Detection of Enzyme-bound Intermediates by Cross-saturation in Nuclear Magnetic Resonance Spectroscopy; an Investigation of the Papain–*N*-Benzoylaminoacetaldehyde Complex

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Summary A hemithioacetal formed between the active site thiol of the proteolytic enzyme papain, and the inhibitor *N*-benzoylaminoacetaldehyde, has been detected by a double resonance experiment in which magnetisation is transferred between the enzyme-bound and free inhibitor.

WHEN the rate of ligand dissociation from an enzyme–ligand complex is slow on the n.m.r. time-scale, identification of ligand resonances in a ^1H n.m.r. spectrum of the complex is a formidable task. If however the rate of ligand dissociation is fast with respect to the rate of relaxation of a ligand nucleus ($1/T_1$), saturation of the magnetisation of a bound ligand nucleus in a double resonance experiment should lead to cross-saturation of the corresponding ligand nucleus in free solution. In this way the chemical shift of an enzyme-bound ligand nucleus can be determined provided it is different from that of the ligand nucleus in free solution.

N-Acylaminoacetaldehydes are potent inhibitors of the proteolytic enzyme, papain.[1] They exist in aqueous solution predominantly in their hydrated form, but it is the

aldehyde and not the hydrated aldehyde which is the effective inhibitor.[2] It is possible however that in addition to forming an enzyme–inhibitor complex, the complex is also in equilibrium with a hemithioacetal formed between the active site thiol group of the enzyme and the aldehyde group of the inhibitor.[1,3]

N-Benzoylaminoacetaldehyde has an inhibition constant $K_I = 3\,\mu$M, and is in slow exchange with papain on the chemical shift time-scale. The low-field ^{1}H n.m.r. spectrum of a solution of *N*-benzoylaminoacetaldehyde and papain is shown in Figure 1a. The singlet at τ 0·39 is that of the aldehydic proton. In a double resonance experiment, the spectrum was scanned with a high power radio frequency (R.F.) source. No effect was observed on the intensity of the aldehydic proton in any region (*e.g.* Figure 1b at τ 7·96) except near τ 4 (Figure 1c) where the aldehydic signal was almost completely lost. No effect on the aldehydic signal was observed in the absence of enzyme when the spectrum was scanned with a high power R.F. source, even at the position of the hydrated aldehyde.

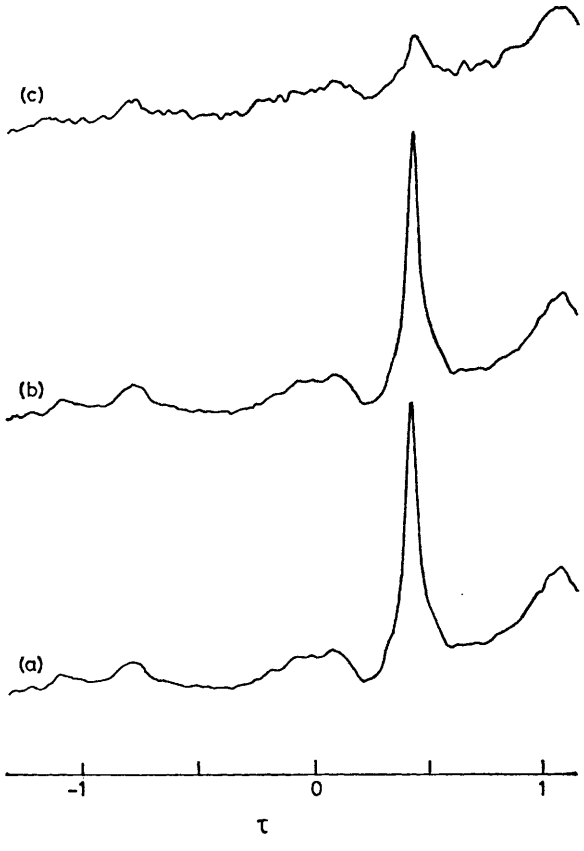

FIGURE 1. The low field ^{1}H n.m.r. spectrum at 270 MHz of *N*-benzoylaminoacetaldehyde (94·6 mM; aldehyde : hydrate ratio, 1 : 6·5) in the presence of papain (1·1 mM) at pH 5·9(a), with a high power R.F. source applied at (b) τ 7·96 and (c) τ 4·00.

