SYNTHESIS AND PROPERTIES OF A FLUORINE-CONTAINING SULFHYDRYL REAGENT FOR ^{19}F NMR STUDIES

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1. Introduction

Nuclear magnetic resonance (NMR) spectroscopy provides a unique, non-destructive method for the study of biochemical processes. The advent of NMR spectroscopy using nuclei other than protons has permitted the extension of such studies to systems using ^{13}C, ^{15}N, ^{19}F, ^{31}P and other nuclei. Of these nuclei, ^{19}F and ^{31}P are the predominant naturally occurring isotopes of these elements and studies using these nuclei have the advantage of not requiring the higher concentrations or isotope enrichment often necessary for the carbon and nitrogen NMR experiments.

Several fluorine-containing probes have been described for the modification of proteins for NMR studies. These include the trifluoroacetyl group to label lysine amino groups [1] and trifluorobromopropanone [2,3], *S,S*-(2,2,2-trifluoro-1,1-dideuteroethyl)-*O*-methyldithioperoxycarbonate [4] and the *S*-trifluoromethylmercuri-group [5] for sulfhydryl residues. Each of these suffers from some disadvantages, including non-specificity of reaction and difficulty in the synthesis or introduction of the label into proteins. We would like to describe properties of a maleimide derivative for use in fluorine NMR studies which is simple to prepare and shows a good deal of specificity for sulfhydryl groups.

Abbreviations: NMR, nuclear magnetic resonance; Hepes, *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid); TFE, 2,2,2-trifluoroethanol; GSH, glutathione; BSA, bovine serum albumin; TLC, thin-layer chromatography

2. Materials and methods

Maleic anhydride was obtained from J. T. Baker Chemicals; *p*-aminobenzotrifluoride, 2-mercaptoethanol and bovine serum albumin were from Sigma. Reduced and oxidized glutathione and the various amino acids were obtained from Calbiochem. All other chemicals were reagent grade.

Ultraviolet spectral measurements were made on a Beckman DB-GT spectrophotometer at 25°C. Proton NMR spectra were obtained on a Bruker HX-90 spectrometer in spinning 5 mm sample tubes. Fluorine NMR spectra were taken on the HX-90 or the Bruker HX-270 spectrometer at the Francis Bitter National Magnet Laboratory, Mass. Inst. Technol. Fluorine chemical shifts are referenced to internal 2,2,2-trifluoroethanol (TFE).

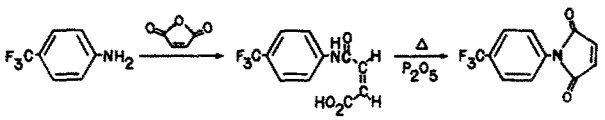

Fig.1. Reaction scheme for the preparation of *N*-(4-trifluoromethylphenyl)maleimide.

The maleimide derivative was prepared by treating maleic anhydride (1.96 g, 20 mmol) in 10 ml tetrahydrofuran dropwise with *p*-aminobenzotrifluoride (3.25 g, 20.2 mmol) (fig.1). The crude maleamic acid which crystallized on cooling the reaction mixture (4.4 g, 92% yield) could be recrystallized from aqueous ethanol to give an analytical sample, m.p. 183–186°C (uncorr.); ultraviolet λ_{max} (ethanol)

226, 270 nm (ε 9550, 8260). The maleamic acid was cyclized by heating 0.10 g derivative in vacuo (bath temp. 85–90°C, 10 mm Hg) in a sublimation vessel in the presence of 0.50 g P_2O_5 (fig.1). The product sublimed as fine needles in about 60% yield, m.p. 150–152°C (uncorr.); ultraviolet λ_{max} (ethanol) 268, 308 nm (ε 7900, 5000). Both compounds gave chemical analyses and proton NMR spectra consistent with the proposed structures.

Reactions of the maleimide with various compounds was carried out in 0.05 M Hepes, pH 7.4. Aliquots of the maleimide in ethanol or acetone were added to solutions of the material to be labeled. Reactions of small molecules could be monitored by thin-layer chromatography (TLC) on silica gel plates (Eastman). Solvent systems used include; ethyl acetate–*n*-hexane (7:3), chloroform–methanol (3:1), ethyl acetate–formic acid–water (7:2:1), and *t*-butanol–methyl-ethyl ketone–water–ammonium hydroxide (4:3:2:1). Materials were visualized by ultraviolet light at 254 nm, iodine vapor or ninhydrin. The reactions could also be followed by monitoring the ultraviolet spectrum in the region 200–225 nm; loss of absorbance in this region paralleled the reactions monitored by TLC.

Bovine serum albumin (BSA), 50 mg/ml in 0.05 M Hepes, pH 7.4, was treated with a 2-fold molar excess of the label for 18–24 h at room temperature. Excess label and degradation products were removed by passage of the reaction mixture through a column of Sephadex G-10 packed in water or by exhaustive dialysis against water. The protein was lyophilized and dissolved in the buffer for NMR measurements. BSA was also labeled under denaturing conditions (6 M guanidine hydrocholoride) using a 10-fold excess of the label.

3. Results and discussion

The maleimide reacted rapidly, at pH 7.4, with 2-mercaptoethanol and cysteine as monitored by ultraviolet spectroscopy or TLC. The ^{19}F-NMR spectra of the maleamic acid, maleimide and adducts with 2-mercaptoethanol and cysteine showed single sharp resonances at 14–15 ppm to lower field of internal 2,2,2-trifluoroethanol (TFE). Upon standing, the cysteine adduct produced a second sharp resonance

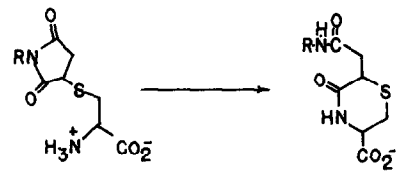

Fig.2. Possible rearrangement of maleimide derivatives.

slightly to lower field of the original resonance of the adduct. This is probably due to a rearrangement of the type shown in fig.2, reported to occur in other maleimide derivatives [6,7]. No reaction was apparent between the maleimide and serine, threonine, lysine, glutamic acid, histidine or tryptophan under the conditions used to label 2-mercaptoethanol or cysteine.

