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Inhibition of Papain by *N*-Acyl-aminoacetaldehydes and *N*-Acyl-aminopropanones

Evidence for Hemithioacetal Formation by a Cross-saturation Technique in Nuclear-Magnetic-Resonance Spectroscopy

M. Robin BENDALL, Ian L. CARTWRIGHT, Peter I. CLARK, Gordon LOWE, and David NURSE

The Dyson Perrins Laboratory, Oxford University

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N-Acyl-aminoacetaldehydes are potent inhibitors of the proteolytic enzyme, papain. Although they exist predominantly in their hydrated form in aqueous solution only the aldehyde is an effective inhibitor. The binding constants for related amides and methyl ketones confirm that it is principally the lower steric requirement of the aldehyde rather than its increased electrophilicity which is responsible for its powerful inhibitory properties. Using nuclear magnetic resonance spectroscopy, evidence is provided for an *N*-acetyl-aminoacetaldehyde-papain complex. Using a cross-saturation technique evidence is also provided for a hemithioacetal, formed from the aldehyde and the active-site thiol group. Hemithioacetal formation has also been detected between *N*-benzoyl-aminoacetaldehyde and papain. This provides the first direct evidence for a tetrahedral adduct with papain and supports the proposed involvement of such intermediates in papain-catalysed hydrolyses.

Papain-catalysed hydrolyses proceed by a reaction pathway which involves minimally three steps:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightleftarrows} ES' \overset{k_3}{\rightarrow} E + P_2 + P_1$$

where ES is the Michaelis complex, ES′ is the acyl-enzyme formed through the thiol group of cysteine-25, P_2 is the acid and P_1 the alcohol or amine moeity of the hydrolysed substrate. There is, however, indirect evidence for the involvement of tetrahedral intermediates in the acylation step, k_2, and if established this would make a tetrahedral intermediate in the deacylation step, k_3, virtually mandatory [1 – 3].

The specificity of papain for structural features in the acyl moiety of the substrate manifests itself very largely in the formation of the acyl-enzyme from the enzyme-substrate complex, *i.e.* the specificity is largely observed in the acylation rate constant, k_2 [4]. This feature of kinetic specificity is common amongst the proteolytic enzymes and in principle can arise from three distinct causes: non-productive binding, a substrate-induced conformational change in the enzyme, or substrate destabilisation in the enzyme-substrate complex [5]. Model building suggested that a non-bonded interaction between the α-CH bond of His-159 in the enzyme and the NH or O of the substrate-leaving group would destabilize the ES complex and so contribute to the observed kinetic specificity. Experimental support for this hypothesis was provided by the fact that when the ester or amide group of the substrate was replaced by a nitrile, the specificity of the enzyme was reflected in their binding constants [4]. This hypothesis further predicted that replacement of the ester or amide group in the substrate by an aldehyde should lead to a potent competitive inhibitor but replacement by a methyl ketone would not.

Westerik and Wolfenden have shown that *N*-acyl-aminoacetaldehydes are indeed potent inhibitors of papain [6] and in this investigation *N*-acyl-aminopropanones have been shown to be poor inhibitors of the enzyme.

Westerik and Wolfenden suggested that the potency of the *N*-acyl-aminoacetaldehydes was probably due to their ability to form a hemithioacetal with the active-site cysteine residue. No support for this hypothesis was provided, nor indeed was any evidence provided to establish whether the aldehyde or

Abbreviation. NMR, nuclear magnetic resonance.

Enzyme. Papain (EC 3.4.22.2).

the hydrated aldehyde was the true inhibitor. These questions have now been investigated by NMR spectroscopy.

MATERIALS AND METHODS

Materials

Papain was prepared from the commercial dried papaya latex [7] and purified to give 100% active material by affinity chromatography [8]. Treatment with a molar equivalent of mercuric chloride solution gives 100% activatable mercuripapain. This material, after activation, was used in the kinetic studies.

Active papain for use in the NMR experiments was prepared by affinity chromatography and concentrated by ultrafiltration in an Amicon Diaflo with successive portions of deuterium oxide (5 × 20 ml). The final concentration used was approximately 1.5 mM.

N-Benzoyloxycarbonyl-glycine *p*-nitrophenylester, obtained from the Sigma Chemical Corporation (London), was used as a 5 mM solution in purified acetonitrile. Acetonitrile (obtained from B.D.H. Ltd) was dried over $MgSO_4$ and distilled from phosphorus pentoxide, the fraction distilling at 81.5–82.0 °C at atmospheric pressure being collected. Aminoacetaldehyde dimethyl acetal was obtained from Ralph N. Emanuel, Ltd. Deuterohydrochloric acid (35% in ^2H_2O) and deuterated sodium hydroxide (40% in ^2H_2O) were obtained from B.D.H. Chemicals Ltd. Deuterium oxide was obtained from the Ryvan Chemical Co. Ltd. Buffer solutions were prepared using demineralised water and B.D.H. AnalaR sodium dihydrogen phosphate and disodium hydrogen phosphate. All buffer solutions were 1 mM in EDTA disodium salt and 0.3 M in sodium chloride. All kinetic studies were performed at pH 6.0. Adjustments of the buffer was made with sodium hydroxide solution.

Syntheses of N-*Acyl-aminoaldehydes*

N-*Benzoyl-aminoacetaldehyde dimethyl acetal.* This was prepared by the method of Brown [9] and obtained as colourless crystals (77%) m.p. 52–54 °C; λ_{max} (EtOH) 228 nm (ε 10400 M^{-1} cm^{-1}), 208 nm (ε 7950 M^{-1} cm^{-1}); τ in [$^2H_6$]dimethyl sulphoxide 1.5 (1H, s, NH), 2.1–2.6 (5H, ArH), 5.47 (1H, t, CH), 6.6 (2H, dd, CH_2), 6.70 (6H, s, OCH_3) ppm; on treatment with ^2H_2O, the singlet (τ = 1.5 ppm) disappeared. Found: C, 63.4; H, 7.3; N, 6.7%: $C_{11}H_{15}NO_3$ requires C, 63.1; H, 7.2; N, 6.7%.

