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Comments on the Proposed Rigidity of Staphylococcal Protease†

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ABSTRACT: It is proposed that the unusual low-field resonance observed in proton magnetic resonance spectra of staphylococcal protease (Markley, J. L., Finkenstadt, W. R., Dugas, H., Leduc, P., Drapeau, G. R. (1975), *Biochemistry 14,* 998) could be reassigned to an impurity of formic acid. This would reduce significantly evidence for the special rigidity of this protein.

In a recent publication (Markley et al., 1975) an unusual resonance was observed in the proton magnetic resonance (^{1}H NMR) spectrum of staphylococcal protease. This resonance (labeled X4), at ca. 8.2 ppm from an external standard of 5% Me_4Si[1] in CCl_4, was said to arise from solvent exchangeable N–H protons. A tentative assignment to nine buried amide N–H_2 groups was made. In order to explain the unusually narrow line width, some regularity in the bonding interactions of the buried amide groups was postulated. Mainly because of the behavior of this resonance, which did not completely exchange with solvent over the pH range 3 to 10 or on heating the protease solutions in D_2O to 80°C, several conclusions about the protease structure and its rigidity toward "breathing motions" were drawn. In this paper evidence will be presented for an alternative explanation for the behavior of peak X4, based on its assignment to an impurity in the sample.

Results and Discussion

The resonance X4 has a chemical shift of 8.16 ppm from external Me_4Si at pH values above 6. It was found experimentally in this work that at 270 MHz and 30°C this external Me_4Si standard resonates at 0.28 ppm downfield[2] from

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[1] Abbreviations used are: Me_4Si, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentane-5-sulfonate.

[2] This value is both temperature and frequency dependent, and will depend on the exact conditions used. There is therefore some uncertainty in the correction factor.

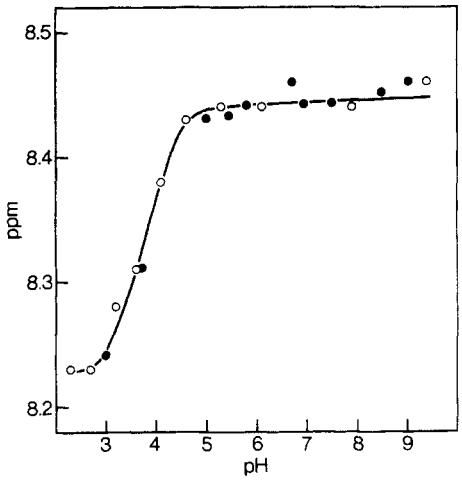

FIGURE 1: Chemical-shift values (relative to DSS) of a dilute solution of formic acid in D_2O (○) and of the resonance X4 of Markley et al. (1975) (●). Spectra were recorded at 30°C.

the more usual internal standard DSS.[1] This factor has therefore been added to the chemical shift values of Markley et al. (1975). The peak X4 is then at 8.44 ppm on this scale. The spectra of Markley et al. indicate that the peak is probably a singlet, and that an upfield shift occurs at low pH values. This suggests that the resonance could arise from formic acid (formate at pH above 4), an impurity observed to the author's knowledge in several proteins. The chemical-shift value of formic acid in dilute aqueous solution was obtained as a function of pH under the conditions used by Markley et al. These are plotted in Figure 1 along with the data of Markley et al. The data are very similar, the pH dependence of formic acid arising from its pK value of ca. 3.8.

If resonance X4 is to be assigned to an impurity, it must now be asked how the apparent solvent exchange of the resonance could have been observed. The solvent exchange was proposed because the resonance was of reduced area in samples of the protein which had been preexchanged by heating in D_2O at pH 5.0. The spectra of the preexchanged protein were thus presumably of different samples from the spectra of nonexchanged material. It would, for example, be possible to reduce the concentration of the impurity of formic acid in a sample by lyophilization or dialysis at pH values of ca. 5.0 or below where a significant fraction exists as the acid (bp 100°C) rather than the anion. Indeed this is proposed as a means of removing the formic acid. Such a procedure (dialysis and lyophilization at pH 3.0) is used (Campbell et al., 1973) to remove the very similar impurity of acetic acid which is known to occur in commercial samples of lysozyme. In its anionic form, acetate is extremely difficult to remove from lysozyme.

In conclusion, the possibility of impurities contributing significantly to spectra of proteins must always be considered. In particular, resonances of those small molecules used routinely in protein preparations (as buffers, in columns, as solvents, etc.) should be searched for. Given the assignment of peak X4 to formic acid, there appears at present to be little ^1H NMR evidence for the greater rigidity toward "breathing motions" of staphylococcal protease when compared to other proteins.

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