# Determination of the Structure of Tetrahedral Transition State Analogues Bound at the Active Site of Chymotrypsin Using $^{18}O$ and $^{2}H$ Isotope Shifts in the $^{13}C$ NMR Spectra of Glyoxal Inhibitors†

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ABSTRACT: The peptide-derived glyoxal inhibitor Z-Ala-Pro-Phe-glyoxal, where Z is benzyloxycarbonyl, is an extremely potent inhibitor of chymotrypsin. When it is bound to chymotrypsin both the glyoxal (RCOCHO) keto and aldehyde carbons are $sp^3$ hybridized with chemical shifts of 100.7 and 91.4 ppm, respectively. However it is has not been shown whether these carbons are bound as hydrates or whether the active-site serine has reacted with them to form the corresponding hemiketal or hemiacetal. In this study we use $^{18}O$ isotope shifts to determine whether one or two exchangeable oxygen atoms are attached to the glyoxal keto or aldehyde carbons when it is free in water or bound to α-chymotrypsin. Both the $^{18}O$ isotope shifts at the free and enzyme-bound aldehyde carbons were ∼0.04 ppm showing that it is hydrated in both the free and bound forms. The $^{18}O$ isotope shift for the free hydrated keto carbon at 96.6 ppm was 0.046−0.049 ppm, but this was reduced to 0.026 ppm when the glyoxal inhibitor was bound to α-chymotrypsin showing that the nonexchangeable serine hydroxyl group has formed a hemiketal with glyoxal keto carbon. Deuterium isotope shifts on the $^{13}C$ NMR signals from the glyoxal inhibitor when it free and hydrated, when it is bound to chymotrypsin, as well as when it forms a model hemiketal confirm that the serine hydroxyl group has formed a hemiketal with the glyoxal keto carbon. The reasons for the different reaction specificities of glyoxal inhibitors for the active-site nucleophiles of serine and cysteine proteases are discussed.

Specific peptide-derived glyoxal inhibitors are formed by converting the peptide α-carboxylate group (RCOOH) to a glyoxal group (RCOCHO). Z-Ala-Pro-Phe-glyoxal[1] has been shown to be an extremely potent reversible inhibitor of the serine protease chymotrypsin with an apparent disassociation constant of 25 ± 8 nM at pH 7.0 (*1*), and Z-Phe-Ala-glyoxal has been shown to be an even more potent reversible inhibitor of the cysteine protease papain with an apparent disassociation constant of 3.3 ± 0.3 nM at pH 7 (*2*). If specific substrate-derived glyoxal inhibitors bind in the same way as substrates in both enzymes, the α-keto carbon of the glyoxal inhibitor is expected to occupy the same position as the hydrolyzed peptide carbonyl carbon of the corresponding substrate. In water the glyoxal keto group is ∼50% hydrated while the glyoxal aldehyde group is ∼100% hydrated. With the use of $^{13}C$ NMR it was shown that on binding to the serine proteases chymotrypsin (*1*) and subtilisin (*3*), both the glyoxal keto and aldehyde carbons were tetrahedrally coordinated. The tetrahedrally coordinated keto carbon of the glyoxal inhibitor had a chemical shift of ∼100.7 ppm, whereas its hydrate had a chemical shift which was ∼4 ppm smaller. However, the hemiketal carbons of chloromethylketone inhibitors had been shown to have chemical shifts that were ∼3 ppm larger than their hydrates (*4*). Therefore, it has been argued (*1*, *3*, *5*) that the hydroxyl group of the active-site serine residue reacts with the keto carbon of the glyoxal inhibitor to form a hemiketal which is analogous to the tetrahedral intermediate formed during substrate catalysis. This conclusion was supported by the fact that there was a much smaller increase of 0.7 ppm for the hydrated aldehyde carbon of the glyoxal inhibitor when it bound to chymotrypsin (*1*). Due to the lower electronegativity of sulfur compared to oxygen the chemical shifts of thiohemiacetal and thiohemiketal carbons are 15−20 ppm smaller than those of the corresponding hydrates. Therefore, with papain (*2*) it was possible to show unambiguously that the thiol group of the active-site cysteine residue reacted with the glyoxal aldehyde carbon to form a thiohemiacetal with a chemical shift that was ∼18 ppm smaller than the hydrate signals at ∼90 ppm. The keto carbon of the glyoxal group was expected to be in the same position as the peptide carbon of the hydrolyzed peptide bond in the analogous substrate. Therefore, this showed that in the cysteine protease papain it was the greater reactivity of the glyoxal aldehyde carbon, and not the spatial alignment of the glyoxal keto carbon and the enzyme nucleophile, which determines whether a thiohemiacetal or thiohemiketal is formed.