N-*Benzoyl-aminoacetaldehyde.* Hydrolysis was performed in 0.1 M deuterohydrochloric acid and the reaction followed by NMR spectroscopy. After hydrolysis, the solution contained both the aldehyde and the hydrated form in the molar ratio 1:6.5.

N-*Acetyl-aminoacetaldehyde dimethyl acetal.* This was prepared by the method of Brown [9] in 80.5% yield, b.p. 78–79 °C (0.2 mm of Hg); n_D^{22} 1.447; τ in [$^2H_6$]dimethyl sulphoxide 2.13 (1H, s, NH), 5.64 (1H, t, CH), 6.72 (6H, s, OCH_3), 6.84 (2H, dd, CH_2), 8.18 (3H, s, CH_3) ppm; on treatment with ^2H_2O, the singlet (τ = 2.13 ppm) disappeared. Found: C, 49.0; H, 9.0; N, 9.5%: $C_6H_{13}NO_3$ requires, C, 49.0; H, 8.9; N, 9.5%.

N-*Acetyl-aminoacetaldehyde.* Hydrolysis was achieved in 0.1 M deuterohydrochloric acid solution by continuous shaking for 24 h. The NMR spectrum showed that hydrolysis was complete and that the solution contained the aldehyde and the hydrate in a molar ratio of 1:5.4. Adjustment to pH 6.0 caused no change.

N-*Benzyloxycarbonyl-L-phenylalanyl-aminopropanone.* *N*-Benzyloxycarbonyl-L-phenylalanyl *N*-hydroxysuccinimide ester (5.96 g, 15 mmol) [10], aminopropanone hydrochloride (1.6 g, 15 mmol) [11] and triethylamine (2.1 ml, 15 mmol) were dissolved in dimethoxyethane (20 ml) and water (10 ml). The solution was stirred for 1 h, cooled, and water (75 ml) added with vigorous stirring. A white solid was precipitated, filtered off and dissolved in ethanol. The solution was concentrated and *N*-benzyloxycarbonyl-L-phenylalanyl-aminopropanone crystallised at 0 °C as white needles (64.5%), m.p. 126–128 °C; λ_{max} (ethanol) 210 nm (ε 12000 M^{-1} cm^{-1}); $[\alpha]_D^{20}$ + 0.6 °; τ in C^2HCl_3 2.7–2.8 (10H, ArH), 3.3 (1H, broad s, NH), 4.45 (1H, d, J = 8.5 Hz, NH), 4.97 (2H, s, CH_2), 5.5 (1H, m, CH), 5.98 (2H, d, CH_2), 6.95 (2H, d, J = 8.0 Hz, CH_2), 8.91 (3H, s, CH_3) ppm. Found: C, 67.8; H, 6.3; N, 7.9%: $C_{20}H_{22}N_2O_4$ requires C, 67.9; H, 6.3; N, 7.9%.

N-*Acetyl-L-phenylalanyl-aminopropanone.* To *N*-Benzyloxycarbonyl-L-phenylalanyl-aminopropanone (3.54 g) was added a saturated solution of hydrogen bromide in acetic acid (10 ml) and further acetic acid (30 ml). After 30 min, when evolution of carbon dioxide had ceased, ether (200 ml) was added. The syrup which formed was triturated with ether and used directly in the next stage. L-Phenylalanyl-aminopropanone hydrobromide (1.5 g, 5 mmol) was suspended in chloroform (25 ml), and cooled to 0 °C. Triethylamine (0.7 ml, 5 mmol), and a solution of acetic anhydride (0.5 ml, 5 mmol) in chloroform (5 ml) were added dropwise to the stirred solution over 30 min. The suspension gradually dissolved, and the reaction mixture was kept stirring overnight. After washing with 0.5 M HCl (4 ml) and water (4 ml) it was dried ($MgSO_4$) and the solvent was removed. The residue was dissolved in acetone/ether and set aside to crystallise. Recrystallisation from acetone/ether gave colourless crystals of *N*-acetyl-L-phenylalanyl-aminopropanone (18.5%), m.p. 129–130 °C; λ_{max} (ethanol), 220 nm (ε 1950 M^{-1} cm^{-1}); $[\alpha]_D^{20}$-0.5 °;

τ in C^2HCl_3, 2.7–2.9 (5H, ArH), 3.13 (1H, broad, s, NH), 3.5 (1, d, $J = 8$ Hz, NH), 5.2 (1H, m, CH), 5.96 (2H, d, $J = 5$ Hz, CH_2) 6.1 (2H, d, $J = 8$ Hz, CH_2), 7.86 (3H, s, CH_3), 8.03 (3H, s, CH_3) ppm. Found: C, 64.1; H, 6.9; N, 10.5%: $C_{14}H_{18}N_2O_3$ requires C, 64.1; H, 6.9; N, 10.7%.

N-*Benzoyl-aminopropanone.* (This was prepared with D. C. Case). 2-Phenyl-5-oxazolone (8 g) [12], lead acetate (2 g) and ethyl orthoacetate (14 g) were heated on a water bath for 40 min. The reaction mixture was cooled, poured into ice-water and filtered. The yellow solid obtained was suspended in 6 M hydrochloric acid (30 ml) and heated on a water bath for 90 min when evolution of carbon dioxide had ceased. The solution was decanted and extracted with chloroform and the extract washed with sodium carbonate and water, dried ($MgSO_4$) and the solvent removed. Trituration with light petroleum (b.p. 40–60 °C) gave a crystalline *N*-benzoyl-aminopropanone which was recrystallised from benzene/light petroleum (b.p. 40–60 °C) and had m.p. 82–83 °C (*cf.* m.p. 82–83 °C [13]) (4.0 g); ν_{max} ($CHCl_3$), 3410 (NH), 1723 (CO) 1660 and 1620 cm^{-1} (CONH); τ in C^2HCl_3 2.08–2.70 (5H, m, ArH), 2.95 (1H, m, NH), 5.66 (2H, d, $J = 5$ Hz, NH · CH_2) 7.60 (3H, s, CH_3) ppm. Found: C, 68.0; H, 6.4; N, 7.8%: $C_{10}H_{11}NO_2$ requires C, 67.9; H, 6.2; N, 7.9%.

