Evidence for Hemiacetal Formation between *N*-Acyl-L-phenylalaninals and α-Chymotrypsin by Cross-Saturation Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: *N*-Acetyl-L-phenylalaninal exists predominantly in its hydrated form in aqueous solution, but the aldehyde and not the hydrate is shown by nuclear magnetic resonance (NMR) spectroscopy to be the effective inhibitor of α-chymotrypsin. NMR spectroscopy also indicates that the initial α-chymotrypsin–*N*-acetyl-L-phenylalaninal complex is in equilibrium with a hemiacetal formed between the aldehyde and the active site serine residue. The rate of the latter equilibration is slow on the NMR time scale but the hemiacetal can be detected by cross-saturation NMR spectroscopy. *N*-Benzoyl-L-phenylalaninal is a more potent inhibitor of α-chymotrypsin than the *N*-acetyl derivative and both the formation of the enzyme–inhibitor complex and the hemiacetal are slow on the NMR time scale, but the hemiacetal in the enzyme can be detected by cross-saturation NMR spectroscopy. The *N*-acyl-L-phenylalaninals also bind to *N*-methylhistidinyl-57-α-chymotrypsin, but clear evidence for hemiacetal formation was not obtained by cross-saturation NMR spectroscopy either because the hemiacetal was not formed or more probably because the rate of dissociation was slow compared with the rate of relaxation of the hemiacetal proton. The dissociation constant of *N*-benzoyl-L-phenylalaninal to dehydroalaninyl-195-α-chymotrypsin was found to be high relative to the dissociation constant to native α-chymotrypsin, supporting the NMR evidence that a hemiacetal with the Ser-195 is formed on association of *N*-benzoyl-L-phenylalaninal with α-chymotrypsin.

The inhibition of trypsin by the leupeptins Pr-LL and Ac-LL (i.e., propionyl- and acetyl-L-leucyl-L-leucyl-DL-argininal) isolated from actinomycetes was the first example of the inhibition of a serine protease by a peptide aldehyde (Aoyagi et al., 1969; Kondo et al., 1969; Kawamura et al., 1969). The aldehydic and guanidino groups were shown to be essential structural features for the potent inhibition of trypsin, but the leupeptins were ineffective inhibitors of α-chymotrypsin (Cht). However, synthetically derived peptide aldehydes in which the argininal residue of the leupeptins was replaced by phenylalaninal, tyrosinal, and tryptophanal were potent inhibitors of α-chymotrypsin (Ito et al., 1972). Similarly peptide aldehydes related to substrates were reported to be potent inhibitors of elastase (Thompson, 1973). Thompson argued that these aldehydes form tetrahedral adducts (hemiacetals) with site reactive serine of the elastase active site and that the enzyme acts to stabilize this adduct because the proposed hemiacetal tetrahedral structure may be close to the transition-state structure in substrate hydrolysis by the enzyme (Thompson, 1973, 1974). This conclusion is based on the prediction that a transition-state structure has a much higher affinity for an enzyme than the ground-state structure of a substrate (Pauling, 1946; Wolfenden, 1972; Lienhard, 1972, 1973). However, while the arguments for aldehyde hemiacetal formation with peptide aldehydes to elastase were credible, the evidence in support of the structure was indirect (Thompson, 1973). Lewis & Wolfenden (1977a) have argued for thiol–hemiacetal formation on association of benzamidoacetaldehyde to the cysteine protease papain, based on an observed secondary deuterium isotope effect.

Evidence to support the formation of a hemiacetal between an aldehyde and a serine protease has so far been provided only for the inhibition of Cht by hydrocinnamaldehyde. The competitive inhibition constant was pH dependent on a group with $pK_a = 7$ which was interpreted as being due to enzyme-catalyzed hemiacetal formation between the aldehyde and the active site serine residue (Schultz & Cheerva, 1975). This conclusion was verified by an NMR double resonance experiment in which the aldehydic proton of hydrocinnamaldehyde in the presence of Cht was cross-saturated on irradiation in the region expected for the hemiacetal formed with the active site serine residue (Lowe & Nurse, 1977). We now report an investigation by NMR spectroscopy of the interaction of *N*-benzoyl- and *N*-acetyl-L-phenylalaninal with α-chymotrypsin (Cht) and *N*-methylhistidinyl-57-α-chymotrypsin (MCht). The binding constant of *N*-benzoyl-L-phenylalaninal to dehydroalaninyl-195-α-chymotrypsin (ACht) is also reported. A more extensive kinetic and thermodynamic study of the interaction of the specific substrate analogue *N*-benzoyl-L-phenylalaninal has been made with Cht and MCht, in which the probable similarities and dissimilarities of the aldehyde–Cht adduct to the transition state in substrate catalysis are discussed (Kennedy & Schultz, 1979).

