Mechanism of Association of a Specific Aldehyde "Transition-State Analogue" to the Active Site of α-Chymotrypsin†

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ABSTRACT: The affinity and rate constants for association of the specific substrate aldehyde analogue *N*-benzoyl-L-phenylalaninal (BzPheal) to native α-chymotrypsin (Cht) and *N*-methylhistidinyl-57-α-chymotrypsin (MCht) were obtained. We find an observed binding dissociation constant (K_I) for BzPheal to be 2.6×10^{-5} M (in 3% Me_2SO, pH 7.8) to native Cht and 3.5×10^{-6} M (in 3% Me_2SO, pH 7.8) to MCht. Evidence is discussed that supports a mechanism in which the unhydrated form of BzPheal is the form that predominantly associates. Calculation of a corrected K_I based on the solution concentrations of unhydrated BzPheal gives a K_I of 2.4×10^{-6} and 3.2×10^{-7} M, respectively, which is 1.35×10^3- to 1.4×10^4-fold smaller than substrate analogues not containing the aldehyde function. Accordingly, the high affinity of BzPheal to Cht indicates a possible transition-state-like mode of association. However, we find the pH dependence of K_I for BzPheal association to Cht shows only a fourfold increase between pH 8.0 and pH 3.0. The lack of a significant pH dependence in K_I supports the existence of a neutral hemiacetal adduct of BzPheal in Cht, rather than a more transition state like oxyanion hemiacetal adduct. The pH dependency of the second-order rate constants for BzPheal association to native Cht correlates with general base catalysis by two groups in the enzyme of pK_a = 4.4 and pK_a = 7.0. Limiting rate constants ($k_2/K_{s(lim)}$) for base catalysis by the groups of pK_a = 4.4 and 7.0 are calculated at 22×10^5 and 19×10^6 M^{-1} s^{-1}, respectively. The pH dependency of the second-order rate constant for BzPheal association to MCht correlates with general base catalysis by a single group of pK_a = 6.8 with $k_2/K_{s(lim)} = 21 \times 10^5$ M^{-1} s^{-1}. Base catalysis may occur in MCht by utilization of the $N^{\delta 1}$ of the His-57, when the $N^{\epsilon 2}$ of His-57 is methylated. The identity of the rate-limiting constants for catalysis by the monobasic form of Cht to that for BzPheal association to MCht may support the group of pK_a = 4.4 being the His-57 imidazole and the group of pK_a = 7.0 being the γ_{COOH} of Asp-102. This mechanism indicates that the Asp-102 in the catalytic charge relay system only contributes a factor of 10 toward the rate of BzPheal hemiacetal formation in native Cht. However, an alternative mechanism in which the ($N^{\epsilon 2}$-methyl) nitrogen of His-57 in MCht or the protonated ($N^{\epsilon 2}$) nitrogen of His-57 in native Cht acts as a general base catalyst with a Brønsted coefficient (β) of 0.07 may be possible.

Enzymes act to catalyze chemical reactions by lowering the free energy required to activate a substrate to its transition state. While this transition-state form of the substrate is highly unstable, transition-state theory predicts that a substrate in its transition-state form has a higher affinity to the enzyme than in its ground-state form (for reviews see Wolfenden, 1972; Lienhard, 1973). Accordingly, it is of interest to study the thermodynamic and kinetic properties for the association to enzymes of compounds that mimic features of substrate transition states in order to gain insights into the nature of the transition state in enzyme catalysis of substrates. While many factors that can account for the lowering of the energy to reach the transition state in enzymic catalysis have been discussed (see Jencks, 1975), there is not yet a clear quantitative understanding of these factors in any one enzyme example.

For the serine and cysteine proteinases, aldehyde analogues of L-α-acylamido amide substrates have been proposed as transition-state analogues (Westerik & Wolfenden, 1972; Thompson, 1973; Rawn & Lienhard, 1974). These aldehyde analogues initially associate to the enzyme active site into a noncovalent Michaelis complex as in IA (unhydrated) and/or IB (hydrated) (Figure 1). The unhydrated aldehyde, complex IA, might further react with the active site nucleophile (Ser-195 in Cht[1]) to form a covalent hemiacetal adduct, complex IIA and/or IIB (Figure 1). In complexes IB, IIA, and IIB, the C-1 carbon of the aldehyde has a tetrahedral sp^3 configuration like that proposed for the reactive carbonyl carbon of substrates in the transition state of protease catalyzed hydrolytic reactions (Blow, 1976; Kraut, 1977). Recent support for association of aldehyde analogues to Cht as a hemiacetal as in IIA or IIB comes from the observation that an aldehyde analogue of the nonspecific substrate hydrocinnamide has been shown to associate to Cht in a covalent hemiacetal linkage with the Ser-195 by a cross-saturation NMR technique (Lowe & Nurse, 1977). More recently similar NMR double resonance experiments have shown that the specific substrate aldehyde analogue, *N*-benzoyl-L-phenylalaninal (BzPheal), forms a covalent hemiacetal as in IIA or IIB (Chen et al., 1979).

In this paper we report on the mechanism of aldehyde hemiacetal formation in native Cht and *N*-methylhistidinyl-57-Cht (MCht), based on the pH dependency of the equilibrium binding constant and rates for association of *N*-benzoyl-L-phenylalaninal (BzPheal). These data support association of BzPheal as the neutral hemiacetal adduct IIB (Figure 1), under base catalysis by two groups of pK_a = 4.4 and 7.0 in native Cht. Conclusions made from our data may relate to the pK_a values for the residues of the charge-relay system, as well as the catalytic efficiency of this system. Furthermore, the homology of the aldehyde hemiacetal adduct to the substrate transition state will be discussed.

Experimental Section

Methylhistidinyl-57-α-chymotrypsin (MCht) and dehydroalaninyl-195-α-chymotrypsin (ACht) were prepared as

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[1] Abbreviations used: Cht, α-chymotrypsin; MCht, ($N^{\epsilon 2}$-methylhistidinyl-57)-α-chymotrypsin; ACht, (dehydroalaninyl-195)-α-chymotrypsin; BzPhe, *N*-benzoyl-L-phenylalanine; BzPheal, *N*-benzoyl-L-phenylalaninal; BzPheol, *N*-benzoyl-L-phenylalaninol; AcPheal, *N*-acetyl-L-phenylalaninal; Me_2SO, dimethyl sulfoxide; S_1', binding site in the enzyme to which the amino acid residue that contributes its α-amine to the hydrolyzable peptide bond in the substrate productively associates.

