A ^{13}C -NMR study of the inhibition of papain by a dipeptide-glyoxal inhibitor

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Z-Phe-Ala-glyoxal (where Z is benzyloxycarbonyl) has been synthesized and shown to be a competitive inhibitor of papain with a $K_i = 3.30 \pm 0.25$ nM. ^{13}C -NMR has been used to show that in aqueous media, Z-Phe-[2- ^{13}C]Ala-glyoxal gives signals at 207.7 p.p.m. and 96.3 p.p.m. showing that both the α -keto carbon and its hydrate are present. When this inhibitor is bound to papain a single signal at 209.7 p.p.m. is observed due to the ^{13}C -enriched carbon. This demonstrates that the glyoxal α -keto carbon is not hydrated when it is bound to papain and that it does not form a thiohemiketal with the thiol group of Cys-25.

Z-Phe-[1- ^{13}C]Ala-glyoxal has also been synthesized and its aldehyde carbon is fully hydrated in aqueous solution giving signals at 88.7 p.p.m. and 90.2 p.p.m. when the α -keto carbon and its hydrate are present respectively. When this inhibitor is bound to papain a single signal at 71.04 p.p.m. was observed due to the ^{13}C -enriched carbon showing that the ^{13}C -enriched aldehyde carbon forms a thiohemiacetal with Cys-25.

Key words: protease, tetrahedral adduct, thiohemiacetal, thiol.

INTRODUCTION

Specific-substrate-derived glyoxal or α -keto- β -aldehyde (RCOCHO) inhibitors of proteases have been synthesized [1] and shown to be potent inhibitors of chymotrypsin [2–4], cathepsin B [3,5], cathepsin L [5], cathepsin S [6] and the proteasome [7]. We have recently [4] used ^{13}C -NMR to study the mechanism of inhibition of chymotrypsin by specific-substrate-derived glyoxal inhibitors. However, there have been no similar studies on the mechanism of inhibition of thiol proteases by glyoxal inhibitors. Therefore one of the main aims of the present work is to undertake such studies with the thiol protease papain.

If specific-substrate-derived glyoxal inhibitors bind in the same way as substrates, then the ketonic glyoxal carbonyl group should be in the same position as the carbonyl of the hydrolysed peptide bond in the corresponding substrate. This has led to the suggestion that the active site thiol of the thiol proteases could form a thiohemiketal intermediate with the ketonic carbonyl group of the glyoxal [3]. Such a thiohemiketal adduct would be analogous to the tetrahedral adduct thought to be formed during catalysis. Substrate-derived aldehyde inhibitors whose carbonyl group is in the same position as the carbonyl of the hydrolysed peptide bond in the corresponding substrate have been shown to be potent inhibitors of papain, forming tetrahedral thiohemiacetals with the thiol group of Cys-25 [8–10]. However, with substrate-derived chloromethylketone [11,12] or diazoketone [13] inhibitors of papain a stable thiohemiketal is not formed; instead, the active-site thiol is alkylated by the methylene carbon adjacent to the inhibitor carbonyl carbon which is analogous to the carbonyl of the hydrolysed peptide bond. It is therefore not clear whether the thiol group of Cys-25 will form a hemiketal or hemiacetal with glyoxal inhibitors. However, due to the lower electronegativity of sulphur compared with oxygen the chemical shifts of thiohemiacetal and thiohemiketal carbons are ≈ 15 p.p.m. smaller than those of the corresponding hydrates. Therefore we intend to prepare specific-substrate-derived glyoxal inhibitors of papain with either the ketonic or aldehyde carbon enriched in carbon-13 and then use ^{13}C -NMR to determine which carbonyl carbon reacts with Cys-25 of papain. This should allow us to determine whether specific-substrate-derived glyoxal inhibitors of papain form tetrahedral

adducts analogous to the tetrahedral intermediate thought to be formed during catalysis.