Experimental Section

Materials

α-Chymotrypsin (Cht)[1] was purchased from Worthington Biochemicals Co. or Sigma Biochemicals Ltd. as a three times

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[1] Abbreviations used: Cht, α-chymotrypsin; MCht, *N*-methylhistidinyl-57-α-chymotrypsin; ACht, dehydroalaninyl-195-α-chymotrypsin; NMR, nuclear magneic resonance; AcPheal, *N*-acetyl-L-phenylalaninal; BzPheal, *N*-benzoyl-L-phenylalaninal; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate.

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crystallized (from four times crystallized chymotrypsinogen) dialyzed salt-free and lyophilized powder.

Dehydroalaninyl-195-α-chymotrypsin (ACht) and *N*-methylhistidinyl-57-α-chymotrypsin (MCht) were prepared and purified by the methods previously described (Schultz et al., 1977).

N-Acetyl-L-phenylalanine methyl ester (AcPheOMe) was prepared from L-phenylalanine (Cambrian Chemicals Ltd.) by standard procedures, mp 89–90 °C (lit. 90–91 °C, Hwang et al., 1952).

N-Acetyl-L-phenylalaninal (AcPheal) was synthesized by the general method of Ito et al. (1975). *N*-Acetyl-L-phenylalanine methyl ester (2.1 g; 9.5 mmol) was dissolved in dry dimethoxyethane (50 mL) and cooled to about −60 °C. A solution of diisobutylaluminum hydride (15 mL; 20% in toluene) was added to the rapidly stirred solution over 30 min, and the reaction mixture was kept at about −60 °C for a further 90 min before adding 2 N HCl (100 mL) and allowing this mixture to warm to 0 °C. The organic layer was separated and the aqueous layer extracted first with ethyl acetate (3 × 50 mL) and then with chloroform (5 × 50 mL). The ethyl acetate extracts and the original organic layer were combined and washed with saturated NaCl solution, dried (Na_2CO_3), and evaporated to yield an oil (0.6 g) which was mainly starting material. The chloroform extracts were washed with saturated NaCl solution and dried ($MgSO_4$), and the solvent was removed to give a white crystalline solid (0.75 g, 41%): mp 107–110 °C from ethyl acetate–ether; τ (Me_2SO-d_6) 0.5 (s, 1 H, CHO), 1.7 (db, 1 H, NH), 2.7 (s, 5 H, C_6H_5), 5.7 (m, 1 H, CH), 6.8 (dd, 1 H, $ArCH_B$), 7.2 (dd, 1 H, J_{AM} = 9.3 Hz, J_{BM} = 5.1 Hz, J_{MX} = 7.3 Hz, J_{AB} = 14 Hz, $ArCH_A$); ν_{max} 1672 (CONH), 1738 (CHO), 3438 cm^{-1} (NH).

N-Benzoyl-L-phenylalaninol (BzPheol) was synthesized from L-phenylalanine (Eastman Chemicals Co.) by procedures previously described (Jones et al., 1974; Hunt & McHale, 1957), mp 168–169 °C (lit. 169 °C, Hunt & McHale, 1957).

N-Benzoyl-L-phenylalaninal (*BzPheal*). Oxidation of BzPheol to BzPheal was carried out by the method previously described (Kennedy & Schultz, 1979), mp 140–141 °C (lit. 143–144 °C, Seki et al., 1972). The optical purity was shown by the synthesis of the D enantiomer by the same procedure, mp 140–141 °C, and the demonstration that the racemic mixture (equimolar mixture melting point of D and L enantiomers) had a melting point depressed by 20 °C.

Methods

NMR Spectroscopy. Proton NMR spectra were obtained on a Bruker 270-MHz Fourier transform spectrometer. Cross-saturation experiments involved application of a 500-ms gated pulse of 0.1–0.5-V rf intensity, immediately prior to the spectral sampling. The bandwidth of the F_2 was sufficiently narrow under these conditions to completely saturate the residual HOD for example without markedly saturating the signal of the free hydrated aldehydes (signal separation was about 0.4 ppm). Adequate spectra were obtained with 125–500 scans in the quadrature detection mode at 25 °C, unless otherwise specified.

NMR Spectra of AcPheal. Cht was dissolved in D_2O and lyophilized several times before dissolving in 0.1 M phosphate buffer, in D_2O. To this solution was added AcPheal dissolved in Me_2SO so that the final concentration of Me_2SO was about 10% (v/v) and the aldehyde concentration was about 0.1 M. MCht solutions were similarly prepared.

