Evidence for Hemiacetal Formation between α-Chymotrypsin and Hydrocinnamaldehyde by Cross-saturation Nuclear Magnetic Resonance Spectroscopy

By GORDON LOWE* and DAVID NURSE
(*The Dyson Perrins Laboratory, Oxford University, South Parks Road, Oxford* OX1 3QY)

Summary Hemiacetal formation between α-chymotrypsin and the inhibitor hydrocinnamaldehyde has been detected by cross-saturation n.m.r. spectroscopy.

HYDROCINNAMALDEHYDE and *trans*-cinnamaldehyde are competitive inhibitors of the proteolytic enzyme, α-chymotrypsin, with K_I = 0·38 and 2·6 mM at pH 7·8 respectively.[1] The inhibition of α-chymotrypsin with hydrocinnamaldehyde shows pH-dependence on a group with a pK_a 7 which led Schultz and Cheerva[2] to suggest that this must be due to the enzyme catalysed formation of the hemiacetal between the aldehyde and the active site serine hydroxy group of the enzyme. Breaux and Bender however pointed out that other interpretations are possible and using

p-dimethylaminocinnamaldehyde found no evidence by u.v. spectroscopy for the formation of a hemiacetal with α-chymotrypsin.[3] The binding of *trans*-cinnamaldehyde to α-chymotrypsin has also been investigated by n.m.r. spectroscopy.[4] Line broadening of the aldehydic proton was observed as the enzyme concentration was increased, and because no change in chemical shift was observed it was concluded that the enzyme–cinnamaldehyde complex was not in equilibrium with the hemiacetal. The validity of this conclusion however depends on the assumption that if a hemiacetal was formed, it would be in fast exchange with the free aldehyde on the n.m.r. time scale. A similar observation was made in an n.m.r. investigation of the binding of *N*-acetylaminoacetaldehyde to papain, in that the aldehydic proton was broadened but not shifted on the addition of papain, demonstrating fast exchange between the free and enzyme bound aldehyde on the n.m.r. time scale.[5] In a double resonance experiment however it was shown that a radiofrequency (r.f.) pulse in a region expected for the hemithioacetal gave rise to cross-saturation of the free aldehydic proton, providing evidence for the presence at equilibrium of a hemithioacetal in slow exchange on the n.m.r. time scale, but in fast exchange relative to the rate of relaxation of the nuclear magnetisation $(1/T_1)$.[5,6]

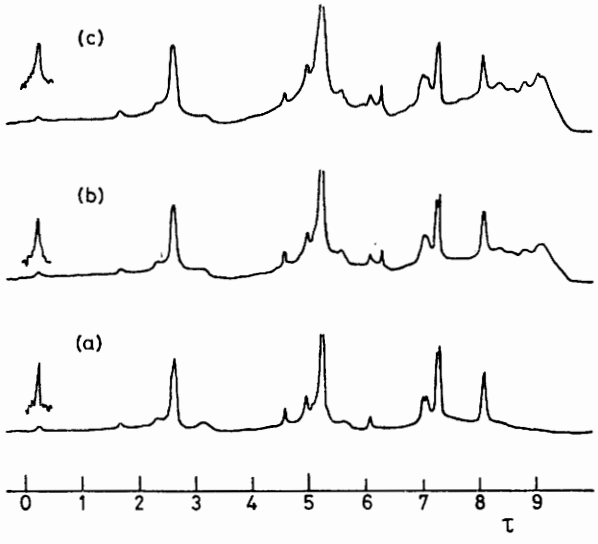

FIGURE 1. (a) The ^{1}H n.m.r. spectrum of hydrocinnamaldehyde (15 mM; aldehyde to hydrate, 1 : 2·7) in 50 mM sodium phosphate buffer, pD 6·9, in D_2O containing 10% (v/v) $(CD_3)_2SO$. The aldehydic proton is also recorded at increased gain (×8) above each spectrum. (b) The ^{1}H n.m.r. spectrum of the same solution in the presence of 0·15 mM α-chymotrypsin and (c) 0·25 mM α-chymotrypsin.

