A ^{13}C -n.m.r. investigation of ionizations within a trypsin–inhibitor complex

Evidence that the $\text{p}K_{\text{a}}$ of histidine-57 is raised by interaction with the hemiketal oxyanion

William U. PRIMROSE,* A. Ian SCOTT, Neil E. MACKENZIE and J. Paul G. MALTHOUSE†

Center for Biological NMR, Department of Chemistry, Texas A & M University, College Station, TX 77843, U.S.A.

The ^{13}C -n.m.r. titration shifts of the α -methylene group of *N*-alkylated imidazoles are shown to be a sensitive probe of the ionization of the imidazolium ion. The ^{13}C -n.m.r. titration shifts of both the intact and denatured/autolysed 2- ^{13}C - and 1- ^{13}C -enriched trypsin–7-amino-3-benzoyloxycarbonylamino-1-chloroheptan-2-one (Z-Lys-CH₂Cl) complexes are compared. The titration shift for the denatured/autolysed complex confirms that this ionization is due to deprotonation of the *N*-alkylated imidazolium ring of histidine-57. In the intact trypsin–inhibitor complex the titration shift due to the 1- ^{13}C -enriched carbon is anomalous. We conclude that this titration shift cannot arise solely from the ionization of the imidazolium ion of histidine-57 and that the $\text{p}K_{\text{a}}$ of the imidazolium ion of histidine-57 is raised in the trypsin–inhibitor complex. The relevance of these studies to the mechanism of action of the serine proteinases is discussed.

INTRODUCTION

It is generally accepted that the serine proteinases catalyse the hydrolysis of small-molecular-mass substrates by a minimal three-step mechanism:

The formation and hydrolysis of the acyl intermediate (ES') is believed to proceed via tetrahedral intermediates (not shown in eqn. 1). Kinetic studies with thiono substrates (Asbóth & Polgár, 1983) imply that transition-state stabilization of the oxyanion of the tetrahedral intermediate is essential for efficient catalysis.

For optimal catalysis, intermediates should not accumulate. However, transition-state analogues offer a powerful method of assessing the effectiveness of enzymic stabilization of such transitory adducts. Specific ^{13}C enrichment of such analogues provides a direct, non-perturbing, 'atomic probe' of enzyme–inhibitor complexes. For example, 'chloromethyl ketone' inhibitors derived from specific substrates alkylate N-3 of the imidazole of histidine-57 (Shaw & Springhorn, 1967; Coggins *et al.*, 1974), and the side-chain hydroxy group of serine-195 forms a hemiketal with the inhibitor 'chloromethyl ketone' (Poulos *et al.*, 1976; Malthouse *et al.*, 1983, 1985). The inhibitor adduct therefore provides a stable model of the catalytic tetrahedral intermediate.

In our earlier studies (Malthouse *et al.*, 1983, 1985) of the trypsin–Z-Lys-CH₂Cl inhibitor complex [(I) in Scheme 1], ^{13}C enrichment of the inhibitor carbonyl group (2- ^{13}C) enabled us to provide direct evidence that a hemiketal was formed and that trypsin can stabilize the oxyanion of the ionized hemiketal (Malthouse *et al.*,

1985). We suggested that this stabilization could arise by a direct electrostatic interaction between the oxyanion and the imidazolium ion of histidine-57, leading to the proposal that the $\text{p}K_{\text{a}}$ of the imidazolium ion of histidine-57 was raised in the trypsin–inhibitor complex. Komiyama & Bender (1979) have pointed out that catalysis cannot proceed via a tetrahedral intermediate if the histidine $\text{p}K_{\text{a}}$ remains at 7 during formation of the tetrahedral intermediate. Kossiakoff & Spencer (1981) have argued that, owing to electrostatic interaction between the oxyanion and the imidazolium ion of histidine-57, the $\text{p}K_{\text{a}}$ of histidine-57 could be raised on formation of a tetrahedral intermediate. Therefore catalysis could proceed via a tetrahedral intermediate if the $\text{p}K_{\text{a}}$ of histidine-57 is raised on formation of a tetrahedral adduct, with histidine functioning as a general acid catalyst for expulsion of the α -amino function of the amino acid leaving group. It is therefore of considerable interest whether the $\text{p}K_{\text{a}}$ of histidine-57 is raised in the trypsin–Z-Lys-CH₂Cl inhibitor complex, as this will establish a precedent for this $\text{p}K_{\text{a}}$ being increased during catalysis by interaction with the oxyanion of a tetrahedral adduct.