NMR Spectra of BzPheal. Due to the limited solubility of this inhibitor, the following procedure was adopted for preparing solutions. A 0.1 M phosphate buffer containing 1 mg/mL of EDTA in D_2O was added with rapid stirring to a Me_2SO-d_6 solution of the inhibitor. The maximal aldehyde concentration in this 20% (v/v) Me_2SO/buffer solution was 9 mM. Weighed amounts of three times crystallized Cht which had previously been dialyzed at pD 3.0 and 4 °C against 0.01 M EDTA (16 h) in D_2O and finally against D_2O (2 × 12 h) before lyophilization were added. Active site titration with *N*-cinnamoylimidazole revealed 70–80% active sites by weight. MCht solutions with aldehyde were similarly prepared in 25% (v/v) Me_2SO-d_6 buffer.

Determination of the Inhibition Constant (K_i) of AcPheal to Native Cht. The inhibition constant was determined by standard steady-state kinetics (Dixon & Webb, 1964) against the substrate *N*-acetyl-L-phenylalanine methyl ester. In a typical experiment, 10 μL of enzyme solution at pH 7.8 was added to 6 mL of substrate solution in 1 mM phosphate buffer at pH 7.8 in a thermostated reaction cell (25 °C) of a Radiometer pH-stat, and the hydrolysis of substrate was followed by the uptake of 0.1 M NaOH. A plot of $1/v_i$ against [I] for two different substrate concentrations (5 and 20 mM) gave straight lines. At their point of intersection, $-K_i$ = [I] (Dixon, 1953).

Determination of Binding Constants to ACht. A minimum estimate of the binding constant of BzPheal to ACht was determined by a proflavin displacement procedure as previously described (Schultz et al., 1977; Brandt et al., 1967). For ACht, a base-line spectrum was obtained with 3.0 mL of a solution containing 2.1×10^{-4} M ACht, 3×10^{-5} M phenylmethanesulfonyl fluoride (to inhibit any residual active Cht) in the appropriate buffer (0.049 M sodium phosphate, 0.095 M NaCl, 12.5% Me_2SO) at pH 7.8 and 25.0 °C against a reference cuvette without the enzyme component. To each cuvette was added 100 μL of proflavin stock solution prepared in the appropriate buffer (pH 7.8) to give a proflavin concentration in the cuvettes of 3.6×10^{-5} M, and the difference spectrum was obtained between 540 and 440 nm.

Rate of Hydration and Racemization of AcPheal. AcPheal (36 mg) in Me_2SO-d_6 (0.2 mL) was added to 100 mM sodium phosphate buffer in D_2O (2 mL, pD 7.8). The NMR spectrum showed that the aldehyde–hydrate equilibrium was established rapidly (<15 min). The optical rotation of the equilibrated sample changed only slightly (<10%) in 5 h.

pD Measurements. Reported pD values represent corrected pH meter readings in D_2O, standardized against H_2O buffers, i.e., pD = "pH" + 0.41.

Hydration Constant for BzPheal. The areas of the aldehyde and hydrate proton signals were measured by either weighing the cut-out NMR signals or utilizing the area measurement capability of the Nicolet data system. Either method gave a hydration constant, K_H(hydrate)/(aldehyde), of 9 ± 1 for a 20% Me_2SO-d_6/0.1 M phosphate, D_2O solution of BzPheal. The constant is quite sensitive to the Me_2SO concentration, so that in 25% Me_2SO-d_6, $K_H \sim 6$. Similar hydration constants were found previously for other acylamidoaldehydes (Lewis & Wolfenden, 1977b).

Results and Discussion

N-Acetyl- and *N*-benzoyl-L-phenylalaninal were insufficiently soluble in aqueous buffer to achieve the required concentration for the NMR experiments; consequently, the inhibitors were added in dimethyl sulfoxide to the buffered enzyme solution. It was shown in a separate study that the hydration of AcPheal added in dimethyl sulfoxide to aqueous buffer was complete in less than 15 min (the time taken to

determine the NMR spectrum), whereas in 5 h, less than 10% racemization (followed polarimetrically) had occurred under the conditions used for the NMR experiments. A low racemization rate of less than 5% in 6 h was obtained for BzPheal. Furthermore, it is shown that the D enantiomer of BzPheal has a K_i of 1.1×10^{-3} M (12.5% Me_2SO) against *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Kennedy & Schultz, unpublished results), while the L enantiomer has a K_i of 3.5×10^{-5} M (12.5% Me_2SO) (Kennedy & Schultz, 1979). Accordingly, even if a small amount of racemization occurs, the bound species will be the L enantiomer. All solutions for NMR spectroscopy were freshly prepared from crystalline aldehyde.

