4**) R = Me (Br—MBADP)

† Melting points: (**1**), 144—146; (**2**), 175—178; (**3**), 220—222; (**4**), 189—190 °C.

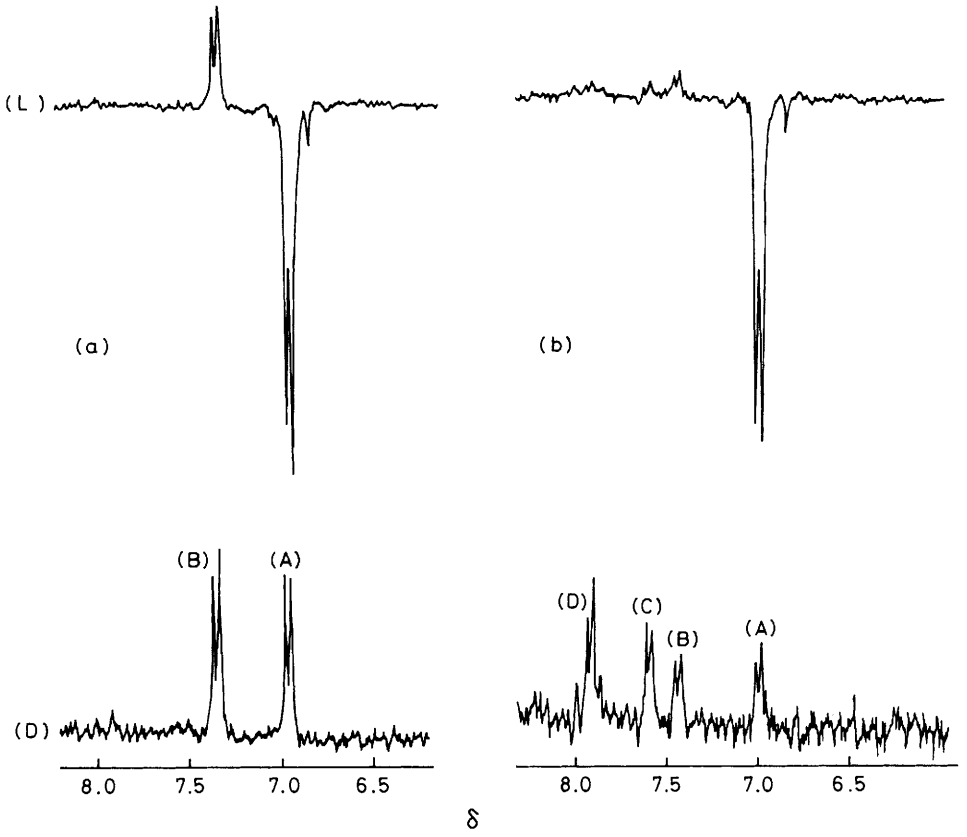
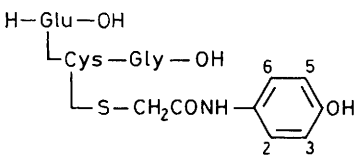
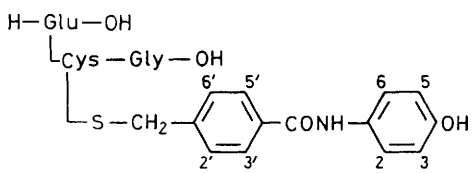


Figure 1. Aromatic region of the dark (D) and light (L) 270 MHz ^{1}H n.m.r. spectra of S-AAP-glutathione (**5**) [(a), 1.6×10^{-4} M] and S-MBAP-glutathione (**6**) [(b), 1.4×10^{-4} M] in D_2O at pH 7.5, in the presence of 4×10^{-4} M 10-carboxyethylflavin, acting as a reversible H atom photoabstractor. Spectra obtained in the Fourier transform mode, by accumulation of 60 free induction decay acquisitions. In the light spectra each radio frequency pulse is preceded by a sequence consisting of a 10 s cooling delay, 0.4 s optical irradiation, and 0.05 s magnetic relaxation delay. The light source is a 5000 W Hg–Xe lamp operated at 3500 W power (for full details, see ref. 4). Assignments: (A) C-3 and C-5 protons; (B) C-2 and C-6 protons; (C) C-2′ and C-6′ protons; (D) C-3′ and C-5′ protons.



(**5**)



(**6**)

studies of biological phenol systems[2,7] (*e.g.* tocopherol, catecholamines, and ion carriers) as well as of lysine residues in proteins following suitable derivatization of the ε-amino groups.[6]

Compounds (**1**)—(**4**) in 2-fold excess reacted rapidly to completion [first half-life of 10 min for (**1**)] with reduced glutathione [H-γ-Glu-Cys(SH)-Gly-OH, 10 mM] at pH 7.4 (0.1 M phosphate buffer). The reaction was carried out in mixed solvents [2% acetonitrile for (**1**) or (**2**), 40% acetone or dimethylformamide for (**3**) or (**4**)]. Excess of reagent was removed by extraction with n-butanol and the aqueous solution was lyophilized. Amino-acid analysis indicated nearly complete *S*-alkylation of glutathione by Br-AAP. The photo-CIDNP spectra of the *S*-AAP- and of the *S*-MBAP-derivatives show strong emission signals of the phenol C-3 and C-5 protons (Figure 1). The pattern and signs of the polarized signals and the absence of a permanent chemical change indicate that the CIDNP effects in Figure 1 are due to a reversible hydrogen atom transfer from the aromatic hydroxy group to the triplet state dye molecule, ^{3}D [equation (1)].