MATERIALS AND METHODS

N- α -Benzyloxycarbonyl-L-phenylalanine was obtained from Sigma-Aldrich Chemical Co., Poole, Dorset, U.K. L-[1- ^{13}C]Alanine (99 atom %) was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Papaya latex (which hydrolyses 3 μmol of *N*-benzoyl-L-arginine ethyl ester/min at pH 6.2 and 25 $^{\circ}\text{C}$) was the Fluka product obtained from Sigma-Aldrich Ireland. All other chemicals used were obtained from Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Synthesis of benzyloxycarbonyl (Z)-Phe-Ala and Z-Phe-[1- ^{13}C]Ala

These were synthesized from Z-Phe and L-Ala-methyl ester using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as a coupling reagent, as described by Sheehan and Ledis [14], and by the mixed anhydride method of Coggins et al. [15]. Yield was 0.41 g (1.11 mmol, 82.3 %) of Z-Phe-Ala. ^{13}C -NMR ($^2\text{H}_6$]DMSO) δ : 17.15 (1C, CH_3), 37.43 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 47.54 (1C, CHCH_3), 55.91 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 65.17 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 126.24–129.24 (10C, $\text{CH}=\text{CH}$), 137.05–138.19 (2C, $\text{CH}=\text{C}$), 155.87 (1C, $\text{O}-\text{CO}-\text{NH}$), 171.50 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}-\text{CO}-\text{NH}$), 174.04 (1C, COOH). Chemical analysis (calculated) for $\text{C}_{20}\text{H}_{22}\text{N}_2\text{O}_5$: C, 64.86; H, 5.95; N, 7.57; O, 21.62. Found: C, 64.80; H, 6.00; N, 7.47; O, 21.73.

The ^{13}C -NMR spectrum of Z-Phe-[1- ^{13}C]Ala in $^2\text{H}_6$]DMSO contained a single signal at 174.03 p.p.m. due to the ^{13}C -enriched carbon atom.

Conversion of Z-Phe-Ala into Z-Phe-Ala-glyoxal, Z-Phe-Ala-[1- ^{13}C]glyoxal and Z-Phe-Ala-[2- ^{13}C]glyoxal

Z-Phe-Ala was converted into Z-Phe-Ala-diazoketone using diazomethane and the mixed anhydride method described by Coggins et al. [15]. The diazoketone was converted into a glyoxal using a procedure modified from that described by Ihmels et al. [16]. A 100 mg (0.252 mmol) quantity of Z-Phe-Ala-diazoketone

Abbreviations used: Ac-Phe-Gly-H, acetyl-Phe-glycinal; T_1 , spin lattice relaxation time; Z, benzyloxycarbonyl.

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was oxidized by dissolving it in a 5 ml solution of 0.1 M dimethyldioxirane in acetone and stirring until no more nitrogen evolved. The reaction mixture was then moistened by addition of $\approx 0.25\%$ (v/v) water and the acetone removed by evaporation under reduced pressure. The moist compound was then dissolved in $[^2\text{H}_6]\text{DMSO}$. ^{13}C -NMR ($[^2\text{H}_6]\text{DMSO}$) δ : 16.48 (1C, CHCH_3), 37.50 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 49.80 (1C, CHCH_3), 55.88 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}$), 65.21 (1C, $\text{C}_6\text{H}_5\text{CH}_2$), 89.06 [1C, $\text{CO}-\text{CH}(\text{OH})_2$], 126.28–129.23 (10C, $\text{CH}=\text{CH}$), 137.02–138.14 (2C, $\text{CH}=\text{C}$), 155.89 (1C, $\text{O}-\text{CO}-\text{NH}$), 171.45 (1C, $\text{C}_6\text{H}_5\text{CH}_2\text{CH}-\text{CO}-\text{NH}$), 206.81 [1C, $\text{COCH}(\text{OH})_2$].