Previous study has shown that the equilibrium dissociation constant of the specific aldehyde *N*-benzoyl-L-phenylalaninal to Cht varies little (factor of 4) between pH 3 and 8, although the rates of equilibration are pH dependent (Kennedy & Schultz, 1979). Similarly with MCht, the affinity constant of *N*-benzoyl-L-phenylalaninal is not found to vary significantly between pH 4 and 8 (Kennedy & Schultz, unpublished results).

N-Acetyl-L-phenylalaninal (AcPheal). AcPheal is a competitive inhibitor of Cht with $K_i = 0.7 \pm 0.2$ mM (Table I). The chemical shift of the aldehydic proton of AcPheal is virtually unaffected by the addition of Cht but the line width, $\nu_{1/2}$, increases as expected for a simple two-state equilibration

$$E + I \rightleftarrows EI$$

where $I_0 \gg E_0$ so that effectively EI $\sim E_0$ for all observations; E_0 and I_0 are the total enzyme and inhibitor concentrations, respectively. Under these conditions

$$\nu_{1/2} = \nu_{1/2I} + \nu_{1/2EI}E_0/I_0$$

where $\nu_{1/2I}$ and $\nu_{1/2EI}$ are the line widths at half-height of the free and enzyme-bound inhibitor, respectively.

A plot of the corrected line width of CH proton of the aldehyde and the hydrated aldehyde against E_0/I_0 at pD 6.5 is shown in Figure 1. The line widths were corrected for additional line broadening due to increased viscosity of the solution upon addition of variable amounts of enzyme. The correction involved adding the line width of the acetyl resonance in the absence of enzyme and subtracting the line width of the acetyl resonance at the appropriate enzyme–inhibitor ratio, to the aldehyde and hydrated aldehyde signals. It is evident that, while the aldehydic proton is progressively broadened as the enzyme concentration is increased, the CH proton of the hydrated aldehyde is not affected, clearly indicating that the aldehyde and not the hydrated aldehyde is the effective inhibitor. The line-width correction eliminates the smaller, nonspecific broadening of the signals due to viscosity changes. At pD 7.9 the effect on the line width of the aldehydic proton is much greater for a given E_0/I_0 ratio (not shown). This could be associated with the expected decrease in K_i at pD 7.9 but might also be due to an increase in the exchange rate between the EI complex and the putative hemiacetal, putting it into the slow to intermediate exchange rate on the NMR time scale. A study of the effect of temperature on the chemical shift of the aldehydic proton supported this suggestion since the chemical shift moved to high field, i.e., in the direction of the chemical shift of the putative hemiacetal. It was not possible, however, to reach the coalescence temperatures for the exchange before the enzyme began to denature (about 45 °C).

In order to provide more convincing evidence for the presence of the hemiacetal formed between AcPheal and the active site serine residue of Cht, a cross-saturation experiment was undertaken (cf. Lowe & Nurse, 1977; Clark et al., 1977; Bendall et al., 1977). The chemical shift of the hemiacetal would be most clearly defined under conditions of slow exchange on the NMR time scale with the bound aldehyde (EI), which is known from the observed line broadening to be in fast exchange with the free aldehyde. The conditions chosen for the cross-saturation experiment are shown in Figure 2. The second radio-frequency pulse was used to scan the whole spectrum but only in the region of δ 5.36 ppm was saturation transfer observed. In Figure 2 are shown the effects of the second radio-frequency pulse at (a) 5.19, (b) 5.36, and (c) 5.76 ppm. The chemical shift of 5.36 ppm where the maximal cross-saturation was observed is in the region expected for the proton of the hemiacetal formed between the aldehyde and the active site serine residue of Cht. Similar results were obtained at pD 8, although the line width and the region of cross-saturation were broader.

N-Benzoyl-L-phenylalaninal (BzPheal). In contrast to AcPheal, the NMR chemical shifts and line widths of BzPheal are not affected by the addition of Cht. Thus, over a range of I_0/E_0 of 8–38 (cf. Figure 3), the chemical shifts of all ^1H NMR signals for both the hydrate form and the aldehyde form of BzPheal are unchanged (±1 Hz) and are essentially the same as the signals in the absence of enzyme. Similarly, the line widths of all signals for both forms of the inhibitor are

Table I: Binding Dissociation Constants Found for BzPheal and Related Compounds to Native and Modified Forms of α-Chymotrypsin at pH 7.8

substrate analogue	enzyme form	K_i (mM)
BzPheal	native Cht	$0.026 \pm 0.001^{a-c}$
BzPheal	native Cht	$0.036 \pm 0.003^{a,b,e}$
BzPheal	ACht	$>1^{a,d,e}$
BzPheal	MCht	$0.0063 \pm 0.0006^{a-c}$
BzPhe	native Cht	27^f
$BzPheNH_2$		3.5^g
AcPheal		0.7 ± 0.2^h