In the present study we use [1- ^{13}C]Z-Lys-CH₂Cl (i.e. ^{13}C enriched in the inhibitor C-1 methylene group) to determine the $\text{p}K_{\text{a}}$ of the histidine-57 in the enzyme–inhibitor complex both before and after denaturation. Using model compounds, we establish that the titration shift of the α -methylene group of *N*-alkylated imidazoles is a sensitive probe of imidazolium ion ionization.

MATERIALS AND METHODS

Materials and general methods were as described in our previous studies (Malthouse *et al.*, 1983, 1985) with [2- ^{13}C]Z-Lys-CH₂Cl.

Abbreviation used: Z-Lys-CH₂Cl, 7-amino-3-benzoyloxycarbonylamino-1-chloroheptan-2-one.

* Present address: Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

† To whom correspondence should be addressed at present address: Department of Biochemistry, University College, Dublin 4, Ireland.

Table 1. Model compounds

$$\text{R}-\text{N}^+\text{H} \rightleftharpoons \text{R}-\text{N} + \text{H}^+$$

α -C, chemical shift (p.p.m.)					
Compound	R	ImH ⁺	Im	Δ p.p.m.*	pK _a †
(1)	–CH ₂ CH ₃	47.03 ± 0.09	44.37 ± 0.03	–2.66	7.256 ± 0.054
(2)	–CH ₂ COCH ₃	59.95 ± 0.15	58.14 ± 0.05	–1.81	6.087 ± 0.136
(3)	–CH ₂ CH ₂ OH	54.06 ± 0.08	51.59 ± 0.02	–2.48	6.795 ± 0.047
(4a)	–CH ₂ CO ₂ –‡	52.62 ± 0.10	51.04 ± 0.14	–1.58	7.111 ± 0.256
(4b)	–CH ₂ CO ₂ H§	50.63 ± 0.13	52.62 ± 0.10	+1.99	2.184 ± 0.195
(5)	–CH ₂ COOCH ₃	50.47 ± 0.03	48.53 ± 0.01	–1.94	6.089 ± 0.016

* Upfield shifts negative.

† All pK_a values determined from α -C titration shifts.

‡ Titration of imidazolium cation.

§ Titration of carboxylic acid.

Synthesis of [1-¹³C]Z-Lys-CH₂Cl

This was prepared as described previously (Malthouse *et al.*, 1985) except that the diazomethane used was generated from N-[¹³C]methyl-N-nitrosotoluene-*p*-sulphonamide.

Synthesis of N-alkylated imidazoles (Table 1)

Derivative (1) was prepared by the method of Wallach (1883), derivative (3) by the method of Giesemann (1956) and derivative (5) by the method of Pellicciari *et al.* (1981); derivative (4) was prepared by acid hydrolysis of derivative (5). Derivative (2) was prepared by adding bromoacetone to a stirred suspension of imidazole (bromoacetone/imidazole molar ratio was 1:2) in dichloroethane at room temperature. A precipitate of imidazole hydrobromide formed within 5 min and, after stirring for 6 h, was filtered off. The remaining organic phase was evaporated under reduced pressure to give an oily residue. Purification by column chromatography (silica gel; methanol/chloroform, 10:1, v/v) gave a yellow liquid, homogeneous by t.l.c.

N.m.r. spectra

Spectra at 7.045 T were recorded on a Bruker WM300 wide-bore spectrometer (sample size 10 ml). All samples contained 12.5% (v/v) ²H₂O. Chemical shifts are reported relative to tetramethylsilane at 0 p.p.m. Because of the short T₁ value for [1-¹³C]Z-Lys-CH₂Cl bound to trypsin, large pulse angles and rapid data acquisition were possible. Spectral conditions were either 8000 time-domain data points, 55 μ s pulse width (112 μ s $\equiv$ 90° pulse) and 0.25 s acquisition time or 4000 time-domain data points, 104 μ s pulse width, 0.12 s acquisition time and 1 W decoupler power. All spectra were weighted by a 10 Hz exponential weighting factor.

RESULTS

[1-¹³C]Z-Lys-CH₂Cl gave a single resonance at 47.5 p.p.m. in [²H₆]dimethyl sulphoxide, but in aqueous solution [12.5% (v/v) ²H₂O, pH 3.2] two resonances were detected, one at 49.1 p.p.m. and the other at 48.1 p.p.m., which were assigned to the enriched methylene group adjacent to the ketone group and its hydrate respectively

(Malthouse *et al.*, 1983). Both species appeared as triplets (J_{C-H} = 151 Hz) in proton coupled spectra.