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[1] Abbreviation: Z, benzyloxycarbonyl.

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The fact that the glyoxal aldehyde carbon is fully hydrated in water, whereas the keto carbon is only ~50% hydrated in solution, shows that glyoxal aldehyde carbon forms a more stable hydrate than the glyoxal keto carbon. However, as we have shown that reactivity and not spatial alignment can determine which glyoxal carbon reacts with a nucleophile, then we cannot dismiss the possibility that in the serine proteases the active-site serine hydroxyl group reacts with the glyoxal aldehyde carbon. If this is true then the chemical shift of 96.6 ppm for the hydrated keto carbon of the glyoxal inhibitor must increase by ~4 to 100.7 ppm when it is bound to chymotrypsin. As this possibility cannot be discounted, it is essential that we find a method of determining whether the active serine hydroxyl group in the serine proteases reacts with the glyoxal keto or aldehyde carbons.

Replacing $^{16}O$ and $^{1}H$ with the heavier stable isotopes $^{18}O$ and $^{2}H$ produces small changes in the $^{13}C$ NMR chemical shift which are proportional to the number of atoms replaced, and the $^{18}O$ shifts are also proportional to the hybridization state of the carbon atom. $^{18}O$ shifts have been used to show reversible hydration of the ketone carbon of an inhibitor bound to pepsin (*6*). $^{18}O$ shifts have also been used to show that the tetrahedral adduct formed when trypsin is alkylated by a chloromethylketone inhibitor is not due to hydration of the ketone carbon but results from the active-site serine hydroxyl group reacting with the inhibitor carbonyl carbon (*7*). Differential $^{18}O$ and $^{2}H$ isotope shifts on $^{13}C$ NMR spectra have enabled the detection of a covalent tetrahedral adduct in reactions catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A synthase (*8*, *9*). In the present study we use $^{18}O$ and $^{2}H$ isotope shifts on $^{13}C$ to determine whether the active serine hydroxyl group of chymotrypsin reacts with the glyoxal aldehyde or keto carbon to form a tetrahedral species.

## EXPERIMENTAL PROCEDURES

*Materials.* L-[1-$^{13}$C]Phenylalanine (99 atom %) and L-[3-$^{13}$C]alanine were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA) and from CDN Isotopes (Pointe-Claire, Quebec, Canada). All other chemicals used were obtained from the Sigma-Aldrich Chemical Co., Dorset, U.K.

*Inhibitor Synthesis*. Z-Ala-Pro-Phe-glyoxal, Z-Ala-Pro-Phe-[2-$^{13}$C]glyoxal, Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal, and Z-[3-$^{13}$C]Ala-Ala-Phe-[2-$^{13}$C]glyoxal were synthesized using the methods described by Djurdjevic-Pahl et al. (*1*).

*Enzyme Solutions.* α-Chymotrypsin (crystallized and lyophilized) was obtained from Sigma-Aldrich Chemical Co., and the amount of fully active protein (69%) was determined as described by Finucane et al. (*10*).

*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. Sample tubes of 5 mm diameter were used with a 2.5 mm insert containing 100% (v/v) $^{2}H_2O$ to obtain a deuterium lock signal. The $^{13}C$ NMR spectral conditions for the samples of chymotrypsin inhibited by Z-Ala-Pro-[2-$^{13}$C]Phe-glyoxal or Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal at 11.75 T were as follows: 131 072 time-domain data points; spectral width 240 ppm; acquisition time 2.163 s; 6.0 s relaxation delay time; 90° pulse angle; 2048 transients recorded per spectrum. Waltz-16 composite pulse $^{1}H$ decoupling with a BLARH100 amplifier was used with 16 dB attenuation during the acquisition time and 34 dB attenuation during the relaxation delay to minimize dielectric heating but maintain the nuclear Overhauser effect. Quantitative $^{13}C$ NMR spectra were obtained without proton decoupling and using an interpulse delay of at least 5 times the spin lattice relaxation time ($T_1$) of the carbon nucleus being quantified. Spectra of protein complexes were transformed using an exponential weighting factor of 5 Hz. Samples of chymotrypsin inhibited by Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal were examined under the same conditions except that the relaxation delay was 0.6 s and 2320 transients were recorded per spectrum. Spectra were transformed using an exponential weighting factor of 10 Hz. All spectra were zero filled to give 131 072 data points. This gave a digital resolution of 0.231 Hz/point or 0.00184 ppm/point.