In order to establish more closely the chemical shift of the enzyme-bound ligand nucleus in equilibrium with the free aldehydic proton, a sub-saturating R.F. power level was used which enabled a narrower band width to be achieved. Under these conditions the maximum cross-saturation was observed at τ 3·81. This chemical shift is certainly consistent with the existence of a hemithioacetal in equilibrium with the enzyme-bound and free aldehydes.[4] The free and enzyme-bound aldehydic resonances probably have similar chemical shifts since papain broadens but does not shift the aldehydic proton of *N*-acetylaminoacetaldehyde which is in fast exchange on the chemical shift time-scale.[2]

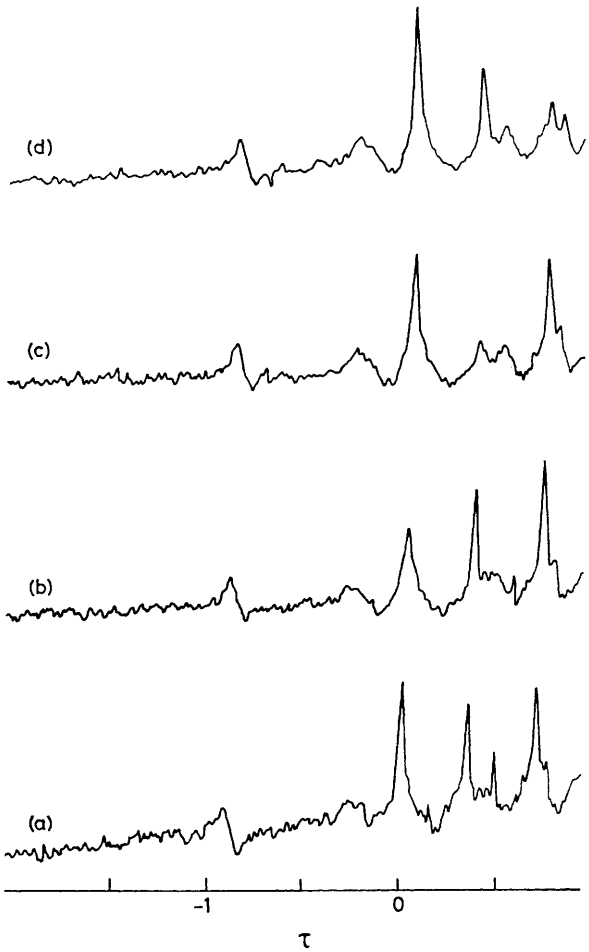

FIGURE 2. The low field ^{1}H n.m.r. spectrum at 270 MHz of *N*-benzoylamino-[1-^{13}C]acetaldehyde (150 mM; aldehyde : hydrate ratio, 1 : 6·5) in the presence of papain (1·1 mM) at pH 5·9 (a), with a high power R.F. source applied at (b) τ 3·49, (c) τ 3·82, and (d) τ 4·13.

An alternative explanation however is that the correlation time of the papain–*N*-benzoylaminoacetaldehyde complex is sufficiently long and an enzymic proton with τ 3·81 is sufficiently close to the bound aldehydic proton, to cause a negative nuclear Overhauser effect.[5] To distinguish between these two possibilities, *N*-benzoylaminoacetaldehyde was prepared with 60 atom % ^{13}C enrichment in the aldehyde. The low-field ^{1}H n.m.r. spectrum of a solution containing *N*-benzoylamino-[1-^{13}C]acetaldehyde and papain is shown in Figure 2a. The aldehydic proton now appears as three lines of approximately equal intensity, the two outer lines arising from coupling to the ^{13}C nucleus [^1J(^{1}H-^{13}C) 182 Hz] and the central line from the [^{12}C]aldehyde.

When the double-resonance experiment was performed, as the high power R.F. source scanned from low to high field, the three aldehydic lines collapsed in turn when the irradiation was at τ 3·49, 3·82, and 4·13, respectively. These observations are consistent only with a cross-saturation phenomenon and not a negative nuclear Overhauser effect. They also indicate that the rate of exchange is faster than the rate of relaxation of the ^{13}C nucleus ($1/T_1$ *ca.* 0·05 s^{-1}).[6]

The chemical shift at which the cross-saturation occurs (τ 3·81) is somewhat lower than that of a simple hemithioacetal,[4] but the local magnetic environment, especially the ring current shift associated with histidine-159 and tryptophan-177 could account for this. The coupling constant [$^1J(^1H\text{-}^{13}C)$] of the enzyme-bound species, determined from the maximal effect of the high power R.F. source on the ^{13}C-coupled lines of the free aldehydic proton was 173 Hz, which is in the expected region for a hemithioacetal.[7] The hydrated form of *N*-benzoylamino-[1-^{13}C]acetaldehyde has a coupling constant $^1J(^1H\text{-}^{13}C)$ = 165 Hz.

Cross-saturation n.m.r. spectroscopy appears to be a promising technique for detecting intermediates in reactions at equilibrium, whether enzyme-catalysed or not, provided that the rate of exchange between sites is in the appropriate time range.

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