Multiple Forms of p-Trifluoromethylbenzenesulphonyl-α-chymotrypsin in Solution

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Summary ^{19}F N.m.r. studies of *p*-trifluoromethylbenzenesulphonyl-α-chymotrypsin indicate that this protein exists in two distinct forms at both pH 3 and 7.

TRIFLUOROMETHYL-SUBSTITUTED benzenesulphonyl fluorides inactivate the enzyme α-chymotrypsin, presumably by reaction with the serine-195 residue at the active site.[1] When the trifluoromethyl group is at the *para* position of the benzenesulphonyl unit, the modified enzyme is an analogue of tosylchymotrypsin, the *X*-ray structure of which has been determined.[2] We have been examining the ^{19}F n.m.r. spectra of the trifluoromethyl-containing enzymes, and we now describe some results for *p*-trifluoromethylbenzenesulphonylchymotrypsin.

Unexpectedly, in the ^{19}F n.m.r. spectra of this derivatized enzyme, two signals instead of one are apparent. Computed spectra, prepared by summing two Lorentzian peaks of variable position, relaxation rates (R_2), and intensities, were compared with the experimental spectra until a good match was obtained. The calculated curves were generated with the parameters given in the Table. Fluorine transverse relaxation curves, obtained by the Carr-Purcell–Meiboom–Gill technique[3] at 22·8 and 51 MHz, were consistent with the high-resolution observations. Over the pH range 3—8, these n.m.r. experiments thus imply the existence of two distinguishable environments for the trifluoromethyl group of this protein. In addition, a major change in the relaxation properties of one of these takes place with variation in solution acidity. The apparent pK of the group regulating this change is *ca.* 4·5.

TABLE. ^{19}F N.m.r. parameters for *p*-trifluoromethylbenzene sulphonylchymotrypsin[a]

pH	Populations[b] 1	Populations[b] 2	Chemical shifts/p.p.m.[c] 1	Chemical shifts/p.p.m.[c] 2	R_2/s^{-1} [d] 1	R_2/s^{-1} [d] 2
3	0·70	0·30	13·5	12·7	47	160
7	0·75	0·25	13·6	12·8	55	16

[a] Samples were *ca.* 1·5 mM derivatized enzyme in 0·05 M KCl solutions. A Bruker WH-90 spectrometer operating at 84·66 MHz was used. Approximately 2K transients were collected for each spectrum. The sample temperature was 23 °C. [b] Estimated uncertainty ±5%. [c] Chemical shifts downfield from 0·005 M internal trifluoroacetate. [d] Estimated uncertainty ±10%.

A number of control or confirmatory experiments were performed. (i) Preparation of the enzyme sulphonate ester employs typically a 10:1 molar excess of *p*-trifluoromethylbenzenesulphonyl fluoride over α-chymotrypsin.‡ However, the ^{19}F n.m.r. spectrum obtained is independent of the reaction time or length of time of the work-up (dialysis). There was no appreciable change in the spectrum of a sample during at least 24 h. (ii) Under the conditions used for the n.m.r. experiments (0·05 M KCl, 25 °C) there is virtually no detectable enzyme activity immediately after dissolution of the modified protein and essentially no increase in activity over periods 2—10 times as long as the duration of the n.m.r. experiments. (Signal averaging is required for 1—10 h, depending upon the type of experiment.) (iii) Quantitative high-resolution experiments employing an internal reference of known concentration show that there is present $0·7 \pm 0·1$ mol of the trifluoromethylbenzenesulphonyl group per mol of protein (on a gravimetric basis). Active site titration[4] indicates that there are 0·78—0·83 mol of active sites per mol of protein on the same basis. (iv) The effects of raising or lowering pH on the spectra are reversible. (v) When the modified enzyme is denatured either by treatment with 10% sodium dodecyl sulphate or by heating above 40 °C, the two peaks of the spectrum of the derivatized enzymes are replaced by a single sharp peak (R_2 *ca.* 1·0 s^{-1}) at 12·7 p.p.m. (vi) *p*-Trifluoromethylbenzenesulphonyl-α-chymotrypsin maleylated according to the procedure of Hartley, *et al.*[5] gives two signals in the ^{19}F n.m.r. spectrum although the transverse relaxation rates for these signals differ slightly from those of the unmodified protein. (vii) N.m.r. results obtained from the *o*- and *m*-trifluoromethylbenzenesulphonyl fluoride-inhibited enzymes are very similar to those described above for the *para* substituted derivative despite the substantial differences in reactivity of these compounds with the enzyme.[1] Experiments i, ii, iii, and vii indicate that the various sulphonyl fluorides react only at the active centre of the enzyme, suggesting that the observed ^{19}F signals are not due to several trifluoromethyl groups bound to a given protein molecule but rather arise from a single group in two magnetically distinguishable environments. Experiments i and iv show that irreversible degradation of the protein samples during preparation or observation is not responsible for our observations; that these derivatives have no catalytic activity precludes autodegradation. The observed relaxation rates indicate that none of the signals observed with 'native' protein samples arises from denatured protein and, since maleylated α-chymotrypsin does not associate,[6] it appears unlikely that protein association is primarily responsible for the presence of two signals in the fluorine spectra.

We have shown that *p*-trifluoromethylcinnamoylchymotrypsin[7] can be prepared by treating the enzyme with *p*-trifluoromethylcinnamoylimidazole. Although in these preparations less than a stoicheiometric amount of acylating agent is used, the ^{19}F n.m.r. spectrum of the acylenzyme at pD 3·6 is very similar to the spectrum of *p*-trifluoromethylbenzenesulphonylchymotrypsin in the number of signals present, their relative intensities, and relaxation times. The observations made with the tosylchymotrypsin analogues thus may be quite general, although there is as yet no evidence that the acylenzyme intermediate(s) involved in the hydrolysis of 'natural' substrates behave in a corresponding way.

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‡ There is evidence that α-chymotrypsin catalyses the hydrolysis of *p*-trifluoromethylbenzenesulphonyl fluoride.[1]

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