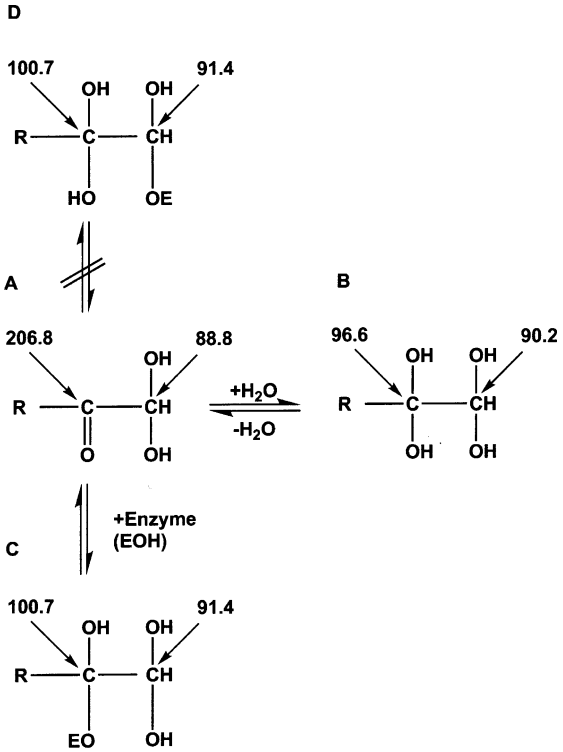
Both $^{1}H$ and $^{13}C$ chemical shifts are quoted relative to tetramethylsilane at 0.00 ppm. In aqueous solutions the chemical shift of 1,4-dioxane at 67.4 ppm (0.04% (v/v)) was used as an internal reference as described previously (*10*). The small amounts of DMSO-$d_6$ in samples had a chemical shift of 38.7 ppm. The signal due to the $^{13}C$-enriched methyl group of the alanine residue in Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal gave a signal at 18.5 ppm in the presence of chymotrypsin which could be used as a secondary reference signal.

## RESULTS

Using quantitative $^{13}C$ NMR spectra of Z-Ala-Pro-[1-$^{13}$C]-Phe-glyoxal and Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal at pH 7 we obtained hydration constants ($K_{HYD}$ = [hydrate]/[nonhydrate]) of 89.3 ± 0.9 and 1.28 ± 0.05 for the aldehyde and keto carbons, respectively, of the glyoxal inhibitors. Therefore, on the basis of reactivity we would predict that the active-site serine hydroxyl group would be ~70 times more likely to react with the glyoxal aldehyde carbon than with its keto carbon. It is therefore not surprising that with the cysteine protease papain, the thiolate ion of the active-site cysteine residue reacts with the glyoxal aldehyde group to form a hemiacetal (*2*). In the serine proteases subtilisin (*3*) and chymotrypsin (*1*, *5*, *11*) we have argued that the active-site serine hydroxyl group reacts with the glyoxal keto group and not its aldehyde group. This argument assumes that the chemical shift of a hemiketal carbon would be ~3 ppm greater than that of its hydrate. However, we cannot dismiss the possibility that the glyoxal keto-hydrate has been bound and that its chemical shift has been perturbed on binding to chymotrypsin. It is also assumed that it is the spatial alignment and not the reactivity that determines which glyoxal carbon reacts with the active-site serine hydroxyl in the serine proteases, whereas it was reactivity and not spatial alignment that determined which glyoxal carbon reacted with the active-site cysteine thiolate ion in the cysteine proteases. It is therefore essential that we use other methods to determine the structure of the tetrahedrally coordinated glyoxal carbons in chymotrypsin inhibitor complexes.

In this study we use $^{18}O$ and $^{2}H$ isotope shifts on the $^{13}C$ NMR chemical shift of the tetrahedral glyoxal keto carbon to determine whether the inhibitor glyoxal carbon has formed a hemiketal or hydrate when it is bound to chymotrypsin.

Scheme 1: Chemical Shifts and Structures of Z-Ala-Pro-Phe-glyoxal in the Presence and Absence of α-Chymotrypsin




Inhibition studies from pH 3−12 found that the enzyme is saturated with inhibitor over this pH range (*11*). $^{1}$H NMR experiments demonstrated that when the glyoxal inhibitor is bound, the active-site histidine is fully protonated over this pH range (*11*). Our $^{13}$C NMR experiments have shown that the glyoxal keto carbon is bound in a tetrahedral form over the pH range of 3−12 (*11*). However, these $^{13}$C NMR studies have also shown that the purported hemiketal hydroxyl group ionizes with a p$K_a$ of ∼4, and so it is not possible to measure deuterium isotope shifts at neutral pH values. There is also significant slow exchange broadening of the hemiketal signals at pHs > 4. However, line widths are minimal at pH 3 facilitating accurate chemical shift measurement. Therefore, both $^{18}$O and $^{2}$H isotope shifts were measured at pH 3.1