N-*Benzoyl-amino[1-^{13}C]acetaldehyde.* [1-^{13}C]Glycine (60 atom % ^{13}C) was converted to *N*-benzoyl-[1-^{13}C]glycine [14] which was esterified with diazomethane. A solution of diisobutyl aluminium hydride in toluene (1.5 molar excess, 2.5 ml) was added over 5 min to a stirred solution of *N*-benzoyl-[1-^{13}C]glycine methyl ester (0.5 g) in dimethoxyethane under N_2 at −60 °C. After addition was complete, the solution was stirred for a further 12 h at −60 °C. 1 M hydrochloric acid (5 ml) was added and the solution allowed to warm up to about 10 °C (1 h) and the dimethoxyethane removed by rotary evaporation. The aqueous solution was extracted with chloroform (3 × 25 ml), the extracts dried ($MgSO_4$) and the solvent removed. Trituration of the residue with ether at −20 °C gave *N*-benzoyl-amino[1-^{13}C]acetaldehyde as a white solid (0.36 g, 81%), m.p. 41–46 °C, ν_{max} ($CHCl_3$) 3430 (NH), 1730 (CHO), 1662 and 1610 cm^{-1} (CONH); ^{1}H NMR τ in C^2HCl_3 0.39 (1H, d, $^1J_{^{13}CH}$ 179 Hz, ^{13}CHO), 2.0–3.2 (6H, m, ArH and NH), 5.79 (2H, t, $J_{NHCH} = {}^2J_{^{13}CH}$ 4.8 Hz, NH · $\mathbf{CH_2}$ · ^{13}CHO; τ (^2H_2O, p^{2}H 6.0) 0.39 (d, $^1J_{^{13}CH}$ 182 Hz, ^{13}CHO), 2.0–2.5 (m, ArH), 5.70 (d, $^2J_{^{13}CH}$ 5 Hz, $\mathbf{CH_2}$ · ^{13}CHO), 4.79 [dt, $^1J_{^{13}CH}$ 169 Hz, $^3J_{HH}$ 5.5 Hz, CH_2 · $^{13}\mathbf{CH}(O^2H)_2$], 6.50 [m, $^2J_{^{13}CH}$ 5 Hz, $^3J_{HH}$ 5.5 Hz, $\mathbf{CH_2}$ · $CH(O^2H)_2$], hydrate to aldehyde ratio 6.5:1.

Papain for Kinetic Experiments

To a mercuripapain solution (1 ml, approximately 0.28 mM enzyme) was added a solution of mercaptoethanol (50 µl, 0.6 M) and Na_2EDTA (2.5 mg) to give a solution approximately 5 mM in EDTA. The solution was kept for about 0.5 h in a closed tube and then placed on a Sephadex G-25 (fine grade) column (30 × 2.5 cm) and eluted with 5 mM EDTA solution. Enzyme concentration was calculated using a value for the molar absorption coefficient, $\varepsilon_{278\,nm}$, of 57000 M^{-1} cm^{-1}. The value so calculated is taken to be the concentration of active enzyme.

Kinetics

Kinetic runs were made with a Pye Unicam SP-1800 double-beam spectrophotometer with the cell compartment thermostatically controlled at 35.0 °C. Buffer solution (2.5 ml) was added to each cuvette followed by a small volume of the inhibitor solution. The active enzyme solution was then added such that the concentration of enzyme was of the order of 0.1 µM. The cuvette was stoppered and allowed to attain thermal equilibrium (10 min). *N*-Benzyloxycarbonyl-glycine *p*-nitrophenyl ester in acetonitrile was then added to give a substrate concentration of about 0.1 mM; the acetonitrile concentration was 2%. Recording of absorbance at 340 nm was immediately commenced.

NMR Spectroscopy

NMR spectra of enzyme-inhibitor complexes were obtained on a Bruker 270-MHz spectrometer equipped with Fourier transform facilities and magnetic discs for storing spectra. Cross-saturation experiments were performed using a 500-ms gated pulse immediately before sampling the spectrum, the H_2 offset was varied until the maximal effect was observed. Solutions similar to those described below were used, the inhibitor concentration being such that an acceptable spectrum could be obtained in less than 1000 scans.

Binding of N-*Benzoyl-aminoacetaldehyde*

Aliquots (50 µl) of 0.202 M *N*-benzoyl-aminoacetaldehyde in 16.7% (v/v) [$^2H_3$]acetonitrile in ^2H_2O (p^{2}H 6) were added and mixed with 4% (w/v) active papain in the same solvent (0.5 ml, pH 6) and the ^{1}H NMR spectra recorded at 270 MHz and 35 °C. The number of scans used was 1000–3000 depending on the concentration of the inhibitor. A spectrum of active papain recorded under the same conditions and stored in the computer was subtracted from the inhibitor-enzyme spectrum.

Binding of N-*Acetyl-aminoacetaldehyde*

Aliquots (50 µl) of 0.208 M *N*-acetyl-aminoacetaldehyde in ^2H_2O (p^{2}H 6) were added and mixed

with 4% (w/v) active papain in ^2H_2O (0.5 ml, pH 6) and the ^1H NMR spectra recorded as above. The enzyme spectrum was subtracted.