Alkylation of trypsin with [1-¹³C]Z-Lys-CH₂Cl

Trypsin was alkylated with [1-¹³C]Z-Lys-CH₂Cl and separated from low-*M_r* materials by gel filtration as previously described for the preparation of the trypsin–[2-¹³C]Z-Lys-CH₂Cl complex (Malthouse *et al.*, 1983, 1985).

At pH 3.2 a single new resonance appeared at 53.8 p.p.m. in the spectrum of [1-¹³C]Z-Lys-CH₂Cl-inhibited trypsin [Fig. 1(a), (i)] that was not present in the spectrum of the inhibitor complex formed with non-enriched Z-Lys-CH₂Cl [Fig. 1(b), (i)]. The difference spectrum [Fig. 1(c), (i)] confirms that only one new resonance is observed.

On increasing the pH, the resonance at 53.8 p.p.m. (peak A) moved downfield and an additional resonance appeared (peak B), which moved upfield [Fig. 1(a), (ii) and (iii)]. On restoration of the pH to 3.2, the resonance at 53.8 p.p.m. (peak A) is still observed but a new resonance at 55.09 p.p.m. (peak B) is detected [Fig. 1(a), (iv)]. Difference spectra obtained by subtracting control spectra of the unenriched trypsin–Z-Lys-CH₂Cl complex from the spectra of the 1-¹³C-enriched complex [Fig. 1(c), (ii), (iii) and (iv)] confirmed that these were the only signals due to ¹³C enrichment [Figs. 1(a) and 1(c), (iv)].

pH-dependence of ¹³C chemical shifts

Detailed studies of the pH-dependence of the chemical shifts of the two ¹³C-enriched species (peaks A and B, Fig. 1) were undertaken. For peak A the downfield shift (+4.55 p.p.m.) with increasing pH was due to a single ionizing group of pK_a 7.99 ± 0.03 (Fig. 2). The upfield shift (–1.89 p.p.m.) of peak B with increasing pH was due to a single ionizing group pK_a 5.36 (Fig. 3). These pK_a values are similar to the pK_a values observed for the intact and denatured trypsin–[2-¹³C]Z-Lys-CH₂Cl complex (7.88 and 5.26 respectively) by Malthouse *et al.* (1985). That peak B arose from the denatured trypsin–inhibitor complex and peak A from the intact trypsin–inhibitor complex was confirmed by tryptic digestion at alkaline

Fig. 1. pH titration of the trypsin-[1- ^{13}C]Z-Lys-CH $_2$ Cl complex

The concentration of inhibited trypsin was 0.9 mM in medium containing 20 mM-glycine, 20 mM-glycylglycine, 20 mM-CaCl $_2$ and 12.5% (v/v) $^2\text{H}_2\text{O}$; the sample volume was 9 ml. The acquisition time was 0.246 s. [1- ^{13}C]Z-Lys-CH $_2$ Cl-inhibited trypsin was used for spectra (i)–(iv) in (a). pH values (numbers of accumulations in parentheses) were 3.2 (70 000), 8.09 (66 000), 9.50 (75 000) and 3.18 (70 000) for spectra (i)–(iv) in (a) respectively. All spectra were obtained with the same sample and were run consecutively (i) → (iv). pH adjustments were made with 0.875 M-NaOH containing 12.5% (v/v) $^2\text{H}_2\text{O}$ [spectra (i)–(iii)] and with 0.875 M-HCl containing 12.5% (v/v) $^2\text{H}_2\text{O}$ [spectrum (iv)] as described by Malthouse *et al.* (1985). Spectra (i)–(iv) in (b) were obtained with Z-Lys-CH $_2$ Cl-inhibited trypsin with no ^{13}C enrichment. pH values (numbers of accumulations in parentheses) were 3.21 (120 000), 8.11 (65 000), 9.50 (80 000) and 3.18 (56 000) for (i)–(iv) in (b) respectively. Sample treatment and concentrations were the same as for spectra (i)–(iv) in (a). Spectra in (c) are difference spectra. These were obtained by subtraction of the non- ^{13}C -enriched trypsin-inhibitor complex [spectra in (b)] from the corresponding ^{13}C -enriched spectra [spectra in (a)] at the same pH. Peaks A and B are the [1- ^{13}C]inhibitor resonances of the intact and denatured trypsin-[1- ^{13}C]Z-Lys-CH $_2$ Cl complexes respectively.