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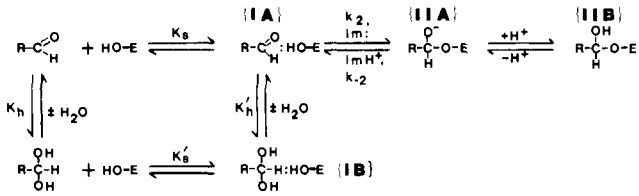

FIGURE 1: Scheme for the association of BzPheal to native Cht. Complexes IA and IB represent noncovalent Michaelis complexes with sp^2 and sp^3 aldehydic C-1 configurations, respectively; complexes IIA and IIB are covalent hemiacetal adducts with the Ser-195 in Cht.

previously described (Schultz et al., 1977).

N-Benzoyl-L-phenylalaninol (BzPheol). The alcohol derivative, BzPheol, of *N*-benzoyl-L-phenylalanine was prepared from L-Phe by procedures previously described (Jones et al., 1974; Hunt & McHale, 1957), mp 168–169 °C [lit. mp 169 °C (Hunt & McHale, 1957)].

N-Benzoyl-L-phenylalaninal (BzPheal). Oxidation of BzPheol to BzPheal was carried out by procedures generally described by Thompson (1977). A solution of 1.1 g (4.2 mmol) of BzPheol and 4.0 g (20 mmol) of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma Chemical Co.) in 25 mL of doubly distilled dimethyl sulfoxide (Me_2SO) was strongly stirred and treated dropwise with 2 mL of 2 M anhydrous H_3PO_4 in Me_2SO. After 3 h, 150 mL of 0.1 M sodium phosphate buffer, pH 8.0, was added and the solution extracted with ethyl acetate. The ethyl acetate was washed with saturated aqueous $NaHCO_3$ and saturated saline, dried over anhydrous Na_2SO_4, and filtered, and the solvent removed under vacuum. A yield of 492 mg (45%) of BzPheal was obtained from benzene–hexane, mp 140–141 °C [lit. mp 143–144 °C (Seki et al., 1972)]. The infrared spectrum and nuclear magnetic resonance spectrum were identical with those reported (Seki et al., 1972).

Determination of Equilibrium Binding Constants ($K_{I(obsd)}$) to Native Cht. Binding dissociation constants of BzPheol and BzPheal to native Cht were determined by standard steady-state inhibition kinetics (Dixon & Webb, 1964) against the substrate *N*-benzoyl-L-tyrosine *p*-nitroanilide. In a typical experiment 100 μL of enzyme in 10^{-3} M HCl was added to a thermostated cuvette at 25.0 °C containing 3.1 mL of a preincubated buffered solution of substrate and the appropriate concentration of inhibitor, the solution rapidly mixed, and the appearance of *p*-nitroaniline followed at 390 nm. Solutions were 12.5% or 3% in Me_2SO, 0.05 M in buffer component (sodium acetate of sodium phosphate), and 0.095 M in NaCl. Substrate concentrations were significantly below the K_m and pseudo-first-order kinetics were observed. The $K_{I(obsd)}$ for inhibitor association were computed from eq 1, where $[I]_0$ is

$$K_{I(obsd)} = \frac{[I]_0}{(k_u/k_i) - 1} \quad (1)$$

the inhibitor concentration, k_u the pseudo-first-order rate constant in the absence of inhibitor, and k_i the pseudo-first-order rate constant in the presence of inhibitor. At pH values below 5.0, the rates were slow and initial rates were substituted for k_u and k_i. The data summarized in Table I represent the mean and standard deviation of between five to ten determinations of K_I at inhibitor concentrations about K_I and three times K_I.

Determination of the Equilibrium Binding Constant to MCht. The K_I for BzPheal binding to MCht was carried out by proflavin displacement as previously described (Schultz et al., 1977; Brandt et al., 1967). However, the equation of Brandt et al. (1967), for determination of K_I for BzPheal association, was modified to account for our conditions of $[BzPheal]_0$ not in great excess of $[E]_0$. Sample solutions contained 3.6×10^{-5} M proflavin, 1.6×10^{-5} M MCht, and $4–8 \times 10^{-5}$ M BzPheal and were 0.05 M in buffer component, 0.1 M in NaCl, and 3% in Me_2SO. Reference solution contained no enzyme. The equilibrium binding constant between MCht and proflavin (K_{EP}) and the $\Delta\epsilon_{465}$ for the MCht–proflavin complex were determined under conditions of $[P]_0 = 3.6 \times 10^{-6}$ M and $[MCht]_0 = 4–10 \times 10^{-5}$ M. We found a $K_{EP} = 4.25 (\pm 0.24) \times 10^{-5}$ M and $\Delta\epsilon_{EP}^{465} = 2.42 \times 10^{-4}$ M^{-1} cm^{-1} at pH 7.8 and in 3% Me_2SO. In the calculation of K_I, binding between proflavin and BzPheal were found to be insignificant because of the low concentrations of BzPheal utilized.

Kinetics of BzPheal Association. Stopped-flow spectrophotometry was employed to study the rates of BzPheal association by observation of the rate of proflavin displacement at 465 nm from the enzyme by BzPheal. Equal volumes of solutions, one containing BzPheal and proflavin, and the other containing enzyme and proflavin, were rapidly mixed in either a Durrum or Aminco stopped-flow spectrophotometer and the pseudo-first-order rate of proflavin displacement followed. By convenient analogy to the determination of the rate constants for substrate acylation and deacylation of Cht by ester or amide substrates via proflavin displacement (Himoe et al., 1969), eq 2 is derived showing the dependency of the observed pseudo-first-order rate constant (k_{obsd}) on BzPheal concentration. In eq 2, k_{obsd} is the first-order rate constant observed

$$k_{obsd} = \frac{k_2[BzPheal]_0}{K_s(1 + [P]_0/K_{EP}) + [BzPheal]_0} + k_{-2} \quad (2)$$

at a particular concentration of BzPheal and calculated from a ln $(A_t - A_\infty)$ vs. time plot made directly from the stopped-flow spectrum, K_{EP} the equilibrium dissociation constant for binding of proflavin to enzyme determined by methods discussed below, $[P]_0$ is the total proflavin concentration, and k_2, k_{-2}, and K_s for BzPheal association defined by the scheme in Figure 1. Equation 2 is simplified to eq 3 at $[BzPheal]_0 << K_s(1 + [P_0]/K_{EP})$, so that a plot of k_{obsd} vs. $[BzPheal]_0$ leads to a straight line with the second-order rate constant

$$k_{obsd} = \frac{k_2[BzPheal]_0}{K_s(1 + [P]_0/K_{EP})} + k_{-2} \quad (3)$$

k_2/K_s calculated from the slope. The K_{EP} was determined experimentally under our conditions according to procedures previously described (Brandt et al., 1967) at pH 7.8 and pH 4.0. Values of K_{EP} at other pH values were then calculated from the values of K_{EP} obtained under our conditions and the pH dependency for K_{EP} to Cht data of Marini & Caplow (1971). The ratio of K_{EP} values found at pH 7.8 and pH 4.0 under our conditions (5% Me_2SO) are identical with the ratio of K_{EP} at pH 7.8 and pH 4.0 found by Marini & Caplow.