*$^{13}$C Isotope Shifts of Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal or Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal in 50% $H_2{}^{18}O$ and 50% $H_2{}^{16}O$.* In this solvent mixture we expect to see a doublet for carbons such as keto carbons which have one oxygen attached or for hemiacetal and hemiketal carbons which have only one solvent-exchangeable oxygen and triplets due to carbons such as hydrates with two exchangeable oxygens attached. The triplet signal is due to the following species being formed: $^{16}$O, $^{16}$O (25%), $^{16}$O, $^{18}$O (50%), and $^{18}$O, $^{18}$O (25%). However, this species was not observed for any of the hydrate signals obtained from Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]-Phe-glyoxal or Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal at 25 °C. However, with Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal, on lowering the temperature to 4 °C, a triplet was seen for the signal at 96.6 ppm (Scheme 1B) and a doublet for the signal at 206.8 ppm (Scheme 1B). The signals at 96.6 and 206.8 ppm had isotope shifts of 0.023 ppm/oxygen atom and 0.052 ppm/oxygen atom, respectively. Essentially identical isotope shifts have been observed for the chloromethylketone carbon and its hydrate in Z-[2-$^{13}$C]Lys-chloromethylketone (*7*), and such $^{18}$O isotope shifts are typical of those expected for $sp^2$ (0.05 ppm) and $sp^3$ (0.02 ppm) hybridized carbons (*8*, *12*). It would appear that oxygen exchange is too rapid at 25 °C for the individual signals from the different isotopomers to be resolved. The failure to detect different isotopomers with Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal at 4 °C suggests that oxygen exchange is much more rapid with the glyoxal aldehyde group compared to the glyoxal keto group.

*$^{13}$C NMR Spectra of α-Chymotrypsin in the Presence of Either an Excess of Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal or an Excess of Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal in 100% $H_2{}^{16}O$ at pH 3.1 and 25 °C.* When α-chymotrypsin was incubated with an excess of Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal (Figure 1A) signals at 88.8 and 90.2 ppm from excess free inhibitor were observed (Scheme 1, parts A and B). A signal at 91.4 ppm due to enzyme-bound inhibitor (Scheme 1, parts C or D) was also observed (*1*, *11*). It is hoped that isotope-labeling experiments will allow us to determine whether the glyoxal aldehyde carbon is bound as a hydrate (Scheme 1C) or as a hemiacetal formed with the active-site serine hydroxyl group of chymotrypsin (Scheme 1D). The signal at 38.7 ppm is due to DMSO-$d_6$ used as a solvent for the inhibitor. The signal at 67.4 ppm is from the 1,4-dioxane used as an internal chemical shift reference.

When α-chymotrypsin was incubated with an excess of Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal (Figure 1B), signals at 206.8 and 96.6 ppm due to excess free inhibitor were observed (Scheme 1, parts A and B). A signal at 100.7 ppm due to the enzyme-bound inhibitor (Scheme 1, parts C or D) is also observed (*1*, *11*). Again it is hoped that isotope-labeling experiments will allow us to determine whether the glyoxal keto carbon is bound as a hydrate (Scheme 1D) or as a hemiketal formed with the active-site serine hydroxyl group of chymotrypsin (Scheme 1C).

*$^{13}$C Isotope Shifts of the $^{13}$C-Enriched Carbon Signals Observed When α-Chymotrypsin Is Incubated with Either Excess Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal or Excess Z-[3-$^{13}$C]-Ala-Pro-[2-$^{13}$C]Phe-glyoxal in 100% $H_2{}^{18}O$ and 100% $H_2{}^{16}O$ at pH 3.1 and 25 °C.* The differential $^{18}$O isotope shift of 0.045 ppm for the signal at 91.4 ppm (Figure 2A) due to the $^{13}$C-enriched aldehyde carbon of Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal bound to chymotrypsin is essentially the same as that observed (Table 1) for the signals (Figure 2, parts B and C) from the excess of free inhibitor at 90.2 ppm (Scheme 1B) and 88.8 ppm (Scheme 1A). The magnitudes of these isotope shifts show that two exchangeable oxygen atoms are attached to the glyoxal aldehyde carbon. This shows that the glyoxal aldehyde carbon does not form a hemiacetal (Scheme 1D) with the nonexchangeable active-site serine hydroxyl group and that it is bound to chymotrypsin as its hydrate (Scheme 1C).