Binding of N-*Acetyl-L-phenylalanylaminopropanone*

N-Acetyl-L-phenylalanylaminopropanone (0.54 mg, 1.0 equiv.) was dissolved in 1.96 mM papain in ^2H_2O (1.1 ml, p^{2}H 6.0) and a further amount of the compound (5.56 mg, 10.3 equiv.) was dissolved in another aliquot of the enzyme solution (1.1 ml). Solutions containing intermediate concentrations of inhibitor were made by adding aliquots (100 µl) of one solution to 0.5 ml of the other. Spectra were recorded after each addition.

RESULTS AND DISCUSSION

The effect of inhibitor concentration on the Michaelis parameters for the papain-catalysed hydrolysis of *N*-benzyloxycarbonyl-glycine *p*-nitrophenyl ester is shown in Tables 1, 2 and 3 for *N*-benzoyl-aminoacetaldehyde, *N*-acetyl-aminoacetaldehyde and *N*-acetyl-L-phenylalanyl-aminopropanone respectively. From these results the inhibition constants (K_i) were determined and are compared with the binding constants of other related inhibitors and substrates in Table 4. Since the acylation step (k_2) is rate-limiting in the papain-catalysed hydrolysis of *N*-benzoyl-glycinamide, K_m can be regarded as a true binding constant and comparable therefore with K_i values for inhibitors. A comparison between *N*-benzoyl-aminoacetaldehyde (K_i = 3 µM), *N*-benzoyl-glycinamide (K_m = 202 mM) and *N*-benzoyl-aminopropanone (K_i > 1 M), shows the very large change in binding dissociation constant associated with these structural changes which cannot be correlated with the electrophilicity of the carbonyl group, but could be associated with the increasing bond length (C-H, 1.12 Å, C-N, 1.47 Å, C-C 1.53 Å) and steric size of the group (H < NH_2 < CH_3) attached to it. Although nitriles might be expected to fit into the active site with even less non-bonded interaction than the aldehydes, from the X-ray crystallographic analysis of a

Table 1. *Kinetic parameters for the competitive inhibition of papain-catalysed hydrolysis of* N-*benzyloxycarbonyl-glycine* p-*nitrophenyl ester by* N-*benzoyl-aminoacetaldehyde*
Conditions: temperature 35.0 °C, 0.1 M phosphate buffer at pH 6.0, ionic strength 0.3 M in sodium chloride and buffer ions, [E] = 0.165 µM, acetonitrile = 2% (v/v). From a plot of K_m^{app} against [I] the binding constant for competitive inhibition of papain, K_i = 0.003 mM, was obtained

[I]	V	K_m^{app}
µM	µM · s^{-1}	µM
0	1.230	7.90
5.02	1.139	22.47
10.02	1.107	32.19
15.02	1.100	46.43
19.99	1.116	65.80
24.94	1.062	71.80

Table 2. *Kinetic parameters for competitive inhibition of papain-catalysed hydrolysis of* N-*benzyloxycarbonyl-glycine* p-*nitrophenyl ester by* N-*acetyl-aminoacetaldehyde*
Conditions: temperature 35.0 °C, 0.1 M phosphate buffer at pH 6.0, ionic strength 0.3 M in sodium chloride and buffer ions, [E] = 0.112 µM, acetonitrile = 2% (v/v). From a plot of K_m^{app} against [I] the binding constant for competitive inhibition of papain, K_i = 0.39 mM, was obtained

[I]	V	K_m^{app}
µM	µM · s^{-1}	µM
0	1.212	7.69
114	1.202	11.34
284	1.216	12.64
563	1.174	17.12
1106	1.198	30.23

Table 3. *Kinetic parameters for competitive inhibition of papain-catalysed hydrolysis of* N-*benzyloxycarbonyl-glycine* p-*nitrophenyl ester by* N-*acetyl-L-phenylalanyl-aminopropanone*
Conditions: temperature 35.0 °C, 0.1 M phosphate buffer at pH 6.0, ionic strength 0.3 M in sodium chloride and buffer ions, [E] = 0.135 µM, acetonitrile = 2% (v/v). From a plot of K_m^{app} against [I] the binding constant for competitive inhibition of papain, K_i = 1.55 mM, was obtained

[I]	V	K_m^{app}
µM	µM · s^{-1}	µM
0	1.170	6.47
746	1.031	8.31
1099	0.809	11.78
1439	0.698	12.35
1767	0.660	13.96

Table 4. *Binding dissociation constants for papain complexes at 35.0 °C and pH 6.0*

Compound	K_i
	mM
$CH_3-CONH-CH_2-CHO$	0.39
$C_6H_5-CONH-CH_2-CHO$	0.003
$CH_3-CO-PheNH-CH_2-CHO$	0.000046[a]
$CH_3-CONH-CH_2-CN$	44[b]
$C_6H_5-CONH-CH_2-CN$	0.46[b]
$CH_3O-CO-PheNH-CH_2-CN$	0.0018[b]
$C_6H_5-CONH-CH_2-CO-CH_3$	>1000
$CH_3-CO-PheNH-CH_2-CO-CH_3$	1.55
$C_6H_5-CONH-CH_2-CONH_2$	202[c]

[a] At pH 5.5 and 25 °C [6].
[b] From [4].
[c] From [25] (= K_m).

papain-inhibitor complex the carbonyl group of the substrate (or inhibitor) should hydrogen bond with the side-chain NH_2 of Gln-19 and the backbone NH of Cys-25 and thereby increase the binding constants [15].