$$\mathrm{ArOH} + {}^3\mathrm{D} \rightarrow \overline{{}^3\mathrm{ArO}^{\bullet} + \mathrm{DH}^{\bullet}} \quad (1)$$

$$\overline{{}^3\mathrm{ArO}^{\bullet} + \mathrm{DH}^{\bullet}} \rightarrow \mathrm{Ar^*OH} + \mathrm{D} \quad (2)$$

This process is followed by recombination of the triplet spin correlated radical pair $\overline{{}^3\mathrm{ArO}^{\bullet} + \mathrm{DH}^{\bullet}}$ to give the observed polarized species Ar*OH,‡[2] where * denotes nuclear polarization [equation (2)]. Under the present experimental conditions the apparent negative enhancement is 5 for *S*-AAP- and 16 for *S*-MBAP-glutathione. The difference in polarization between (**5**) and (**6**) in Figure 1 could be due to the greater accessibility of the aromatic hydroxy group in *S*-MBAP-glutathione to the 10-carboxyethylflavin molecule. Sufficient accessibility is a prerequisite in the present method as the

‡ The opposite polarization of the escape branch of the reaction in equation (1) is largely lost before product formation because of the short nuclear spin relaxation times (10^{-3}—10^{-5} s) in free radicals.

creation of nuclear polarization by H-atom transfer requires genuine molecular contact of the excited dye with its substrate.§ Under the present irradiation conditions the H atom photo-transfer cycle is highly reversible, since no polarization decrease was observed after repeated measurements.

The ring methyl ^{1}H n.m.r. signals of *S*-AADP- and *S*-MBADP-glutathione displayed enhanced absorption by factors of 3.2 (δ 2.19) and 2.9 (δ 2.22), respectively. The sign of this effect is consistent with previously reported enhanced absorption of the methyl groups in 2,6-dimethylphenol.[2] The chemical shifts of the methyl protons of the *S*-AADP- and *S*-MBADP-derivatives are thus well-separated from those originating from any aromatic residues of a native protein.

Papain, which contains a single active site thiol group,[8] was inhibited by Br-AAP. The enzyme (1 mg/ml) in a reducing medium when treated with (**1**) (8 mM) at pH 8.0 at 23 °C showed a 90% decrease in activity after 24 h. Small solutes were extracted with n-butanol followed by ultrafiltration. The enzyme derivative displayed an intense doublet emission signal at δ 6.93, which was not present in the photo-CIDNP spectrum of papain, and which we attribute to a covalently bound *S*-AAP group on cysteine 25. The bulkier Br-MBAP and Br-AADP did not inhibit the enzymatic activity of papain under similar conditions.

An important application of photo-CIDNP in the field of protein ^{1}H n.m.r. involves the study of the accessibility of susceptible surface residues. Accessibility changes due to conformational transitions and to self-association in single protein systems, interactions with substrates, inhibitors, and other protein–protein interactions can be studied in depth by taking advantage of the special chemical specificity and sensitivity of the photo-CIDNP method in proteins, as shown by recent studies.[4] On the basis of the present results we envisage the application of this CIDNP technique to the study of the immediate surroundings of protein thiol groups including those in cysteinyl proteins (*e.g.* phosphokinases, thiol proteinases, neurotoxins,[9] and serum albumin) and those generated by specific disulphide reductions.

Received, 13th May 1983; Com. 611

§ That equation (1) represents a true H atom transfer, and not an electron transfer is shown by the strong decrease in the CIDNP effect or by its complete disappearance observed in basic solutions of phenols (*e.g.* 4-methylphenol,[2,7] *N*-acetyltyrosine,[5b,7] tyrosyl-tyrosine,[3,7] and insulin[7]). The same conclusion is also indicated by the absence of photo-CIDNP in *N*-acetyl-*O*-methyl-tyrosine.

References

1 A. Light, R. Frater, J. R. Kimmel, and E. L. Smith, *Proc. Natl. Acad. Sci. USA*, 1964, **52**, 1276.
2 K. A. Muszkat and M. Weinstein, *J. Chem. Soc., Chem. Commun.*, 1975, 143; *Z. Phys. Chem. (Frankfurt am Main)*, 1976, **101**, 105.
3 K. A. Muszkat, *J. Chem. Soc., Chem. Commun.*, 1977, 872; K. A. Muszkat and C. Gilon, *Nature*, 1978, **271**, 685; *Biochem. Biophys. Res. Commun.*, 1978, **79**, 1059.
4 K. A. Muszkat, S. Weinstein, I. Khait, and M. Vered, *Biochemistry*, 1982, **21**, 3775, and references cited therein; *Biopolymers*, 1983, **22**, 387.
5 (a) L. J. Berliner and R. Kaptein, *Biochemistry*, 1981, **20**, 799, and references cited therein; (b) F. J. Muller, C. G. v. Schagen, and R. Kaptein, *Methods Enzymol.*, 1980, **66E**, 385.
6 C. L. Lerman and M. Cohn, *Biochem. Biophys. Res. Commun.*, 1980, **97**, 121.
7 K. A. Muszkat, S. Weinstein, and I. Khait, unpublished results.
8 J. Drenth, J. N. Jasonius, R. Koekoek, and B. G. Wolthers, *Adv. Protein Chem.*, 1971, **25**, 79.
9 H. S. Kim and N. Tamiya, *Biochem. J.*, 1981, **199**, 211.