The differential $^{18}$O isotope shift (0.026 ppm) for the signal at 100.7 ppm (Figure 2D) was approximately half the isotope shift (0.049 ppm) obtained for the signal at 96.6 ppm (Figure 2E) due to the $^{13}$C-enriched hydrated keto carbon of the excess free inhibitor Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal (Table 1). The magnitude of the isotope shift for the signal at 100.7 ppm shows that it only has one oxygen atom attached, while the ∼2-fold larger isotope shift of the hydrate signal at 96.6 ppm is expected as it has two oxygen atoms

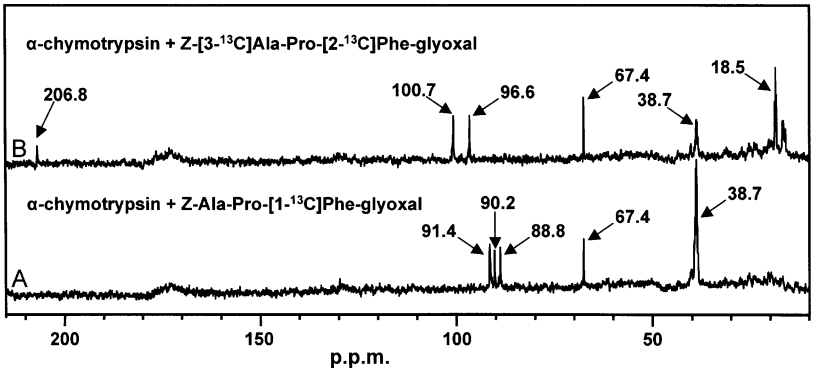



FIGURE 1: $^{13}C$ NMR spectra of α-chymotrypsin in the presence of an excess of Z-[3-$^{13}C$]Ala-Pro-[2-$^{13}C$]Phe-glyoxal (spectrum B) and Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal (spectrum A). Acquisition and processing parameters were as described in the Experimental Procedures section. Sample conditions were (B) 0.5 mL of 0.99 mM α-chymotrypsin in $H_2{}^{16}O$ containing 1.98 mM Z-[3-$^{13}C$]Ala-Pro-[2-$^{13}C$]Phe-glyoxal, 0.04% dioxane, 0.8% $Me_2SO$, pH 3.04; (A) 0.4 mL of 0.97 mM α-chymotrypsin in $H_2{}^{16}O$ containing 2.30 mM Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal, 0.04% dioxane, 4.5% $Me_2SO$, pH 3.12.