[a] At 25 °C, 0.05 M sodium phosphate, and 0.1 M NaCl. [b] From Kennedy & Schultz (1979). [c] Solution contains 3% Me_2SO. [d] Determined via proflavin displacement methods as described in Methods. [e] Solution contains 12.5% Me_2SO. [f] From Bender & Kemp (1957). [g] From Kaufman & Neurath (1949). [h] At 25 °C, 1 mM phosphate; K_i obtained against *N*-acetyl-L-phenylalanine methyl ester as described in Methods.

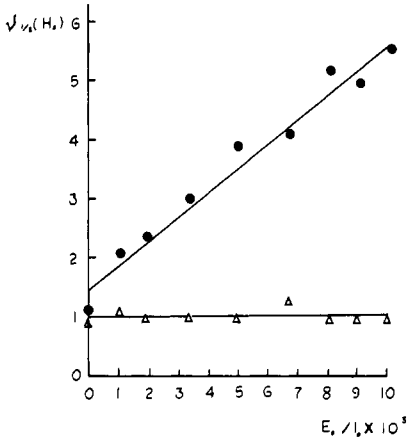

FIGURE 1: Plots of the corrected line widths of resonances of *N*-AcPheal as a function of the α-chymotrypsin–inhibitor ratio (E_0/I_0) at 25 °C, p*D* 6.5, and I_0 = 104 mM. $\nu_{1/2}$ is the line width (at half-height) of the aldehyde proton (●) and the hydrated aldehyde proton (△) plus (in each case) the line width (at half-height) of the acetyl resonance in the absence of enzyme minus the line width (at half-height) of the acetyl resonance at the appropriate enzyme–inhibitor ratio.

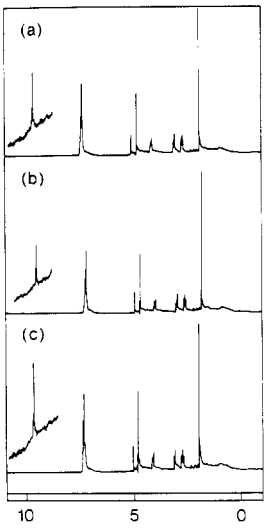

FIGURE 2: The ^1H NMR spectrum of *N*-AcPheal (104 mM) in the presence of α-chymotrypsin (0.69 mM) at 25 °C and pD 6.5 in D_2O containing 10% (v/v) Me_2SO-d_6, with a high-power radio-frequency pulse applied at (a) 5.19, (b) 5.36, and (c) 5.76 ppm. The aldehydic proton (9.58) is recorded at increased gain (×16) above each spectrum.

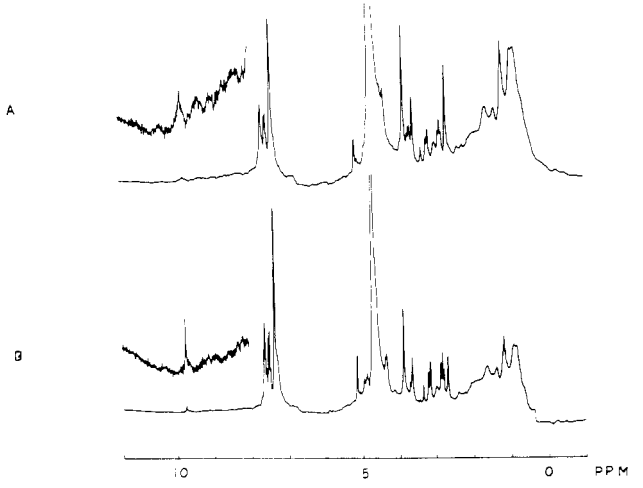

FIGURE 3: ^1H NMR spectra of BzPheal and Cht (13:1) at pD 6.4 (A) and (31:1) at pD 4.9 (B). The Cht concentration was 0.3–0.4 mM. Insets of aldehyde signals at 9.78 ppm from external DSS are recorded at eightfold gain. Note broad protein signal superimposed under sharper aldehyde signal.

unchanged over this I_0/E_0 range, although a slight broadening of the signals is observed on addition of 0.3–0.4 mM Cht to an enzyme-free solution. Thus, in the absence of Cht at pD 6.4, the apparent line widths at half-height for the aldehydic proton, the hydrate proton $-CH(OH)_2$, and the aromatic benzyl protons are 7.0, 9.5, and 11.0 Hz, respectively. On addition of 0.3–0.4 mM Cht, these signals broaden to 7.5, 11.0, and 12.0 Hz, respectively. Intrinsic line widths for these signals under our instrumental conditions are actually much smaller because included in these values are partially resolved splittings and (for the aromatic signal) chemical shift nonequivalence. A splitting of the hydrate proton is observed ($J \sim 5$ Hz to the α proton) even in the presence of enzyme. The spectrum of the inhibitor is essentially unchanged between pD 4.7 and 7.0.