pH as described by Malthouse *et al.* (1985) for [2- ^{13}C]Z-Lys-CH $_2$ Cl-inhibited trypsin. On denaturation/autolysis the intensity of resonance A decreased and there was a concomitant increase in the intensity of peak B. For the trypsin-[2- ^{13}C]Z-Lys-CH $_2$ Cl complex it has been demonstrated that inclusion of Ca $^{2+}$ prevents any detectable autolysis at high pH values (Malthouse *et al.*, 1985). However, at pH 9.5 the amplitudes of peaks A and B were comparable [Fig. 1(c), (iii)], but on integration peak B was found to be only 20% of the enriched-carbon intensity. The narrow linewidth of peak B (20 ± 5 Hz at pH 9.5) relative to peak A (linewidth approx. 66 ± 5 Hz at pH 9.5) is expected if the ^{13}C -enriched methylene group has a greater librational freedom in the denatured enzyme. The residual proteolytic activity of both the 1- ^{13}C - and the 2- ^{13}C -enriched inhibitor adducts were similar (activity less than 0.4% of non-inhibited enzyme

activity), and the greater linewidth of the enriched methylene group meant that suitable signal/noise ratio was only obtained after 6 h as opposed to 20 min for the C-2 enriched carbon (Malthouse *et al.*, 1985). Therefore the amount of autolysis is greater during the present experiments, but is negligible in the experiments with the 2- ^{13}C -enriched inhibitor adduct. With [2- ^{13}C]Z-Lys-CH $_2$ Cl it has been shown that in the denatured species the C-2 carbon is largely in the form of an sp^2 ketone (Malthouse *et al.*, 1985).

Comparison of the ^{13}C titration shifts of the intact and denatured trypsin-Z-Lys-CH $_2$ Cl complexes with those of model compounds

Examination of the model compounds in Table 1 shows that all the α -methylene groups of *N*-alkylated imidazole undergo an approx. -2 p.p.m. downfield shift on

Fig. 2. pH titration of the trypsin-[1-¹³C]Z-Lys-CH₂Cl complex (peak A, Fig. 1)

Acquisition parameters and sample conditions were as described in the legend for Fig. 1(a). The continuous line was calculated by using the equation

$$\delta_{\text{obs}} = (S_1 + S_2 \cdot K_a/[H]) / (1 + K_a/[H])$$

and the fitted parameters $pK_a = 7.99 \pm 0.03$, $S_1 = 53.84 \pm 0.02$ and $S_2 = 58.39 \pm 0.13$.

Fig. 3. pH titration of the trypsin-[1-¹³C]Z-Lys-CH₂Cl inhibitor complex (peak B, Fig. 1)

Acquisition parameters and sample conditions were as described in the legend for Fig. 1(a). Some data points were also obtained with samples where the amplitude of peak B was increased (the amplitude of peak A being attenuated) by adding active trypsin (10%, w/w) and incubating at alkaline pH to allow autolysis/denaturation (see the text). The continuous line was calculated as described in Fig. 2 legend and the fitted parameters $pK_a = 5.36 \pm 0.03$, $S_1 = 55.04 \pm 0.04$ and $S_2 = 53.14 \pm 0.01$.

deprotonation of the imidazole. The -1.89 p.p.m. downfield shift with increasing pH for the denatured/autolysed 1-¹³C-enriched inhibitor adduct is therefore typical of the α -methylene group of an *N*-alkylated imidazole, and the deprotonation shift of the α -methylene group of the denatured 1-¹³C-enriched inhibitor adduct (-1.89 p.p.m.) is essentially the same as that of its analogous model compound [see Table 1, compound (2), $\Delta p.p.m. = -1.81$ p.p.m.]. It has been shown that 'chloromethyl ketone' inhibitors that are analogues of specific substrates alkylate N-3 of histidine-57 (Shaw & Springhorn, 1967; Coggins *et al.*, 1974). The titration shift of the denatured/autolysed trypsin-[1-¹³C]Z-Lys-CH₂Cl complex confirms that alkylation of histidine has occurred and that the covalent bond between the C-1 of the methylene group of the inhibitor and N-3 of histidine-57 survives denaturation/autolysis.