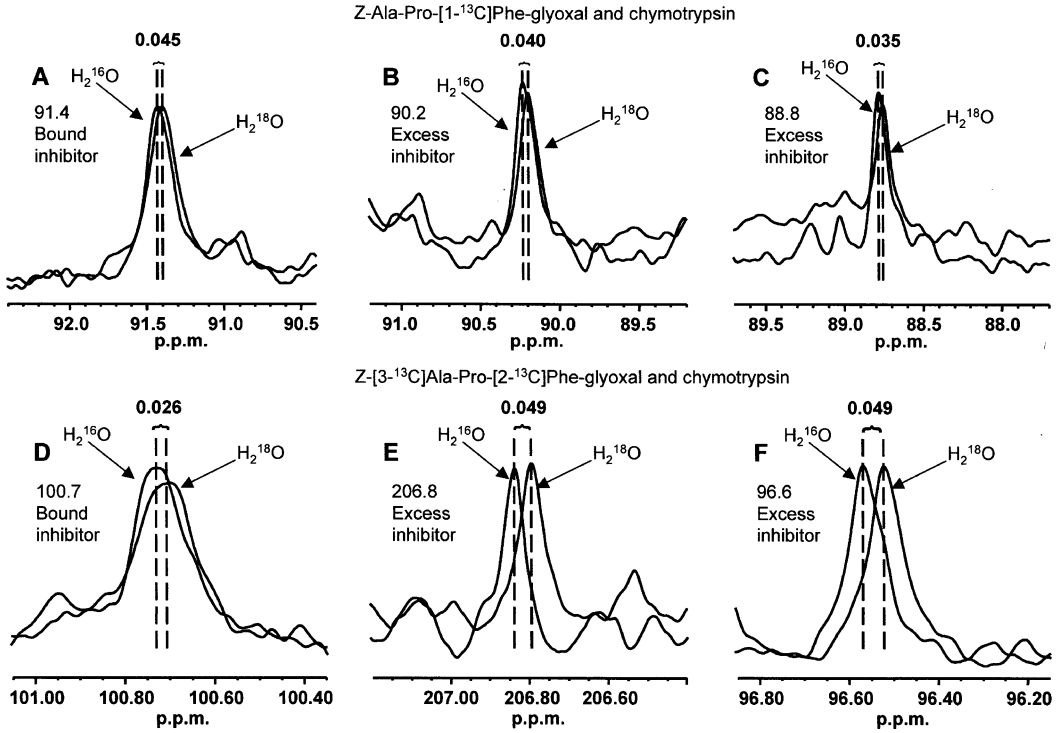



FIGURE 2: $^{18}O$ isotope effects on the $^{13}C$-enriched NMR signals of Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal (spectra A−C) and Z-[3-$^{13}C$]Ala-Pro-[2-$^{13}C$]Phe-glyoxal (spectra D−F) in the presence of α-chymotrypsin. Acquisition and processing parameters were as described in Experimental Procedures section. Spectra in $H_2{}^{16}O$ and $H_2{}^{18}O$ are presented. Both samples had volumes of 0.4 mL, and they contained 0.99 mM α-chymotrypsin and 0.5% dioxane. Spectra A−C contained 2.30 mM Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal and 4.5% $Me_2SO$. Spectra in $H_2{}^{16}O$ and $H_2{}^{18}O$ had pH values of 3.14 and 3.11, respectively. Spectra D−F contained 2.48 mM Z-[3-$^{13}C$]Ala-Pro-[2-$^{13}C$]Phe-glyoxal and 1% $Me_2SO$. Spectra in $H_2{}^{16}O$ and $H_2{}^{18}O$ had pH values of 3.13 and 3.11, respectively.

attached to the $^{13}C$-enriched glyoxal keto carbon (Scheme 1B). These results show that there is only one solvent-exchangeable oxygen atom present in tetrahedrally coordinated glyoxal keto carbon at 100.7 ppm. The chemical shift value of this carbon shows that it is tetrahedrally coordinated and is typical of a tetrahedral carbon with two oxygen ligands. Unlike hydrated carbonyls, alcohol oxygens are not usually solvent exchangeable under ambient conditions. Therefore, these results support our earlier assignment (*1*, *11*) of the signal at 100.7 ppm to a hemiketal species (Scheme 1C) formed by the addition of the active-site serine hydroxyl group to the $^{13}C$-enriched 2-$^{13}C$ glyoxal keto carbon (Scheme 1A) of Z-[3-$^{13}C$]Ala-Pro-[2-$^{13}C$]Phe-glyoxal.

*$^{13}C$ Isotope Shifts of the $^{13}C$-Enriched Carbon Signals Observed When α-Chymotrypsin Is Incubated with Either Excess Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal or Excess Z-[3-$^{13}C$]-Ala-Pro-[2-$^{13}C$]Phe-glyoxal in 100% $^2H_2O$ and 100% $^1H_2O$ at pH 3.1 and 25 °C.* Differential deuterium isotope shifts on hydroxyl-bearing carbon atoms in sugars usually lie in the range of 0.15−0.2 ppm, but the anomeric carbon with two oxygens attached can have a smaller isotope shift in the range of 0.11−0.15 (*13*, *14*). The $^2H$ isotope shift for the signal at 91.4 ppm (Figure 3A) due to the $^{13}C$-enriched aldehyde carbon of Z-Ala-Pro-[1-$^{13}C$]Phe-glyoxal bound to chymotrypsin is essentially the same as that observed (Table 2) for the signals from the excess of free inhibitor at 90.2 ppm (Figure 3B) and slightly larger than that observed for

Table 1: $^{18}O$ Isotope Shifts in 100% $H_2{}^{18}O$ at pH 3.1 and 25 °C

| inhibitor | enzyme +/− | signal ppm | isotope shift ppm |
|---|---|---|---|
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | + | 91.4 | 0.045 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal[a] | + | 90.2 | 0.040 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal[a] | + | 88.8 | 0.035 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal[b] | − | 206.8 | 0.052 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal[a] | + | 206.8 | 0.049 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal[b] | − | 96.6 | 0.046 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal[a] | + | 96.6 | 0.049 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | + | 100.7 | 0.026 |

[a] Signals from excess free inhibitor in the presence of chymotrypsin. [b] Sample at 4 °C, isotope shift measured in 50% $H_2{}^{18}O$.

the signal at 88.8 ppm (Figure 3C). The magnitude of this shift is similar to that observed for a single hydroxyl group. However, the hydrated aldehyde carbon of the free inhibitor has two exchangeable hydroxyl groups directly bonded to it, and so it is clear that the differential deuterium isotope shift is smaller for hydrates than for alcohols. To confirm this we dissolved the inhibitor in $CH_3OH$ and $CH_3O^2H$. When the alcohol reacts with the aldehyde a new chiral center is formed, and so two diastereomeric hemiacetals are formed at 96.6 and 96.4 ppm with only one exchangeable hydroxyl group. The isotope shifts for these hemiacetals were therefore approximately half that observed for the free hydrate at 90.2 ppm and for the signal from the enzyme-bound aldehyde carbon at 91.4 ppm (Table 2). This confirms that the glyoxal aldehyde carbon does not form a hemiacetal (Scheme 1D) with the nonexchangeable active-site serine hydroxyl group and that it is bound to chymotrypsin as its hydrate (Scheme 1C).

For Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal the differential deuterium isotope shift of ~0.15 ppm due to the two hydroxyl groups of the hydrated glyoxal keto carbon (Figure 3E) was significantly smaller than that observed for the hydrated glyoxal aldehyde carbon (Table 2). However, the differential deuterium isotope shifts for the diastereomeric hemiketals formed in methanol were approximately half those observed for the corresponding hydrates (Table 2), showing that the differential isotope effect does reflect the number of hydroxyl groups attached to the glyoxal keto carbon. The differential deuterium isotope shift of 0.100 ppm for the signal at 100.7 ppm (Figure 3D) from the $^{13}$C-enriched keto carbon of the bound glyoxal inhibitor was smaller than that observed for the hydrate glyoxal keto carbon, but it was similar in magnitude to the shift observed for the corresponding hemiketal formed with methanol (Table 2). These results show that the nonexchangeable active-site serine hydroxyl group forms a hemiketal with the keto carbon of the glyoxal inhibitor, and so the glyoxal inhibitor bound to chymotrypsin has the structure shown in Scheme 1C.

## DISCUSSION

The fact that the active-site hydroxyl group of serine-195 reacts with the less reactive glyoxal keto carbon and not the more reactive glyoxal aldehyde carbon shows that reaction specificity is not determined by the intrinsic chemical reactivity of the groups involved. In contrast, the active-site thiolate ion of cysteine-25 of papain reacts with the more