Solutions in the stopped-flow spectrophotometer after mixing were 4.8×10^{-5} M in proflavin, 1×10^{-5} M in native Cht or 5×10^{-6} M MCht, 0.09 M in NaCl, 0.049 M in buffer (sodium acetate or sodium phosphate), and 5% in Me_2SO. $[BzPheal]_0$ was varied between 2×10^{-4} to 8×10^{-4} M. The rate constants reported in Tables II and III represent the mean and standard error calculated from the least-squares slope of the data plotted according to eq 3. Determinations of k_{obsd} at each BzPheal concentration were carried out at least three times and over four different concentrations of BzPheal were utilized between 2×10^{-4} and 8×10^{-4} M in the plot at each pH.

Table I: Binding Constants Found for BzPheal and Structurally Related Compounds to Native and Active Site Modified Forms of Chymotrypsin[a]

substrate analogue	enzyme form	$K_{I(obsd)}$ (mM)	Me_2SO concn
BzPheal	Cht	0.026 ± 0.001[b] (0.0024)[f]	3%
BzPheal	Cht	0.036 ± 0.002[b] (0.0033)[f]	12.5%
BzPheal	ACht	>1.0[c]	12.5%
BzPheol	Cht	50[b]	12.5%
BzPheal	MCht	0.0035 ± 0.0004 (0.00032)[f]	3%
$BzPheNH_2$	Cht	3.5[d]	0
BzPhe	Cht	27[e]	0

[a] pH 7.8; aqueous buffer is 0.1 M in NaCl and 0.05 M in sodium phosphate; at 25 °C. [b] $K_{I(obsd)}$ obtained kinetically against the substrate *N*-benzoyl-L-tyrosine *p*-nitroanilide. [c] $K_{I(obsd)}$ obtained by proflavin displacement method (Chen et al., 1979). [d] From Neurath & Schwert, 1950. [e] From Bender & Kemp, 1957. [f] If K_I is based on unhydrated BzPheal concentration (see text).

Results

The equilibrium dissociation constants obtained for the binding of BzPheal and structurally related Cht substrates and substrate analogues at pH 7.8 are given in Table I. A direct comparison of the equilibrium dissociation constant (K_I) of BzPheol to that of BzPheal in 12.5% Me_2SO and the K_I of BzPhe and $BzPheNH_2$ to that of BzPheal in 3% Me_2SO shows that the $K_{I(obsd)}$ for BzPheal is between 135- and 1400-fold smaller than that for structurally analogous compounds without the aldehyde function. The literature values of K_I for BzPhe and $BzPheNH_2$ reported in Table I were obtained in the absence of Me_2SO. A comparison of the K_I for BzPheal association to Cht in 3% and 12.5% Me_2SO (Table I) shows an increase in K_I of approximately 35%. Similar increases in equilibrium dissociation constants by increased Me_2SO concentration have been reported (Fink, 1974). Based on this increase, the K_I for BzPhe and $BzPheNH_2$ in 3% Me_2SO would be estimated to be about 10% larger.

The calculation of K_I for BzPheal association in Table I is based on the total BzPheal concentration (hydrated + unhydrated). However, recent double resonance NMR spectroscopy experiments have clearly shown that it is the unhydrated forms of the aldehydes AcPheal and hydrocinnamaldehyde that predominantly associate with Cht (Chen et al., 1979; Lowe & Nurse, 1977). Accordingly, it is strongly inferred that the unhydrated form of BzPheal preferentially associates. Kinetic experiments with BzPheal, discussed below, support this conclusion for BzPheal under the conditions of our experiment. As the unhydrated BzPheal is the species that appears to primarily associate, then the true K_I for BzPheal association is equal to $K_{I(obsd)}/(1 + K_h)$, where K_h is the hydration association constant in water (Thompson, 1973; Lewis & Wolfenden, 1977a). As K_h is ~10 for BzPheal (Chen et al., 1979),[2] the true K_I ($K_{I(unhydrated)}$) for BzPheal association is 2.4×10^{-6} M to native Cht and 3.2×10^{-7} M to MCht in 3% Me_2SO. This indicates a 1.4×10^3- to 1.5×10^4-fold better association of BzPheal than the other analogous substrates or substrate analogues listed in Table I. Thus BzPheal has a significantly greater affinity to the Cht active site than respective substrates, in agreement with the expectation of a better affinity for a transition-state analogue. In contrast, hydrocinnamaldehyde, the aldehyde analogue of a *nonspecific substrate* hydrocinnamide, shows only a 7-fold better affinity to Cht than hydrocinnamide (Schultz & Cheerva, 1975).

[2] In 5% Me_2SO, $K_h \sim 10$ (D. Gorenstein, private communication).

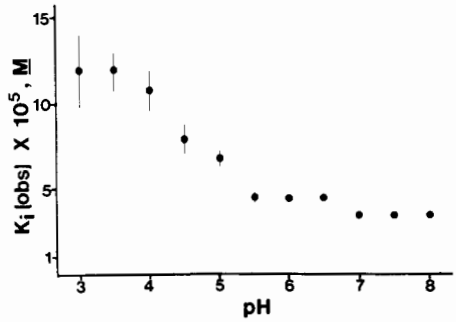

FIGURE 2: pH dependency of $K_{I(obsd)}$ for association of BzPheal to native Cht. Solution conditions: 0.09 M in NaCl, 0.048 M in buffer component (buffer is sodium acetate at pH 3.0–5.5 and sodium phosphate at pH 5.5–8.0), 12.5% Me_2SO, and 25 °C.

In MCht the catalytically essential $N^{\epsilon 2}$ of His-57 is modified by methylation (Nakagawa & Bender, 1970). Table I shows the $K_{I(obsd)}$ for BzPheal binding to MCht is approximately seven times smaller than the $K_{I(obsd)}$ for binding to native Cht. The result demonstrates that the $N^{\epsilon 2}$ of His-57 is not required for high affinity of BzPheal, even though this nitrogen participates in the transition state in proposed mechanisms for Cht catalysis (Blow, 1976; Kraut, 1977).