The titration shift ($+4.8$ p.p.m. downfield) of the enriched carbon in the intact trypsin-[1-¹³C]Z-Lys-CH₂Cl complex is not observed in any of the model compounds (Table 1), and is discussed later below.

pH effects on the linewidth at the trypsin-[1-¹³C]Z-Lys-CH₂Cl complex

Experimental linewidths of peak A from Fig. 1 were 50 Hz (pH 3.2), 58 Hz (pH 8.1) and 66 Hz (pH 9.5). The increased linewidths of peak A at pH 8.1 (approx. 8 Hz) and at pH 9.5 (approx. 16 Hz) are essentially the same as observed for the 2-¹³C-enriched trypsin-inhibitor complex (Malthouse *et al.*, 1985).

Since the pH-dependent line broadening was essentially the same for both the 1-¹³C and 2-¹³C enriched atoms of the trypsin-inhibitor complex, then, as argued by Malthouse *et al.* (1985), the line broadening observed at alkaline pH values cannot be attributed to an increase in the effective rotational correlation time of the trypsin-inhibitor complex.

DISCUSSION

The results from our studies of the [1-¹³C]- and [2-¹³C]-Z-Lys-CH₂Cl-inhibited trypsin are summarized in Scheme 1. Previous studies of the trypsin-[2-¹³C]Z-Lys-CH₂Cl complex have shown that on denaturation the ketonic species (V) and (VIII) of Scheme 1 predominate (Malthouse *et al.*, 1985), and the pK_a 5.26 for their interconversion was assigned to the ionization of the imidazole ring of alkylated histidine-57. This pK_a value was 0.74 pH unit lower than the pK_a of the similar model compound (compound 2, Table 1). As shown in Table 1, all the α -methylene resonances undergo a characteristic approx. -2 p.p.m. downfield shift on deprotonation of the imidazole ring. The denatured trypsin-[1-¹³C]Z-Lys-CH₂Cl complex showed a similar -1.9 p.p.m. downfield shift on deprotonation (pK_a 5.36). Therefore the assignment of the pK_a of approx. 5.3 to the imidazole ring of *N*-alkylated histidine-57 in the denatured trypsin-inhibitor complex is confirmed.

From the data in Table 1, it is clear that the ¹³C titration shift of the α -methylene resonance of *N*-alkylated imidazoles is a sensitive probe for the ionization state of imidazole. All the α -methylene titration shifts (Table 1) exhibited a -1.8 to -2.66 p.p.m. downfield shift on deprotonation of the imidazolium cation.

The pK_a values obtained from 1-¹³C and 2-¹³C titration shifts of the intact trypsin-inhibitor complex were 7.99 and 7.88 respectively. These pK_a values show that the titration shifts either result from a single group of pK_a approx. 7.9 or from the ionization of two groups with essentially the same pK_a values.

These titration shifts could arise from: (a) formation of zwitterionic species [species (iii) in Scheme 2], the pK_a of approx. 8 reflecting ionization of the hemiketal hydroxy group; (b) formation of the neutral species

Scheme 1. Structures and chemical shifts at $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -enriched Z-Lys-CH₂Cl and its intact and denatured complexes with trypsin

Scheme 2. Ionizations of the intact trypsin-Z-Lys-CH₂Cl inhibitor complex

[species (ii) in Scheme 2], the pK_a of approx. 8 reflecting ionization of the imidazolium ion of histidine-57; (c) formation of both the neutral and zwitterionic species (ii) and (iii) of Scheme 2.

On the basis of the titration shifts of the α -methylene resonances of the model compounds (Table 1), formation of species (ii) of Scheme 2 [i.e. possibility (b)] should be characterized by downfield titration shift of -1.8 to -2.6 p.p.m. When histidine-200 of carbonic anhydrase was alkylated with bromo[$1\text{-}^{13}\text{C}$]acetate, a -1.7 p.p.m. downfield shift due to deprotonation of the N -alkylated imidazolium ion of histidine-200 was observed (Schoot Uiterkamp *et al.*, 1978). We therefore conclude that the

$1\text{-}^{13}\text{C}$ titration shift at $+4.6$ p.p.m. cannot be assigned solely to deprotonation of the imidazolium ring of histidine-57 [i.e. possibility (b)].