Westerik and Wolfenden determined the inhibition constant for *N*-benzoyl-aminoacetaldehyde and quoted a value of 0.025 mM [6]. In the present work a value of 0.003 mM was obtained after allowance was made for the fact that it is the aldehyde alone that is the inhibitor and not its hydrate which exists in solution in equilibrium with it. This follows from observation of the NMR spectrum of *N*-acetyl-aminoacetaldehyde (Fig. 1) when enzyme is added (Fig. 2). The resonances associated with the aldehyde (at $\tau = 0.3$ and 5.8 ppm) are broadened, but the resonances of the hydrate (at $\tau = 4.8$ and 6.7 ppm) are not effected. Hence K_i has been calculated using the actual concentration of aldehyde present in solution. For *N*-benzoyl-aminoacetaldehyde, the ratio determined experimentally from the NMR spectrum is 6.5:1 in favour of the hydrate. Since Westerik and Wolfenden assumed that all the aldehyde added was present in solution their value which is approximately 8 times larger than that in Table 4, would, if corrected be in good agreement with the value shown. Westerik and Wolfenden proposed that aldehydes, structurally related to good substrates, might form stable hemithioacetals by addition of the thiol group of Cys-25 [6]. The formation of hemithioacetals in aqueous solution occurs with association constants in the range $10-60\ M^{-1}$; association constants for hemithioketals are about 100-fold less [16].

Papain-aldehyde and papain-ketone complexes have been investigated by NMR spectroscopy at pH 6.0 and 35.0 °C. The spectra of *N*-acetyl-aminoacetaldehyde show broadening of the aldehyde proton resonance over a range of concentration ($[I]_0/[E]_0$, 7–20), but no change in chemical shift is observed. If a hemithioacetal is formed and is in fast exchange with the free aldehyde, a large upfield chemical shift would be expected, since the resonance for this proton calculated from Shoolery's effective shielding constants [17] should occur at $\tau = 4-5$ ppm. The inhibition constant experimentally determined for *N*-acetyl-aminoacetaldehyde means that the rate of exchange between bound and unbound aldehyde is rapid compared with the NMR time scale and hence it can be inferred that the observed line width ($\Delta\nu_{obs}$) is the weighted average of the line width of the free ($\Delta\nu_I$) and enzyme-bound ($\Delta\nu_{EI}$) inhibitor.

$$\Delta\nu_{obs} = \frac{[EI]}{[I]_0}(\Delta\nu_{EI} - \Delta\nu_I) + \Delta\nu_I .$$

A plot of $\Delta\nu_{obs}$ against $[EI]/[I]_0$ is shown in Fig. 3. The good correlation confirms that the value of K_i determined by kinetic methods is correct, since [EI] is calculated from a knowledge of K_i, $[E]_0$, and $[I]_0$.

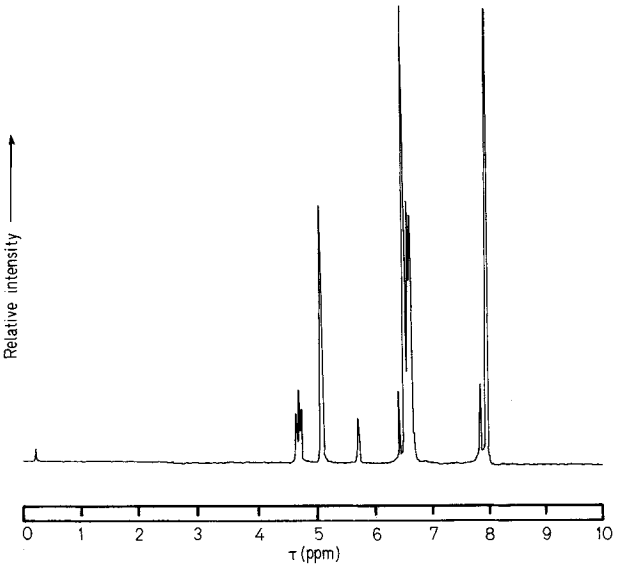

Fig. 1. *The ^1H NMR spectrum of* N-*acetyl-aminoacetaldehyde in ^2H_2O at 35.0 °C at 270 MHz*

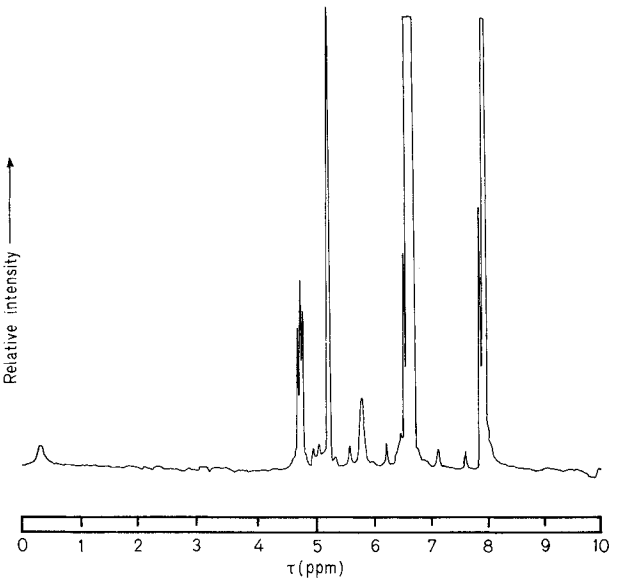

Fig. 2. *An ^1H NMR spectrum of* N-*acetyl-aminoacetaldehyde in the presence of papain in ^2H_2O at 35.0 °C at 270 MHz.* The enzyme spectrum has been subtracted

Similar experiments were attempted with *N*-benzoyl-aminoacetaldehyde, but no broadening or shift of the aldehyde resonance was observed. Since there is no doubt that *N*-benzoyl-aminoacetaldehyde exhibits inhibitory action, the rate of exchange must be slow compared with the chemical shift difference between the aldehydic proton in the bound and free states and thus the only resonances detected are those of the unbound inhibitor. *N*-Acetyl-L-phenylalanyl-aminopropanone, however, showed broad-