Z-Phe-Ala-[1- ^{13}C]glyoxal and Z-Phe-Ala-[2- ^{13}C]glyoxal were synthesized by the same procedure except that *N*-[^{13}C]methyl-*N*-nitrosotoluene-*p*-sulphonamide was used to generate the diazomethane used in the synthesis of Z-Phe-Ala-[1- ^{13}C]glyoxal and [1- ^{13}C]Ala-ethyl ester was used for the synthesis of Z-Phe-Ala-[2- ^{13}C]glyoxal. In $[^2\text{H}_6]\text{DMSO}$ the ^{13}C -enriched carbon of Z-Phe-Ala-[2- ^{13}C]glyoxal gave signals at 95.42 p.p.m. and 206.98 p.p.m. while the ^{13}C -enriched carbon of Z-Phe-Ala-[1- ^{13}C]glyoxal gave signals at 89.29 p.p.m., 90.46 p.p.m. and 199.45 p.p.m. The signals from the ^{13}C -enriched carbons in water are described and assigned in the Results and discussion section.

Isolation and quantification of fully active papain

Papain was isolated from papaya latex essentially as described by Baines and Brocklehurst [17]. Papain was also further purified by covalent chromatography [18]. The concentration of fully active papain was determined by thiol titration with 2,2'-dipyridyl disulphide [19]. Papain was shown to be free of any contaminating chymopapains by equal thiol titrations at pH 4 and pH 8 [17, 19]. Its identity was confirmed by the pH dependence of its reactivity towards 2,2'-dipyridyl disulphide [20] and its catalytic activity (k_{cat}/K_m) towards *N*-benzoyl-L-arginine-*p*-nitroanilide [21]. For NMR experiments with Z-Phe-[2- ^{13}C]Ala-glyoxal, papain was prepared by the method of Baines and Brocklehurst [17] and it had 0.38 mol of thiol/mol of protein. For NMR experiments with Z-Phe-[1- ^{13}C]Ala-glyoxal the papain was also further purified by covalent chromatography [18] and it had 0.71 mol of thiol/mol of protein.

Inhibition of papain by Z-Phe-Ala-glyoxal

The inhibition of the papain catalysed hydrolysis of *N*-benzoyl-L-arginine-*p*-nitroanilide by Z-Phe-Ala-glyoxal was studied at 25 °C in 0.1 M sodium phosphate buffer, pH 7.03, containing 1.67% (v/v) DMSO. K_i values for the inhibitor were determined using the method of Henderson [22]. Stock solutions of ≈ 10 mM substrate and 0.4 mM inhibitor were dissolved in water and DMSO respectively. Final concentrations of the enzyme, substrate and inhibitor were 0.37 μM , 0.52–3.14 mM and 0.5–4.0 μM respectively.

NMR spectroscopy

NMR spectra at 11.75 T were recorded with a Bruker Avance DRX 500 standard-bore spectrometer operating at 125.7716 MHz for ^{13}C -nuclei. Unless stated otherwise 10 mm-diameter sample tubes were used. The spectral conditions for the samples of chymotrypsin inhibited by Z-Phe-[1- ^{13}C]Ala-glyoxal at 11.75 T were: 16384 time-domain data points; spectral width, 240 p.p.m.; acquisition time, 0.270 s; 1.73 s relaxation delay time; 90° pulse angle; 512–8192 transients were recorded per spectrum. Waltz-16 composite pulse ^1H decoupling with a BLARH100 amplifier was used with 16 dB attenuation during

Table 1 Dissociation constants of aldehyde (R-H) and glyoxal (R-CHO) inhibitors of proteases

Enzyme	Inhibitor	pH	K_i (M)	Reference
Chymotrypsin	Z-Val-Phe-CHO	7.4	$(5.5 \pm 0.6) \times 10^{-6}$	[3]
Chymotrypsin	Z-Val-Phe-H		5.4×10^{-5}	[3]
Cathepsin B (human)	Z-Phe-Ala-CHO	6.4	$(7.7 \pm 0.8) \times 10^{-8}$	[3]
Cathepsin B (human)	Z-Phe-Ala-H		2.1×10^{-7}	[3]
Papain	Z-Phe-Ala-CHO	7.0	$(3.3 \pm 0.3) \times 10^{-9}$	Present study
Papain	Ac-Phe-Gly-H	7.0	1×10^{-8}	[9]
Papain	Ac-Phe-Gly-H	7.0	2.6×10^{-8}	[10]

the acquisition time and 34 dB attenuation during the relaxation delay to minimize dielectric heating but maintain the nuclear Overhauser effect. All spectra were transformed using an exponential weighting factor of 50 Hz. For samples of papain inhibited by Z-Phe-[2- ^{13}C]Ala-glyoxal at 11.75 T the spectral conditions were: 32768 time-domain data points; spectral width, 240 p.p.m.; acquisition time, 0.541 s; 7.46 s relaxation delay time; 90° pulse angle; 512–6144 transients were recorded per spectrum. All spectra were zero filled to give 64000 data points before being transformed.