Reduced glutathione (GSH) reacted more slowly than 2-mercaptoethanol or cysteine. The fluorine spectrum of labeled GSH is shown in fig.3. The

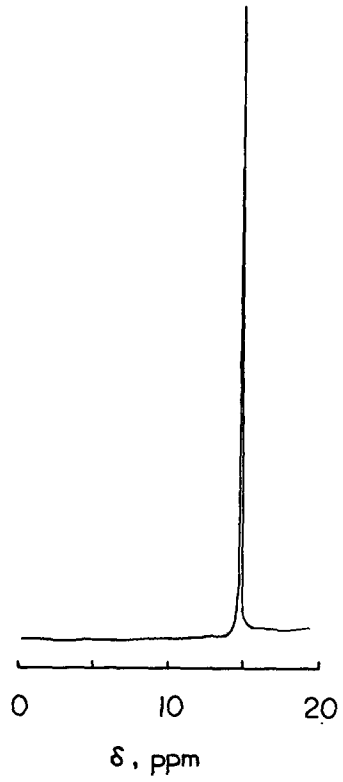

Fig.3. ^{19}F NMR spectrum of 254 MHz of labeled glutathione (10 mg/ml in 0.05 M Hepes, pH 7.4).

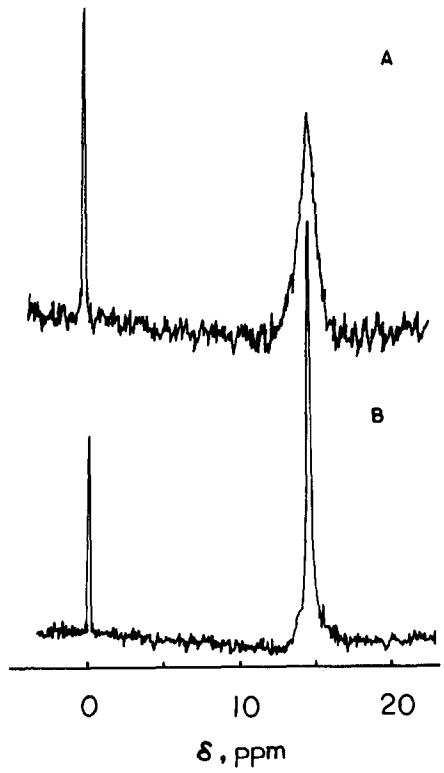

Fig.4. ^{19}F NMR spectra at 254 MHz of bovine serum albumin labeled with the maleimide. (A) BSA labeled in the native state. (B) Sample from (A) in the presence of 6 M guanidine hydrochloride.

resonance (~ 15 ppm from TFE) is fairly sharp, indicating the absence of structure in the tripeptide. Oxidized GSH did not react with the label.

BSA which shows less than one reactive cysteine residue per molecule under native conditions [7,8] gave the ^{19}F spectrum shown in fig.4 after labeling with the maleimide. There is one broad resonance about 15 ppm to low field of TFE. The width at half-height of this peak is about 230 Hz. Treatment of this sample with 6 M guanidine hydrochloride has little effect on the chemical shift of the resonance, however, the resonance sharpens dramatically (width at half-height is about 60 Hz) (fig.4B) and appears to be comprised of at least two overlapping resonances

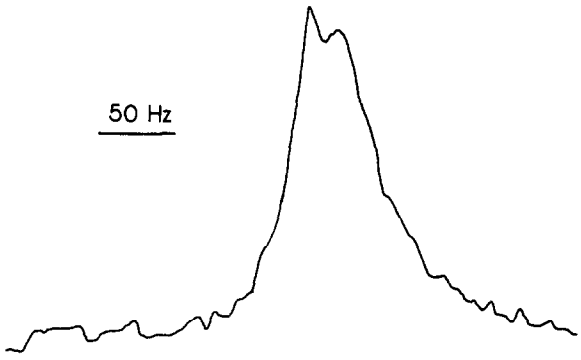

Fig.5. Expanded spectrum of labeled BSA in the presence of 6 M guanidine hydrochloride.

(fig.5). BSA which had been labeled under denaturing conditions gave essentially the same spectrum as BSA labeled under normal conditions. Although BSA contains 6 cysteine residues in all [8,9], it appears that if more than one is reactive, all have approximately the same chemical environment as measured by the fluorine probe. The fact that the BSA resonance does not give a single sharp resonance after denaturation of the sample suggests the retention of some tertiary structure in the BSA molecule in the region about the probe. The loss of structure would be expected to be manifested in free movement of the probe and result in sharp resonances. It is possible that an intramolecular rearrangement of the type described above (fig.2) has crosslinked the protein, causing retention of structure about the reactive cysteine(s). Such a reaction has been postulated to occur in BSA labeled with a fluorescent maleimide derivative [7]. It is interesting that labeled GSH, which also has a free amino group available for reaction, does not appear to undergo this transformation. This indicates that the tertiary structure of the protein is important in determining whether or not a crosslinking reaction occurs.

These studies indicate that this label is specific for SH-groups and that it may be useful in the study of protein structure and protein interactions by ^{19}F-NMR. In particular, it may be possible to use this reagent to crosslink certain protein molecules with proximal nucleophilic groups and to study the effect of such a crosslink by using the linking molecule as a reporter.

Acknowledgements

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