The pH dependency of $K_{I(obsd)}$ for BzPheal binding to native Cht between pH 3.0 and pH 8.0 is shown in Figure 2. This plot while exhibiting an apparent pK_a of association at pH 4 shows $K_{I(obsd)}$ increasing by only a factor of 4 over the pH range studied. The lack of a significant pH dependency in $K_{I(obsd)}$ may be surprising as substrate catalysis and the association of boronic acid transition-state analogues both show a dependency on a group of $pK_a \sim 7$, identified as the pK_a of the catalytically essential charge relay system in Cht (Hess, 1971; Blow, 1976; Philipp & Bender, 1971). However, in BzPheal association studied herein, no large variation in K_I with pH is observed. The lack of significant pH dependency in $K_{I(obsd)}$ demonstrates that the basic form of the charge-relay system, which probably participates in the transition state of substrate catalysis (Blow, 1976; Kraut, 1977), is not required for the high affinity of the aldehyde BzPheal to Cht. This result correlates with the high affinity observed for BzPheal to MCht, in which the charge-relay system is modified by methylation of His-57 instead of protonation at pH <7.

The rates of BzPheal association to native Cht and MCht were determined by following the rate of displacement of proflavin from the enzyme active site by BzPheal at 465 nm in a stopped-flow spectrophotometer. First-order rate constants for proflavin displacement were observed at constant enzyme concentration and BzPheal concentration between 2×10^{-4} 8×10^{-4} M under conditions of $[E]_0 << [BzPheal]_0 << K_s(1 + [P]_0/K_{EP})$. Plots of $k_{(obsd)}$ vs. BzPheal concentration showed a linear dependence as predicted by eq 3, leading to the calculation of $k_2/K_{s(obsd)}$. These values of $k_2/K_{s(obsd)}$ are based on the total BzPheal concentration (hydrated + unhydrated). However, if the calculation is based on the concentration of unhydrated BzPheal, assuming the mechanism in which the unhydrated BzPheal is the predominant associating species (see above), then the real values of k_2/K_s are tenfold higher. The second-order rate constants calculated on the basis of the unhydrated BzPheal concentration are designated $k_2/K_{s(calcd)}$.

The values of $k_2/K_{s(calcd)}$ are reported in Tables II and III, with the error calculated from the standard error in the slope of the linear regression line obtained from the plot of eq 3.

Table II: Rate Constant for the Association of BzPheal to Native Cht[a]

pH	$10^{-5} \times k_2/K_{s(calcd)}$[b] ($M^{-1}$ s^{-1})	$10^{-3} \times k_2$[c] (s^{-1})	k_{-2} (s^{-1})
8.0	(148)[d]	(80)	(42)
6.5	61.4 ± 5.0	28.3 ± 2.3	22.3 ± 2.2
6.0	33.3 ± 0.9	15.0 ± 0.4	12.2 ± 0.8
5.5	28.9 ± 0.8	13.0 ± 0.4	11.0 ± 0.7
5.0	20.7 ± 1.3	9.30 ± 0.58	11.6 ± 0.8
4.5	9.18 ± 0.60	4.10 ± 0.27	5.9 ± 0.4
4.0	6.04 ± 0.31	2.70 ± 0.14	5.3 ± 0.3
3.0	0.868 ± 0.032	0.39 ± 0.02	0.83 ± 0.04

[a] Aqueous buffer solution 0.1 M in NaCl, 0.05 M in buffer component (acetate at pH 3.0–5.5, phosphate at pH 6.0 and 6.5), 5% in Me_2SO, at 23 °C. [b] Based on the concentration of unhydrated BzPheal (see text). [c] Calculated utilizing a value of K_s of 4.5 mM and $k_2/K_{s(calcd)}$. [d] Calculated utilizing the k_2/K_s value obtained from the theoretical line of Figure 3.

Table III: Rate Constants for the Association of BzPheal to MCht[a]

pH	$10^{-5} \times k_2/K_{s(calcd)}$[b] ($M^{-1}$ s^{-1})	$10^{-2} \times k_2$[c] (s^{-1})
7.8	17.8 ± 1.0	80
7.5	16.6 ± 0.9	75
7.0	14.3 ± 0.8	64
6.5	7.09 ± 0.18	32
6.0	3.76 ± 0.14	17
5.5	0.739 ± 0.025	3.3
5.0	0.310 ± 0.011	1.4
4.0	0.118 ± 0.006	0.53

[a] Aqueous buffer solution 0.1 M in NaCl, 0.05 M in buffer component (sodium acetate at pH 4.0–5.5, sodium phosphate at pH 6.0–7.8), 5% in Me_2SO, at 23 °C. [b] Based on the concentration of unhydrated BzPheal (see text). [c] Calculated utilizing a value of K_s of 4.5 mM and $k_2/K_{s(calcd)}$.

At pH values greater than 6.5, the rate of association to native Cht is too fast to observe accurately. However, at pH values below 7 the association is observable, and a plot of $k_2/K_{s(calcd)}$ vs. pH (Figure 3) shows increasing rates with an apparent sigmoidality between pH 5 and pH 6.

The rate constants k_2 in Table II and III were calculated from $k_2/K_{s(calcd)}$, assuming a K_s value of 4.5 mM for BzPheal in 5% Me_2SO. This value of K_s is based on the K_s for $BzPheNH_2$ (Table I) corrected to 5% Me_2SO as discussed above. The calculation also assumes a lack of variation in K_s with pH (other than the increases in K_s with pK_a = 2.7 and 8.7 due to dissociation of the Ile-16:Asp-194 ion pair (Hess, 1971)) in agreement with earlier results that show no substantial pH dependency in the noncovalent association of neutral substrates to the active site of Cht (Johnson & Knowles, 1966). Calculation of k_{-2} could not be made from the plot of eq 3 as the Y-axis intercepts were near zero in our coordinate system. However, calculations of k_{-2} in Table II were made from eq 6 (see below), with k_2 and K_s calculated as above, and $K_{I(unhydrated)}$ calculated from the $K_{I(obsd)}$.

Rate constants for noncovalent association of substrates to Cht (step k_1) have been calculated at 6×10^7 to 2.4×10^8 M^{-1} s^{-1} (Hess, 1971; Hirohara et al., 1977). The $k_2/K_{s(calcd)}$ value of pH 8.0 (Table II) approach these diffusion rate-limiting reaction rates. The slower association for BzPheal below pH 7 supports the existence of a rate-controlling step following initial noncovalent association, which correlates with hemiacetal formation being rate controlling (step k_2, Figure 1).