Both ^1H and ^{13}C chemical shifts are quoted relative to tetramethylsilane at 0.00 p.p.m. In aqueous solutions the chemical shift of the α -carbon of glycine was used as a chemical reference as described previously [23]. For non-aqueous solvents either 10% tetramethylsilane was used as an internal standard or an appropriate solvent signal was used as a secondary reference [24].

All aqueous samples contained 10% (v/v) $^2\text{H}_2\text{O}$ to obtain a deuterium lock signal, as well as 10 mM sodium phosphate buffer to help maintain stable pH values. All samples were at 25 °C.

RESULTS AND DISCUSSION

Inhibition of papain by Z-Phe-Ala-glyoxal

The inhibition of the papain-catalysed hydrolysis of *N*-benzoyl-L-arginine-*p*-nitroanilide by Z-Phe-Ala-glyoxal at pH 7.0 was analysed using eqn. (1) [22]:

$$I_t/(1 - v_i/v_o) = \{K_i[(S_t + K_m)/K_m]v_o/v_i\} + E_t \quad (1)$$

where E_t , S_t and I_t are the total concentrations of enzyme, substrate and inhibitor respectively, and v_o and v_i are initial rates of substrate catalysis in the absence and presence of the inhibitor respectively. Plots of $I_t/(1 - v_i/v_o)$ versus v_o/v_i were linear and a plot of the slopes of these graphs versus the substrate concentration was linear with a positive non-zero slope, demonstrating that the inhibition was competitive [22] and that $K_i = 3.30 \pm 0.25$ nM at pH 7.03 and 25 °C. Specific-substrate-derived glyoxal inhibitors are 3–10-fold more effective inhibitors than their aldehyde equivalents [3] and so it is expected that Z-Phe-Ala-glyoxal (Z-Phe-Ala-CHO) will be a more effective papain inhibitor (Table 1) than the similar aldehyde inhibitor acetyl-Phe-glycinal (Ac-Phe-Gly-H).

^{13}C -NMR signals obtained from Z-Phe-[1- ^{13}C]Ala-glyoxal and Z-Phe-[2- ^{13}C]Ala-glyoxal in water

In fully aqueous media at pH 5.5 (Figure 1, trace f) the intensity of the α -keto-carbon of Z-Phe-Ala[2- ^{13}C]glyoxal at 207.7 p.p.m. (Scheme 1, structure 1) was approximately half that of its hydrate at 96.3 p.p.m. (Scheme 1, structure 2). The signal at 38.7 p.p.m. is due to the $[^2\text{H}_6]\text{DMSO}$ used to dissolve the inhibitor (see legend to Figure 1 for the percentage of $[^2\text{H}_6]\text{DMSO}$ in samples). The aldehyde carbon of Z-Phe-[1- ^{13}C]Ala-glyoxal was fully hydrated

Figure 1 ^{13}C -NMR spectra of papain, Z-Phe-[1- ^{13}C]Ala-glyoxal, Z-Phe-[2- ^{13}C]Ala-glyoxal and the inhibitor complexes they form at pH 5.5