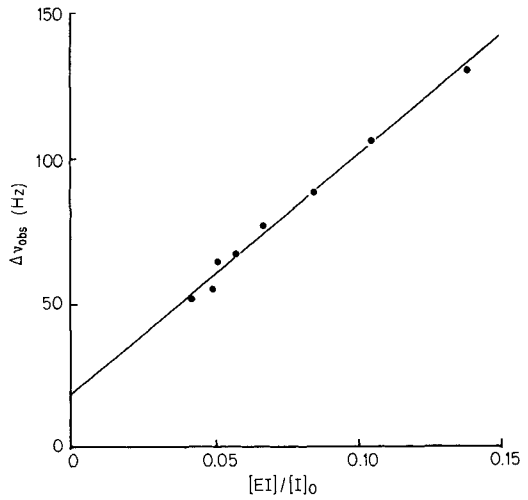

Fig. 3. *A plot of the observed line width at half height ($\Delta\nu_{obs}$) of the aldehyde ^1H resonance against the fraction of papain-bound inhibitor ([EI]/[I]$_0$) for inhibition by* N-*acetyl-aminoacetaldehyde at 35.0 °C and pH 6.0 using* K_i = *0.39 mM determined kinetically*

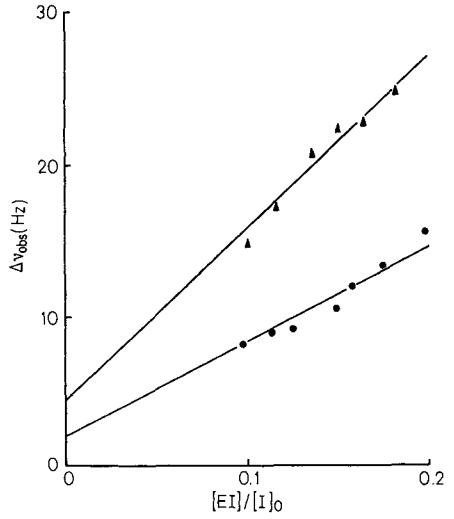

Fig. 4. *A plot of the observed line width at half height ($\Delta\nu_{obs}$) for the methyl (●) and methylene (▲) ^1H resonances ($-NH-CH_2-CO-CH_3$) against the fraction of papain-bound inhibitor ([EI]/[I]$_0$) for inhibition by* N-*acetyl-*L-*phenylalanyl-aminopropanone at 35.0 °C and pH 6.0 using* K_i = *1.55 mM determined kinetically*

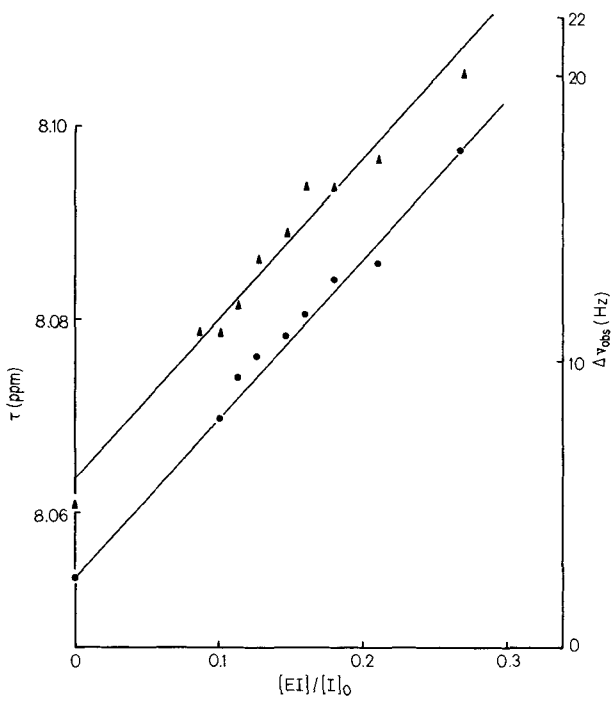

Fig. 5. *A plot of the observed line width at half height ($\Delta\nu_{obs}$, ●) and chemical shift (τ, ▲) of the acetyl ^1H resonance of* N-*acetyl-*L-*phenylalanyl-aminopropanone against the fraction of papain-bound inhibitor ([EI]/[I]$_0$) at 35.0 °C and pH 6.0 using* K_i = *1.55 mM determined kinetically*

Table 5. *The calculated line width at half height ($\Delta\nu$) and the change in chemical shift ($\Delta\tau$) of three resonances of* N-*acetyl-*L-*phenylalanyl-aminopropanone when bound to papain at 35 °C at pH 6.0 and ionic strength 0.3 M*

Resonance	$\Delta\nu$	$\Delta\tau$
	Hz	ppm
CH_3-CONH	58	0.19
$-CH_2-CO-CH_3$	34	0
$-CH_2-CO-CH_3$	68	0

ening of the $-CH_2-CO-CH_3$ resonance at τ = 5.84 ppm and of the $-CH_2-CO-CH_3$ resonance at τ = 7.79 ppm, but once again, no shifts were observed compared with the free ketone. A plot of the observed line width at half height ($\Delta\nu_{obs}$) against the fraction of bound inhibitor ([EI]/[I]$_0$) for the methyl and methylene resonances is shown in Fig. 4. The good correlation shows that the value of K_i determined kinetically is confirmed by NMR spectroscopy. The *N*-acetyl group of this inhibitor showed both line broadening and a change in chemical shift on addition of papain; these data are plotted in Fig. 5. Again a good correlation is obtained using the kinetically determined K_i value. From the data in Fig. 4 and 5 the line width and change in chemical shift of the inhibitor resonances can be calculated and are shown in Table 5.