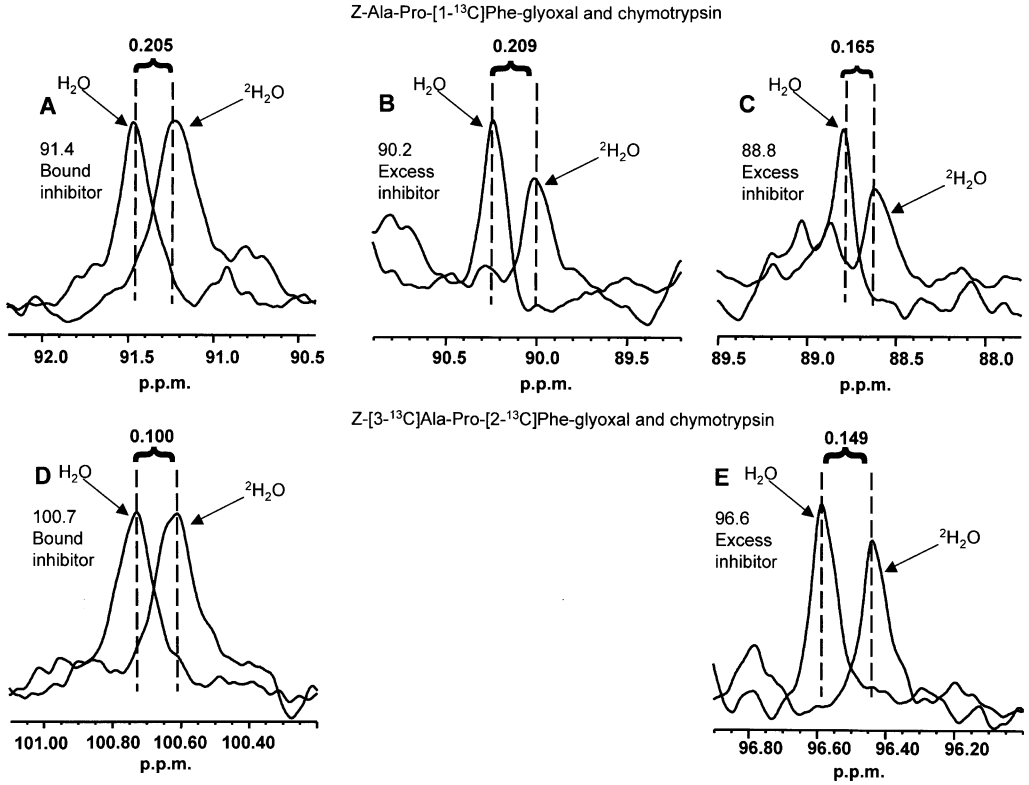



FIGURE 3: $^2H$ isotope effects on the $^{13}$C-enriched NMR signals of Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal (spectra A−C) and Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal (spectra D and E) in the presence of α-chymotrypsin. Acquisition and processing parameters were as described in Experimental Procedures section. Spectra in $H_2O$ and $^2H_2O$ are presented. Both samples had volumes of 0.5 mL, and they contained 0.99 mM α-chymotrypsin and 0.5% dioxane. Spectra A−C contained 2.30 mM Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal and 4.5% $Me_2SO$. Spectra in $H_2{}^{16}O$ and $H_2{}^{18}O$ had pH values of 3.12 and 3.10, respectively. Spectra D and E contained 1.98 mM Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal and 0.8% $Me_2SO$. Spectra in $H_2{}^{16}O$ and $H_2{}^{18}O$ had pH values of 3.04 and 3.07, respectively.

Table 2: $^{2}H$ Isotope Shifts in 100% $^{2}H_2O$ at pH 3.1 and 25 °C

| inhibitor | enzyme, methanol, or water | signal ppm | isotope shift ppm |
|---|---|---|---|
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | enzyme | 91.4 | 0.205 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | water[a] | 90.2 | 0.209 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | water[a] | 88.8 | 0.165 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | methanol | 96.6 | 0.109 |
| Z-Ala-Pro-[1-$^{13}$C]Phe-glyoxal | methanol | 96.4 | 0.110 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | water[a] | 96.6 | 0.149 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | water | 96.6 | 0.155 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | methanol | 97.8 | 0.086 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | methanol | 98.0 | 0.079 |
| Z-[3-$^{13}$C]Ala-Pro-[2-$^{13}$C]Phe-glyoxal | enzyme | 100.7 | 0.100 |

[a] Signals from excess free inhibitor in the presence of chymotrypsin.

reactive glyoxal aldehyde carbon to form a stable thiohemiacetal (*2*). It is the glyoxal keto carbon and not the glyoxal aldehyde which is expected to be bound in a position equivalent to the carbonyl carbon of the labile peptide bond of the equivalent peptide substrate. Therefore, it is clear that with the cysteine protease papain it is the reactivity of the aldehyde group and not the geometry of the enzyme−inhibitor complex which determines where the active-site thiolate ion reacts. In contrast with the serine protease chymotrypsin it is clear that it must be geometry and not reactivity that ensures that the active-site serine hydroxyl group reacts with the glyoxal keto carbon.

From studies with thiono and oxygen ester substrates it has been argued that although stabilization of the oxyanion of a tetrahedral intermediate is important for the serine proteases it is not significant in the cysteine proteases (*15*, *16*). One of the hydrogen-bond donors in the proposed oxyanion hole of papain is the side chain of Gln-19. However, mutation of Gln-19 produced significant reductions in $k_{cat}$ suggesting that the side chain of Gln-19 is hydrogen bonded to the oxyanion (*17*, *18*). Therefore, it appears that oxyanion stabilization in the oxyanion hole does occur in the cysteine proteases, but it is more effective in the serine proteases (*18*). It has also been suggested that with aldehyde inhibitors the neutral hemiacetal hydroxyl group may be located outside the oxyanion hole (*19*, *20*). However, X-ray crystallographic studies showed that when the aldehyde inhibitor leupeptin was bound to papain, the thiohemiacetal hydroxyl group was located in the oxyanion hole (*21*). This has led to the suggestion that there might be two possible configurations of the thiohemiacetal: one with the hemiacetal hydroxyl group in the oxyanion hole as in leupeptin, and the other where it hydrogen bonds to the active-site histidine (*22*). A similar mechanism was first proposed for hemiacetals formed with the serine proteases (*23*, *24*). It was also proposed that aldehyde hydrogen could be accommodated in the oxyanion hole of the cysteine protease papain and that the thiohemiacetal hydroxyl group could hydrogen bond to the active-site histidine (*19*). Gln-19 hydrogen bonds to the oxyanion, and its mutation to alanine and other amino acids decreases catalysis suggesting that oxyanion stabilization is essential for catalysis (*18*). However, the binding of aldehyde inhibitors was slightly improved by such mutations, suggesting that the thiohemiacetal hydroxyl group is not located in the oxyanion hole (*22*). This implies that thiohemiacetals are not good models of the transition state in papain-catalyzed reactions (*25*). Likewise it has been shown that glyoxal inhibitors form a thiohemiacetal with the cysteine protease papain which does not mimic the catalytic tetrahedral intermediate (*2*). However, our current results show that with the serine protease chymotrypsin, glyoxal inhibitors react with the active-site serine hydroxyl group to form a hemiketal which is analogous to the tetrahedral intermediate formed during substrate catalysis.

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