Acquisition parameters were as described in the Materials and methods section. An exponential weighting factor of 50 Hz was used for all spectra. The number of transients recorded per spectrum were: 1024 for spectrum a; 2048 for spectrum b; 8192 for spectra c–e; 512 for spectrum f and 6144 for spectrum g. Spectrum a is of 0.55 mM Z-Phe-[1- ^{13}C]Ala-glyoxal, spectrum b is of 0.44 mM fully active papain, spectrum c is of 0.44 mM fully active papain and 0.29 mM Z-Phe-[1- ^{13}C]Ala-glyoxal, spectrum d is of 0.44 mM fully active papain and 0.58 mM Z-Phe-[1- ^{13}C]Ala-glyoxal, spectrum e is the same sample as that used for spectrum d except that it has been incubated with 1.8 mM 2,2'-dipyridyl disulphide (see text for experimental details), spectrum f is of 0.81 mM Z-Phe-[2- ^{13}C]Ala-glyoxal, and spectrum g is of 0.32 mM fully active papain and 0.32 mM Z-Phe-[2- ^{13}C]Ala-glyoxal. Samples a and c–g contained 0.5, 0.27, 0.58, 0.58, 0.5 and 0.74% (v/v) $[\text{D}_6]\text{DMSO}$ respectively. Samples a–e had 3 ml volumes, and samples f and g had 2 ml volumes.

in fully aqueous media at pH 5.5 with signals at 88.7 p.p.m. and 90.2 p.p.m. (Figure 1, trace a), which were assigned to structures 1 and 2 in Scheme 1 respectively. The intensity and chemical shifts of the signals from Z-Phe-[1- ^{13}C]Ala-glyoxal and Z-Phe-[2- ^{13}C]Ala-glyoxal were unchanged from pH 3 to 10. As in our earlier studies [4] the glyoxals underwent internal Cannizzaro reactions at pH values > 10 involving both C-1 to C-2 hydride and C-2 to C-1 alkyl shifts to give the corresponding α -hydroxy-carboxylic acid (RCHOHCOOH). At pH 5.5 this gave signals at 178.9 p.p.m. and 74.6 p.p.m. due to the ^{13}C -enriched carboxy- and α -hydroxy-carbons respectively.

^{13}C -NMR signals from Z-Phe-[1- ^{13}C]Ala-glyoxal, Z-Phe-[2- ^{13}C]Ala-glyoxal and papain at pH 5.5

On adding Z-Phe-[1- ^{13}C]Ala-glyoxal (Figure 1, trace a) to papain (Figure 1, trace b) a new signal at 71.04 p.p.m. was detected (Figure 1, trace c). The chemical shift of this signal is very similar to those of the two diastereomeric thiohemiacetal carbons (76.91 p.p.m. and 75.49 p.p.m.) formed when *N*-acetyl-L-cysteine is added to Z-Phe-[1- ^{13}C]Ala-glyoxal at pH 5.5. Therefore the signal at 71.04 p.p.m. is assigned to the thiohemiacetal formed when the thiolate ion of Cys-25 reacts with the ^{13}C -enriched aldehyde carbon of Z-Phe-[1- ^{13}C]Ala-glyoxal. Examination of the signal at 71.04 p.p.m. showed that only one signal was present, demonstrating that only one diastereoisomer was present and that thiohemiacetal formation was stereospecific. Linewidths of

Scheme 1 Structures and chemical shifts of Z-Phe-[1- ^{13}C]Ala-glyoxal and Z-Phe-[2- ^{13}C]Ala-glyoxal in water containing 10% (v/v) $^2\text{H}_2\text{O}$ at pH 5.5

50 Hz have been observed when only one diastereomeric hemiacetal is formed with papain [9,10]. The signal at 71.04 p.p.m. had a linewidth of 48.1 ± 3.5 Hz giving further support to our suggestion that only one diastereomeric thiohemiacetal is formed when papain reacts with Z-Phe-Ala-glyoxal. Addition of an excess of Z-Phe-Ala[1- ^{13}C]glyoxal led to the signal at 71.04 p.p.m. reaching its maximum intensity and to the reappearance of the signals at 88.7 p.p.m. and 90.2 p.p.m. due to the free inhibitor (Figure 1, trace d). As separate signals are observed for the free and bound inhibitor this shows that they are not in fast exchange on the NMR timescale. When chymotrypsin was inhibited by Z-Ala-Pro-[1- ^{13}C]Phe-glyoxal there was a slow pH-dependent irreversible breakdown of the inhibitor to give the hydroxy-acid which had a chemical shift in the range of 72.85–74.93 p.p.m. [4]. These chemical shifts are similar to that of the signal at 71.04 p.p.m. assigned to the formation of a thiohemiacetal. Therefore it was important to confirm that the signal at 71.04 p.p.m. is not due to formation of an hydroxy-acid. 2,2'-Dipyridyl disulphide is an active-site-directed reagent which rapidly covalently modifies the active-site thiol group of papain [20]. Addition of this reagent displaced the bound inhibitor at 71.04 p.p.m. leading to an increase in intensity of the signals at 88.7 p.p.m. and 90.2 p.p.m. due to the free inhibitor (Figure 1, trace e). This confirmed that Z-Phe-Ala[1- ^{13}C]glyoxal did not break down to give hydroxy-acid and that it is a reversible inhibitor of papain at pH 5.5. The signals at 122.0 p.p.m., 123.2 p.p.m., 139.7 p.p.m. and 150.0 p.p.m. (Figure 1, trace e) were due to the added 2,2'-dipyridyl disulphide.