Since BzPheal binds about 20 times more tightly to the enzyme than AcPheal (Table I), the negligible effect of added Cht on the spectra of BzPheal suggests that the rate of chemical exchange between the bound inhibitor and the inhibitor free in solution is slow on the NMR time scale; i.e.,

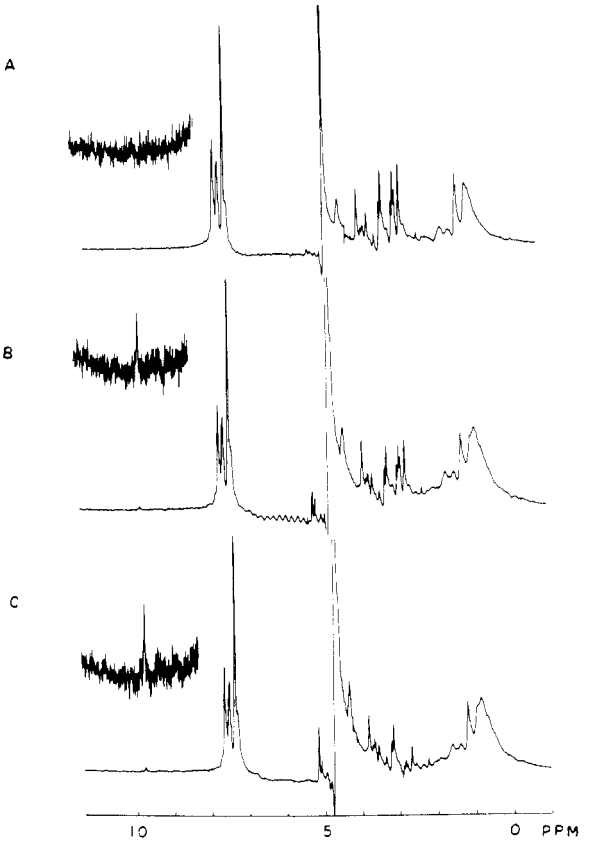

FIGURE 4: ^1H NMR spectra of BzPheal and Cht (31:1) at pD 6.5 with application of rf pulse F_2 at 5.60 ppm (0.8 V) (A), 5.29 ppm (0.3 V) (B), and 3.1 ppm (0.3 V) (C). Insets of aldehyde region are recorded at 16-fold gain. Note change of hydrate signal intensity at 5.17 ppm. The Cht concentration was 0.3–0.4 mM.

the rate constant for dissociation of the complex, k_1, $\ll 2\pi\Delta$, where Δ is the chemical-shift difference between the bound and unbound states. Under our conditions, only the signals of the free form of the inhibitor are observed.

Providing that the rate of chemical exchange is fast relative to the longitudinal rate of relaxation, evidence for this bound form of the inhibitor may still be obtained from a cross-saturation experiment. When the BzPheal/Cht solution (pD 4.7–7.0) is irradiated with a second radio-frequency pulse at 5.29 ppm from external DSS, a diminution of the aldehyde signal is observed. At sufficiently high rf power levels (0.8 V) complete saturation of the aldehyde signal can be achieved (Figure 4). At less than maximal saturation levels, a bandwidth of ca. 100–150 Hz is observed over which the second irradiation pulse causes saturation transfer. Irradiating at the resonance position for the hydrate proton (5.17 ppm) provides only minimal cross-saturation with the aldehyde. Since the spectral region centered around 5.29 ppm for maximal cross-saturation overlaps partially with the hydrate signal, this slight aldehyde signal attenuation produced by irradiating at 5.17 ppm is not thought to arise from any true cross-saturation mechanism between free hydrate and aldehyde. In the absence of added Cht no cross-saturation of the aldehyde proton is found upon irradiation in any region of the spectrum. In addition, in the presence or absence of Cht, no cross-saturation of the hydrate proton is observed when the aldehyde proton is irradiated.

A signal at 5.29 ppm is consistent with the expected chemical shift of the hemiacetal. Although it could also be consistent with a bound hydrate form of the inhibitor, other studies by Lowe & Nurse (1977), the stopped-flow kinetic

evidence of Kennedy & Schultz (1979), and those results discussed earlier on AcPheal effectively rule out the binding of the hydrate forms to Cht.