The resonances in the ^{1}H n.m.r. spectrum of hydrocinnamaldehyde (Figure 1a) centred at τ 7·0, and those at 7·2 and 8·1, are assigned to the A_2B_2 multiplets of the methylene protons of the aldehyde and hydrated aldehyde respectively.† The methylene protons adjacent to the aldehyde are expected to exchange slowly with solvent deuterons leading to a diminished signal intensity at τ 8·1 $[CH_2{\cdot}CH(OH)_2]$ since the aldehyde and hydrated aldehyde are in equilibrium. The ratio of the aldehyde to the hydrated aldehyde can nevertheless be estimated as 1 : 2·7 from the relative areas under the two A_2B_2 multiplets. The hydrated aldehyde resonance $[{\cdot}CH(OH)_2]$ is expected to occur under the DOH peak, but that of the aldehyde (·CHO) is at τ 0·2. On addition of α-chymotrypsin, the aldehyde resonance broadens, as does the A_2B_2 multiplet of the methylene protons of the aldehyde centred at τ 7·0 (Figure 1b and c). A plot of the line-width (at half height) against the ratio of enzyme to inhibitor concentration gave a straight line as expected, since the inhibitor concentration is considerably greater than K_I.[4] The line-width of the resonances (at 7·2 and 8·1) of the A_2B_2 multiplet of the hydrated aldehyde are not significantly broadened on adding α-chymotrypsin (Figure 1b and c) indicating that the binding of the hydrated aldehyde to the enzyme is much weaker.

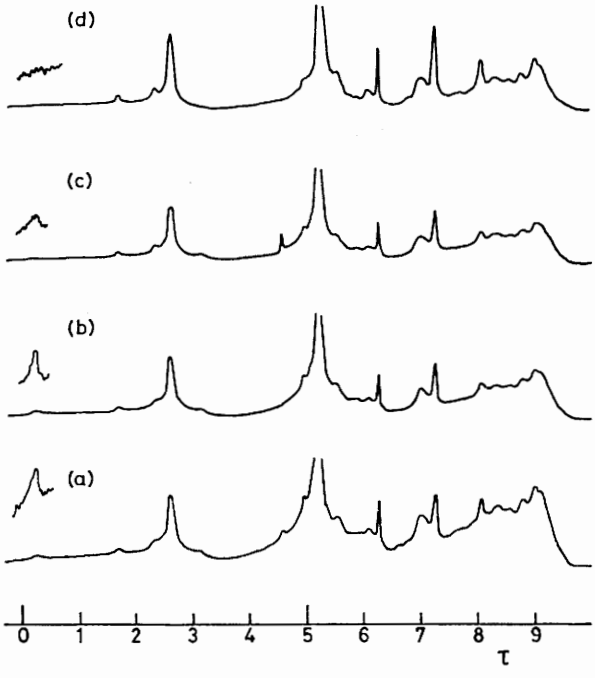

FIGURE 2. (a) The ^{1}H n.m.r. spectrum of hydrocinnamaldehyde (12·6 mM) in 50 mM sodium phosphate buffer, pD 6·9, in D_2O containing 8·4% (v/v) $(CD_3)_2SO$ in the presence of α-chymotrypsin (0·45 mM). Spectra (b), (c), and (d) were obtained from the same solution with a second r.f. pulse applied at τ 4·6 with power levels of 0·1, 0·2, and 0·4 V respectively. The aldehydic proton resonance is also recorded at increased gain (×8) above each spectrum.

When a solution of hydrocinnamaldehyde (as in Figure 1a) is irradiated with a second r.f. pulse in the range between τ 4·6 and the DOH peak, no diminution of the aldehyde signal intensity is observed, demonstrating that the rate of exchange of the hydrated aldehyde with the free aldehyde is too slow (compared with the rate of relaxation, $1/T_1$) to cause any observable cross-saturation of the aldehydic proton. When a similar double resonance

† ^{1}H N.m.r. spectra were obtained at 27 °C on a Bruker 270 MHz spectrometer in the Fourier transform mode, storing 1000 scans in a 4K block.

experiment is performed in the presence of α-chymotrypsin however, saturation of the aldehydic proton does occur when the second r.f. pulse is at τ 4·6. The spectra in Figure 2 show the effect of increasing the r.f. power at this frequency. As is shown in Figure 2d, it is possible to saturate completely the aldehydic proton resonance in this way. Although in principle this observation could be ascribed to a negative nuclear Overhauser effect,[7] the coincidence of the chemical shift (τ 4·6) with that expected for a hemiacetal, together with the complete saturation of the aldehydic proton resonance makes cross-saturation almost certainly the cause, vindicating the suggestion of Schultz and Cheerva.[2]

A similar experiment was performed with *trans*-cinnamaldehyde (8·8 mM) in the presence of α-chymotrypsin (2·34 mM). Scanning of the ^{1}H n.m.r. spectrum with a second r.f. pulse, failed to produce any cross-saturation of the aldehydic proton (0·3 p.p.m.). No evidence is provided therefore for hemiacetal formation between *trans*-cinnamaldehyde and α-chymotrypsin, in complete accord with the u.v. spectroscopic observations of Breaux and Bender.[3]

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