The sigmoidality of the pH dependency of k_2/K_s between pH 3.0 and pH 6.5 best supports a mechanism of base catalysis by two acid groups in the enzyme. Accordingly, the theoretical

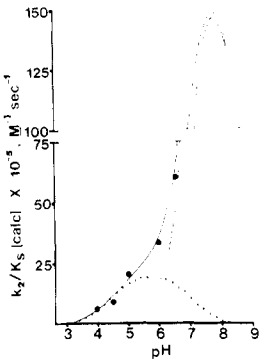

FIGURE 3: pH dependency of second-order rate constant (k_s/K_s) for association of BzPheal as a hemiacetal to native Cht. (●) Observed constant; (—) calculated line between pH 3.0 and pH 6.5 based on basic catalysis by groups of pK_{a1} = 4.4 and pK_{a2} = 7.0 with $k_2/K_{s(lim)1} = 22 \times 10^5$ M^{-1} s^{-1} and $k_2/K_{s(lim)2} = 19 \times 10^6$ M^{-1} s^{-1}, calculated line also corrected for increase in K_s due to acid groups of pK_a = 2.7 (Asp-194) and pK_a = 8.7 (Ile-16) (Hess, 1971; Fersht, 1972); (····) extension of calculated line from pH 6.5–8.8; (●) contribution of the monobasic form of enzyme to observed rate constant; (···) $k_2/K_{s(lim)1}$ value; (- - -) contribution of dibasic form of enzyme to observed rate constant. Solution conditions: 0.1 M in NaCl, 0.05 M in buffer component (phosphate or acetate), 5% in Me_2SO, at 23 °C. All rate constants would be smaller by a factor of 10 if calculation was based on the total BzPheal concentration (see text).

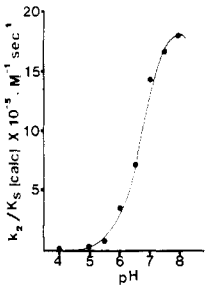

FIGURE 4: pH dependency of $k_2/K_{s(calcd)}$ for association of BzPheal to MCht. (●) Observed rate constant; (—) calculated line based on catalysis by a single group of pK_a = 6.8 with $k_2/K_{s(lim)} = 21 \times 10^5$ M^{-1} s^{-1} (calculated line also corrected for increase in K_s due to groups of pK_a = 2.7 (Asp-194) and 8.7 (Ile-16); see text). Solution conditions same as in Figure 3. All rate constants would be smaller by a factor of 10 if calculation was based on the total BzPheal concentration (see text).

line through the data of Figure 3 was calculated by eq 4, which is based on a mechanism of general base catalysis by groups of pK_{a1} = 4.4 and pK_{a2} = 7.0 with a $k_2/K_{s(calcd)}$ limiting rate

$$\frac{k_2}{K_{s(calcd)}} = \frac{\dfrac{k_2/K_{s(lim)2}}{1 + [H^+]/K_{a2}} + \dfrac{k_2/K_{s(lim)1}}{1 + K_{a2}/[H^+] + [H^+]/K_{a1}}}{1 + [H^+]/K_{a3} + K_{a4}/[H^+]} \quad (4)$$

constant of 22×10^5 M^{-1} s^{-1} for the monobasic form and 19×10^6 M^{-1} s^{-1} for the dibasic form, the values of pK_{a1}, pK_{a2}, $k_2/K_{a(lim)1}$, and $k_2/K_{s(lim)2}$, for the monobasic and dibasic forms, respectively, being chosen to give a good fit to the data. The K_{a3} and K_{a4} of eq 4 refer to the pK_a's of the γ-COOH of Asp-104 and the α-NH_3^+ of Ile-16 (pK_a = 2.7 and 8.7, respectively), which control the ability of substrates to bind in step K_s (Hess, 1971; Fersht, 1972).

Figure 4 shows the pH dependency for $k_2/K_{s(calcd)}$ in association to MCht. This pH dependency conforms to a mechanism in which association is catalyzed by a single basic group of pK_a 6.8 with $k_2/K_{s(lim)}$ of 21×10^5 M^{-1} s^{-1}. The catalysis of association by a group of pK_a 6.8 in MCht is not surprising, as it has been reported that MCht can catalyze

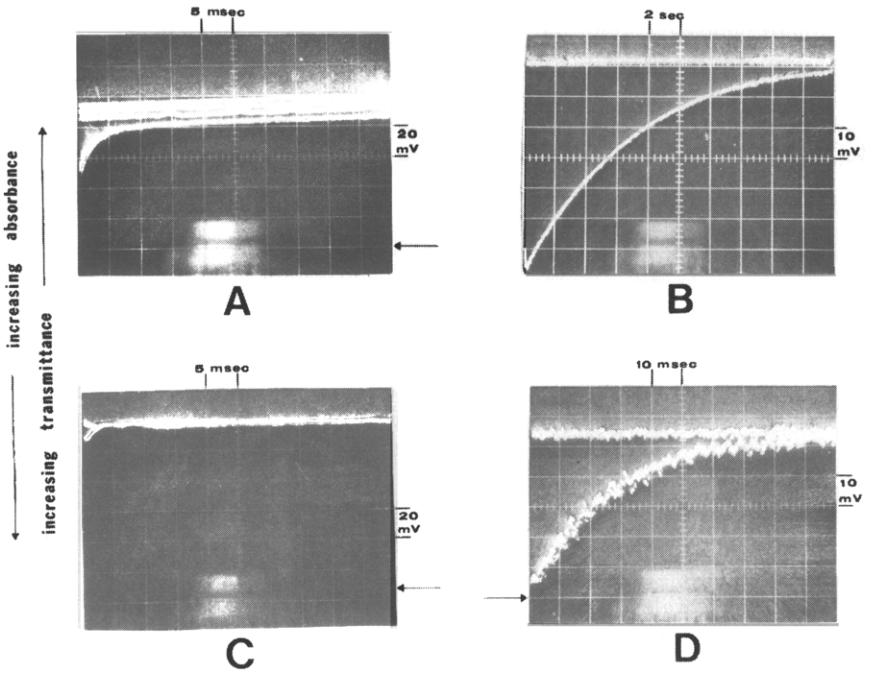

FIGURE 5: Stopped-flow spectra showing biphasic association rates under conditions of unhydrated BzPheal concentrations limiting. (A) Fast time scale (5 ms/cm) and $\{BzPheal\}_{unhydrated} < [E]_0$. Bottom trace shows fast phase of which only a small part is observable at this pH; ← indicates start of reaction at time zero (lower base line). Upper traces show successive scans on reaction at 2–3-s intervals. The slow increase in the apparent end point indicates a slow reaction phase leading to the displacement of proflavin on a slow time scale. Reaction concentrations: $\{BzPheal\}_0 = 5 \times 10^{-5}$ M; $\{Cht\}_0 = 1 \times 10^{-5}$ M; at pH 7.8. (B) Slow time scale (2 s/cm) at concentrations identical with A. Trace directly shows slow reaction phase indicated by increasing end point in A. (C) Under conditions of $\{BzPheal\}_{unhydrated} > \{E\}$. Trace shows reaction is complete in less than 2 ms at this pH, with end point constant on successive 2-s interval sweeps. ← indicates lower base line at time zero. Concentrations: $\{BzPheal\}_0 = 5 \times 10^{-4}$ M; $\{Cht\}_0 = 1.0 \times 10^{-5}$ M. At pH 7.8. (D) Trace for BzPheal association to MCht under conditions of $\{BzPheal\}_{unhydrated} > \{MCht\}_0$. Trace represents the composite of three consecutive runs. → indicates base line at time zero. Solution concentrations: $\{BzPheal\}_0 = 4 \times 10^{-4}$ M; $\{MCht\}_0 = 5 \times 10^{-6}$ M. At pH 7.5.

