^{19}F NMR PROBES FOR PROTEINS; *S*-TRIFLUOROMETHYLMERCURI-PAPAIN

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1. Introduction

The potential uses of ^{19}F NMR spectroscopy in the study of proteins are very considerable and it is desirable to develop a variety of reagents which can be used to incorporate fluorine into proteins at specific functional groups. The use of ^{19}F NMR probes covalently attached to proteins has until recently been limited to the trifluoroacetonyl group attached to cysteine residues [1–6] and to the trifluoroacetyl group attached to lysine residues [7].

In general, probes should be of small size. We have investigated the use of several reagents for attaching small ^{19}F NMR probes to cysteine 25 of papain. Thiol-disulphide exchange with bis(trifluoromethyl) disulphide was rapid and one equivalent of this reagent gave more than 90% inhibition in 5 min when mixed with the active enzyme at pH 3–5 [8]. However, the broad ^{19}F resonance of the trifluoromethylthio probe attached to the enzyme rapidly decreases in intensity leaving a single resonance due to trifluoromethanethiol. Apparently the mixed disulphide with the enzyme is rapidly hydrolysed to the sulphenic acid of papain [9–11]. The reagent may be suitable as a ^{19}F NMR probe with other proteins as the hydrolysis of the disulphide bond in this case could conceivably have been catalysed by histidine 159. Attempts to form a mixed disulphide by the reaction of papain with bis(2,2,2-trifluoro-1,1-dideuteroethyl) disulphide were only partially successful since the equilibrium favoured the symmetrical rather than the mixed disulphide. However, this probe has been incorporated into papain by reaction with *SS*-(2,2,2-trifluoro-1,1-dideuteroethyl) *O*-methyl dithioperoxycarbonate [12]. This type of reagent is very useful for making mixed disulphides with papain in general [6] and the reagent type has been independently demonstrated by others [13,14].

This paper describes a third type of small ^{19}F NMR probe which may be attached to the cysteine residue of papain, the trifluoromethylmercuri probe.

2. Materials and methods

Papain was prepared, purified and assayed by methods previously described [6]. ^{19}F NMR spectra were determined on a Bruker HFX 90 spectrometer as previously described [6]. Unless otherwise stated, chemical shifts are relative to external neat trifluoroacetic acid.

Trifluoromethylmercuric trifluoroacetate was prepared by S. W. Jackson by the dry distillation of mercuric trifluoroacetate [15], ^{19}F resonances (H_2O) −47.8 p.p.m. (*s* with sidebands, CF_3Hg, $J_{^{19}F-^{199}Hg}$ 2354 Hz) and −4.4 p.p.m. (*s*, CF_3CO_2). Trifluoromethylmercuric bromide was prepared by S. W. Jackson from the trifluoroacetate by treatment with excess aqueous sodium bromide and extraction with ether, ^{19}F resonances (MeOH) −44.2 p.p.m. (*s* with sidebands, $J_{^{19}F-^{199}Hg}$ 1885 Hz) ([16] −44.7 p.p.m. and 1928 Hz).

100% active papain (10 μmol) in water (100 ml) was inhibited with trifluoromethylmercuric bromide (1.25 equiv., 4 mg). The solution was concentrated by ultrafiltration to give 4% (w/v) *S*-trifluoromethylmercuripapain (5.85 ml). Solutions of *S*-trifluoromethylmercuri-papain containing various concentrations of NaCl and NaBr were obtained by dilution with appropriate salt solutions and subsequent ultrafiltration.

100% active papain (prepared by affinity chromatography using 0.1 M sodium trifluoroacetate (CF_3CO_2-Na) (pH 4–6) instead of 20 mM EDTA (pH 4.6) [17] buffer) was inhibited with trifluoromethylmercuric trifluoroacetate (1.25 equiv.) and dialysed and concentrated by ultrafiltration with 10 mM CF_3CO_2Na to 4% (w/v). ^{19}F NMR measurements on this *S*-trifluoromethyl-mercuri-papain were inconsistent except when a freshly dialysed (with 10 mM CF_3CO_2Na) preparation was used. A control experiment, in which the ^{19}F spectrum of freshly dialysed solution was determined before and after a pH measurement, demostrated that the inconsistency was due to leakage of KCl from the reference compartment of the combined pH-electrode into the enzyme solution. The problem was overcome by using a CF_3CO_2Na salt bridge between the reference electrode and the solution.

The pH-dependences of the ^{19}F NMR spectrum of *S*-trifluoromethylmercuri-papain in 10 mM, 50 mM and 0.1 M CF_3CO_2Na were determined at 25°C and the results are shown in fig.2. The respective concentration of trifluoroacetic acid was used for pH adjustment. Several spectra at different pH values were repeated after the addition of more trifluoromethylmercuric trifluoroacetate (1 equiv.) and the invariability of the chemical shift of the ^{19}F resonance of the enzyme-bound mercurial proved that slow chemical exchange prevailed.