Of the *N*-acyl-aminoacetaldehydes available only the weakest inhibitor, *N*-acetyl-aminoacetaldehyde, was in fast exchange with papain on the NMR time scale. Although therefore no evidence of a change in chemical shift was observed for the bound aldehydic protein, it could be that the more potent inhibitors such as *N*-benzoyl-aminoacetaldehyde or *N*-acetyl-L-phenylalanyl-aminoacetaldehyde form a hemithioacetal with the thiol group of cysteine-25. It was also

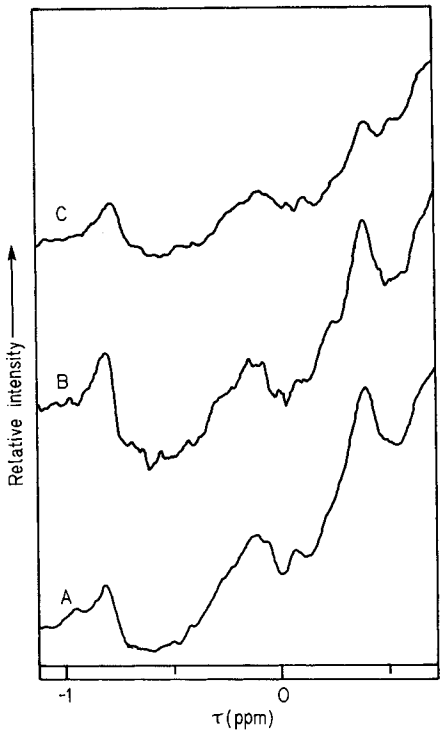

Fig. 6. *The low-field ^1H NMR spectrum of* N-*acetyl-aminoacetaldehyde (140 mM) in the presence of papain (1.3 mM) at pH 5.9 (A), with a high power radio frequency source H_2 applied at* $\tau = 2.06$ *ppm (B), and* $\tau = 3.97$ *ppm (C)*

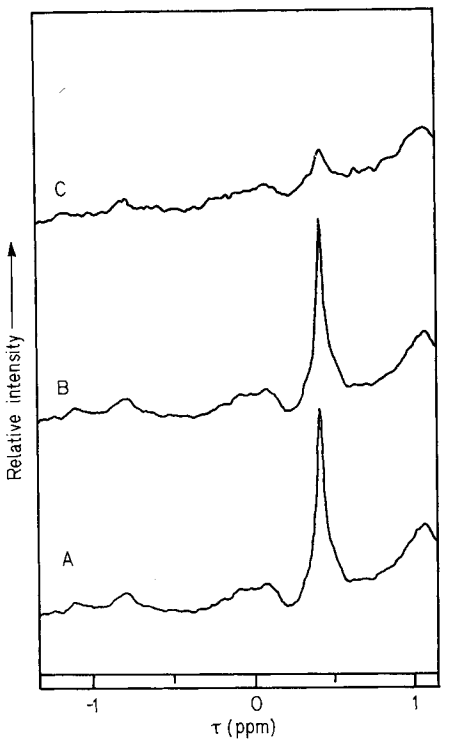

Fig. 7. *The low-field ^1H NMR spectrum of* N-*benzoyl-aminoacetaldehyde (94.6 mM) in the presence of papain (1.1 mM) at pH 5.9 (A), with a high-power radio frequency source H_2 applied at (B)* $\tau = 7.96$ *ppm and (C) 4.00 ppm*

possible that a hemithioacetal was formed between the enzyme thiol group and *N*-acetyl-aminoacetaldehyde but that the interconversion of bound aldehyde and hemithioacetal was slow and so did not affect the spectrum of the free aldehyde. To test this possibility a double-resonance technique was used. If a hemithioacetal was formed and was in slow exchange compared with the chemical shift difference between its resonance and that of the free aldehydic proton, it would be expected to have a ^1H resonance between 4 and 5 ppm [17]. If the sample is irradiated in this region with sufficient power to saturate the resonance, provided the rate of exchange is fast compared with the rate of relaxation, cross-saturation of the aldehyde ^1H resonance should be observed. The use of this technique has been the subject of a preliminary communication [18].

The NMR spectrum of a solution of papain (1.3 mM) containing *N*-acetyl-aminoacetaldehyde (140 mM) at pH 5.9 is shown in Fig. 6A, the aldehydic proton is at $\tau = 0.4$ ppm. In a double-resonance experiment the effect of a high-power radio frequency source (H_2) on the intensity of the aldehydic proton was investigated throughout the spectrum. When H_2 was close to 4 ppm the aldehydic proton signal virtually disappeared. NMR spectra with H_2 at 2.06 and 3.97 ppm are shown in Fig. 6B and 6C respectively.

A similar double-resonance experiment was performed on a solution of papain (1.1 mM) containing *N*-benzoyl-aminoacetaldehyde (94.6 mM) at pH 5.9 (Fig. 7A). The aldehydic proton ($\tau = 0.39$ ppm) is not broadened since it is in slow exchange with the enzyme on the NMR time scale. The effect of a high-power (saturating) radio frequency source, H_2, on the intensity of the aldehydic proton was investigated throughout the spectrum. When H_2 was at 4.00 ppm the aldehydic signal again virtually disappeared (Fig. 7C) whereas at other τ values (*e.g.* 7.96 ppm, Fig. 7B) the aldehyde signal was not affected. By lowering the power level of H_2 below saturation, a narrower band width could be achieved and hence a more precise definition of the chemical shift could be obtained for the intermediate responsible for the cross-saturation effect on the unbound aldehydic proton. In Fig. 8 spectra are shown with H_2 at subsaturation levels between 3 and 4 ppm. The chemical shift at which cross-saturation is optimal is at $\tau = 3.81$ ppm.