substrate hydrolysis at rates three to five orders of magnitude slower than native Cht with an apparent pH dependency similar to native Cht (Henderson, 1971). However, it is surprising that the $k_2/K_{s(lim)}$ found for BzPheal association to MCht is only tenfold less than the limiting value of k_2/K_s found for BzPheal association to native Cht.

Utilizing the value $K_h = 10$ (Chen et al., 1979)[2] to compute the concentration of unhydrated BzPheal present in aqueous solutions of BzPheal, we found that in stopped-flow experiments in which the calculated concentration of unhydrated BzPheal was equal to or lower than the enzyme concentration, biphasic first-order kinetics were observed (Figure 5). The first-order rate constant found for the slow reaction phase ($k_{(obsd)(slow)} = 0.3$ s^{-1} at $[BzPheal]_0 = 5 \times 10^{-5}$ M) is in the range expected for dehydration of alkyl aldehydes nonenzymatically (Bell, 1966). Accordingly, we believe that this slow reaction phase may represent the rate-limiting dehydration of hydrated BzPheal to unhydrated BzPheal, which becomes rate limiting for BzPheal association after the initially present unhydrated aldehyde concentration is reduced significantly by association to the enzyme. These observations may then correlate with the NMR evidence showing that it is the unhydrated form of aldehyde inhibitors that are in equilibrium with a Ser-195 hemiacetal form of the enzyme–aldehyde complex (Chen et al., 1979; Lowe & Nurse, 1977), and the pathway at the top of Figure 1 is the predominant pathway for aldehyde association.

Discussion

Mechanism of Aldehyde Association. The formation of a covalent hemiacetal adduct between BzPheal and the Ser-195 γ-O is supported by the following observations: (i) the relatively poor affinity ($K_I > 2$ mM) found for BzPheal to dehydroalaninyl-195-α-chymotrypsin by proflavin displacement (Chen et al., 1979); (ii) the observation in the presence of Cht of cross-saturation of the BzPheal NMR aldehydic signal on irradiating the sample with a radio frequency pulse in the region of the NMR absorption of the hemiacetal proton peak (Chen et al., 1979) in a result identical with that reported for hydrocinnamaldehyde association (Lowe & Nurse, 1977); (iii) the rate constant for association to native Cht below pH 7 and to MCht between pH 3 and pH 8 is several orders of magnitude slower than that expected for a simple noncovalent association process; and (iv) the observation that the alcohol derivative (BzPheol) which contains an sp^3 configuration about its C-1 carbon, but can only associate noncovalently, does not have a high affinity to native Cht (Table I).

In addition, the NMR result showing that the unhydrated solution forms of the aldehyde inhibitors AcPheal and hydrocinnamaldehyde are in equilibrium with covalently bound Ser-195 hemiacetal adduct of these aldehydes (Lowe & Nurse, 1977; Chen et al., 1979), and the observation of a slow-rate controlling step with a first-order rate constant in the range expected for uncatalyzed aldehyde dehydration at unhydrated BzPheal concentration limiting (Figure 5), show that it is the unhydrated form of BzPheal that strongly associates.

Accordingly, the pathway depicted in eq 5 is the simplest

$$E + BzPheal_{(unhydrated)} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} IA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} IIA \text{ and/or } IIB \qquad (5)$$

to explain our data. Based on this mechanism eq 6 is derived,

$$K_I = \frac{K_s}{1 + (k_2/k_{-2})}, \text{ where } K_s = k_{-1}/k_1 \qquad (6)$$

which shows the dependency of K_I on K_s and the ratio k_2/k_{-2}.

In a previous paper on the association of hydrocinnamaldehyde to native Cht, we argued that the stable complex of hydrocinnamaldehyde and enzyme was the hemiacetal anion, structure IIA (Figure 1; Schultz & Cheerva, 1975). We further argued that formation of the hemiacetal anion is general base catalyzed by His-57 (pK_a = 7) and, by microscopic reversibility, step k_{-2} is general acid catalyzed (Schultz & Cheerva, 1975). Accordingly, K_I would be expected to have the pH dependency described by eq 7. The data on hydrocinnamaldehyde association gave a good correlation with eq

$$K_I = \frac{K_s}{1 + (K'/[H^+])}, \text{ where } K' = \frac{k_{2(lim)}}{k_{-2(lim)}}K_a \qquad (7)$$

7 with values of $K_a = 10^{-7}$ M, K_s = 5.5 mM, and $k_{2(lim)}/k_{-2(lim)}$ = 5 (Schultz & Cheerva, 1975). However, the pH dependency of $K_{I(obsd)}$ for the specific aldehyde analogue BzPheal does not easily fit eq 7, unless one assumes an extremely low K_s (less than 1×10^{-4} M) and different pK_a values for the groups controlling step k_2 and step k_{-2}. A low K_s value is not supported by the observed association rate constants, which are slower than expected for simple noncovalent association. Accordingly, an explanation for the pH dependency of binding by a one proton transfer process as in eq 7 is not supportable for BzPheal, and either the mechanism of association for BzPheal and hydrocinnamaldehyde differ, or the pH dependency observed in K_I for hydrocinnamaldehyde association is not due to general base–general acid catalysis as proposed (Schultz & Cheerva, 1975). It may be that the observed pH dependence in $K_{I(obsd)}$ for hydrocinnamaldehyde association is due to a rather significant perturbation on the noncovalent association constant, K_s, by a group of $pK_{a(app)} \sim 6$, and the ratio k_2/k_{-2} actually has no pH dependency. This then would be an analogous situation to that described for association of the more specific aldehyde inhibitor BzPheal, described below, for which it is clearly shown that $K_{I(obsd)}$ and thus the ratio k_2/k_{-2} has no significant pH dependency.

