Two-dimensional $^{19}F\{^1H\}$Overhauser Effects in Proteins

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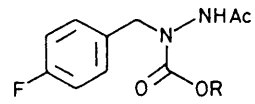
Two-dimensional heteronuclear Overhauser effect n.m.r. spectroscopy is shown to be practical with a fluorine-labelled protein; the experiment reveals through-space proton–fluorine interactions at the active site of an acylated form of α-chymotrypsin.

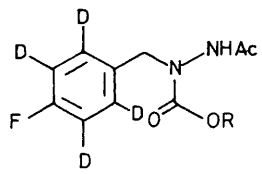
The observation of nuclear Overhauser effects (n.O.e.s) by two-dimensional nuclear magnetic resonance (2D-n.m.r.) is a powerful method for studying the solution structure of biological molecules. This has been well illustrated by experiments involving proton–proton n.O.e.s in proteins,[1] oligosaccharides,[2] and nucleic acids.[3] Work to date has been carried out on relatively small molecules because with increasing size there is an increase in (a) the number of resonances in the ^1H n.m.r. spectrum and (b) the linewidth of each resonance. These factors combine to produce a ^1H spectrum that is largely intractable for all but the smallest proteins (M.W. ≤ 20 000). Furthermore, the rapid transverse relaxation responsible for (b) also causes low sensitivity in 2D experiments on large molecules.

^{19}F N.m.r. spectra of proteins can be obtained following biosynthetic incorporation of fluorinated amino acids or after chemical modication with a fluorinated reagent.[4] Such spectra are generally well resolved, even for quite large proteins, and consequently ^{19}F n.m.r. spectroscopy affords a useful way of investigating protein structure and dynamics.[4] Fluorine relaxation in labelled proteins is dominated by proton–fluorine dipolar interactions, which can be studied using the heteronuclear $^{19}F\{^1H\}$ n.O.e.[5] A potentially convenient and informative way of mapping the ^{19}F–^1H through-space interactions in fluorine-containing proteins is to carry out a 2D $^{19}F\{^1H\}$ n.O.e. experiment. This communication describes such an experiment and its application to a covalently modified derivative of the proteolytic enzyme α-chymotrypsin. To date, this protein is larger than any for which n.O.e.s have been studied by 2D-n.m.r.

α-Chymotrypsin (M.W. *ca.* 24 500) reacts stoicheiometrically with the carbazate (**1**) to produce a reasonably stable, catalytically inactive, enzyme derivative.[6] Studies of fluorine and deuterium relaxation in the enzyme modified with (**1**) or



(**1**)



(**2**)

R = *p*-$NO_2C_6H_4$

with the deuteriated analogue (**2**) indicate that the fluorinated aromatic ring is highly immobilized within the protein structure.[7] The pulse sequence $(\pi/2)^1H$-t_1-$(\pi/2)^1H$-τ-$(\pi/2)^{19}F$-F.I.D.(t_2) was used to generate a two-dimensional map of proton–fluorine Overhauser effects in both modified enzymes. The phases of the first proton pulse and the receiver were cycled to provide quadrature detection in both dimensions.[8] A field gradient pulse to quench transverse magnetisation before data acquisition was not necessary because of the rapid transverse relaxation of both protons and fluorine in this system. Also, decoupling during the acquisition period was not used since effects due to H–F spin-coupling are expected to be small and, in any event, will only affect the magnitude of the n.O.e. in the aromatic part of the correlation map. The mixing time τ was set to 0.35 s and should be small enough to discriminate strongly against the effects of spin diffusion since a transient n.O.e. experiment, involving inversion of all proton spins, showed a minimum at 0.55 s in the curve for recovery of the fluorine magnetisation.

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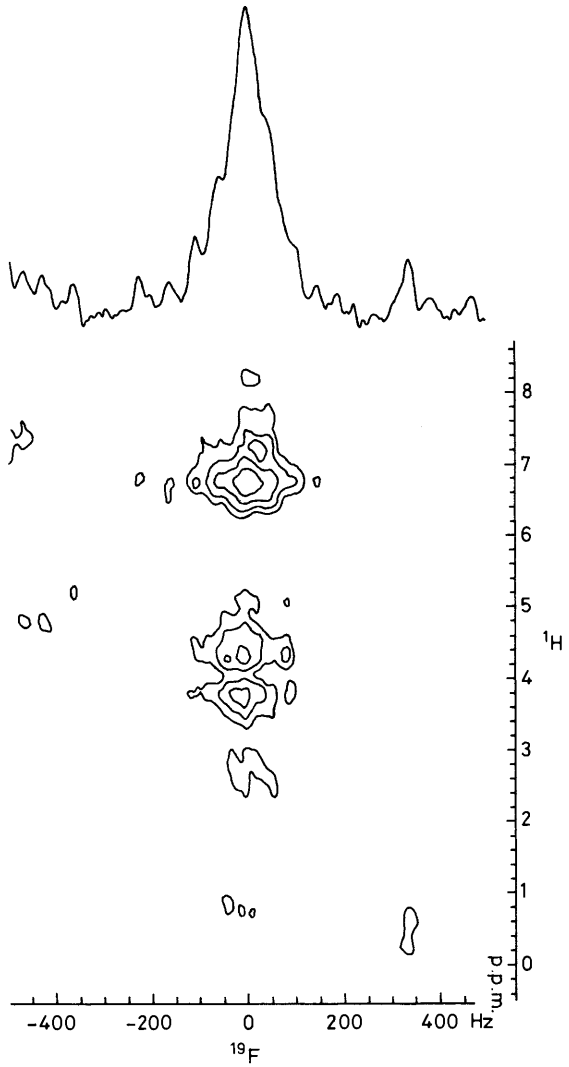