Since the pK_a values determined with [$1\text{-}^{13}\text{C}$]- and [$2\text{-}^{13}\text{C}$]-Z-Lys-CH₂Cl-inhibited trypsin are within 0.1 pH unit, it is not possible to distinguish between possibilities (a) and (c) on the basis of pK_a values. It is known that for amines the ^{13}C titration shifts of carbons β to the amino group often show larger titration shifts than the α -carbon (Batchelor *et al.*, 1975; Sarneski *et al.*, 1975). We have already shown that such a shift on ionization of the imidazolium ring of histidine-57 could not explain the $2\text{-}^{13}\text{C}$ titration shift of the inhibited enzyme (Malthouse *et al.*, 1985). However, if the hemiketal ionization produces a β -shift of $+6.6$ p.p.m. downfield at the inhibitor $1\text{-}^{13}\text{C}$ then this shift, combined with a -2 p.p.m. downfield shift due to ionization of the imidazolium ion of histidine-57, could explain the observed $1\text{-}^{13}\text{C}$ titration shift of $+4.6$ p.p.m. downfield if $pK_1 = pK_2$ (Scheme 2). Alternatively, if pK_1 is greater than 11 (i.e. not detected in the present studies) then both the $1\text{-}^{13}\text{C}$ and $2\text{-}^{13}\text{C}$ titrations must be due to ionization of the hemiketal hydroxy group (pK_2 approx. 8).

Unfortunately, the instability of analogous aliphatic hemiketals precludes determination of their titration shifts. Also, ^{13}C titration shifts depend on numerous factors, e.g. bond polarizability, nuclear charge and molecular geometry (Batchelor *et al.*, 1975; Jardetzky & Roberts, 1981) and therefore their prediction by 'ab initio' methods is unreliable.

Robillard & Shulman (1974) observed that the titration shift of the histidine-aspartic acid hydrogen-bonded proton of chymotrypsin inhibited by a 'chloromethylketone' inhibitor titrated with pK_a 8.4 but the titration shift was only one-third of that observed in the free enzyme. This suggests that the pK_a of histidine-57 may be approx. 8 in the present study. However, it is also possible that ionization of the hemiketal could affect the chemical shift of the histidine-aspartic acid hydrogen-bonded proton with the histidine imidazole ring remaining protonated. Robillard & Shulman (1974) also found that with negatively charged boronic acid derivatives the histidine-aspartic acid hydrogen-bonded proton did not titrate up to pH 9.5. This demonstrates that the histidine-57 pK_a can be raised with negatively charged tetrahedral adducts.

We conclude that in the trypsin-Z-Lys-CH₂Cl complex the pK_a of the imidazole ring of histidine-57 is either approx. 8 (in this case a fraction will also have a pK_a greater than 11; see below) and is perturbed or masked by the ionization of the hemiketal hydroxy group (pK_a approx. 8), or the pK_a of histidine-57 is greater than 11 and is not observed in the present study, the $1\text{-}^{13}\text{C}$ and $2\text{-}^{13}\text{C}$ titration shifts being due to the titration of the hemiketal hydroxy group. If the ionizations of the hemiketal hydroxy group and the imidazolium ion are linked as in Scheme 2 and if $pK_1 = pK_2$, then, on ionization of the cationic species (i), equal amounts of the neutral species (ii) and zwitterionic species (iii) (Scheme 2) will be formed ($K_T = [\text{species (ii)}]/[\text{species (iii)}] = K_1/K_2$). Therefore both pK_3 and pK_4 must be greater than approx. 11 as no other ionizations are detected. Consequently approx. 50% of histidine-57 will have a pK_a of approx. 8, the remaining 50% will have a pK_a greater than 11. Alternatively, if the pK_a values for both the $1\text{-}^{13}\text{C}$ - and $2\text{-}^{13}\text{C}$ -enriched adducts represent hemiketal ionization

then the pK_a of histidine-57 is greater than 11. The present studies and the previous studies (Malthouse *et al.*, 1985) with model compounds demonstrate that neither pK_a determined with the 1- ^{13}C - or 2- ^{13}C -enriched inhibitor can be solely attributed to the ionization of the imidazolium ion of histidine-57. We therefore conclude that the pK_a of at least 50% of the imidazolium ring of histidine-57 is greater than 11. Thus the electrostatic interaction between the hemiketal oxyanion and the imidazolium ions of histidine-57 stabilizes the zwitterionic species (iii) (Scheme 2) such that at least 50% of the imidazolium ion titrates with a pK_a greater than 11 and at least 50% of the hemiketal hydroxy group ionizes with a pK_a of approx. 8. Therefore it would appear possible that the pK_a of histidine-57 could be raised substantially (at least 3 pH units) on formation of a tetrahedral intermediate during substrate catalysis, which would facilitate general acid catalysis of leaving-group expulsion.

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