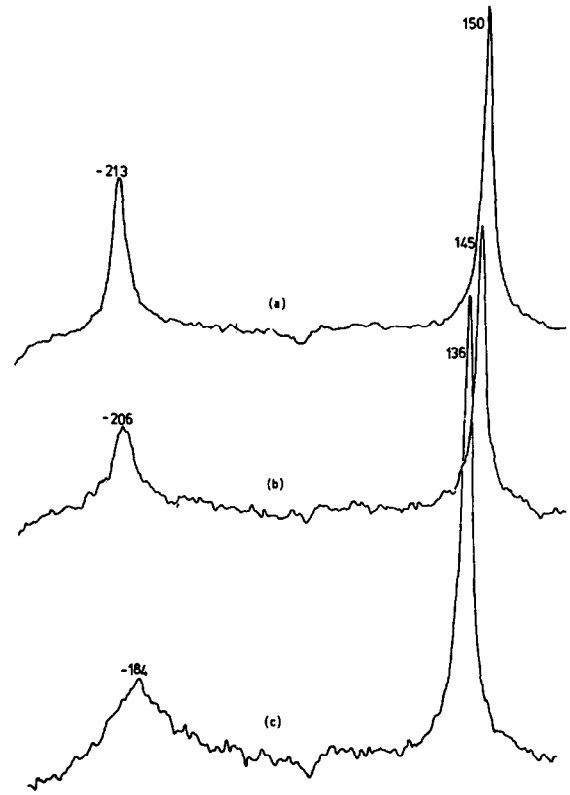

Fig.1. ^{19}F NMR spectra of active papain inhibited by CF_3HgBr (1.5 equiv.) in solutions at pH 5.0 containing (a) no additional chloride ion, (b) 10 mM chloride ion, and (c) 30 mM chloride ion. Chemical shifts (in Hz) are relative to external CF_3HgBr in chloroform.

3. Results and discussion

The ^{19}F NMR spectrum of a solution of papain containing 1.5 equiv. of trifluoromethylmercuric bromide, at pH 5.0, showed two resonances (fig.1a). The line width and chemical shift of the resonances are seen to depend on the chloride ion concentration present in solution (fig.1b and c). These resonances were absent in ^{19}F spectra of trifluoromethylmercuric bromide in the presence of *S*-(2-hydroethyl)thiopapain ($HOCH_2CH_2S$- attached to cysteine 25 [6]) indicating that both resonances depend on cysteine 25 bound species. Consequently, these resonances must represent distinct enzyme bound species with one or possibly both undergoing intermediate chemical exchange with reagent in solution. It is possible for example that the lower field and broader resonances has the mercury atom liganded to the imidazole of histidine 159 and a chloride ion as well as to cysteine 25, with the higher field and narrow line presumably being bound to cysteine 25 only. A similar but less dramatic dependence on bromide ion concentration was also observed. It was necessary therefore to choose a counter ion with less nucleo-

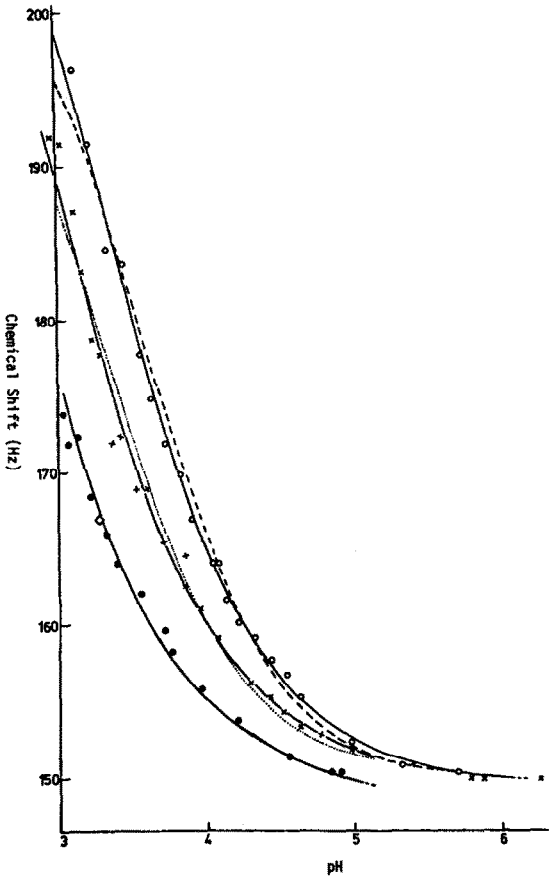

Fig.2. The pH-dependence of the ^{19}F resonance of *S*-trifluoromethylmercuri-papain in 10 mM CF_3CO_2Na (•), 50 mM CF_3CO_2 Na (×), 0.1 M CF_3CO_2 Na (○), and in 10 mM CF_3CO_2Na containing a molar excess of $CF_3HgO_2CCF_3$ (◇). The broken curves are theoretical for pK_a 3.34 and a shift of 52 Hz (50 mM CF_3CO_2Na) and pK_a 3.58 and a shift of 58 Hz (0.1 M CF_3CO_2Na). Continuous curves are theoretical for pK_1 2.70 and pK_2 4.6 (10 mM CF_3CO_2Na), pK_1 3.08 and pK_2 4.7 (50 mM CF_3CO_2Na) and pK_1 3.35 and pK_2 4.7 (0.1 M CF_3CO_2Na). For the three continuous curves, the shift corresponding to pK_1 is 65 Hz and for pK_2 is 4 Hz. Chemical shifts are relative to external CF_3HgBr in chloroform.

philic character towards mercury than halide ions; trifluoroacetate ions were selected.