A reasonable explanation of the pH independence of $K_{I(obsd)}$ for BzPheal association is that two proton transfers occur on BzPheal association leading to the protonated tetrahedral complex IIB as the major association species, rather than the anion species IIA. Thus step k_2 may be depicted as a two step proton transfer process in which the His-57 imidazole first abstracts the proton from the γ-O of Ser-195 generating the γ-O^- nucleophile, and in a second step the solution donates a proton to the oxygen anion on C-1 of the aldehyde (in IIA) resulting in the capture of the stable neutral tetrahedral adduct IIB (Figure 1). Accordingly, step k_2 is general base–specific acid catalyzed and step k_{-2} (microscopic reverse) is specific base–general acid catalyzed. As specific base–general acid catalysis is kinetically indistinguishable from general base catalysis (Jencks, 1969a), both k_2 and k_{-2} have the format $k_x = k_{(lim)}/(1 + [H^+]/K_a)$. The term $[1 + [H^+]/K_a]$ cancels out in the ratio k_2/k_{-2} and thus $K_{I(obsd)}$ according to eq 6 would show little dependency on hydrogen ion concentration. The small variation in $K_{I(obsd)}$ with pH (Figure 2) may be simply due to small increases in K_s with decreasing pH (Fersht & Requena, 1971).

Alternatively, a two-proton transfer can occur in a concerted rather than in a stepwise fashion. In a concerted process the His-57 in step k_2 acts both to abstract the γ-OH proton from the Ser-195 and simultaneously donates that proton to the developing oxygen anion of incipient IIA, generating IIB in a single step. In step k_{-2} the proton is carried by the His-57 from the hydroxy oxygen on IIB back to the Ser-195 simultaneously with the breaking of the Ser-195 aldehydic carbon covalent bond. Again, both steps k_2 and k_{-2} require the basic form of the His-57, and the ratio k_2/k_{-2} and thus $K_{I(obsd)}$ show no clear pH dependency. However, according to current hypotheses for the Cht mechanism (Blow, 1976; Kraut, 1977) this concerted mechanism must stereochemically place the oxygen anion generated in incipient IIA in the substrate leaving group site (S_1') rather than in the oxyanion hole (near the Ser-195 and Gly-193 peptide bonds), which is proposed to stabilize the carbonyl oxyanion in the tetrahedral transition state of substrate hydrolysis by Cht (Blow, 1976).

The base catalysis observed in the association of BzPheal to MCht may be due to the participation of the $N^{\delta 1}$ of His-57, which is rotated 180° about its C_α–C_β bond with respect to its productive position in native Cht. This argument was previously made by others to explain the low (10^5 lower) catalytic activity of MCht toward ester substrates (Henderson, 1971; Wright et al., 1972). However, it has been recently argued that the methylated $N^{\epsilon 2}$ in MCht can act as a general base catalyst with the unmodified $N^{\delta 1}$ remaining oriented toward the Asp-102 as in the unmodified native structure, if the Brønsted coefficient (β) for base catalysis is ~0.38 (Byers & Koshland, 1978; see Discussion below). It is observed that the second-order rate constant is surprisingly high for BzPheal association to MCht, $k_2/K_{s(lim)}$ for hemiacetal formation in MCht being as fast as the k_{cat}/K_m values observed for hydrolysis of specific methyl ester substrates of native Cht at optimum pH (Cohen, 1969). This limiting rate constant is only tenfold less than the limiting rate constant calculated for BzPheal association to native Cht.

pK_a of Catalytically Essential Active Site Residues. We have shown that the pH dependency of k_2/K_s for BzPheal association to native Cht fits a scheme in which groups in the enzyme with pK_a = 4.4 and pK_a = 7.0 control the rate of association into a covalent hemiacetal adduct. Recent spectral evidence may assign the lower pK_a to the imidazole side chain of His-57, and the higher pK_a to the γ-COOH of Asp-102 (Hunkapiller et al., 1973; Koeppe & Stroud, 1976). Accordingly, the rate of $k_2/K_{s(lim)1}$ for catalysis by the group with pK_a = 4.4 may reflect general base catalysis by the neutral His-57 imidazole side chain. However, the imidazole moiety will not be "actively" hydrogen bonded to the γ-COOH of Asp-102, as the γ-COOH has the higher pK_a and is in its protonated (unactive) form during catalysis by the monobasic form of native Cht between pH 4.4 and 7.0. Similarly in MCht, the Asp-102 to His-57 hydrogen bond is prevented by the intercalation of the $N^{\epsilon 2}$-methyl group, if the mechanism is assumed in which the imidazole side chain can rotate 180° about its C_α–C_β bond and the $N^{\delta 1}$ is directed toward the Ser-195 γ-OH (Henderson, 1971; Wright et al., 1972). Accordingly, it is suggested that base catalysis of BzPheal association by the monobasic form of native Cht and by MCht may both involve the imidazole of His-57 which is not hydrogen bonded to the γ-COO^- of Asp-102. In agreement with this rotated imidazole model for MCht and the assignment of pK_{a1} to His-57 and pK_{a2} to Asp-102 in native Cht, we find the value of $k_2/K_{s(lim)1}$, the limiting rate constant for BzPheal association to the monobasic form of native Cht, is identical with the value of $k_2/K_{s(lim)}$ found for BzPheal association to MCht. Again, in both cases catalysis is suggested to occur by the imidazole of His-57 not hydrogen bonded to the Asp-102. Given this assignment of pK_a = 4.4 to His-57 and pK_a = 7.0 to Asp-102 in native Cht, and the high limiting rate constant of 22×10^5 M^{-1} s^{-1} found for MCht and the monobasic form of native Cht, it is indicated that the Asp-102 γ-COO^- in the charge relay system accelerates hemiacetal

formation in Cht by a factor of only 10 over that of the His-57 imidazole acting alone.