The signal at 71.04 p.p.m. due to the thiohemiacetal carbon had one directly bonded proton ($J_{\text{CH}} = 170.1 \pm 2.7$ Hz), a linewidth of 48.1 ± 3.5 Hz and a spin lattice relaxation time (T_1) of 1.65 ± 0.19 s.

A ≈ 3 -fold excess of Z-Phe-[2- ^{13}C]Ala-glyoxal (Figure 1, trace f) was added to papain and unbound materials were removed by ultrafiltration using an Amicon PM10 membrane. Only one new signal was detected, at 209.7 p.p.m., due to the ^{13}C -enriched carbon of the protein-bound inhibitor (Figure 1, trace g). The absence of a thiohemiketal signal at ≈ 80 p.p.m. (Figure 1, trace g) shows that the thiolate ion of Cys-25 does not react with the ^{13}C -enriched carbon of the inhibitor. The absence of a hydrate signal at ≈ 96 p.p.m. and the presence of a signal at 209.7 p.p.m. shows that the ^{13}C -enriched carbon of the bound inhibitor is not hydrated and that it is sp^2 hybridized.

The signal at 209.7 p.p.m. due to the α -keto carbon had no directly bonded protons, a linewidth of 28.1 ± 0.6 Hz and a spin lattice relaxation time (T_1) of 2.59 ± 0.21 s.

General conclusions

As Z-Phe-Ala-glyoxal is a specific-substrate-derived inhibitor of papain, the Phe and Ala amino acid residues are expected to bind

Scheme 2 Expected binding of Z-Phe-Ala-glyoxal to papain and reaction products if the thiolate ion of Cys-25 reacts with α -keto or aldehyde glyoxal carbons

in the S_2 and S_1 subsites respectively, in the same way as the corresponding substrate. Therefore the inhibitor ketone carbonyl is expected to be in same position as the peptide carbonyl which is hydrolysed in an analogous substrate [10,25,26]. It should therefore be ideally placed to facilitate nucleophilic attack (Scheme 2, reaction A) by the thiolate ion of Cys-25 to form a thiohemiketal tetrahedral adduct which is analogous to the tetrahedral intermediate formed during substrate catalysis. However, our results demonstrate that a thiohemiketal is not formed; instead, the thiolate ion of Cys-25 reacts (Scheme 2, reaction B) with the glyoxal aldehyde carbon to form a tetrahedral thiohemiacetal adduct (Scheme 3). This demonstrates that it is the greater chemical reactivity of the inhibitor aldehyde carbon and not the spatial alignment of the inhibitor α -keto carbon and enzyme which determines which glyoxal carbon reacts with the thiolate ion of Cys-25. Likewise with peptidyl epoxide inhibitors alkylation of the papain thiolate ion is by the carbon adjacent to the carbon equivalent to the carbonyl of a substrate [27]. In this case it was suggested that steric hindrance prevented alkylation at the carbon equivalent to the carbonyl of a substrate. A similar result has also been obtained with specific-substrate-derived diazo-ketone [13] or chloromethylketone inhibitors of papain [11,12]. In both these cases it has been suggested that alkylation on sulphur is facilitated by transient thiohemiketal formation and by the ability of sulphur to participate in three-membered cyclic transition states [28]. A similar process could also contribute to the formation of stable thiohemiacetal adducts when papain reacts with glyoxal inhibitors. However, our studies with model compounds show that while the glyoxal α -keto carbon has a low reactivity towards thiol groups, the glyoxal aldehyde carbon has a high reactivity towards thiol groups. Therefore it is unlikely that there is transient thiohemiketal formation prior to thiohemiacetal formation when the glyoxal inhibitor reacts with papain.