The loss of the aldehydic proton signal in both *N*-acetyl-aminoacetaldehyde and *N*-benzoyl-aminoacetaldehyde when a solution containing papain was irradiated with a high-power radio frequency source H_2 in the region of 4.0 ppm is certainly consistent with the existence of a hemithioacetal in equilibrium with the enzyme-bound and free aldehyde. An alternative

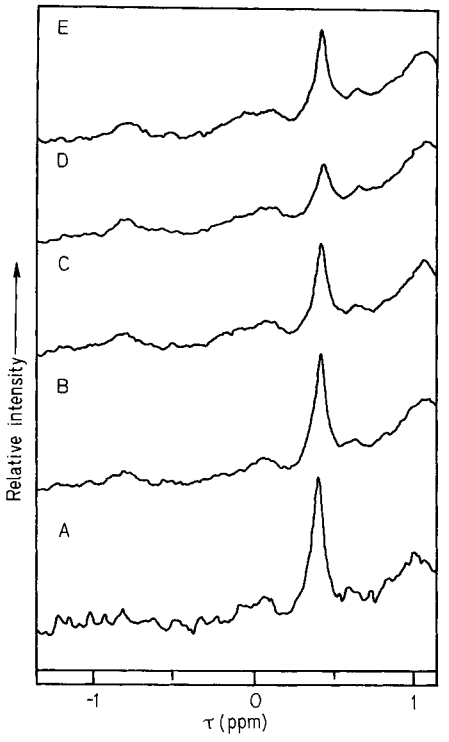

Fig. 8. *The low-field ^1H NMR spectrum of* N-*benzoyl-aminoacetaldehyde (94.6 mM) in the presence of papain (1.1 mM) at pH 5.9 at a power level H_2, slightly below saturation, applied at (A) τ = 3.29, (B) 3.47, (C) 3.65, (D) 3.81, and (E) 4.00 ppm*

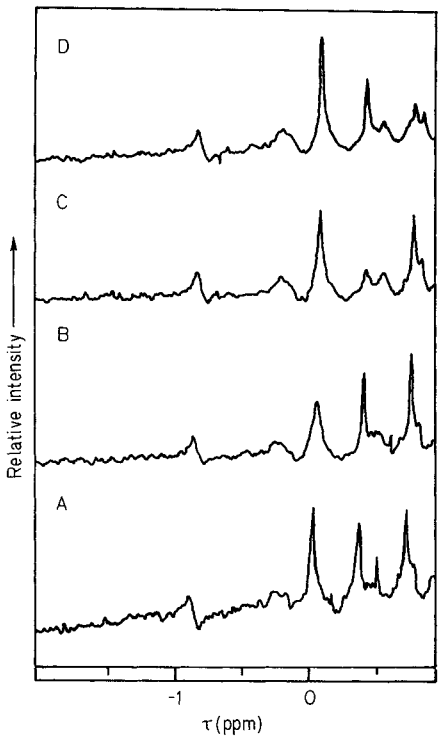

Fig. 9. *The low-field ^1H NMR spectrum of* N-*benzoyl-amino[1-^{13}C]-acetaldehyde (150 mM) in the presence of papain (1.1 mM) at pH 5.9 (A), with a high power radio frequency source applied at (B) τ = 3.49, (C) 3.82 and (D) 4.13 ppm*

explanation, however, is that the correlation time of the papain-aldehyde complex is sufficiently long and the proximity of an enzymic proton (τ = 3.81 ppm) sufficiently close to the bound aldehydic proton to cause a negative Overhauser effect [19]. To distinguish between these two explanations *N*-benzoyl-aminoacetaldehyde was prepared with 60 atom % ^{13}C enrichment in the aldehydic group. The ^{1}H NMR spectrum of a solution of papain (1.1 mM) containing the *N*-benzoyl-amino[1-^{13}C]acetaldehyde (150 mM) is shown in Fig. 9A. The aldehydic proton now appears as three lines of approximately equal intensity, the two outer lines arising from coupling to the ^{13}C nucleus ($^1J_{C-H}$ = 182 Hz) and the central line from the normal unlabelled aldehyde. When the double-resonance experiment was performed, as the high-power radio frequency source (H_2) scanned from low to high field, the three aldehydic lines collapsed in turn. At 3.49 ppm the low-field signal virtually disappeared (Fig. 9B) (this is superimposed on a rather broad additional signal which is unaffected in each spectrum), at 3.82 ppm the central line virtually disappeared (Fig. 9C) and at 4.13 ppm the high-field signal virtually disappeared (Fig. 9D). 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 ($1/T_1 \approx 0.05\ s^{-1}$) [20]. Although the chemical shift at which H_2 causes cross-saturation of the aldehydic proton to occur is below that expected for a simple hemithioacetal, its local magnetic environment, especially the ring current shift associated with histidine-159 and tryptophan-177, could easily account for this. The coupling constant, $^1J_{C-H}$, for the bound species is found to be 173 Hz which is in the expected region for a hemithioacetal [21]. For the hydrated form of *N*-benzoyl-amino[1-^{13}C]acetaldehyde the coupling constant $^1J_{C-H}$ = 165 Hz. Direct spectroscopic evidence is therefore provided for the existence of both bound aldehyde and hemithioacetal. This strongly supports the proposed involvement of tetrahedral intermediates in papain-catalysed substrate hydrolysis.

Although cross-saturation in NMR spectroscopy has been successfully applied to correlate resonances in the oxidised and reduced states of cytochrome *c* [22], in the oxy and deoxy forms of haemoglobin [23], and in the aromatic protons of a slowly rotating tyrosine residue in ferrocytochrome *c* [24], as far as we are aware this is the first application of the technique for the investigation of an enzyme-inhibitor interaction. The technique appears to have considerable potential for the detection of intermediates in enzyme-catalysed reactions.

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M. R. Bendall, School of Science, Griffith University, Nathan, Brisbane, Queensland, Australia 4111

I. L. Cartwright, G. Lowe*, and D. Nurse, The Dyson Perrins Laboratory, University of Oxford, South Parks Road, Oxford, Great Britain, OX1 3QY

P. I. Clark, Department of Chemistry, University College of Swansea, University of Wales, Singleton Park, Swansea, Great Britain, SA2 8PP

* To whom correspondence should be addressed.