Two-dimensional Tritium–Proton Nuclear Overhauser Effects in Proteins

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Two-dimensional $^3H\{^1H\}$ NOE experiments have been used to examine an α-tritiated tosyl derivative of chymotrypsin, demonstrating the feasibility of such experiments with a material of M_r 25 000 and revealing through-space dipolar interaction at the active site of the enzyme.

Observations of $^1H\{^1H\}$ nuclear Overhauser effects play a central role in studies of the structures of biological macromolecules in solution.[1] The approaches used to provide the requisite assignment of the proton spectrum of the system of interest, and the estimation of internuclear distances needed for tertiary structure determination from multidimensional NOE experiments have developed rapidly. However, these methods begin to falter when proteins are larger than *ca.* M_r 15 000 because, with increasing size, spectral crowding and the linewidths of individual resonances both increase, thereby reducing resolution. With larger structures it is necessary to enrich specific positions with carbon-13 or nitrogen-15[2] or introduce an extrinsic nucleus such as fluorine-19[3] to obtain structural information. Tritium NMR spectra from appropriately labelled materials are useful for addressing many questions in a small molecule context[4] but there have been few examples of tritium NMR studies with protein systems.[5] We report the facile observation of tritium signals from a M_r 25 000 protein and the detection of $^3H\{^1H\}$ Overhauser effects that provide indications of local structure about the tritium-containing reporter group.

The proteolytic enzyme α-chymotrypsin reacts stoichiometrically with tosyl fluoride to give an inactive protein by virtue of attachment of a tosyl group to the critical serine-195 residue at the active site.[6] The native enzyme and the tosylated derivative have been studied extensively in the crystalline state and in solution.[7] In the present work tosyl fluoride was labelled with tritium in the methyl group by catalytic dechlorination of 4-(dichloromethyl)benzenesulphonyl fluoride with 100% T_2 gas over 10% Pd on carbon. Although the starting materials were pure, such reductions invariably lead to some scrambling, and the sample used to modify the enzyme (specific activity 42.7 Ci mmol^{-1}) contained a mixture of the non-, mono-, di- and tri-tritiated species in the ratio of 4 : 52 : 38 : 6, respectively, as judged by ^3H and ^1H NMR spectroscopy. There was no evidence of tritiation at positions other than the methyl group. Treatment of chymotrypsin with this material produced an inactive protein which, after extensive dialyses to replace the inactivation medium with a deuteriated solvent mixture, was examined by tritium NMR at 320.14 MHz.

A ^3H NMR spectrum of [^3H]-tosylchymotrypsin could be obtained by collection of 64 transients following a 90° pulse with a delay of 7.5 s (approximately 12× the observed tritium T_1) between each acquisition, to afford a tritium NMR spectrum of [^3H]tosylchymotrypsin with a signal-to-noise ratio of *ca.* 20. The spectrum consisted of a single broad line ($w_{\frac{1}{2}}$ = 55 Hz), the shape of which was unaltered by proton decoupling. Each isotopomer of the modified protein is expected to have a different chemical shift due to isotope effects[4] but the intrinsic linewidths in this case were sufficiently broad that these signals were overlapped. The observed tritium resonance showed an appreciable NOE when the sample was irradiated at the proton frequency and two-dimensional $^3H\{^1H\}$ NOESY spectra with phase sensitivity in both dimensions were obtained using a standard pulse sequence.[8] A number of cross peaks were observed in the $^3H\{^1H\}$ NOESY data and their relative intensities as a function of mixing time were examined. Fig. 1 records some of these results.