Aldehydes Binding to MCht. The chemical shifts and signal line widths for all protons of both AcPheal and BzPheal are essentially unaffected by added MCht at pD 6.4 (E_0/I_0 = 0.0025–0.0050 and E_0/I_0 = 0.026–0.052, respectively). In addition only marginal cross-relaxation effects were observed for the AcPheal/MCht solutions. At low, second rf power levels (0.3 V) only a 10–20% reduction in the aldehyde signal intensity was observed upon irradiation in the hemiacetal region (5.3 ± 1.5 ppm). However, this small effect (almost within the experimental error) disappeared at higher power levels (1.5 V) where only a nonselective reduction in the aldehyde signal level was observed due to the broad-band nature of the second rf field at these power levels. Additional MCht failed to further reduce the aldehyde signal intensity upon cross-irradiation, and at a higher temperature (44 °C) even the small effect observed at 0.3 V power levels disappeared. These marginal cross-saturation effects were absent in the BzPheal/MCht samples at power levels between 0.3 and 1.5 V.

The lack of any clear evidence for chemical exchange or cross-saturation in these aldehyde/MCht mixtures cannot be due to lack of binding of the inhibitors to this modified enzyme since, for BzPheal at least, this inhibitor binds four times tighter to modified MCht than to native Cht (Table I). Since BzPheal already binds to Cht in the NMR slow exchange limit, its binding to MCht (and the binding of AcPheal to MCht) most likely also fall within the slow exchange limit, assuming that the tighter binding to the modified enzyme reflects a slower dissociation rate.

The failure to observe any significant cross-saturation between the putative hemiacetal signal and the free aldehyde signal could be due to a lack of hemiacetal formation with the MCht. More probably, the rate of saturation transfer between the bound hemiacetal and free aldehyde could simply be much slower than the longitudinal relaxation rate of either the bound hemiacetal or bound aldehyde. Thus, the equilibrium magnetization of the hemiacetal (or bound aldehyde) could conceivably completely recover after the saturation pulse before any significant dissociation of the bound inhibitor occurs. Since $I_0 \gg E_0$, observation of significant cross-saturation effects on protons of the unbound inhibitor requires rapid (on the relaxation rate time scale) cycling of free inhibitor between free aldehyde, bound aldehyde, and bound hemiacetal. If *any* step in this cycle is much slower than the longitudinal relaxation rate, then no cross-saturation will be observed. As already discussed, the tighter binding of BzPheal to MCht than to Cht is likely to be due to either slower dissociation of the bound aldehyde or slower catalysis of the breakdown of the bound hemiacetal to bound aldehyde. Kennedy & Schultz (1979) have estimated that the Cht catalyzed rate for this latter reaction is between 5 and 40 s^{-1}. If this rate were only slightly slower (factor of 10),[2] then cross-saturation would not occur, based upon reasonable estimates for the longitudinal relaxation rates of a bound inhibitor to a protein the size of

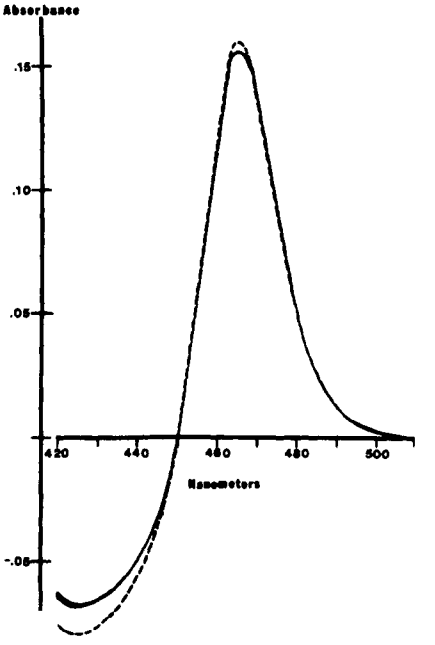

FIGURE 5: Difference spectra for the binding of proflavin to ACht in the absence and presence of BzPheal, at pH 7.8, 25 °C, 0.1 M NaCl, 0.5 M sodium phosphate, and 12.5% Me_2SO; [ACht] = 2.1×10^{-4} M, [proflavin] = 3.6×10^{-5} M, and 3×10^{-5} M in phenylmethanesulfonyl fluoride, (- - -) in the absence of BzPheal and (—) in the presence of 2×10^{-3} M BzPheal. K_i of proflavin to ACht and $\Delta\epsilon_{465}$ are given in Schultz et al. (1977). The experiment shows no significant displacement of proflavin from the active site by BzPheal.