S-trifluoromethylmercuri-papain prepared in the absence of chloride gave reproducible spectra provided a trifluoroacetate salt bridge was used when measuring the pH of solutions. Measurements in 50 mM CF_3CO_2Na were continued up to pH 9, but the resonance underwent very considerable broadening as pH 8.5 was approached, suggesting that it is necessary to keep the pH below the pK_a of the thiol to which the mercurial is attached. The determinations in 50 mM and 0.1 M CF_3CO_2Na were fitted to theoretical curves for a single ionisation (fig.2 broken curves). It was independently shown that the pH-dependence of the ^{19}F resonance of *S*-trifluoroacetonyl-papain [6] was not influenced by the CF_3CO_2Na concentration [18]. Presumably the dependence of the ^{19}F resonance of *S*-trifluoromethylmercuri-papain on trifluoroacetate concentration results from direct interaction of trifluoroacetate ions with the enzyme-bound mercurial. The enzyme model shows that a mercury atom attached to cysteine 25 could also complex with histidine 159. Mercury has a high affinity for neutral amines but not protonated amines [19]. Thus even weak complexing with histidine 159 would, by stabilising the neutral form, lower the observed pK_a, whereas complex formation of trifluoroacetate ions with the bound mercurial would decrease the stability of the interaction with histidine 159 and so raise its pK_a.

Inspection of a model of papain shows that if the probe is fully extended down the enzyme cleft towards aspartic acid 158, the CF_3 group would be close to the carboxyl of that residue. Therefore the probe should detect its ionisation, though perhaps to a lesser degree than the ionisation of histidine 159. Figure 2 shows that the probe is in fact affected by a second ionisable group as there are small but consistent divergences of the experimental data from the theoretical curves for a single ionisation. Theoretical curves can be drawn for two non-interacting ionisable groups which fit the data in fig.2. (solid lines) better than theoretical curves for single ionisations (broken lines). However, the probe could cause interaction between aspartic acid 158 and histidine 159, since it could hydrogen-bond to the former and complex with the latter [6]. Theoretical curves which fit the data well can also be calculated assuming two such interacting groups. Nevertheless, irrespective of whether the groups interact or not, the data strongly suggest that the probe detects two ionisable groups with pK_a s < 5, which is in agreement with the conclusions drawn previously by the authors [6,12] with regard to the low pK_a of histidine 159.

Provided care is taken to exclude halide, the

trifluoromethylmercuri probe should be a useful addition to the range of ^{19}F NMR probes capable of attachment to cysteine residues in proteins.

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References

[1] Heustis, W. H. and Raftery, M. A. (1972) Biochemistry 11, 1648–1654.
[2] Heustis, W. H. and Raftery, M. A. (1973) Biochemistry 12, 2531–2535.
[3] Lee, T-t, Heustis, W. H. and Raftery, M. A. (1973) Biochemistry 12, 2535–2539.
[4] Heustis, W. H. and Raftery, M. A. (1975) Biochemistry 14, 1886–1892.
[5] Bode, J., Blumenstein, M. and Raftery, M. A. (1975) Biochemistry 14, 1146–1152, 1153–1160.
[6] Bendall, M. R. and Lowe, G. (1976) Eur. J. Biochem. 65, 481–491.
[7] Heustis, W. H. and Raftery, M. A. (1971) Biochemistry 10, 1181–1186.
[8] Bendall, M. R. and Lowe, G., unpublished results.
[9] Galzer, A. N. (1970) Ann. Rev. Biochem. 39, 101–130.
[10] Campbell, P. and Kaiser, E. T. (1972) Biochem. Biophys. Res. Commun. 48, 866–872.
[11] Allison, W. S. and Swain, L. C. (1973) Arch. Biochem. Biophys. 155, 405–410.
[12] Bendall, M. R. and Lowe, G. (1976) Eur. J. Biochem. 65, 493–502.
[13] Smith, D. J. and Kenyon, G. L. (1974) J. Biol. Chem. 249, 3317–3318.
[14] Smith, D. J., Maggio, E. T. and Kenyon, G. L. (1975) Biochemistry 14, 766–771.
[15] Aldrich, P. E. (1962) US Patent 3,043,859, (Chem. Abs. (1952) 57, 14944h).
[16] Rausch, M. D. and Van Wazer, J. R. (1964) Inorganic Chemistry 3, 761–766.
[17] Blumberg, S., Schechter, I. and Berger, A. (1970) Eur. J. Biochem. 15, 97–102.
[18] Bendall, M. R. and Lowe, G., unpublished results.
[19] Wirth, T. H. and Davidson, N. (1964) J. Amer. Chem. Soc. 86, 4314–4329 and references there cited.