Figure 1. Contour plot of the 2D proton–fluorine n.O.e. spectrum of N^2-acetyl-N^1-(4-fluorobenzyl)carbazoyl-α-chymotrypsin [enzyme inactivated with (**1**)] and its projection on the fluorine chemical shift axis. The spectrum was obtained by ^{19}F observation at 282 MHz (^{1}H frequency 300 MHz) on a Nicolet NT-300 using a 10 mm dedicated ^{19}F probe. The sample was 2 mM in D_2O at pD 4, 25 °C and was prepared from chromatographically purified enzyme derivative. Signal averaging was carried out by the collection of 800 transients at each value of t_1. The delay t_1 was incremented 64 times. The interval between pulse trains was greater than five times the proton or fluorine T_1 and the total time for collection of the data was about 40 h. An exponential line-broadening factor of 30 Hz was used in each dimension and in the proton (t_1) dimension the 64 data points were zero-filled to 256. The proton chemical shift axis was referred to the HOD signal at 4.7 p.p.m. by direct observation of the proton spectrum.

Figure 1 shows a contour plot of the 2D n.O.e. spectrum, and its projection onto the ^{19}F axis, for enzyme modified with (**1**). The projection of this contour map onto the ^{1}H axis is shown in Figure 2(a) above a projection of the map obtained for enzyme modified with (**2**) Figure 2(b). Comparison of the two projections indicates that the signal at *ca.* 6.8 p.p.m. in (a) arises from protons *ortho* to the fluorine in (**1**) since this feature of the correlation map is removed by deuteriation of the fluorophenyl ring. It is also clear that a number of protons in the range 3—5 p.p.m. and at *ca.* 0.8 p.p.m. are close enough to the fluorine nucleus to contribute substantially to its

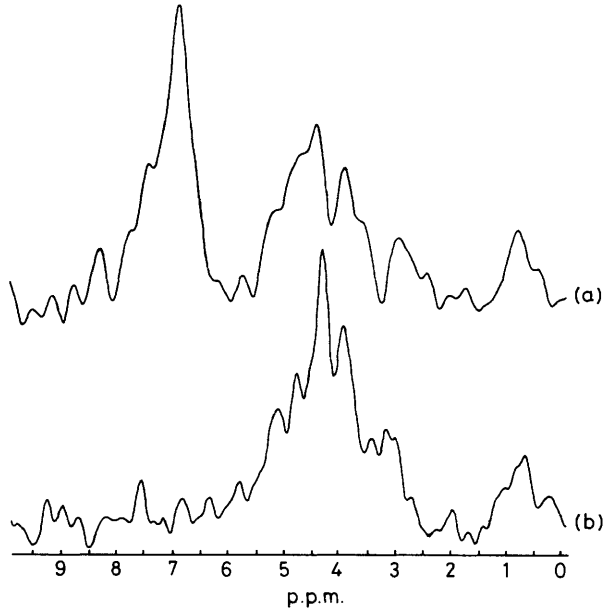

Figure 2. Projections on the proton chemical shift axis of the 2D ^{19}F{^{1}H} n.O.e. spectra for α-chymotrypsin inactivated with (**1**) (a) and (**2**) (b). Projection (a) corresponds to the contour plot shown in Figure 1. The same conditions and procedures described in Figure 1 for enzyme inactivated with (**1**) were used to obtain the 2D spectrum for enzyme inactivated with (**2**).

relaxation. (These effects are reproduced when the experiment is repeated with a different proton carrier frequency). The intensities of the signals in (a) with respect to the signal at 6.8 p.p.m. suggest that the interacting protons of the protein are, on average, only slightly further than 0.26 nm (the *ortho* H–F distance) away from the fluorine nucleus.

Assuming that the fluorophenyl ring occupies the 'tosyl pocket' of α-chymotrypsin,[9] as seems likely from its observed slow rotation,[7] then examination of a molecular model of the enzyme suggests that the fluorine in this derivative can reside close enough to appropriate protons of the residues Ser-190, Cys-191, Cys-220, Val-213, Trp-215, and Gly-226 to account for the n.O.e. data.

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