Cht. No cross-saturation between the free hydrate and free aldehyde is observed since the longitudinal relaxation rate of the aldehyde or hydrate proton is much faster than the rate of dehydration of BzPheal hydrate (~0.3 s^{-1}; Kennedy & Schultz, 1979) or hydration of the aldehyde (pseudo-first-order rate constant, ~3 s^{-1}).

It is especially significant in support of the above agrument that even in Cht/BzPheal, cross-saturation effects can be markedly reduced by slowing the rate of the enzyme-catalyzed hemiacetal to aldehyde reaction. At pD ≥6.4, $E_0 \sim 0.3$ mM, and $I_0 \sim 4$ mM, complete saturation of the free aldehyde proton signal is achieved by irradiating the hemiacetal region of the BzPheal/Cht complex with sufficiently high rf power (0.8 V; see Figure 4). At pD 4.9 but otherwise similar conditions, only a 30% reduction in the aldehyde signal intensity can be achieved, and this signal reduction is independent of the power level between 0.3 and 0.7 V. This partial elimination of the cross-saturation effect at lower pD is probably due to the decreased rate of the breakdown of the hemiacetal. Kennedy & Schultz (1979) have estimated that this rate should fall by a factor of about two between pH 6.5 and 5.0; thus even in the native enzyme system, the cycling rates are similar to the relaxation rates. MCht is a much poorer catalyst for hydrolysis of specific substrates than Cht (Byers & Koshland, 1978; Henderson, 1971), and it seems likely therefore that the MCht catalyzed rate for the hemiacetal breakdwn (chemically analoguous to the breakdown of the enzyme tetrahedral intermediate) is also slower than the Cht catalyzed reaction.[2]

Presumably the small (if real) cross-saturation effects for AcPheal/MCht reflect the faster rate for the breakdown of the hemiacetal and/or dissociation of the bound aldehyde. We are assuming that AcPheal binds less well (and dissociates faster) than BzPheal to MCht, just as it does to Cht. The disappearance of the cross-saturation effect for AcPheal/MCht at 44 °C could be attributed to a greater increase in the

[2] From the work of Kennedy & Schultz (1979) the following rate constant comparison can be made. At pH 6.0, k_{-2} is 12 s^{-1} for Cht. At pH 7.8, a k_{-2} value of 5.6 s^{-1} can be calculated for MCht (K_i and hence k_{-2} values are unfortunately not available from the above work at pH 6.0). This calculation for k_{-2} assumes a K_s of 2.2 mM, which is suggested by the fact that substrates bind two times better to MCht than to Cht. If the pH dependency of k_{-2} follows that of k_2 (see Table III in Kennedy & Schultz, 1979), then k_{-2} at pH 6.0 is 1.2 s^{-1}. This is approximately ten times slower than k_{-2} in Cht at pH 6.0.

longitudinal relaxation rates relative to the saturation transfer rates.

Binding BzPheal to Anhydrochymotrypsin (ACht). In ACht the catalytically essential Ser-195 is modified to dehydroalanine (Ako et al., 1974). Figure 5 shows that BzPheal does not displace proflavin from the active site in ACht at concentrations of BzPheal equal to 2×10^{-3} M, and thus the K_i of BzPheal to ACht must be considerably greater than this concentration. The possible conformational heterogeneity alledged for ACht preparations may give a somewhat poorer binding constant to ACht than expected for binding to native Cht (Schultz et al., 1974). However, it was recently shown that *N*-acetyl-L-tryptophan binds with identical affinity to ACht as to native Cht (Schultz et al., 1977). An ACht impurity designated ACht(X) (Matta et al., 1977) does not exist in our sample as our crude ACht is routinely retreated with phenylmethanesulfonyl fluoride prior to affinity chromatography (Schultz et al., 1977). This procedure eliminates any ACht(X) from the binding fraction to the affinity column (Matta et al., 1977). We believe that differences in binding equilibrium constants due to possible heterogeneity of ACht would be insignificant in comparison with the difference (at least two orders of magnitude) found in K_i between BzPheal binding to native Cht and ACht in 12.5% Me_2SO.

After correction for the different Me_2SO concentrations reported for the inhibitors, BzPheal is estimated to bind 1000 and 140 times tighter to Cht than BzPhe and $BzPheNH_2$, respectively (Table I). However since only 10 ± 1% of the inhibitor is present in the aldehyde (90 ± 1% as the hydrate) as determined spectroscopically, and since only the aldehyde form binds effectively, the binding constant differences are approximately 10000- and 1400-fold. The absence of effective binding of BzPheal to ACht combined with the cross-saturation studies with Cht provides clear evidence that the binding of BzPheal to Cht is accompanied by hemiacetal formation which contributes considerably to the overall binding dissociation constant.

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