As the sulphur atom of Cys-25 reacts with C-1 of both Z-Phe-Ala-glyoxal and Z-Phe-Ala-chloromethylketone inhibitors it is reasonable to expect that they will both be bound in a similar way. If this is true then the C-2 glyoxal carbonyl oxygen will form hydrogen bonds with the backbone NH of Cys-25 and the side-chain NH_2 of Gln-19, as observed in the Z-Phe-Ala-chloromethylketone inhibitor derivative formed with papain [25]. The chemical shift of the α -carbonyl carbon of the P_1 glyoxal inhibitor residue (i.e. the C-2 glyoxal keto-carbon) confirms that it is sp^2 hybridized in its complex with papain (Figure 1, trace g), demonstrating that the tight binding by this inhibitor is not

Scheme 3 Structure and chemical shifts of the papain-glyoxal inhibitor adduct formed at pH 5.5

dependent on there being tetrahedral geometry at this carbon. The 2 p.p.m. increase in chemical shift at the C-2 glyoxal keto-carbon on binding to papain (Figure 1, traces f and g) could reflect more effective hydrogen bonding of the carbonyl oxygen on binding to papain [29]. If the glyoxal inhibitor binds in a similar way to its chloromethylketone analogue then the oxygen atom of the C-2 keto-oxygen atom will be 3.6 Å from both the backbone NH of Cys-25 and the side-chain NH_2 of Gln-19. From model building experiments we estimate that the oxygen atom of the thiohemiacetal will also be 3.6 Å from the side-chain NH_2 of Gln-19 and 2.2 Å from ND1 of His-159. However, it is much further away (4.2 Å) from the backbone NH of Cys-25. Therefore both the keto and thiohemiacetal oxygens of the bound glyoxal inhibitor should be stabilized by hydrogen bonding. The additional hydrogen bonding to the thiohemiacetal oxygen may explain the greater potency of glyoxal inhibitors relative to their aldehyde analogues [5,6].

Earlier studies had proposed that with peptide aldehyde inhibitors a neutral thiohemiacetal was formed with papain and that the thiohemiacetal hydroxyl group might not be in the oxyanion hole [10]. However, the crystal structure of the complex between papain and the aldehyde inhibitor, leupeptin, showed that the oxygen atom of the thiohemiacetal is located in the oxyanion hole where it can hydrogen bond to the backbone amide nitrogen of Cys-25 and to the side-chain amide nitrogen of Gln-19 [30]. However, the removal of the hydrogen-bonding interaction with Gln-19 by site-directed mutagenesis led to a small improvement in binding of leupeptin and the aldehyde inhibitor Ac-Phe-Gly-H, which led to the conclusion that the thiohemiacetal formed between papain and a peptide aldehyde cannot be considered a good model for the transition state in substrate hydrolysis [31]. Likewise, although peptide glyoxals are potent inhibitors of papain they cannot be regarded as transition-state analogues of the tetrahedral intermediates formed during substrate catalysis. This is because the tetrahedral adduct is formed with the carbon adjacent to the carbonyl carbon which is in an equivalent position to the substrate carbonyl (Scheme 3). Therefore peptide glyoxals are not suitable inhibitors for studying how the thiol proteases stabilize tetrahedral intermediates during catalysis. However, an inhibitor which reacts to form a tetrahedral adduct which is a good transition-state analogue may well not be a good papain inhibitor because a major part of its binding energy may have been used to convert the substrate carbonyl carbon from an sp^2 hybridized state to an sp^3 hybridized state. Therefore, as our work shows, potent inhibitors do not have to mimic the tetrahedral transition state thought to occur in the thiol proteases.

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