An alternative proposal may be put forth to explain our data similar to a recent proposal to explain catalysis of substrates by MCht at rates 10^5 less than in native Cht by Byers & Koshland (1978). These authors estimate the pK_a of the methylated $N^{\epsilon 2}$ nitrogen in the imidazole ring of His-57 to be ~ -6.9 (Byers & Koshland, 1978). This is indeed a very poor base. However, if the Brønsted coefficient (β) is equal to 0.38 (whereas in nonenzymic ester hydolysis it is typically 0.5), the rates of base catalyzed ester hydrolysis in MCht can be explained without rotation of the His-57 side chain to a position that allows the unmodified ($N^{\delta 1}$) nitrogen to act as a base catalyst. Accordingly, if base catalysis of hemiacetal formation in Cht is characterized by a Brønsted coefficient (β) of 0.07 and a pK_a of -7 is assumed for the protonation of the *N*-methyl ($N^{\epsilon 2}$) nitrogen of the imidazole group in His-57, then the high activity toward BzPheal of MCht relative to native Cht can be accounted for. Similarly, the reactivity of the monobasic form of native Cht between pH 4.4 and 7.0 toward BzPheal would be explained by the catalysis of the already singly protonated ($N^{\epsilon 2}$) nitrogen in the imidazolium form of His-57, with a $pK_a \sim -7$ for acceptance of a second proton, and an assumed Brønsted coefficient < 0.1 for base catalysis of hemiacetal formation. A low Brønsted coefficient indicates little dependency on the nature of the base and a transition state for hemiacetal formation in which the proton that is to be lost still closely associated with the Ser-195 rather than with the general base (Bender, 1971). In step k_{-2}, the Brønsted coefficient for general acid catalysis would be close to 1. There exists mixed support for an assumption of $\beta \sim 0.07$ in nonenzymic studies of general base (or general acid) catalyzed nucleophilic addition to aldehydes (Jencks, 1969b). Hydration of acetaldehyde is acid catalyzed with a value of α equal to 0.54 (Bell & Higginson, 1949). However, the hydrolysis of acetals (a model for step k_{-2} in Figure 1) is acid catalyzed with a value of α near 1.0 (Jencks, 1969b). The reverse reaction will be base catalyzed with a value of β near 0.

In our discussion above of the possibility of base catalysis by the methylated or protonated $N^{\epsilon 2}$ nitrogen we have not addressed the following issues: (i) which group is protonated with $pK_a = 6.8$ in MCht (the $N^{\delta 1}$ nitrogen of His-57 or γ-COO^- of Asp-102); (ii) the charge on the charge relay system in active MCht; and (iii) whether the active form of MCht already contains a single proton between the Asp-102 and the $N^{\delta 1}$ of His-57 or whether this interaction is devoid of a proton. These issues are complicated and may not be simply solved with the data on hand.

A seeming advantage of the Byers and Koshland mechanism is that the His-57 need not rotate in MCht and the His-57 and Asp-102 can assume their rather normal pK_as of 6.8 and 4.4, respectively, in native Cht. However, steric objections raised by Byers and Koshland to the initially discussed model, of base catalysis by the $N^{\delta 1}$ nitrogen after rotation about C_α–C_β of His-57 in MCht from its normal position in fully active Cht, may not be that strong. His-57 is at the dimer interface in the crystallographic structure of MCht and the X-ray crystallographers of MCht suggest that in solution where the dimer interactions do not occur, rotation of the imidazole group and catalysis by the $N^{\delta 1}$ nitrogen may be possible (Wright et al., 1972). Furthermore, other steric restraints that appear in the crystalline model may also not be as severe if multiple active site residues can assume conformations slightly different from their static positions in the crystallographic model. Multiple small structural changes in Cht have been recently shown to occur by the X-ray diffraction technique on substrate analogue association other than toluenesulfonylamide or pipsylamide (Tulinsky et al., 1978). Furthermore, the recently corrected X-ray crystallographic data on native Cht shows the previously postulated hydrogen bond between the Ser-195 β-OH and the $N^{\epsilon 2}$ of His-57 does not preexist in native Cht (Matthews et al., 1977). The formation of this hydrogen bond was a criterion Byers and Koshland attempted to satisfy in their criticism of the rotated imidazole model for MCht catalysis (Byers & Koshland, 1978).

Dissimilarity of the Aldehyde–Enzyme Complex to Proposed Transition States. The lack of a significant pH dependency in $K_{I(obsd)}$ for BzPheal association to native Cht, and the high affinity of BzPheal to MCht, show that an integral basic form of the His-57 imidazole side chain is not required for the high affinity of BzPheal to Cht, even though an unmodified basic form of the His-57 imidazole is necessary for fast (catalyzed) hemiacetal formation. A priori, one may have predicted that, as the His-57 participates in currently proposed transition-state structures for Cht-catalyzed hydrolyses of substrates (Blow, 1976; Kraut, 1977), its physical participation would be essential to the high affinity of a "transition-state analogue". The lack of His-57 participation in the thermodynamic stability of the BzPheal–Cht complex shows that, even though the aldehyde association adduct mimics features of the substrate transition state, the conformation of the enzyme complementary to the covalently associated aldehyde is not the proposed transition-state conformation. A similar conclusion was recently put forth by Byers and Koshland, based on the higher affinity of phenylethaneboronic acid to MCht than to Cht (Byers & Koshland, 1978). In addition, proposed transition states usually indicate an oxyanion form for the carbonyl oxygen of the labile substrate bond as in IIA. However, our findings indicate IIB is the likely stable form of the aldehyde–Cht association complex. Accordingly, other explanations are possible for the high affinity of BzPheal to Cht other than the similarity in the aldehyde–Cht complex to features of the proposed transition state (Schultz & Cheerva, 1975; Thompson, 1974; Westerik & Wolfenden, 1972). Some investigators have argued for the existence of a tetrahedral covalent *intermediate* on the pathway of cysteine and serine protease-catalyzed hydrolysis (Kraut, 1977). It may well be that the hemiacetal adduct between BzPheal and Cht mimics such an intermediate, and is more stable than the proposed intermediate in substrate reactions because of the chemical ability of the aldehyde functional group to give stability to an sp^3 configuration.

Summary

In summary, this work indicates that the association complex of BzPheal with native Cht is the hemiacetal complex IIB, where the hemiacetal adduct is protonated and contains no negative charge. Furthermore, the His-57 imidazole is not intimately involved in the stabilization of the complex IIB. Accordingly, the complex of BzPheal with Cht has substantial differences in structure and charge from the proposed transition state in Cht-catalyzed hydrolyses. The formation of IIB is initiated by binding of the unhydrated aldehyde to form complex IA, from which hemiacetal complex IIB is formed under base catalysis by two groups of $pK_a = 4.4$ and 7.0 in native Cht and a single group of $pK_a = 6.8$ in MCht. A cogent argument may be made from these data that the group of $pK_a = 4.4$ in native Cht is the His-57 and that of $pK_a = 7.0$ is the Asp-102. Alternatively, assuming a pK_a of -7 for the methylated-protonated $N^{\epsilon 2}$ and the diprotonated $N^{\epsilon 2}$ of His-57 in MCht or Cht, respectively, and a Brønsted coefficient of

0.07 for base catalysis of hemiacetal formation in Cht, the high reactivity of MCht and the monobasic form of Cht in hemiacetal formation may be explained.

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