The mixing time dependence of the cross peaks observed indicates that the tritium nuclei of the labelled enzyme are relaxed by direct dipolar interactions with protons having shifts of 2.21, 7.09, 3.72 and 7.78 ppm. The first two groups of protons reside on the tosyl group and correspond to the residual ^1H on the methyl group and the aromatic ring, respectively, as demonstrated by experiments with a specifically deuteriated–tritiated inactivator. Residual protons in the solvent also contribute appreciably to the relaxation of the methyl tritons of the tosyl group since the intensity of the cross

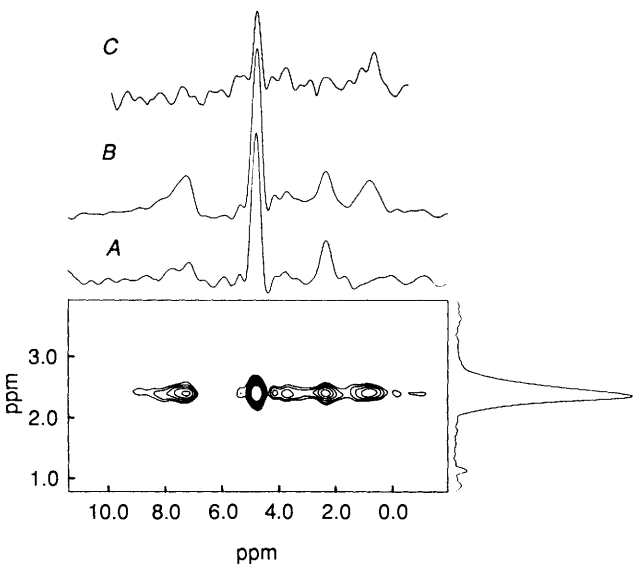

Fig. 1 Phase-sensitive $^3H\{^1H\}$ two-dimensional NOE spectrum of [^3H]tosylchymotrypsin, prepared as described in the text, obtained at 320 MHz and 25 °C with a mixing time of 300 ms. The protein concentration was 0.96 mmol dm^{-3} and the total tritium content 74 mCi, corresponding to an estimated total tritium concentration of 1.4 mmol dm^{-3}. The solvent consisted of 95% D_2O containing 0.05 mol dm^{-3} KCl at pH 7.0. Data for 64 t_1 increments (416 scans each) were recorded and processed using FTNMR (Provided by Hare Research, Inc., Woodinville, WA.) The acquisition time for the raw data set was 61 h. Projections to the proton and tritium axes are shown; the proton shift axis appears horizontally. (Curve *A*) projection to the proton axis of the 2D-NOE spectrum obtained sample when the mixing time was 75 ms. (Curve *B*) projection to the proton axis of the 2D-NOE spectrum obtained sample when the mixing time was 300 ms; a contour plot of these data is shown. (Curve *C*) proton projection obtained with a sample in which the aromatic and residual methyl protons of the tosyl group had been largely replaced by deuterium (mixing time 300 ms)

peak at 4.76 ppm varied with the isotopic composition of the solvent. Other cross peaks appear at 0.78, 2.91 and 7.49 ppm with longer mixing times, showing a characteristic lag phase in their development,[9] and must arise from spin-diffusive processes. However, their appearance indicates that groups characterized by these shifts, while not in direct dipolar contact with the tritiated methyl group, probably are present within the same spin 'island' as this group[10] and are, therefore, within the vicinity of the enzyme's active site.

Consideration of the crystal structure of tosylchymotrypsin shows that in the solid state the tosyl group occupies a cavity on the enzyme surface defined by Cys-191 and Asp-194 on one side of the aromatic ring and a stretch of polypeptide between residues 215 and 221 on the other side. Should this structure be retained in solution, protons of Gly-216, Ser-217 and Cys-220 would be less than 0.4 nm from the tritons of the methyl group. Considering the shifts of the directly interacting nuclei indicated by our NOE studies, proximity of protons from these residues is consistent with our results. Our observations of contact between the tritons of the inhibited enzyme and solvent-derived protons suggests that these protons must reside within the tosyl pocket with a sufficient lifetime to assume the correlation time of the protein. In the crystal the amide NH positions of Gly-216 and Ser-217 are within 0.3 nm of the tosyl methyl groups and these may be responsible for the observed effect.

We are attempting to account for these and related observations of [^{3}H]-tosylchymotrypsin by the construction of a model of the protein structure and dynamics in the vicinity of the tosyl group. For the present our results show that multidimensional NMR studies of fairly large molecules by the use of introduced tritium as a heteronuclear probe are feasible.

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